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DROUGHT PHENOTYPING IN CROPS: FROM THEORY TO PRACTICE

Topic Editors
Philippe Monneveux,
Jean-Marcel Ribaut and Antonia Okono

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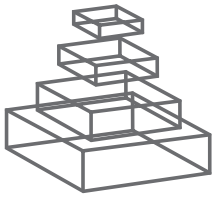

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DROUGHT PHENOTYPING IN CROPS: FROM THEORY TO PRACTICE

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Illustration: Recomposition of two original artworks by Rhoda Okono entitled Desert bloom Meztnopali and Autumn in Africa. Recomposition by G Antonio Luna Avila. The illustration features all the 14 crops covered in the book.

This topic is a unique attempt to simultaneously tackle theoretical and practical aspects in drought phenotyping, through both crop-specific and cross-cutting approaches. It is designed for – and will be of use to – practitioners and postgraduate students in plant science, who are grappling with the challenging task of evaluating germplasm performance under different water regimes.

In Part I, different methodologies are presented for accurately characterising environmental conditions, implementing trials, and capturing and analysing the information this generates, regardless of the crop.

Part II presents the state-of-art in research on adaptation to drought, and recommends specific protocols to measure different traits in major food crops (focusing on particular cereals, legumes and clonal crops).

The topic is part of the CGIAR Generation Challenge Programme's efforts to disseminate crop research information, tools and protocols, for improving characterisation of environments and phenotyping conditions. The goal is to enhance expertise in testing locations, and to stimulate the development and use of traits related to drought tolerance, as well as innovative protocols for crop characterisation and breeding.

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Facing the challenges of global agriculture today: what can we do about drought?

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It is estimated that the planet's demand for food and feed crops will almost double by 2050 (Foley et al., 2011). Globally, rain-fed agriculture is practised in 80% of the total agricultural area and generates 62% of the world's staple food (FAOSTAT, 2011). Taking into consideration global water scarcity and increases in demand for non-agricultural uses of water, expansion of the area under irrigation in developing countries does not appear to be a realistic scenario to address the challenge of food security.

According to the latest climate change scenarios, 20-year extreme annual daily maximum temperature will likely increase by about 1–3°C by mid-21st century, and by about 2–5°C by the late 21st century, depending on the region and emissions scenario (IPCC, 2012). Based on historical data collected in Africa on more than 20,000 trials (1999–2007), each "degree day" spent above 30° reduced yield by 1% under optimal conditions, and that penalty rose up to 1.7% under water-limited conditions (Lobell et al., 2011). The impact of a changing climate is not only about temperature increase, but it is also affecting the magnitude of rainfall and its distribution, and therefore its availability at critical times of the crop cycle (Feng et al., 2013): in fact, while the total amount of rain increased in Africa over the last few years, the erratic and unpredictable nature of the drought and floods cycle also increased (Douglas et al., 2008). As such, improving the drought tolerance of crops, increasing the efficiency of water use and enhancing agricultural water productivity under rain-fed conditions is a number one priority today in a growing number of countries.

The recent genomics and bioinformatics revolutions offer real opportunities for dissecting drought tolerance into component traits, and then using genomic approaches to select plants with favorable alleles at the underlying genes. Although major achievements have been reported recently by the private sector, the development of effective systems for breeding complex traits such as drought tolerance continues to be a major challenge in the public sector, despite significant investments in research and development. Adoption of molecular breeding in developing countries remains very limited. This is due mainly to a shortage of well-trained personnel, inadequate high-throughput capacity, poor phenotyping infrastructure, and a lack of information systems or adapted analytic tools (Ribaut et al., 2010).

Created in 2003, the CGIAR Generation Challenge Programme (GCP) is a time-bound initiative ending in 2014. GCP's mission is to use plant genetic diversity, advanced genomic science and comparative biology to develop tools and technologies that will support plant breeders in the developing world in their efforts to produce better crop varieties for resource-poor farmers in drought-prone environments. Generic facilitating technologies developed by GCP include standardized phenotyping protocols, whole-plant physiology modeling, molecular breeding simulation studies, decision-support tools, procedures for creating low-cost trait diagnostics and high-throughput array-based genotyping systems. Since 2009, GCP has been coordinating the Integrated Breeding Platform (IBP). IBP is a one-stop shop where breeders can access the analytical tools and support services to manage their projects, find new knowledge and training opportunities, and access fora for discussion with peers.

Drought tolerance is the main target trait of the Programme, and genomics-assisted breeding for better crop production under water-limited conditions is at the heart of the research supported by GCP during its second phase. Good genetic studies are impossible without reliable phenotypic data, and plant phenotyping must be conducted locally. Most national breeding programmes from developing countries working in partnership in the GCP network have in common a scarcity of suitable field infrastructure for collection of accurate phenotypic data, especially for stresses such as drought. Therefore, GCP recognizes that accurate and reliable phenotyping is the main bottleneck in drought-tolerance research, and is allocating significant resources to improve crop phenotyping in target environments under different water regimes.

To achieve this objective, geographic information system (GIS) tools and soil water balance models have been used to describe the drought scenario faced by the crops in different target GCP environments, and to compare and cluster the phenotyping locations for GCP projects. Facilities and expertise in the different locations have been evaluated, needs have been prioritized, and today GCP is investing about four million US dollars to improve the local infrastructure of partners involved in GCP projects.

Complementary to the effort to improve infrastructure is the need to develop tools and protocols for improving characterization of environments and plant phenotypes, enhancing expertise in testing locations, and stimulating the development and use of innovative drought tolerance-related traits and protocols (e.g., carbon isotope discrimination, spectroradiometry, thermal imaging). This manual contributes to this effort.

The first part of this manual addresses—from a generic perspective—global issues and challenges related to environment selection and characterization, experimental field design, trait selection, and data analysis and management. The second part of the manual is crop-specific for a set of GCP target crops. Each article presents the state-of-the-art of research on drought tolerance and the protocols that are more specifically used to measure different traits for each of those crops.

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Phenotyping for drought tolerance of crops in the genomics era

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Improving crops yield under water-limited conditions is the most daunting challenge faced by breeders. To this end, accurate, relevant phenotyping plays an increasingly pivotal role for the selection of drought-resilient genotypes and, more in general, for a meaningful dissection of the quantitative genetic landscape that underscores the adaptive response of crops to drought. A major and universally recognized obstacle to a more effective translation of the results produced by drought-related studies into improved cultivars is the difficulty in properly phenotyping in a high-throughput fashion in order to identify the quantitative trait loci that govern yield and related traits across different water regimes. This review provides basic principles and a broad set of references useful for the management of phenotyping practices for the study and genetic dissection of drought tolerance and, ultimately, for the release of drought-tolerant cultivars.

Keywords: drought tolerance, phenomics, genomics, QTL, breeding, yield, phenology, modeling

INTRODUCTION

Crops are exposed to the ravages of drought in various ways and to different extents. Regrettably, global climate change will increase the occurrence and severity of drought episodes, not least due to the higher evapotranspirative demand created by rising temperatures. Altogether, these changes have already been shown to offset a significant portion of the increases in average yields that during the past three decades arose from technology, CO_2 fertilization and other factors (Lobell et al., 2011). Therefore, food security in the twenty-first century will rely increasingly on the release of cultivars with improved resistance to drought conditions and with high yield stability (Swaminathan, 2005; Borlaug, 2007; Pennisi, 2008; Luo, 2010; Tester and Langridge, 2010; Reynolds et al., 2011; Serraj et al., 2011; Chapman et al., 2012).

In this challenging scenario, molecular approaches offer novel opportunities for the dissection and more targeted manipulation of the genetic and functional basis of yield under drought conditions (Forster et al., 2000; Sinclair et al., 2004; Bohnert et al., 2006; Mackill, 2006; Tuberosa and Salvi, 2006; Jenks et al., 2007; Nelson et al., 2007; Ortiz et al., 2007a; Vij and Tyagi, 2007; Leung, 2008; Xu and Crouch, 2008; Ashraf, 2010; Mittler and Blumwald, 2010; Yadav et al., 2011; Deikman et al., 2012). Additionally, the "-omics" platforms now allow for extensive mining of the transcriptome (Rabbani et al., 2003; Poroyko et al., 2007; Degenkolbe et al., 2009; Ergen and Budak, 2009; Sreenivasulu et al., 2010; Deokar et al., 2011; Hiremath et al., 2011), metabolome (Fernie and Schauer, 2009) and proteome (Timperio et al., 2008). Although, some may not consider "-omics" data as phenotypes *sensu stricto*, they should be treated as such, considering that they represent crucial steps that are progressively removed from genes to their ultimate phenes (Houle et al., 2010; Furbank and Tester, 2011). Not with standing the deluge of molecular data produced in the past decade, the applicable results reported so far with non-conventional approaches have not met expectations (Edmeades et al., 2004; Araus et al., 2007, 2008; Collins et al., 2008; Xu and Crouch, 2008; Heffner et al., 2009; Passioura, 2010; Sinclair, 2011), partly because the progress in high-throughput, quality phenotyping has lagged behind.

Before analyzing the factors that affect the quality of phenotypic data collected under water-limited conditions, it is important to define the nomenclature and mechanisms of crop adaptation to drought and clarify their functional basis. Most of the examples and references provided in this review refer to cereals, which, as compared to other crops, have been more extensively investigated under drought conditions. Nevertheless, most concepts presented herein are equally valid for other crops as well.

DROUGHT ADAPTATION: CONCEPTS, NOMENCLATURE, AND MECHANISMS

In agriculture, the term "drought" refers to a condition in which the amount of water available through rainfall and/or irrigation is insufficient to meet the transpiration needs of the crop. The examples presented in this review provide some general guidelines on the different mechanisms that allow plants to withstand and eventually mitigate the negative effects of water deficit. In general, a clear distinction should be made between traits that help plants to survive a severe drought stress and traits that mitigate yield losses in crops exposed to a mild or intermediate level of water stress. Modern breeding activities, including phenotyping conditions, have predominantly targeted the latter levels of stress. Although, yield remains an elusive and neglected concept in most molecular studies carried out under water-limited conditions, it is an appropriate way to gauge the overall phenotypic value of any accession.

THE FUNCTIONAL BASIS OF DROUGHT RESISTANCE

Among the several definitions of drought resistance that have been provided during the past decades, the original one formulated by Levitt (1972) retains its validity and offers a rational approach to classify the strategies that allow plants to mitigate the negative effects of water deficit. Levitt (1972) classified the different mechanisms or strategies of drought resistance into two broad categories: dehydration avoidance and dehydration tolerance. In this respect, morpho-physiological features [e.g., deep roots, early flowering, deposition of epicuticular waxes, osmotic adjustment (OA), etc.] that enable the plant, or parts thereof, to maintain hydration are classified under dehydration avoidance. Conversely, features (e.g., remobilization of stem water-soluble carbohydrates (WSC), accumulation of molecular protectants, etc.) that allow the plant to maintain, at least partially, proper functionality in a severely dehydrated state are classified under dehydration (desiccation) tolerance. Carefully planned experiments conducted under controlled conditions allow us to separate the action of loci imparting avoidance from those providing tolerance to drought (Yue et al., 2006). Several reviews and dedicated volumes have addressed the mechanisms underlying drought resistance and the strategies that can improve yield under such conditions (Blum, 1988, 1996, 2009, 2011; Ludlow and Muchow, 1990; Ceccarelli and Grando, 1996; Passioura, 1996, 2007, 2010; Richards, 1996; Turner, 1997; Ribaut, 2006; Fischer et al., 2003; Boyer and Westgate, 2004; Chaves and Oliveira, 2004; Tuberosa, 2004; Araus et al., 2008; Kumar et al., 2008; Morison et al., 2008; Reynolds and Tuberosa, 2008; Farooq et al., 2009; Passioura and Angus, 2010; Yang et al., 2010; Sadok and Sinclair, 2011; Sinclair, 2011; Cairns et al., 2012; Mir et al., 2012).

The first step is to define the population of environments to be targeted, also identified as the TPE (target population of environments). Differences in TPE are largely determined by long-term patterns of genotype-by-environment interactions (GEI). The identification and characterization of a TPE is facilitated by the use of crop simulation models based on historic records of weather data. Simulation can describe a TPE by the frequency of occurrence of specific abiotic stresses and be based on the soil moisture profile along the crop cycle (Chapman et al., 2003). In Mediterranean environments, wheat and barley usually experience terminal drought caused by high temperatures during the grain-filling period (Araus et al., 2008). Nevertheless, within each TPE and GEI are frequently observed relating to yearly fluctuations in environmental factors (e.g., rainfall, temperature, etc.), diseases (e.g., foliar disease), and/or parasites (e.g., insects). Ideally, phenotyping for drought tolerance and yield stability should be carried out across a broad range of environments present within the TPE. During past decades, these multienvironment trials have been instrumental in increasing yield potential and also in maintaining yield stability under drought-stressed conditions in temperate maize (Tollenaar and Wu, 1999; Duvick, 2005; Tollenaar and Lee, 2006) as well as in other crops (Lafitte et al., 2006; Crossa et al., 2007; Acuna et al., 2008). In a few cases, they have also allowed for the identification of major QTLs consistently affecting yield across a range of water availability (Bernier et al., 2007, 2009; Maccaferri et al., 2008; Venuprasad et al., 2009a,b, 2012; Vikram et al., 2011; Dixit et al., 2012; Ghimire et al., 2012).

WATER-USE EFFICIENCY AND GRAIN YIELD UNDER WATER-LIMITED CONDITIONS

Water-use efficiency (WUE) is the amount of dry matter produced [grain yield (GY) in the case of grain crops when considering seasonal WUE] per unit of water lost through evapotranspiration. A classical formula that highlights the critical role of WUE in determining GY in crops grown in water-limited conditions was suggested by Passioura (1977):

$$GY = W \times WUE \times HI$$

where W is the total amount of water transpired by the crop and evaporated from the field and HI is the harvest index, i.e., the ratio between GY and total biomass. Salekdeh et al. (2009) identify phenotyping protocols that address each formula's factors, describe their key features and illustrate their integration with different molecular approaches. When using this formula, one should consider the possible interdependence of these variables, with the result that selection for improving WUE in order to increase GY may be partially counterbalanced by a reduction in the amount of water extracted from the soil. In fact, a number of traits influence both W and WUE.

The most important factor is matching the phenological development pattern of the crop and the seasonal rainfall pattern (Richards, 1996; Turner, 1997; Araus et al., 2003; Morison et al., 2008; Soltani and Sinclair, 2012). Early vigor potentially improves both W and WUE, while deep roots and/or osmoregulation under appropriate conditions increase water extraction from the soil (Blum, 1988, 2011; Ludlow and Muchow, 1990; Richards, 2006; Reynolds and Tuberosa, 2008; Sadok and Sinclair, 2011). However, we should keep in mind that farmers eventually harvest grain and not WUE, which means that a lower WUE may actually be desirable when WUE is negatively associated with GY, as is well-known in cereals differing in their intrinsic WUE (Blum, 2005, 2006, 2009). Therefore, WUE should not be equated to drought tolerance. The best example is provided by a population of related progeny such as recombinant inbred lines (RILs) that differ in their capacity to extract soil moisture due to differences in root depth, and hence greater capacity to access moisture stored in deeper soil layers. Because, WUE is higher in genotypes characterized by low stomatal conductance, often resulting from a lower water status, the genotypes that are more wasteful (i.e., with a lower WUE) and able to extract more water from the soil (Merah, 2001; Rebetzke et al., 2002; Blum, 2006, 2009, 2011), whilst maintaining higher stomatal conductance, will have higher yield. Conversely, under conditions of limited soil moisture, low WUE resulting from excessive evapotranspiration will not allow sustained accumulation of dry matter and its partitioning to reproductive organs (Monneveux and Ribaut, 2006; Richards, 2006; Tambussi et al., 2007; Barnabas et al., 2008; Sinclair et al., 2008). This finding introduces an essential concept for interpreting cause–effect relationships between morphophysiological traits and GY under drought conditions: the sign and magnitude of this relationship at the whole-plant or QTL level are

not universal and can change widely according to the dynamics (i.e., frequency and timing) and intensity of the drought episode/s (Collins et al., 2008; Sinclair et al., 2010; Tardieu, 2012).

An alternative formula to address properly the factors influencing WUE in crops grown under water-limited conditions has been proposed by Richards (1991):

$$\text{WUE (biomass)} = \text{TE}/(1 + E_s/T)$$

where TE is the transpiration efficiency (above ground dry weight/transpired water), E_s is the water lost by evaporation from the soil surface and T is water lost through transpiration by the crop. Analysis of the variables in this formula provides a useful framework for identifying the agronomic and breeding strategies, and hence phenotyping targets, most suitable for optimizing WUE and maximizing yield in environments that differ in rainfall distribution during the crop cycle.

At the leaf level, "intrinsic WUE" indicates the ratio of the instantaneous rates of CO_2 assimilation and stomatal transpiration. Condon et al. (2002) discussed the factors influencing intrinsic WUE and how an increased intrinsic WUE can be achieved through either lower stomatal conductance, higher photosynthetic capacity, or both. The same authors caution about the possible penalties in terms of yield through manipulation of each variable. They conclude that to achieve more widespread gains in cereal yield derived from greater intrinsic WUE, it is necessary to decouple intrinsic WUE and low crop growth rate. In practical terms, WUE becomes more important when crops grow predominantly on stored soil moisture (Condon et al., 2002), as reflected by the release of wheat cultivars Drysdale and Rees (Richards, 2006), specifically selected for target areas where wheat is grown under such conditions.

WHICH TRAITS SHOULD BE TARGETED?

The morphophysiological traits and the corresponding QTLs that affect yield in drought conditions can be categorized as constitutive (i.e., also expressed under well-watered conditions) or drought-responsive (i.e., expressed only under pronounced water shortage; Lafitte and Edmeades, 1995; Blum, 2006). While drought-responsive traits/QTLs usually affect yield only under rather severe drought conditions, constitutive traits/QTLs can affect yield at low and intermediate levels of drought stress as well. The response of QTLs for drought-adaptive traits (e.g., accumulation of osmolytes, relocation of WSC, etc.) to drought is probably due to regulation of the expression of the underlying structural genes in response to signaling cues such as abscisic acid (ABA) accumulation (Bray, 2002) that are reinforced by cellular dehydration. Under appropriate soil moisture conditions, the presence of QTLs for traits usually classified as constitutive but difficult to measure (e.g., root depth) can be revealed by the collocation of QTLs for traits indicative of the water status of the plant such as ABA concentration, stomatal conductance, canopy temperature depression (CTD), etc., (Lebreton et al., 1995; Tuberosa et al., 2002b; Reynolds et al., 2009, 2011). Experimental evidence indicates that the progress achieved by breeders during the last century can mainly be accounted for by changes in constitutive traits that affect dehydration avoidance rather than drought-responsive traits (Blum, 2005, 2006, 2011). In this respect, emphasis is increasingly being placed on phenotyping traits that constitutively enhance yield *per se* (Blum, 2009; Passioura, 2010), rather than on characteristics that enhance plant survival under extreme drought (Bartels et al., 2006), in view of a possible negative trade-off under less severe circumstances (Blum, 1996, 2005, 2006; Passioura, 2002, 2007, 2010; Sinclair, 2011).

The traits to be considered as potential selection targets for improving yield under water-limited conditions must be genetically (i.e., causally) correlated with yield, and should have a greater heritability than yield itself (Blum, 1988, 2011; Monneveux and Ribaut, 2006). Additional desirable features are the presence of sufficient genetic variability and lack of yield penalties under favorable conditions. Ideally, measurement of the target trait should be non-destructive, rapid, accurate, and inexpensive. It should also be possible to measure the trait using a small number of plants and without lengthy procedures to calibrate sensors to individual plants. Finally, rather than reporting on short-term features at the cellular level, the nature of the secondary trait should be integrative across the growing cycle, or part thereof, and relate to higher levels of functional organization (e.g., the canopy level rather than the single leaf), thereby providing information on the long-term ecophysiological performance of the crop. General information and examples are now provided on a number of traits that have been investigated for their influence on drought resistance and/or WUE.

EARLY VIGOR

Early vigor under conditions of low evapotranspiration may allow annual crops to optimize WUE and limit the loss of water due to direct evaporation from the soil surface. This leaves more stored water available for later developmental stages when soil moisture becomes progressively exhausted and increasingly limiting for yield (Slafer et al., 2005; Richards, 2006; Rebetzke et al., 2007; Richards et al., 2007). Early establishment also reduces the occurrence of inhibition of stomatal conductance as a consequence of root-borne signaling such as from ABA through the xylem flow (Davies et al., 2000; Ren et al., 2007) caused by shallow and superficial roots (Blum, 1996; Giuliani et al., 2005). As a trade-off, excessively vigorous canopy development may cause early depletion of soil moisture. The optimal degree of vigor will thus depend on the environmental characteristics of the TPE. Early vigor has been exploited to improve WUE and yield in wheat (Asseng et al., 2003; Richards, 2006; Rebetzke et al., 2007). QTLs for the growth rate of wheat seedlings (Spielmeyer et al., 2007) are being targeted at CSIRO (Commonwealth Scientific and Industrial Research Organization, Australia)[1].

ROOT ARCHITECTURE

Roots exhibit an astounding level of morphological plasticity in response to soil physical conditions (Passioura, 1983; Bengough et al., 2006; Gerald et al., 2006; Ito et al., 2006; Kato et al., 2007; Lynch, 2007; Forde, 2009; Siopongco et al., 2009), a peculiarity that allows plants to adapt better to the chemical and physical properties of the soil, particularly under drought conditions

[1] http://www.csiro.au/files/files/p2ki.pdf

(Bacon et al., 2002; Yu et al., 2007). The concept of root ideotype should be elaborated only after gaining a detailed understanding of: (1) the factors that limit the availability of soil moisture to the crop; and (2) the metabolic cost sustained by the plant to develop and maintain a more vigorous root system. Notably, recurrent selection for increased GY in drought-stressed tropical maize was associated with a decrease in root mass (Bolaños and Edmeades, 1993). Accordingly, the effects of root size and architecture on final yield will depend on the distribution of soil moisture and the level of competition for water resources within the plant community (King et al., 2009). Therefore, when additional stored moisture is available in deeper soil layers, selection for faster growing and deeper roots could enhance water harvest and help stabilize yield under drought conditions.

The importance of a deep and vigorous root system for higher yield has been recognized in bean (Mohamed et al., 2002), soybean (Sadok and Sinclair, 2011), chickpea (Varshney et al., 2011), lettuce (Johnson et al., 2000), maize (Tuberosa et al., 2003, 2007b, 2011b; Hammer et al., 2009; Landi et al., 2010; Hund et al., 2011), barley (Forster et al., 2005), wheat (Manschadi et al., 2006, 2010; Wasson et al., 2012), and especially, in rainfed rice (Nguyen et al., 1997; Price and Tomos, 1997; Ali et al., 2000; Babu et al., 2003; Courtois et al., 2003, 2009; Steele et al., 2006, 2007; Kamoshita et al., 2008; Witcombe et al., 2008; Bernier et al., 2009; Henry et al., 2011). However, other experiments in rice have shown a lack of correlation between root features and drought resistance (Pantuwan et al., 2002; Subashri et al., 2009).

The main drawback to the study of root features and their use as selection criteria relates to the difficulty of phenotyping field-grown plants (Richards, 2008). A number of techniques allow for the estimation of root mass and its distribution in the soil profile. These techniques require different amounts of labor and plot destruction for sample collection. The fastest but most destructive technique measures the vertical pulling strength required to uproot the plant, as a proxy for root mass and architecture (Lebreton et al., 1995; Sanguineti et al., 1998; Landi et al., 2002). Recently, a high-throughput, albeit equally destructive approach also known as "shovelomics," has been deployed to investigate several root architectural features in field-grown maize (Trachsel et al., 2011). Other less destructive but much more time-consuming techniques such as excavation and coring methods have also been used to estimate root mass and distribution (Nissen et al., 2008).

Minirhizotrons provide a non-destructive, *in situ* method for directly viewing and studying fine roots (Johnson et al., 2001; Smit and Groenwold, 2005). Tube installation is critical, and steps must be taken to ensure good soil/tube contact without compacting the soil. Tube installation causes some degree of soil disturbance and has the potential to create artifacts in root data collection and analysis, resulting in biased values. Therefore, a waiting period of a few months between tube installation and image collection is recommended to allow roots to recolonize the space around the tubes and to permit nutrients to return to pre-disturbance levels (Johnson et al., 2001). The frequency of image collection depends upon the root parameters being measured or calculated, and the time and resources available for collecting images and extracting data.

In maize, a fast non-destructive method to estimate root mass has relied on the use of a hand-held capacitance meter (van Beem et al., 1998; McBride et al., 2008). The accuracy of this method was tested by comparing the results with direct measurements taken on uprooted plants grown in the greenhouse and in the field. The significant correlation (r from 0.56 to 0.73) between the methods suggests the feasibility of using capacitance meters for routine, non-destructive observations repeated over time. Despite this possibility, the method has not been widely applied.

Heterogeneity in soil structure and composition hinders the acquisition of accurate values for root features in field-grown plants. As an alternative to root phenotyping in field experiments, a number of studies have measured roots in plants grown under controlled conditions (Arihara and Crosbie, 1982; Price et al., 1997a, 2002b,c; Landi et al., 1998, 2001a; Tuberosa et al., 2002b; de Dorlodot et al., 2005, 2007; Kimurto et al., 2005; Zhu et al., 2006, 2011; Hochholdinger and Tuberosa, 2009; Zaman-Allah et al., 2011a; Ren et al., 2012). This allows more rapid and accurate analysis of root features. A major shortcoming of these studies is the unnatural environment in which the roots grow, suggesting great caution in extrapolating the results to field-grown plants. In maize, a significant, albeit weak, positive association has been reported between seminal root traits in hydroponics and root pulling resistance in the field (Landi et al., 2001a; Tuberosa et al., 2002b). A reasonable compromise to avoid both the unnatural conditions present in hydroponics and/or aeroponics and the difficulty of studying roots in the field is offered by growing plants in pots, columns and/or observation chambers filled with soil (Azhiri-Sigari et al., 2000; Wade et al., 2000; Zaman-Allah et al., 2011a). Pot experiments also allow for a precise measurement of the amount of water provided to each plant, hence water use and WUE (Price et al., 2002b), and to estimate the capacity of roots to penetrate a wax layer of high mechanical impedance mimicking a soil hardpan, often the main constraint that limits access of roots to soil moisture in deeper soil layers (Cairns et al., 2004; Nhan et al., 2006; Acuna et al., 2007). In rice, an enhanced capacity to penetrate a soil hardpan is considered an essential feature for the development of deeper roots under rainfed lowland conditions (Fukai and Cooper, 1995) and is a key factor in drought adaptation in areas where water supply is limited (Siopongco et al., 2009).

Gel- or soil-filled chambers, soil sacs, pouches, paper rolls, X-ray microtomography, and magnetic resonance imaging (MRI) have also been used to investigate bi- and tri-dimensional root architecture (Bengough et al., 2004; Sanguineti et al., 2007; Hargreaves et al., 2009; Norton and Price, 2009; Ruta et al., 2010; Tracy et al., 2010; Bovina et al., 2011; Clark et al., 2011; Rascher et al., 2011; Singh et al., 2011; Alhosein et al., 2012; De Smet et al., 2012; Hamada et al., 2012; Mace et al., 2012). These experiments are particularly suited to the discovery of QTLs that are prevalently expressed in a constitutive fashion and which, as such, are more likely to influence root architectural features (e.g., root angle) across different soil conditions.

FLOWERING TIME

Flowering time is recognized as the most critical factor to optimize adaptation, hence yield, in environments differing in water

availability and distribution during the growing season (Richards, 2006). Positive associations between plasticity of yield and flowering time across different levels of water availability have been reported in different crops (Sadras et al., 2009). Therefore, in addition to phenology *per se* (i.e., mean time to a phenological stage), plasticity of phenological development merits consideration as a distinct trait influencing crop adaptation and the outcome of any QTL experiment where the effects of phenology on yield are not duly recognized and accounted for (Pinto et al., 2010; Sabadin et al., 2012).

Many studies have investigated the genetic basis of flowering time, reflecting the economic importance of this trait. In annual crops, the genetic basis of flowering time is more complex in temperate species (e.g., barley, wheat, rye, etc.) as compared to tropical species (e.g., rice, sorghum, maize, etc.), due to the presence in the former group of verbalization genes influencing flowering time in response to low temperatures. In cereals, the switch from the vegetative to the reproductive phase is controlled, according to the species, by several genes responsive to verbalization and/or daylength as well as by loci for earliness *per se* (Salvi et al., 2002, 2007, 2011; Distelfeld et al., 2009).

In maize, a valuable selection target for improving drought resistance is provided by the anthesis-silking interval (ASI), a trait of intermediate heritability that is usually negatively correlated with GY under drought conditions (Bolaños and Edmeades, 1996; Monneveux and Ribaut, 2006). Because ASI can be phenotyped quite easily and effectively under the right experimental conditions, substantial breeding efforts have targeted this trait through conventional breeding (Chapman and Edmeades, 1999) or, once QTLs have been identified (Ribaut et al., 1996; Li et al., 2003a; Hao et al., 2008), with marker-assisted selection (MAS) (Ribaut et al., 2004; Ribaut and Ragot, 2007). The negative association reported between the effects of QTLs that have been shown to influence both leaf elongation and ASI suggests turgor maintenance as a possible common mechanism accounting for the correlation (Welcker et al., 2007).

CARBON ISOTOPE DISCRIMINATION

Carbon isotope discrimination ($\Delta^{13}C$) measures the ratio of stable carbon isotopes ($^{13}C/^{12}C$) in the plant dry matter compared to the ratio in the atmosphere (Condon et al., 1990). Because of differences in leaf anatomy and the mechanisms of carbon fixation in species with the C_3 or C_4 pathway, studies on $\Delta^{13}C$ have wider implications for C_3 species where the variation in $\Delta^{13}C$ is larger than in C_4 species and has a greater impact on crop yield (Condon et al., 1990, 2006). Commonly, but not always (Turner et al., 2007), $\Delta^{13}C$ is negatively associated with WUE over the period of dry mass accumulation (Condon et al., 1990, 2004; Araus et al., 2002; Rebetzke et al., 2002; Xu et al., 2007; Royo et al., 2008).

Under drought stress, $\Delta^{13}C$ is a good predictor of stomatal conductance (Condon et al., 2002) and WUE in different crops (Turner, 1997; Tambussi et al., 2007). A number of studies conducted in bread wheat under varying conditions of water availability have shown that the correlation between $\Delta^{13}C$ and final GY varies from positive, when ample water is available to the crop, to negative in drought conditions, with no correlation at all in intermediate conditions (Condon et al., 1993, 2004). These results can be interpreted based on the influence of both stomatal conductance and photosynthetic activity on $\Delta^{13}C$, and on the fact that biomass production is limited in wet years by a lower stomatal conductance—an advantage under drought conditions (Turner, 1997). $\Delta^{13}C$ measured in grains correlates positively with growth cycle duration (Araus et al., 1997) and negatively with leaf temperature (Richards et al., 2002). Therefore, the relationship between $\Delta^{13}C$ and GY depends on the environmental conditions, the phenology of the crop and the plant organ (e.g., leaf or grain) from which the samples are collected (Araus et al., 1997; Merah et al., 2001; Condon et al., 2004).

High genetic variation for grain $\Delta^{13}C$ has been reported in C_3 species (Turner, 1997; Chen et al., 2012), with high heritability (e.g., from 0.76 to 0.85 in durum wheat; Merah et al., 2001) and a low GEI (Richards, 1996; Rebetzke et al., 2008a). For these characteristics, $\Delta^{13}C$ is an attractive breeding target for improving WUE and yield, while the high cost required to measure each sample makes it an interesting candidate for MAS.

STOMATAL CONDUCTANCE

Stomatal conductance plays a pivotal role in regulating the water balance of the plant and determining $\Delta^{13}C$ and WUE (Condon et al., 2002; Richards et al., 2002, 2007; Sinclair et al., 2008, 2010). A retrospective study conducted by Fischer et al. (1998) on a historical series of successful bread wheat cultivars released by CIMMYT from 1962 to 1988 showed a strong positive correlation between stomatal conductance and GY ($r = 0.94$; Fischer et al., 1998), indicating the possibility of raising the yield potential, hence the amount of water used by the crop, through an indirect selection for stomatal conductance and/or leaf temperature.

Given the laborious nature of measuring stomatal conductance, identifying the corresponding QTLs would allow for the implementation of MAS. In fact, it is difficult to accurately measure stomatal conductance in a reasonably large number of plants while properly accounting for the fluctuation in the main environmental factors known to affect stomatal conductance during the day (wind, solar radiation, humidity, etc.). A number of studies have reported QTLs for stomatal conductance (Lebreton et al., 1995; Price et al., 1997b, 2002a; Sanguineti et al., 1999; Ulloa et al., 2000; Takai et al., 2006; Khowaja and Price, 2008).

A more attractive and integrative way to indirectly monitor stomatal conductance through an extended time-period is based on the measurement of the natural oxygen isotope composition ($d^{18}O$) in leaf and grain materials (Barbour et al., 2000; Ferrio et al., 2007). Compared with stomatal conductance, measuring $d^{18}O$ in plant material offers four advantages: (1) it provides an integrated measure of stomatal conductance and leaf temperature over the period that the analyzed tissue was formed; (2) it avoids a number of experimental problems typical of measuring stomatal conductance; (3) it allows for the collection of a large number of samples, and (4) requires very little labor in the field. In the historical series of CIMMYT wheat cultivars tested under irrigated conditions (see above), leaf $d^{18}O$ was strongly correlated with stomatal conductance ($r = -0.93$; Barbour et al., 2000). In this case, GY was more strongly correlated with leaf $d^{18}O$ ($r = -0.90$) as compared to leaf $d^{13}C$ ($r = -0.71$). However, the

authors caution that $d^{18}O$ is a questionable yield predictor when stomatal conductance and GY are not strongly correlated.

CANOPY TEMPERATURE DEPRESSION

CTD as measured by thermal imaging is the difference in temperature between the canopy surface and the surrounding air. CTD is a highly integrating trait resulting from the effects of several biochemical and morphophysiological features acting at the root, stomata, leaf, and canopy levels. In the field, genotypes with a cooler canopy temperature under drought stress, or a higher CTD, use more of the available water in the soil to avoid excessive dehydration (Blum, 1988; Ludlow and Muchow, 1990; Reynolds et al., 2007, 2009). Infrared thermometry can report subtle differences in leaf temperature in both field and controlled conditions (Blum et al., 1982; Jones et al., 2003, 2009; Chaerle et al., 2007; Winterhalter et al., 2011a,b). Importantly, data collection is fast and non-destructive.

CTD is useful mainly in hot and dry environments typical of countries with a Mediterranean climate. Measurements should preferably be made on recently irrigated crops on cloudless and windless days with high vapor pressure deficits. Under these conditions and provided that data are collected when the canopy is sufficiently expanded to cover the soil, CTD can be a good predictor of wheat GY ($r = 0.6$–0.8; Reynolds and Pfeiffer, 2000). In bread wheat, yield progress was found to be associated with cooler canopies (Fischer et al., 1998) and significant genetic gains in yield have been reported in response to direct selection for CTD (Reynolds et al., 1999, 2009; Brennan et al., 2007). The addition of CTD as a selection criterion in wheat nursery improved considerably the identification of the highest yielding materials (van Ginkel and Ogbonnaya, 2007). These results are in keeping with the conclusions of Olivares-Villegas et al. (2007): "*Canopy temperature epitomises a mechanism of dehydration avoidance expressed throughout the cycle and across latitudes, which can be utilized as a selection criterion to identify high-yielding wheat genotypes or as an important predictor of yield performance under drought*."

Grant et al. (2006) investigated the robustness and sensitivity of thermal imaging for detecting changes in stomatal conductance and leaf water status in a range of plant species (grapevine, bean and lupin) under greenhouse or controlled environment conditions. In particular, they compared absolute leaf temperatures and thermal indices of plant stress with stomatal conductance and water potential. Thermal imaging successfully distinguished between irrigated and non-irrigated plants of different species, with strong correlations between thermal indices and stomatal conductance as measured with a leaf pyrometer. Their results also highlighted factors such as leaf angle that should be addressed when using thermal imaging for indirect measurement of the level of drought stress of the tested materials. Additionally, these results are valuable for the design of protocols for application in crop production or ecosystem monitoring.

ABSCISIC ACID CONCENTRATION

One of the main factors influencing leaf temperature via an effect on transpiration through stomatal conductance is the concentration of ABA in the leaf tissue and, ultimately, in guard cells (Wasilewska et al., 2008; Sirichandra et al., 2009). Therefore, ABA is a fundamental component of the mechanisms allowing the plant to match the water demand with the water supply and to optimize growth and survival in response to both daily and more long-term environmental fluctuations (Zhang and Davies, 1990; Xiong et al., 2007). Indeed, an increase in ABA concentration is a universal response observed in plants subjected to drought and other abiotic stresses (Quarrie, 1991; Setter, 2006). Additionally, ABA modulates the expression of a large number of genes whose products protect the cell from the harmful effects of dehydration (Bray, 2002; Seki et al., 2007).

ABA has been shown to affect many of the traits that influence the water balance of the plant through both dehydration avoidance and dehydration tolerance (Thompson et al., 2007). In maize seedlings subjected to artificially induced conditions of water deprivation, an increased ABA concentration enhanced the root/shoot ratio (Spollen et al., 2000; Sharp, 2002; Sharp et al., 2004), an adaptive change beneficial for increasing water uptake. It has also been shown that ABA facilitates water uptake into roots as the soil begins to dry, particularly under non-transpiring conditions, when the apoplastic path of water transport is largely excluded (Hose et al., 2001). Under terminal drought, tolerant pearl millet [*Pennisetum glaucum* (L.) R. Br.] have high leaf ABA and reduced transpiration at high vapor pressure deficit, a feature that highlights the important role of constitutive water-conserving mechanisms in maximizing yield under such conditions (Kholova et al., 2010a,b). The positive role on yield of a conservative water use, rather than deep or profuse rooting, has also been highlighted in chickpea (Zaman-Allah et al., 2011a,b).

In cereals, an accumulation of ABA has been implicated as one of the factors that influence reproductive fertility (Saini and Westgate, 2000; Landi et al., 2001b; Setter et al., 2001; Boyer and Westgate, 2004; McLaughlin and Boyer, 2007; Yang et al., 2007; Tang et al., 2008; Zhang et al., 2009) and endosperm development (Ober et al., 1991; Tuberosa et al., 1992; Setter et al., 1996; Mambelli and Setter, 1998; Seiler et al., 2011). In rice, selection for reduced ABA root signaling has been advocated as a means for better exploitation of subsoil water under mild or transient water deficit (Siopongco et al., 2008, 2009).

Sensitivity to ABA is also of interest for its implications on the adaptive response of plants to drought (Cominelli et al., 2005). Genetic variability for sensitivity to ABA has been reported in maize (Frascaroli and Tuberosa, 1993). Gametophytic selection carried out by spraying maize silks with an ABA solution before pollination led to significant effects on early vigor and other agronomic traits (Frascaroli and Landi, 1996; Landi et al., 2000).

Due to the availability of ABA-specific monoclonal antibodies (Quarrie et al., 1988) that allow for the cost-effective measurement of a large number of samples, several studies have been devoted to the identification of QTLs for ABA concentration and the analysis of their associated effects on other drought-related traits and yield (Lebreton et al., 1995; Tuberosa et al., 1998, 2002a; Sanguineti et al., 1999; Reymond et al., 2003; Giuliani et al., 2005; Landi et al., 2005, 2007; Rahman et al., 2011). Altogether, these studies do not provide a unifying picture of the role of ABA in determining yield, perhaps not unexpectedly in view of the different species and genetic backgrounds involved. Nevertheless, it is worth noting that the evaluation of an historical series of

maize hybrids released in the past 60 years has shown a significant decrease in the capacity to accumulate ABA in response to a given level of water stress (Sanguineti et al., 2006) and, consequently, a negative correlation ($r = -0.62$) between the capacity to accumulate ABA at the seedling stage (a trait never selected for by breeders) and GY.

OSMOTIC ADJUSTMENT

OA is a metabolic process entailing a net increase in intercellular solutes in response to water stress (Morgan, 1984; Zhang et al., 1999; Serraj and Sinclair, 2002). As soil moisture declines, OA favors turgor maintenance, and hence the integrity of metabolic functions. Importantly, OA can bias estimates of the value of relative water content, as has been shown in wheat and barley (Boyer et al., 2008).

OA has been implicated in sustaining yield under conditions of water deficit in oilseed *Brassica* species (Kumar and Singh, 1998), chickpea (Basu et al., 2007), cotton (Saranga et al., 2001), rice (Babu et al., 1999; Jongdee et al., 2002; Praba et al., 2009), sorghum (Tangpremsri et al., 1995), maize (Chimenti et al., 2006), tef (Ayele et al., 2001), barley (Gonzalez et al., 2008), and wheat (Ali et al., 1999; Blum et al., 1999; Salem et al., 2007; Ehdaie et al., 2008; Fan et al., 2008; Izanloo et al., 2008). Yet the value of OA as a desirable selection target from a breeding standpoint has been questioned (Munns, 1988; Palta et al., 2007), based on the notion that drought-tolerant genotypes endowed with a higher capacity to adjust osmotically are likely to be characterized by slow growth, and hence biomass production, due to the metabolic requirements of osmolyte biosynthesis. Under conditions of severe dehydration, a higher capacity to accumulate osmolytes may help plants withstand a prolonged drought spell and undergo a more prompt and complete recovery upon rehydration. Even though, the interpretation of osmotic relations in genetically engineered plants can be cumbersome (Blum et al., 1996), transformation experiments have shed light on the mechanisms by which plants may benefit from an altered capacity to accumulate osmolytes (Umezawa et al., 2006). Similarly to other drought-adaptive traits, the trade-off between the metabolic requirements of OA and the potential benefits for the crop varies on a case-by-case basis as a function of the crop, and the dynamics and severity of the drought episodes.

CHLOROPHYLL CONCENTRATION, STAY-GREEN, AND DELAYED LEAF SENESCENCE

A well-sustained source capacity is a key factor to maximize yield potential during both vegetative and reproductive phases, particularly under source-limiting conditions that commonly characterize drought-stressed crops. Therefore, delaying leaf senescence maintains transpiration and increases cumulative photosynthesis over the crop life cycle (Borrell et al., 2001; Jiang et al., 2004; Vadez et al., 2011). This is a strategy that is adequate for soils with appreciable water reserves but may otherwise cause severe stress at the end of the growth season due to increased transpiration.

The traits that have been monitored most frequently to obtain indirect estimates of photosynthetic potential are chlorophyll concentration, stay-green and delayed senescence, all of which are interconnected (Tuinstra et al., 1998; Thomas and Howarth, 2000; Shukla et al., 2004). In US Corn Belt maize, stay-green has improved significantly and steadily during the past six decades of breeding, particularly under favorable conditions (Duvick, 2005). Additionally, stay-green traits in maize correlate closely to GY, and multiple intervals of stay-green QTLs overlap with yield QTLs (Zheng et al., 2009). Although, stay-green in maize seems more likely to be related to nitrogen use, in sorghum it has been related to maintenance of a more favorable water status as related to root features (Gallais and Hirel, 2004; Blum, 2006; Mace et al., 2012). In sorghum, four major QTLs that control stay-green and GY have been identified (Harris et al., 2007) and near isogenic lines (NILs) for these QTLs have been derived, providing an opportunity for a detailed analysis of stay-green physiology and positional cloning of the underlying genes (Vadez et al., 2011).

REMOBILIZATION OF WATER-SOLUBLE CARBOHYDRATES

Remobilization of WSC from the stem and leaves can mitigate the negative effects on grain filling caused by post-anthesis drought tolerance (Blum, 1988, 1998; Araus et al., 2002; Reynolds et al., 2007; Rebetzke et al., 2008b). QTLs for stem-reserve remobilization have been reported in bread wheat (Salem et al., 2007; Snape et al., 2007; Yang et al., 2007). Rebetzke et al. (2008b) phenotyped three wheat mapping populations for WSC concentration (WSC-C) and for WSC mass per unit area (WSC-A). Genotypes with high WSC-C were commonly shorter, flowered earlier and produced significantly fewer tillers than those of low WSC-C. This resulted in similar yields, lower final biomass, and fewer grains per m^2, but greater dry weight partitioning to grain and kernel weight in high versus low WSC-C genotypes. In contrast, lines high for WSC-A produced more fertile tillers associated with similar or greater anthesis and maturity biomass, grain number and yield, yet similar kernel weight or size compared with genotypes with low WSC-A, thus suggesting an important role for WSC-A in assuring stable yield and grain size in wheat.

This overview of drought-adaptive traits, far from being exhaustive, indicates that genetic variability in drought tolerance and WUE can be traced to the interaction of a multitude of quantitatively inherited morphophysiological features, whose effects on yield can vary greatly both in terms of magnitude and direction according to the prevailing drought scenario and other yield constraints. Therefore, the adoption of drought-adaptive traits as selection criteria for yield should be exercised cautiously and only after acquiring a clear understanding of the factors limiting yield in the TPE. Identifying the QTLs underpinning such traits and interpreting their cause–effect relationships allow us to partially disentangle this complexity to an extent and, eventually, make it amenable to a more direct and effective manipulation for breeding purposes. In both cases, good phenotypic data are essential to success.

COLLECTING GOOD PHENOTYPIC DATA

Plant scientists attempting to improve resistance to drought face two contrasting and apparently irreconcilable requirements. The first is to simplify "the system" in order to facilitate elucidation of the function of the relevant loci for the target traits (i.e., the reductionist approach). The second is to evaluate the broader value of such findings in a breeding and agronomically

sound context (i.e., the holistic approach), where the physiology, epistatic interactions and pleiotropic effects of complex traits inevitably limit and blur the identification of the main factors leading to specific phenotypes (e.g., drought-resistant versus drought-susceptible). In a way, the reductionist approach is like trying to understand the subject of an entire puzzle when only a few pieces are available. On the other hand, the holistic approach selecting, for example, for yield *per se* will provide a complete picture of the puzzle (i.e., the phenotype). However, it will often not allow us to tease the puzzle apart to the extent that we would need to apply targeted approaches such as MAS and/or genetic engineering, because of our incomplete understanding of the number and function of the single pieces such are the QTLs for yield. Valuable opportunities to begin to reconcile this conundrum are provided by bioinformatics (Sawkins et al., 2004) and modeling (Hammer et al., 2004, 2006; Cooper et al., 2009; Tardieu and Tuberosa, 2010; Sinclair et al., 2010; Messina et al., 2011). Both modeling and high-throughput phenotyping for drought-adaptive features are at the very core of DROPS (DROught-tolerant yielding PlantS; www.drops-project.eu), an ongoing EU-funded project aiming at improving our understanding and capacity to ameliorate yield and yield stability under water-limited conditions.

Yet the objective of this review is not to dwell on the merits and pitfall of the reductionist and holistic approaches (see also Passioura, 2010). Rather, it seeks to introduce and discuss a number of major issues on phenotyping that are relevant for both approaches. These issues should be considered seriously in planning and managing experiments under drought conditions, collecting and analyzing the data and, eventually, in interpreting the results properly.

Given the myriad of factors that can influence the quality of phenotypic data, this review only addresses the most important ones. Although, it is possible to define general rules, each experiment has its own "phenotyping story" and the results should be dealt with and interpreted accordingly. What follows is equally relevant for the improvement of crop performance under water-limited conditions and, more generally, for experiments in the field or under controlled conditions aimed at dissecting the physiological and genetic basis of crop adaptation to water-limited conditions. However, given the importance of field evaluation for breeding purposes, phenotyping under field conditions is emphasized.

WHAT DOES "GOOD PHENOTYPING" MEAN?

Good phenotyping is pivotal for reducing the genotype–phenotype gap, especially for quantitative traits, which are the major determinants of drought resistance. Keeping a good record of meteorological parameters (rainfall, temperatures, wind, evapotranspiration, light intensity, etc.) allows for more meaningful interpretation of the results and identification of the environmental factors limiting yield (Sadras, 2002). Equally important, though often neglected or ignored, are the physical-chemical properties of the soil, particularly those influencing the water balance of the crop under decreasing moisture conditions (Cairns et al., 2011).

The basic attributes of good phenotyping carried out with appropriate genetic materials are accuracy and precision of measurements, coupled with relevant experimental conditions that are representative of the TPE. Accuracy involves the degree of closeness of a measured or calculated quantity to its actual (true) value. Accuracy is closely related to precision, also termed reproducibility or repeatability, the degree to which further measurements or calculations show the same or similar results. For a number of traits such as stomatal conductance, flow of xylem sap, etc., measured with mechanical or electronic devices, accuracy and precision in measurements require calibration of the instrument prior to data collection. Failure to so do will produce biased results with a difference between the mean of the measurements and the true reference value. A further complexity of phenotyping a large number of genotypes (e.g., a mapping population or an association mapping panel) for drought-adaptive features is exemplified by those traits such as stomatal conductance and tissue water potential, the value of which can vary considerably within a rather short timeframe due to changing environmental conditions.

An important distinction should be made between experiments aimed at (1) collecting data useful to dissect the genetic basis of target traits or (2) breeding activities for the release of improved cultivars. In both cases, an adequate choice of materials will be essential for successfully meeting the desired objectives. A notable case that clearly underscores the importance of good phenotyping is provided by QTL cloning (Salvi and Tuberosa, 2007). In this respect, the ideal scenario is when the alternative QTL alleles can be unequivocally scored phenotypically and the trait itself is mapped as one of the markers.

PHENOTYPING IS KING AND HERITABILITY IS QUEEN

Good phenotyping means not only the collection of accurate data to minimize the experimental "noise" introduced by uncontrolled environmental and experimental variability, but also the collection of data that are relevant and meaningful from a biological and agronomic standpoint, under the conditions prevailing in farmers' fields within the TPE. Although, hundreds of accurate studies reporting thousands of drought-responsive genes and QTLs can be found in the literature, the relevance of these data to "real" field conditions is often marginal and even questionable; only seldom has it been appropriately addressed and discussed. In the early stages following their development, evaluation of transgenic materials is limited to experiments carried out in greenhouses, a condition that underlines the importance to mimic as close as possible the drought stress conditions in fields (Saint Pierre et al., 2012).

Collecting accurate phenotypic data that are relevant to the TPE has always been a major challenge for the improvement of quantitative traits. The success of this endeavor is intimately connected with the heritability of the trait, namely the portion of the phenotypic variability accounted for by additive genetic effects that can be inherited through sexually propagated generations (Falconer, 1981). Trait heritability varies greatly (from 0 to 1) according to: (1) the genetic makeup of the materials under investigation; (2) the environmental conditions under which such materials are grown and evaluated; and (3) the

accuracy and precision of the phenotypic data. With only a few notable exceptions (e.g., flowering time and carbon-isotope discrimination), most of the traits determining the performance of crops under drought conditions usually have low (0.3–0.4) or, at best, intermediate (0.4–0.7) heritability. This impairs our capacity to dissect their genetic basis properly and, more importantly, reduces the effectiveness of phenotypic selection (Falconer, 1981). Despite this, careful evaluation and appropriate management of the experimental factors that lower the heritability of traits, coupled with a wise choice of the genetic material, can provide effective ways to increase heritability, and hence the response to phenotypic selection.

Once a sound association has been established between a marker and a locus affecting a target trait, the problems encountered in the conventional selection of quantitative traits, particularly the lowly-heritable ones, can been partially overcome through the use of markers linked to QTLs for the target trait. This enables individuals to be scored based on their genetic make-up rather than their phenotypic features (Peleman and Van der Voort, 2003; Langridge, 2005). Paradoxically, the probability of identifying the relevant chromosomal regions and accurately estimating their effects relies on good phenotyping of the genetic materials originally used to establish the phenotype–genotype associations. In other words, the effectiveness of marker-based approaches intimately depends on how well and how accurately the target trait has been assessed phenotypically in mapping populations. In fact, a low heritability impairs the probability of detecting the presence of QTLs (Bernardo, 2004), thereby increasing Type II errors (i.e., false negatives). An accurate and relevant phenotyping is of even greater importance when applying genome-wide selection, an approach that disregards QTL identification and relies on the molecular profiling and accurate phenotyping of each progeny (Bernardo and Yu, 2007; Bernardo, 2008; Heffner et al., 2009).

EXPERIMENTAL DESIGN, DEDICATED SOFTWARE, AND STATISTICAL APPROACHES

It is widely recognized that a substantial part of the increased efficiency of modern breeding is due to the accurate phenotyping of large numbers of plots, this scale-up being made possible by more sophisticated and high-throughput experimental machinery as well as the streamlining and automation of tedious manual operations. Thus, the labeling of a large number of plots and samples, data collection and storage, and keeping track of pedigrees, etc., are now facilitated by the use of electronics (e.g., bar-coding) and dedicated software (e.g., spreadsheets, databases, etc.). Additionally, the effectiveness of field experiments and the management and interpretation of phenotypic data can be enhanced greatly through the utilization of the most appropriate experimental designs to allow for better control of within-replicate variability and to reduce or remove spatial trends. Equally important are statistical approaches to analyzing the data, particularly for investigating the effects of GEI (van Eeuwijk et al., 2005; van Eeuwijk, 2006; Malosetti et al., 2008; Mathews et al., 2008; Messmer et al., 2009) and epistasis (Gao and Zhu, 2007; Jannink, 2007). Coping with the temporal variability of drought-adaptive features can be dealt with through in-depth analysis of QTL-by-environment interactions (van Eeuwijk et al., 2005; Vargas et al., 2006; Burgueno et al., 2008) or by identifying intrinsic characteristics of each genotype relating to its interaction with particular environmental conditions, which requires the development of models able to identify these variables and to simulate the behavior of genotypes in a broad range of environments (Tardieu, 2003; Yin et al., 2003; Reymond et al., 2004; Cooper et al., 2009; Sinclair et al., 2010).

A number of studies have shown the importance of epistasis in determining the genetic architecture of yield and other quantitative traits (Li et al., 2003b; Maccaferri et al., 2008; Zhao et al., 2008; Frascaroli et al., 2009; Messmer et al., 2009; Ravi et al., 2011). However, mapping two-way epistatic interactions requires adequately large mapping population, and detecting higher order epistasis is practically out of reach. Once different sets of NILs become available for loci that are known to interact epistatically, it will be possible to produce different combinations at will for further testing and characterization of the effects of such epistatic interactions.

MONITORING PLANT–SOIL WATER RELATIONS

A sound interpretation of the results of an experiment conducted under conditions of water shortage requires a good characterization of the soil–plant–atmosphere continuum (SPAC), which, in turn, relies on accurate monitoring of the water status of both soil and plant. From an experimental standpoint, an important issue is to what extent genotypic differences in drought-adaptive traits measured in phenotyping platforms at different water regimes reflect genotype performance across watering regimes under field conditions. Along this line, encouraging results have recently been reported in maize (Chapuis et al., 2012).

Regrettably, a unique means of measuring water status that can be applied in all possible situations is not available. Choosing the most appropriate method depends on the objective being pursued, such as understanding drought-adaptive mechanisms, selecting for drought resistance, investigating water movements, or managing irrigation treatments (Boyer, 1995; Kirkham, 2004; Jones, 2007). At the plant level, greater emphasis has traditionally been devoted to water potential rather than sustained turgor, the primary reason for sustained function under drought (Blum, 2006, 2009). Hence, examples of sustained function at low water status as the main reason for drought tolerance are comparatively few. Maintenance of high leaf water potential and turgor under dry conditions indicates dehydration avoidance (Blum, 1988; Ludlow and Muchow, 1990). Similarly, the relative water content of the leaf also provides important information on the water status of the plant, offering the advantage of collecting a high number of samples in a short time (Sanguineti et al., 1999), an important prerequisite for QTL studies trying to link variation in physiological parameters to variation in yield. The precautions to be adopted for measuring relative water content have been discussed by Blum[2]. Although, all components of leaf water relations change during the day as irradiance and temperatures vary, the change is small for about 2 h at and after solar noon. Therefore,

[2]http://www.plantstress.com/methods/index.asp

this is an appropriate time window for investigating leaf water relations in a large number of genotypes[2].

It is equally important to monitor changes in soil moisture, preferably at different depth of the rhizosphere, during the growth and reproductive cycle of the crop. Root water uptake is one of the pivotal processes within the SPAC. While the gravimetric method (i.e., weighing samples of soil columns before and after oven drying) provides accurate, albeit time-consuming, measurement of soil moisture, other methods such as the neutron probe, the capacity method and the "I-sensor" allow for quicker and less labor-intensive measurement (Nagy et al., 2008; Cayci et al., 2009).

During recent decades, progress in microelectronics has allowed the development of several dielectric-based soil water monitoring techniques, namely time-domain reflectometry (TDR), and single and multisensor capacitance probe (SCP/MCP) systems (Fares and Polyakov, 2006; Vereecken et al., 2008). These techniques have greatly simplified the real-time determination of water content on a fine spatial and temporal scale. Because of their relatively low cost and ease of operation, MCP systems have met widespread acceptance as a means of closely monitoring soil moisture by collecting high-resolution soil-water content data in the rhizosphere. Despite their success, MCP systems have shown some temperature and salinity effects in different soil types, suggesting that further research is needed to eliminate such effects for these capacitance systems to take their place as leading soil water monitoring sensors.

TDR has been one of the most widely used techniques to determine soil volumetric water content thanks to its high precision, non-ionizing radiation and low influence of soil salinity, bulk density and texture (Noborio, 2001). However, compared to the neutron probe, most of the TDR equipment available does not allow detailed measurement along the soil profile. Also, the use of conventional TDR probes requires drilling holes or opening trenches in the soil to install the probes, limiting the number of points measured in the soil profile (Manieri et al., 2007). More recently, two-dimensional geoelectrical tomography has been used for monitoring soil-water redistribution due to water uptake by lupin roots (Werban et al., 2008). The resulting average water content from two-dimensional geoelectrical tomography agreed well with the values determined by the TDR measurements model.

WHAT SEVERITY OF WATER SHORTAGE?

Unlike yield under conditions of severe drought stress (>70% reduction from yield under well-watered conditions) yield under more moderate water shortage (up to approximately 50% reduction) reflects more closely yield potential under favorable conditions (Blum, 2006). Therefore, drought resistance *per se* is expected to play a progressively more important role than yield potential as the severity of drought escalates, with genotype ranking for yield changing considerably once the mean yield falls below 20–30% of yield potential (Blum, 2006) as a result of water scarcity. Consequently, germplasm evaluation in areas where drought severity fluctuates widely should preferably be carried out under well-watered conditions and at different levels of drought stress (e.g., intermediate and severe). In maize, this approach has been adopted to identify QTLs for yield across a broad range of water availability (Malosetti et al., 2008; Messmer et al., 2009) and to develop superior hybrids in sub-Saharan Africa (Bänziger et al., 2006).

Retrospective studies conducted with an historical series of maize hybrids showed that screening in multiple sites at high plant densities provides substantial yield gains across a broad range of environments, although, rates of gain in well-watered conditions are more than twice as high as those in water-stressed environments (Duvick, 2005; Campos et al., 2006). In wheat, four decades of breeding at CIMMYT have clearly indicated the importance of selecting and managing key environments differing in their yield potential to identify the best performing genotypes across a broad range of environments. The so-called "shuttle breeding" which was instrumental for the success of the Green Revolution (Borlaug and Dowswell, 2005), remains a key factor in developing more broadly adapted cultivars (Ortiz et al., 2007b; Trethowan and Crossa, 2007). Recently, a QTL with a major and consistent effect on GY in multiple elite genetic backgrounds under both water-stressed and non-stressed conditions has been described (Vikram et al., 2011). Consistency of the QTL effect across different genetic backgrounds makes it a suitable candidate for use in marker-assisted breeding.

PHENOTYPING IN THE FIELD

Assuming that both the type and the number of treatments (genotypes, irrigation volumes, etc.) to be evaluated are adequate for the specific objectives of each experiment, the following general factors should be evaluated carefully to ensure the collection of meaningful phenotypic data in field experiments conducted under water-limited conditions:

- Experimental design
- Heterogeneity of experimental conditions between and within experimental units
- Size of the experimental unit and number of replicates
- Number of sampled plants within each experimental unit
- Genotype-by-environment-by-management interaction.

The relative impact of each factor on the quality of the phenotypic data to be collected will vary greatly according to each experiment. As an example, an excessive heterogeneity in soil characteristics (depth, moisture, pH, etc.), and/or compaction among field plots will inevitably increase the experimental error and will jeopardize an accurate evaluation of yield. Mapping the soil in experimental nurseries for environmental factors that decrease phenotypic accuracy (Cairns et al., 2004, 2011; Rossel et al., 2006; Patzold et al., 2008) and adopting suitable experimental designs can partially mitigate the negative effects of high soil heterogeneity.

For experimental activities carried out under drought conditions, the additional factors discussed below should receive due attention when planning and conducting the experiments.

VARIATION IN PHENOLOGY

In environments where escape is the predominant cause of drought resistance, the presence of large differences in flowering

time among genotypes will inevitably bias the interpretation of the influence of drought-adaptive traits on yield under drought conditions (Soltani and Sinclair, 2012). Likewise, the presence of large differences in plant height and/or root mass among the progeny of a mapping population or accessions of a panel suitable for association mapping studies, may lead to an overestimate of QTL effects owing to competition between neighboring plots, especially when their surface area is small. These QTL effects will most likely decrease once phenotypic evaluation has been carried out with more phenologically homogeneous materials. Surprisingly, this issue has not yet been addressed with dedicated experiments.

INTERACTIONS WITH OTHER STRESSES

Obtaining an accurate estimate of drought resistance *per se* implies the absence of other biotic or abiotic stress agents that influence plant growth and function. Typical case scenarios are those involving factors that cause mechanical damage to roots (e.g., nematodes, root-worms, etc.), impair root growth (e.g., soil acidity, boron toxicity, salinity, etc.), and/or reduce water availability to the crop (e.g., presence of weeds), and source capacity (e.g., foliar diseases, insect damage to the canopy, etc.). When one or more of the above-mentioned constraints affects the experimental plots, genetic variability among the progeny in resistance to these stress agents will inevitably bias an accurate evaluation of drought resistance. Likewise, important and more subtle interactions may occur when the effects of water deficit are evaluated in the presence of other abiotic stress factors (e.g., high temperatures, high ozone, low nutrients, etc.) that hasten leaf senescence and/or enhance the role of specific adaptive mechanisms, such as the relocation of stem WSC in cereals, that normally play a less predominant role in determining yield.

Nevertheless, it should be noted that drought hardly ever occurs in the absence of other stress factors (Sadras, 2002; Sinclair et al., 2007). An example of this is provided by the conditions of terminal drought stress frequently concomitant to high temperatures that wheat and rice experience during grain filling (Pinto et al., 2010; Jagadish et al., 2011; Lopes et al., 2012; Yang et al., 2012). A partial solution to this problem, at least for traits other than GY and its components, which are best evaluated under field testing, is to collect phenotypic data from plants grown in controlled facilities (greenhouse, growth chamber etc.). This will allow for an accurate control of the main environmental parameters—temperature, air humidity, light, etc.,—governing water flow in the SPAC, and hence the water balance. This is particularly important for omics-profiling studies where even small fluctuations in environmental conditions can substantially alter gene expression. On a broader scale, environmental characterization can be improved through the use of geographic information systems (GIS) for crop monitoring (Kahinda et al., 2008), for water balance models (Reshmidevi et al., 2008) and for their combination.

MANAGING THE DYNAMICS AND INTENSITY OF DROUGHT EPISODES

The ability to control the timing, frequency and intensity of drought episodes is a key factor in mimicking the environmental conditions prevailing in the TPE and, consequently, in successfully selecting for improved drought resistance. To this end, an increasing number of public and private breeding programmes have conducted field trials in locations characterized by very low rainfall during the growing season, a condition under which the dynamics and intensity of drought episodes can be tightly controlled through the frequency and volume of irrigation treatments. Trials in dry sites also offer the distinct advantage of a lower incidence of biotic constraints which, if unaccounted for, can bias the evaluation of the role of other traits and corresponding QTLs in the adaptive response to moisture-limited conditions.

The option of field testing in dry areas is not always available to many of those engaged in drought-related experiments. Therefore, rainout shelters offer the possibility of investigating the adaptive response of crops to a desired level of drought stress, avoiding the vagaries of unpredictable rainfall patterns. There are basically two types of rainout shelter: static and moveable. Further details on the merits and pitfalls of these devices are provided by Blum[2]. Major drawbacks to the use of rainout shelters are high construction and operating costs, particularly for the movable type, as well as the usually rather limited area protected by a shelter which, in turn, limits the number and size of experimental plots that can be tested. This is a significant problem when dealing with large mapping populations or panels of accessions suitable for association mapping studies.

INFLUENCE OF THE GROWTH STAGE

An important aspect for phenotyping traits in the most relevant way from a breeding point of view is the identification of the critical stage at which variability in the target traits plays a more prevalent role in final performance. This is the stage at which the correlation between the trait and final yield is highest, and thus becomes more diagnostic. For example, in maize some biochemical factors, such as the concentration of sucrose in the placental-chalazal area of the kernel, exert a particularly strong and timely effect on reproductive fertility around flowering but not a week earlier or later (Boyer and Westgate, 2004; McLaughlin and Boyer, 2004). Similarly, genetically-based differences in the concentration of ABA in leaves of field-grown maize have been shown to peak around the time of flowering or shortly after (Landi et al., 1995; Pekic et al., 1995). Due consideration should also be given to fluctuations in the heritability of target traits exhibited during the growth cycle (see below).

A critical factor in improving the relevance of infrared thermography to measure canopy temperature is the timing of the measurements of temperature differences between treatments. Under field conditions, even well-watered healthy plants may shut their stomata before solar noon, especially under conditions of high evapotranspirative demand. This is particularly relevant when different genotypes are evaluated for their capacity to exploit an avoidance strategy. In this case, the timing of the measurements to allow good discrimination among genotypes needs to be determined for specific conditions and may need considerable readjustment during subsequent samplings as the water stress progresses during the day. An additional factor

to be considered when measuring canopy temperature is the effect of leaf wilting, folding or rolling under stress (Leinonen et al., 2006; Grant et al., 2007). For instance, plant canopy architecture will influence leaf temperature not only through the angle of leaves to the light source, but also through the degree of self-shading in the canopy (Zheng et al., 2008). To a certain extent, the influence of self-shading can be reduced if the most suitable view angle is used, although, different opinions have been expressed in this regard (Grant et al., 2006).

When phenotyping occurs at flowering or shortly after, additional bias is introduced if the tested genotypes differ considerably in flowering time and/or maturity. In such cases, phenotyping all accessions on the same date will provide data collected from plants at different physiological stages, a circumstance that could introduce significant bias in the interpretation of cause–effect relationships between traits and yield. A partial solution is to sow the accessions on two or three dates based on the maturity group (e.g., early and late). Clearly, this procedure will increase the cost of the trial.

TIMING OF MEASUREMENT AND SAMPLE COLLECTION

For morphophysiological traits that fluctuate widely during the circadian cycle (e.g., water status, ABA content, stomatal conductance, leaf rolling, leaf temperature, etc.) choosing the most appropriate time for measurement and/or sample collection is very critical. Additionally, measurement of traits that are time-consuming to record (e.g., stomatal conductance) in a large number of plants introduces a covariate effect proportional to the duration of data or sample collection. In this respect, remote sensing holds great potential to minimize or eliminate altogether effects on trait expression due to the circadian rhythm and corresponding changes in environmental factors.

CTD is a notable indicator of the amount of water extracted from the soil and lost through foliar evapotranspiration into the atmosphere. Therefore, this trait provides an indirect estimate of root architecture (size and depth) and functionality (e.g., permeability to water as a function of aquaporines, etc.) in accessing soil moisture, and can be used as a fast, inexpensive screening of root features (Reynolds et al., 2009). However, to be diagnostic, canopy temperature should be measured under conditions of high evapotranspirative demand and in absence of wind (Blum, 1988), since even a slight breeze can alter the level of evapotranspiration instantaneously and, consequently, alter the leaf temperature. Balota et al. (2007) have investigated the effects of the timing of measuring CTD on breeding selections of wheat in relation to growth stage, time of day and weather. Although, under dry conditions long-term mean CTD at noon and yield were found to be correlated in two growing seasons, the relation of short-term CTD readings to GY was highly variable (Balota et al., 2007). Poor correlation was associated with days of low solar irradiance, high wind speed and rain events. Interestingly, genotype effects on CTD were detected for all hours of day and night. Genotype-by-hour interaction was non-significant at night, suggesting that night-time measurements may provide more stable conditions for CTD comparison among genotypes.

PHENOTYPING IN CONTROLLED ENVIRONMENT FACILITIES

Although, GY and its components are best phenotyped in field trials, measuring secondary traits in plants grown in controlled environment facilities (e.g., greenhouse, growth chamber, etc.) takes advantage of an accurate control of the main environmental parameters of moisture stress, air humidity, temperature, light, etc., that vary greatly in field experiments. However, the conditions under which plants are grown should be relevant to the conditions prevailing in the field (Izanloo et al., 2008). When the materials under test differ in flowering time, the use of plants grown under controlled conditions facilitates the collection of phenotypic data and samples at the same growth stage and under similar conditions. Additionally, a tight control of growing conditions allows for more accurate assessment of the constitutive capacity of different genotypes to accumulate drought-adaptive compounds in response to a given level of water deficit. For example, the accumulation of osmolytes and/or ABA is highly influenced by water status, which can vary considerably among genotypes tested in the field under similar water regimes (Tuberosa et al., 1994; Rauf et al., 2009).

More uniform conditions in terms of water status can be achieved through exposing plants to a solution with a known concentration of polyethylene-glycol (PEG). This approach can be of particular interest as a way of exposing different genotypes to a given level of dehydration (Sanguineti et al., 2006; Verslues et al., 2006; Texeira et al., 2008; Ruta et al., 2010). Unlike in field conditions where different genotypes are likely to experience different stress intensities, plants grown in a PEG solution are exposed to predetermined and rather uniform water stress, a condition that facilitates a more correct interpretation of the cause–effect relationships of the association between traits. However, the use of PEG requires good aeration of the solution to avoid hypoxia and verification of the absence of possible contaminants. Additionally, plants absorb PEG, particularly when it is of a low molecular weight (<6000), which can alter the hydraulic properties of the leaf[2]. Therefore, great caution should be adopted in taking results obtained under such highly artificial conditions and extrapolating them to field conditions.

In most circumstances, the collection of phenotypic data in experimental conditions that are remote from those prevailing in the field may lead to biased and potentially misleading conclusions. At the molecular level, an interesting example is provided by transcriptomics studies (Atienza et al., 2004; Rensink, 2005) wherein plants or plant parts such as detached leaves undergo high-intensity stress treatments in a rather short time, i.e., "shock-like" treatments. These conditions preclude the identification of long-term responses in gene expression that play a more predominant role in adaptation to field aridity (Passioura, 2010). In barley, changes in gene expression were monitored in leaves of plants grown in soil and subjected to slow-drying conditions for 7 and 11 days (7d-WS and 11d-WS, respectively) with the changes obtained under "shock-like" conditions imposed with a 6 h dehydration treatment (Talamè et al., 2007). Among all transcripts that showed a significant change in regulation in at least one of the conditions tested, 57% were exclusively affected in the dehydration shock treatment, 6% at 7d-WS and 14% at 11d-WS. Irrespective of the low percentage of transcripts (10%) with

similar expression changes between shock- and slow-stress treatments, a portion of these transcripts shared a common expression trend under the different drought treatment conditions, as evidenced by low correlations between the fast-occurring and the 7d-WS and 11d-WS treatments ($r = 0.32$ and 0.41, respectively). From a practical standpoint, these results suggest that the information obtained under artificial conditions of water deficit induced over a very short period of time (e.g., a few hours) should be treated very cautiously when used to identify candidate genes for QTLs of field-related traits with a drought-adaptive role.

HARNESSING PHENOTYPIC VARIABILITY

A number of options are available to utilize the information collected through phenotypic evaluation of germplasm resources (Gur and Zamir, 2004; Dreccer et al., 2007; Reynolds et al., 2007; Richards et al., 2007; Ortiz et al., 2008; Bernardo, 2009; Di Bianco et al., 2011; Tuberosa et al., 2011a). A well-informed choice of the parental lines based on a thorough phenotypic characterization of the main traits imparting drought resistance allows for the creation of new populations where segregants that combine drought-adaptive and other desirable features of parental lines can be identified and selected (Reynolds et al., 2005). This so-called "strategic crossing" has been deployed extensively and successfully at CIMMYT, as shown by the fact that several newly released improved wheat accessions have been selected from crosses between parental lines chosen based on their morpho-physiological features (Reynolds et al., 2005, 2011; Ortiz et al., 2007b).

An effective breeding programme relies on the availability of sufficient genetic variability for the target traits. Under this aspect, landraces and wild accessions provide valuable opportunities to enhance the variability for drought-adaptive features and, eventually, yield (Moncada et al., 2001; Talamè et al., 2004; Tan et al., 2008). There is rapidly growing interest in wild relatives of crops and landraces as sources of agronomically superior alleles among those that were left behind by the domestication bottleneck and modern agriculture (Tanksley and McCouch, 1997; Lippman et al., 2007; Reynolds et al., 2007; Feuillet et al., 2008). Advanced-backcross QTL analysis (ABQA) and introgression libraries (ILs) allow for proper and effective dissection of the phenotypic variability contributed by non-commercially viable parental lines (Talamè et al., 2004; Tan et al., 2008; Salvi et al., 2011). Once a desirable QTL feature contributed by unadapted materials tested under drought conditions has been identified, the main issue is to evaluate to what extent the introgression of the target segment in elite materials might cause a yield penalty under favorable conditions. Regarding target traits, landraces and wild relatives have been screened most commonly to identify accessions with an outstanding expression of secondary traits such as root mass, OA, leaf anatomy, etc., thought to play an important role in conferring resistance to drought (Grando and Ceccarelli, 1995; Peleg et al., 2007, 2008).

TOWARD HIGH-THROUGHPUT PHENOMICS

High-throughput phenotyping helps standardize and improve the collection of phenotypic data and facilitates the creation of repository databases useful for QTL meta-analyses (Lippman et al., 2007; Welcker et al., 2011). Unlike a decade ago, our present capacity to conduct high-throughput molecular profiling far outweighs our capacity to collect reliable phenotypic data (Sinclair and Purcell, 2005). The best example is provided by the burst in single nucleotide polymorphism (SNP) discovery and profiling in a number of crops (Rostoks et al., 2005; Kota et al., 2008; Ganal et al., 2009; Waugh et al., 2009; Mondini et al., 2011; Rafalski, 2011; Trebbi et al., 2011). Nevertheless, the past years have witnessed a growing awareness of the need for increasingly integrated, multidisciplinary and field-oriented research in order to mitigate the negative effects of water shortage (Edmeades et al., 2004; Tuberosa et al., 2007a).

High-throughput phenotyping of plants in pots allows for tight control of the water shortage imposed on different genotypes and of the homogeneity of the severity of stress, a condition that is seldom achieved under field conditions, particularly when the genotypes under test differ in phenology and/or biomass. However, a number of distinct limitations characterize pot experiments and should be carefully considered and managed to obtain meaningful results relevant to field conditions (Passioura, 2006).

Phenotyping under controlled conditions is relatively straightforward when scoring traits in a binary fashion, such as for photoperiod sensitivity, and when environmental conditions do not have much effect on the target trait or are easily defined (e.g., light versus darkness). However, it quickly becomes more complex when the target traits are quantitatively assessed, as in the case of growth, and when environmental conditions that vary during the day (e.g., temperature, light intensity, soil water status, etc.) influence the target trait (e.g., the rate of leaf elongation). In this case, the phenotype is rather dynamic and better defined by a series of response curves to environmental stimuli (Tardieu et al., 2003, 2005; Hammer et al., 2004; Tardieu, 2012), an approach that is very time-consuming and requires a tight control of environmental conditions.

Hence, it is important to: (1) measure the physical variable/s (e.g., pot weight, soil moisture etc.) that quantify the level of water stress; and (2) add a precise amount of water to each pot. High-throughput phenotyping platforms allow for the automation of these procedures that have already been adopted by a number of private companies and large public institutions to streamline and standardize the collection of highly accurate phenotypic data in glasshouse-grown plants (Granier et al., 2006; Rajendran et al., 2009). State-of-the art technology including imaging, robotic and computing equipment, allows for the continuous phenotypic measurement of thousands of plants automatically and non-destructively[3]. Regrettably, the installation and operating cost of these platforms is very high.

For certain traits, the high-throughput collection of phenotypic features can be streamlined by the use of digital imaging and measurement of canopy features by means of near-infrared spectroscopy and spectral reflectance, as discussed below.

DIGITAL IMAGING

Digital image analysis provides an inexpensive and rapid way of precisely measuring plant features whose measurement would

[3]See the "Plant Accelerator" at http://www.plantphenomics.org/TPA

otherwise require a great deal of time. A notable example is provided by the measurement of canopy features (Marti et al., 2007; Campillo et al., 2008; Elsayed et al., 2011; Winterhalter et al., 2011b; Fiorani et al., 2012). Digital images offer a series of advantages over other methods of light interception estimation, including the possibility of directly processing images by computer. Video image analysis allows for a dynamic, inexpensive and non-destructive assessment of canopy features and crop growth (Beverly, 1996; Campillo et al., 2008; Cairns et al., 2011; Elsayed et al., 2011; White et al., 2012). Digital imaging is equally valuable for measuring root characteristics in experiments that are often constrained by the lack of suitable methods for continuous, non-destructive measurements (Himmelbauer et al., 2004; Blouin et al., 2007). Additionally, digital image analysis (Kimura et al., 1999; Armengaud et al., 2009) allows for accurate analysis at higher resolution scales, an important prerequisite to investigate the kinetics of the processes regulating root growth. In this respect, a non-invasive technique, based on digital image sequence processing, has been applied for quantifying highly resolved spatio-temporal processes within the root growth zone in the model plant Arabidopsis (Chavarria-Krauser et al., 2008; Iyer-Pascuzzi et al., 2010).

NEAR-INFRARED SPECTROSCOPY AND SPECTRAL REFLECTANCE

Remote sensing via near-infrared spectroscopy and spectral reflectance of plant canopies are promising components of high-throughput phenotyping platforms (Montes et al., 2007) and provide interesting opportunities for collecting integrative traits with high temporal resolution (Gutierrez et al., 2010). Spectral reflectance in the visible and near-infrared regions of the electromagnetic spectrum is collected from the canopy of the crop by sensors that can be mounted on tractors (Montes et al., 2007) or using digital cameras mounted on hand-held devices (Casadesus et al., 2007). Remote sensing has advanced our understanding of the changes in leaf reflectance and leaf emittance according to species, leaf thickness, canopy shape, leaf age, nutrient status and, importantly, water status (Hatfield et al., 2008). Based on this information, various vegetative indices for crop canopies have been formulated to quantify agronomic parameters (e.g., leaf area, crop cover, biomass, yield, etc.). Retrieving meaningful information from the plot spectra relies on the use of calibration models for prediction of the phenotypic values. Under well-managed experimental conditions, spectral reflectance has been used to monitor plant photosynthetic pigment composition, water status assessment and the early detection of abiotic stress (Babar et al., 2006, 2007; Guo et al., 2008; Gray et al., 2010).

SIMULATING VIRTUAL PHENOTYPES

As we inch our way forward to unravel gene functions in a piecemeal fashion (i.e., gene-by-gene) and try to understand how these functions ultimately affect the phenotype, there is a growing interest in models that allow us to simulate virtual phenotypes deriving from all possible combinations of different factors—alleles, environmental variables, etc. In a way, modeling represents a step toward a more comprehensive systems biology approach (Dingkuhn et al., 2005; Yin and Struik, 2008; Tardieu and Tuberosa, 2010) aimed at predicting phenotypic performance of an otherwise intractably large number of treatments, such as the genotypes obtained by combining different gene/QTL alleles, irrigation volumes and frequency, temperatures, etc., (Hoogenboom et al., 2004; Cooper et al., 2007; Heinemann et al., 2008; Letort et al., 2008; Sinclair et al., 2010).

The assumption is that gene networks are regulated in a coordinated way to allow plants to react predictably to a range of environmental conditions (Sadok et al., 2007; Chenu et al., 2008; Jansen et al., 2009; Chapuis et al., 2012). Crucial to the success of this approach is the possibility of monitoring the phenotype of each accession in a precise and rapid way for the target trait (e.g., leaf elongation) in response to closely controlled environmental variables such as temperature, evaporative demand, soil water status, etc. Clearly, this kind of study is best conducted under controlled conditions. In maize, the QTL parameters of these responses were calculated for lines of mapping populations and were then analyzed genetically (Reymond et al., 2003; Welcker et al., 2007), allowing simulation of leaf growth in novel inbred lines as defined by their QTL alleles (Sadok et al., 2007). Therefore, this approach allows for the identification of QTLs of plant responses that, in principle, should not include a GEI. It theoretically allows prediction of the performance of any "virtual genotype" with a given combination of alleles in any climatic scenario. This possibility opens up a promising avenue, but is limited at present to very simple traits and genetic systems.

More integrative models simulate crop development as a function of environmental conditions. Consequently, they allow for the evaluation of the effects of individual traits on the seasonal dynamic of water use and carbon assimilation of crops (Chapman et al., 2003; Yin et al., 2004). However, their algorithms remain relatively crude, so the effects of genes or QTLs cannot usually be simulated at the crop level except for constitutive traits such as phenology (Chapman et al., 2003; Yin et al., 2005), for binary traits related to environmental triggers, such as flowering response to photoperiod (Hoogenboom et al., 2004) or when QTL models at the organ level can be combined with crop models (Chenu et al., 2008; Tardieu and Tuberosa, 2010). Their main function until now has been to evaluate whether a given trait will have a positive effect over a long series of climatic scenarios. For instance, Hammer et al. (2005) simulated the effect of stay-green, a trait considered as conferring drought tolerance, across 547 location-season combinations. As expected, this trait had a positive effect under mid-season or terminal stress, but a negative effect under severe terminal stress.

A factor that affects the prediction capacity of modeling is the unaccounted complications caused by non-linear effects associated with genes acting in networks when selection is conducted on a population of individuals segregating for the genes contributing to the network (Peccoud et al., 2004). Notwithstanding the promising features of modeling, an accurate prediction across genotypes still remains a difficult undertaking.

CONCLUSIVE REMARKS AND PERSPECTIVES

Taking full advantage of germplasm resources and the opportunities offered by genomics approaches to improve drought resistance will require a better understanding of the physiology and genetic basis of drought-adaptive traits. Clearly, an accurate and

cost-effective phenotyping will be instrumental in this respect. The utilization of techniques/approaches that allow for a precise control of the water regime (e.g., irrigated trials in dry regions, rainout shelters, etc.) and a reduction of the experimental noise coupled with the adoption of high-throughput platforms will streamline the collection of good phenotypic data while increasing the cost-effectiveness of phenotyping. This, in turn, will help to lift, at least partially, the "statistical fog" that surrounds QTLs and impairs our capacity to properly gauge their effects and predict the potential of novel combinations of QTL alleles.

However, no matter how accurate our phenotyping will be, the vast majority of the QTLs determining the measured phenotype will remain undetected. By analogy, I refer to this as the "iceberg effect." Similar to an iceberg, where most of mass lies below the sea surface and thus is not visible, the majority of the genetic factors controlling quantitative traits will equally defy detection because their effects are simply too small to be evidenced at a statistically significant level. Therefore, notwithstanding the implementation of new crossing schemes (e.g., multiparental crosses: Blanc et al., 2006, 2008) and approaches (e.g., association mapping: Buckler et al., 2009; Lu et al., 2010, 2012; Maccaferri et al., 2011; Varshney et al., 2012) that facilitate the identification and cloning of QTLs, the targeted manipulation of yield will remain a daunting undertaking.

As compared to MAS, genome-wide selection, while bypassing QTL identification (Bernardo, 2009), relies even more so on accurate phenotyping. As the cost of genotyping and sequencing keeps dropping (Varshney et al., 2009; Feuillet et al., 2011), cost-effective phenotyping will become increasingly strategic for further dissecting drought-adaptive traits and tailoring cultivars better suited for farming under drought-prone conditions. Hopefully, the information presented in this review will help raising interest in phenotyping as well as due awareness and appreciation of its pivotal role.

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I.4 screening experimental designs for quantitative trait loci, association mapping, genotype-by environment interaction, and other investigations

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Crop breeding programs using conventional approaches, as well as new biotechnological tools, rely heavily on data resulting from the evaluation of genotypes in different environmental conditions (agronomic practices, locations, and years). Statistical methods used for designing field and laboratory trials and for analyzing the data originating from those trials need to be accurate and efficient. The statistical analysis of multi-environment trails (MET) is useful for assessing genotype × environment interaction (GEI), mapping quantitative trait loci (QTLs), and studying QTL × environment interaction (QEI). Large populations are required for scientific study of QEI, and for determining the association between molecular markers and quantitative trait variability. Therefore, appropriate control of local variability through efficient experimental design is of key importance. In this chapter we present and explain several classes of augmented designs useful for achieving control of variability and assessing genotype effects in a practical and efficient manner. A popular procedure for unreplicated designs is the one known as "systematically spaced checks." Augmented designs contain "*c*" check or standard treatments replicated "*r*" times, and "*n*" new treatments or genotypes included once (usually) in the experiment.

Keywords: multi-environment trials, augmented experimental designs, genotype × environment interaction, quantitative trait loci (QTL)

INTRODUCTION

Conventional breeding will continue to make significant contributions to efforts to maintain the rate of crop improvement for food production and nutrition in order to meet the increase in human population growth. However, biotechnological methods, such as linkage analysis for detecting quantitative trait loci (QTLs), marker-assisted selection (MAS), association mapping, genomic selection, etc., will also be required. It is of paramount importance that the statistical methods used for designing field and laboratory trials and for analysing the data originating from those trials be accurate and efficient.

Crop breeding programs using conventional approaches, as well as new biotechnological tools, rely heavily on data resulting from the evaluation of genotypes in different environmental conditions (agronomic practices, locations, and years). The incidence of genotype-by-environment interaction (GEI) is a consequence of QTL-by-environment interaction (QEI) and marker effect-by-environment interaction, and this affects conventional breeding as well as MAS and genomic selection breeding strategies. The series of field trials known as multi-environment trials (METs) are vital for: (i) studying the incidence of GEI and assessing the stability of quantitative traits; (ii) mapping QTL and QEI; and (iii) finding associations among molecular markers and quantitative trait variation based on linkage disequilibrium analysis. To detect and quantify the presence of QEI is of vital importance for understanding the genetic architecture of quantitative traits.

All biotechnological methods are based on molecular marker data and phenotypic data. Phenotypic data are vitally important for assessment of the within-environment error structure for each of the trials that will be used later in the MET analysis. The MET statistical analysis is useful for assessing GEI, mapping QTLs, and studying QEI. Large populations are required for scientific study of QEI, and for determining the association between molecular markers and quantitative trait variability. Therefore, appropriate control of local variability through efficient experimental design is of key importance.

Spatial variability in the field is a universal phenomenon that affects the detection of differences among treatments in agricultural experiments by inflating the estimated experimental error variance. Researchers wishing to conduct field trials are faced with this dilemma. They tackle the problem by using an appropriate statistical design and layout for the experiment, and by using suitable methods for statistical analysis. *A priori* control of local variability in each testing environment is usually determined from the experimental design used to accommodate the genotypes to the experimental units. However, *a posteriori* control of the residual effect based on a model that provides a good fit to the data can effectively complement the control of local variability provided by the experimental design (see e.g., Federer, 2003a). Recently, efficient experimental designs (both unreplicated and replicated) have been developed, assuming that observations are not independent in that contiguous plots in the

field may be spatially correlated (Martin et al., 2004; Cullis et al., 2006).

Commonly, field trials used for linkage analyses or association mapping analyses are of 200 or more genotypes in size. These may consist of individuals from segregating F_2 and F_3 populations, recombined inbred lines (RILs), accessions from a genebank, advanced breeding cultivars, or individuals from any segregating population. Usually, QTL mapping is done on large numbers (500 or more) in as many locations or conditions as possible, for estimating QEI and examining the stable or unstable part of the chromosome that influences the trait under study. Thus, seed availability and land and labor costs are crucial factors to be considered when establishing METs for QTL and QEI analyses, and association mapping.

The class of augmented designs is especially useful for achieving control of variability and assessing genotype effects in a practical and efficient manner. In the early stages of a breeding program, a plant breeder is faced with evaluating the performance of large numbers of genotypes. Frequently, the seed supply is limited, but even if it is not, the large number of genotypes can necessitate using a single experimental unit per genotype.

A popular procedure for unreplicated designs is the one known as "systematically spaced checks." In this procedure, a standard check genotype is systematically spaced every certain number of experimental units. Several statistical procedures have been devised over the years to compare the yield of a new genotype with the standard variety. This procedure can require an inordinate amount of space, labor, and other resources devoted to check plots of a single standard genotype. Yates (1936) has shown that the number of check plots should be of the order of the square root of the number of (new genotype) test plots. In conducting METs, Sprague and Federer (1951) have shown that a cost-efficient procedure for maximizing genetic advancement involves using two replicates at each location for single crosses of maize, three replicates for top crosses, and four replicates for double crosses.

A third class of procedure used in the screening of genotypes for yield and other characteristics is that of "augmented experimental designs." These designs contain *c* check or standard treatments replicated *r* times, and *n* new treatments or genotypes included once (usually) in the experiment. Some of the *c* checks could be promising new genotypes (treatments) in the final stages of testing. Any standard experimental design may be used for the check treatments and then the block sizes or the number of rows and columns are increased to accommodate the new treatments. This class of design has several desirable qualities, including the following:

1. The number of checks can be any kind and number *c*.
2. The number of new entries can be any number *n*.
3. The new treatments can be considered as random or as fixed effects.
4. Survivors in the final stages of screening may be used as checks along with some standard checks. The dual use of these genotypes as checks and as their final evaluation is an efficient use of resources.
5. Some of the designs in this class allow for screening when other factors are present, thereby revealing genotype-by-factor interactions.
6. Non-contenders can be discarded prior to harvest, since they do not affect computation of blocking effects and variances.

Various augmented experimental designs are discussed in the following sections. These are augmented block (Federer, 1956, 1961), augmented row–column (Federer and Raghavarao, 1975; Federer et al., 1975), augmented resolvable row–column (Federer, 2002), augmented split plot (Federer, 2005b), and augmented split block (Federer, 2005a).

When the field layout is in a row–column formation, either for the entire experiment or within each complete block, an experimental design can be developed that controls variability in two directions for any number of genotypes and replicates. The row–column experimental designs have two block components, i.e., blocks in rows and blocks in columns. When the entire experiment is laid out in a row–column arrangement, the "latinised" designs assure that entries do not occur more than once in a row or a column of the experiment. Also, neighbor restricted designs restrict randomization of entries in such a way that certain groups of entries do not occur together, so that genotypic interference due to different maturity or plant height can be avoided.

Analysis of designed, spatially laid out experiments needs to take account of the design restrictions encountered. The actual spatial variation that occurs during the course of conducting field experiments may not be taken into account in the experimental design or in the standard statistical analysis selected before the experiment was conducted. Hence, to achieve appropriate statistical analysis for the data obtained from the experiment, it is necessary to determine the type and nature of the spatial variation present in the experiment. This often means selecting from a family of plausible statistical analyses. Federer (2003a) presented a number of methods useful for "exploratory model selection," to account for the variation that is present in the results of an experiment rather than what the variation pattern was expected to be. He used various forms of trend analysis on a variety of examples to determine the model that explained the variation present in each experiment. Several publications have been written using various forms of trend analysis for a variety of situations (Wolfinger et al., 1997; Federer, 2002, 2003a,b; Federer and Wolfinger, 2003).

AUGMENTED BLOCK EXPERIMENTAL DESIGNS

Augmented block experimental designs fall into two categories, complete blocks and incomplete blocks for the check genotypes or treatments. A randomized complete block design (RCBD), with *r* replicates or blocks, is used for the *c* check genotypes to start the construction of an augmented randomized block. Then, the *r* blocks are expanded to include the *c* checks plus *n*/*r* new genotypes in each block. If *n* is not a multiple of *r*, then fewer or more new genotypes would appear in some of the blocks. The *c* checks and *n*/*r* new genotypes are randomly allotted to the experimental units (plots) in each block. Genotype numbers are randomly assigned to the new genotypes, but this is not necessary in the early stages of screening since each new genotype is a random event in itself.

To illustrate an augmented RCBD, let $c = 3$ checks, $r = 4$ blocks, and $n = 13$ new genotypes. A plan is:

Block 1	Block 2	Block 3	Block 4
[A 1 4 B C 9]	[C 5 B 6 13 A]	[12 B A 2 3 C]	[7 A 8 C B 10 11]

A partitioning of the degrees of freedom in an analysis of variance (ANOVA) table for this design is:

Source of variation	Degrees of freedom
Total	25
Correction for mean	1
Block, B	3
Genotype	15
Check	2
New	12
Check versus new	1
B × check	6

In the first stage of screening, there may be a very large number of new genotypes with n of 8,000, 30,000, or even over 100,000. In these cases, the block size may become larger than is considered necessary to retain relative homogeneity within each block. The class of experimental designs known as an "incomplete block design" (ICBD) can then be used. The incomplete blocks of an ICBD may be in complete blocks, resolvable, or they may not. An appropriate ICBD for c checks, r replicates of the checks, incomplete blocks of size k, s incomplete blocks within a complete block, and b incomplete blocks is selected for the check genotypes. Then the b incomplete block sizes are increased to include n/b new genotypes in each incomplete block. To illustrate, let $c = 15$ checks arranged in $r = 5$ replicates and $b = rs = 25$ incomplete blocks of size $k = 3$. Let $n = 300$ new genotypes, and then $n/b = 300/25 = 12$. By enlarging the 25 incomplete blocks from $k = 3$ to $k = 15$ to accommodate $3 + 12 = 15$ experimental units, the 300 new genotypes can be put into these 25 incomplete blocks. The 12 new genotypes and the three checks are randomly allotted to the 15 experimental units in each of the 25 incomplete blocks. The blocks of genotypes are randomly allotted to the incomplete blocks in the field layout. The 15 check genotypes may, for example, be two standard genotypes and 13 promising and surviving new genotypes from previous screening cycles.

A randomized form of an ICBD may be obtained from a software toolkit such as Gendex (2009). Using the parameters $k = c + n/b = 15$, $v = c + n/r = 75$, and $r = 5$, a randomized form of an ICBD is obtained. Then the n/r numbers for v that appear in an incomplete block are replaced by genotype numbers to accommodate the $n = 300$ new genotypes, but retaining k of the check treatments in each incomplete block according to the plan for checks only.

A partitioning of the degrees of freedom in an ANOVA table for the above example is:

Source of variation	Degrees of freedom
Total	375
Correction for mean	1
Block, *R*	4
Genotype	314
Check	14
New	299
Check versus new	1
Incomplete blocks within *R*	20
Intrablock error	36

When the new genotypes are unreplicated, they do not contribute to the estimation of the block and error variances and the estimation of the block effects (Federer and Raghavarao, 1975). Only the replicated check treatments do this. Computer codes for analysing the results from augmented block designs have been given by Wolfinger et al. (1997) and Federer (2003a).

AUGMENTED COMPLETE BLOCK DESIGN FOR A QTL MAPPING STUDY

A typical QTL experiment in maize consists of F_2 plants obtained from the cross of two maize inbred lines referred to as parent 1 (P_1) and parent 2 (P_2). Subsequently, the F_2 plants can be selfed to produce, say, 900 independent F_5 lines. These 900 new entries (RILs) will be genotyped with molecular markers and genetic data, and the respective phenotypic data will be used for QTL and QEI mapping. These lines may be crossed to an inbred tester from an opposite heterotic group to obtain testcross seeds. The check entries may include the parents P_1 and P_2, the F_1 from the cross $P_1 \times P_2$ and two other checks ($check_1$ and $check_2$) the breeder wishes to include. One possible augmented complete block design (CBD) may consist of 20 blocks of size 45 augmented by P_1, P_2, F_1, and $check_1$ and $check_2$. Thus, the block size comprises a total of 50 entries (45 new entries comprising testcross F_5 lines and five other entries that will be repeated in every block). The same or a different group of test lines in the incomplete block can be used in all the sites where the experiment is planted, but with different randomization of the incomplete blocks. In this case, the augmented RCBD has $c = 5$ checks (P_1, P_2, F_1 $check_1$ and $check_2$), $r = 20$ blocks, and $n = 900$ new genotypes. A possible plan is:

Block 1				
P_1...1, 2...	P_2...14, 15...	F_1...20, 21...	$check_1$...30, 31...	$check_2$...44, 45...
Block 2				
P_1...46, 47...	P_2...54, 55...	F_1...60, 61...	$check_1$...70, 71...	$check_2$...89, 90...
.				
.				
Block 20				
P_1...460, 470...	P_2...540, 550...	F_1...600, 610...	$check_1$...700, 710...	$check_2$...890, 900...

The distribution of the repeated checks in the field should avoid, as much as possible, appearance of the same replicated check more than once in the same row or column. This latinised augmented CBD may help to reduce bias due to unexpected soil trends running across columns or rows.

A partitioning of the degrees of freedom in an ANOVA table for this design in each site is:

Source of variation	Degrees of freedom
Total	1000
Correction for mean	1
Block, B	19
Genotype	904
Check	4
New	899
Check versus new	1
B × check	76

Supposing that the trial were established in three different sites, then the partition of the degrees of freedom in the ANOVA table would be as follows:

Source of variation	Degrees of freedom
Total	3000
Correction for mean	1
Site	2
Block within site, B(S)	57
Genotype	904
Check	4
New	899
Check versus new	1
Genotype × site	1808
Check × site	8
New × site	1798
(Check versus new) × site	2
B(S) × check	228

AUGMENTED INCOMPLETE-COMPLETE BLOCK DESIGN FOR AN ASSOCIATION MAPPING STUDY

This example supposes that 200 diverse bread wheat accessions from a genebank are to be used for an association mapping study. The accessions will be used to examine the possible relationship between various phenotypic traits (such as grain yield, resistance to leaf and yellow rust, bread making quality, protein content, etc.) and the molecular markers located along the seven chromosomes of the three genomes of wheat (A, B, and D). Ten sites with contrasting environmental conditions would be used to allow good discrimination of the 200 accessions. Differential environmental conditions must be used in order to obtain a good discrimination for resistance to different potential rust pathogens as well as for the other traits.

It is assumed that $c = 15$ checks can be arranged in $r = 5$ replicates and $b = 25$ incomplete blocks of size $k = 3$ are formed. The 200 accessions can be accommodated in 25 incomplete blocks of size 11 by enlarging the incomplete blocks from $k = 3$ to $k = 11$ by adding $n/b = 200/25 = 8$ new entries in each incomplete block.

The ANOVA table of the combined analysis across ten environments is:

Source of variation	Degrees of freedom
Total	2750
Correction for mean	1
Site	9
Block within sites, R(S)	40
Genotype	214
Check	14
New	199
Check versus new	1
Genotype × site	1926
Check × site	126
New × site	1791
Check versus new × site	9
Incomplete blocks within R × S	200
Intrablock error within sites	560

AUGMENTED ROW–COLUMN EXPERIMENTAL DESIGNS

Augmented row–column designs can be constructed either by adding rows and/or columns or by enlarging the intersections of the rows and columns of a square or rectangle. Considering the latter option, a 5 × 5 Latin square can be used for five checks A, B, C, D, and E, augmented with 250 new genotypes, adding 10 new genotypes to each row–column intersection as follows to obtain the schematic plan before randomization:

A	1–10	B	11–20	C	21–30	D	31–40	E	41–50
B	51–60	C	61–70	D	71–80	E	81–90	A	91–100
C	101–110	D	111–120	E	121–130	A	131–140	B	141–150
D	151–160	E	161–170	A	171–180	B	181–190	C	191–200
E	201–210	A	211–220	B	221–230	C	231–240	D	241–250

A randomization plan would be obtained for the Latin square and then the 11 entries in each row–column intersection would be randomly allotted to the 11 experimental units in each intersection. The new genotypes are randomly assigned to the numbers 1–250. A partitioning of the degrees of freedom in an ANOVA table is:

Source of variation	Degrees of freedom
Total	275
Correction for the mean	1
Row	4
Column	4
Genotype	254
Check	4
New	249
Check versus new	1
Error	12

An alternative row–column plan would be to set up a 25 row by 15 column rectangle as shown below.

If the variation in rows and in columns can be explained by linear, quadratic, and perhaps cubic tends and their interactions, then two checks would have been sufficient to obtain row and column

solutions to adjust the new treatments, and 325 new treatments could have been included. An equal number of rows and columns results in the minimum number of check genotypes. For example, using a 20×20 square, 40 plots could be allocated to two check genotypes and 360 to new genotypes. There still would be more than 20 degrees of freedom associated with the error mean square. Another scenario supposes that one standard check genotype and four promising new genotypes in the final stage of evaluation are used. Utilizing new genotypes in their final stage of testing allows dual use of the results and efficient experimentation, eliminating the inclusion of too many check plots.

A randomization plan would involve randomly allocating the rows and columns in the above plan to the rows and columns in the experimental area, randomly assigning the letters A–E to the checks, and randomly allotting the numbers 1–250 to the new genotypes. A partitioning of the degrees of freedom in an ANOVA table is:

Source of variation	Degrees of freedom
Total	375
Correction for the mean	1
Row	24
Genotype	254
Check	4
New	249
Check versus new	1
Column (eliminating genotype)	14*
Error	82*

**Need correction for confounding effects.*

Federer et al. (1975) discuss a number of other arrangements including one used by Dr. A. Mangelsdorf. The Mangelsdorf design has a nice balanced property and was used for METs.

The first plan given above within this section is row–column–check connected in that solutions are obtainable for all effects. The plan immediately above is row–check connected and column–check connected but is not row–column–check connected. This means that functions of the column effects, such as linear, quadratic, cubic, etc., regressions are used in the analysis of such designs. In order to have a plan that is row–column–check connected, two of the transversals of the square or rectangle need to be adjacent to each other, a feature that an experimenter may consider as undesirable. Computer codes illustrating this type of analysis are given by Federer (2003b), Federer and Wolfinger (2003), and Wolfinger et al. (1997).

AUGMENTED RESOLVABLE ROW–COLUMN EXPERIMENTAL DESIGNS

Experimental designs such as a lattice square or a lattice rectangle may be used to construct augmented lattice square and augmented lattice rectangle plans (Federer, 2002, 2003b). For such plans, row blocking and column blocking are included in each complete block, thus making the design resolvable. Since the proportion of experimental units in relation to the number of checks is less in an augmented lattice square, this is the plan that will be illustrated. There are $k \times k$ experimental units in each complete block, and $2k$, $3k$, etc., check genotypes may be used. To construct such a plan, a lattice square plan is obtained first for $v = k^2$ treatments. The complete blocks where treatments 1 to k and $k+1$ to $2k$

A	1	2	B	3	4	C	5	6	D	7	8	E	9	10
11	A	12	13	B	14	15	C	16	17	D	18	19	E	20
21	22	A	23	24	B	25	26	C	27	28	D	29	30	E
E	31	32	A	33	34	B	35	36	C	37	38	D	39	40
41	E	42	43	A	44	45	B	46	47	C	48	49	D	50
51	52	E	53	54	A	55	56	B	57	58	C	59	60	D
D	61	62	E	63	64	A	65	66	B	67	68	C	69	70
71	D	72	73	E	74	75	A	76	77	B	78	79	C	80
81	82	D	83	84	E	85	86	A	87	88	B	89	90	C
C	91	92	D	93	94	E	95	96	A	97	98	B	99	100
101	C	102	103	D	104	105	E	106	107	A	108	109	B	110
111	112	C	113	114	D	115	116	E	117	118	A	119	120	B
B	121	122	C	123	124	D	125	126	E	127	128	A	129	130
131	B	132	133	C	134	135	D	136	137	E	138	139	A	140
141	142	B	143	144	C	145	146	D	147	148	E	149	150	A
A	151	152	B	153	154	C	155	156	D	157	158	E	159	160
161	A	162	163	B	164	165	C	166	167	D	168	169	E	170
171	172	A	173	174	B	175	176	C	177	178	D	179	180	E
E	181	182	A	183	184	B	185	186	C	187	188	D	189	190
191	E	192	193	A	194	195	B	196	197	C	198	199	D	200
201	202	E	203	204	A	205	206	B	207	208	C	209	210	D
D	211	212	E	213	214	A	215	216	B	217	218	C	219	220
221	D	222	223	E	224	225	A	226	227	B	228	229	C	230
231	232	D	233	234	E	235	236	A	237	238	B	239	240	C
C	241	242	D	243	244	E	245	246	A	247	248	B	249	250

appear together in a row or in a column are deleted. For $2k$ check genotypes, treatments $2k+1$, $2k+2$, . . ., k^2 are deleted in each of the r blocks. The $rk\,(k-2)$ new treatments are inserted into the deleted treatment spaces of the lattice square. To illustrate, with $k=7$ and $r=7$, a plan would be as shown at the bottom of the page.

The symbol × indicates where one of the $rk\,(k-2)=245$ new genotypes would be entered. Row linear and quadratic effects and column linear and quadratic effects can be estimated (Federer, 2002). Checks 1–7 appear once with checks 8–14 in rows and in columns, but do not appear with each other. The diagonal elements need not be adjacent, as illustrated below.

A partitioning of the degrees of freedom in an ANOVA is:

Source of variation	Degrees of freedom
Total	343
Correction for the mean	1
Replicate or block	6
Genotype	258
Check	13
New	244
Check versus new	1
Check × block	78
Row linear within block	7
Column linear within block	7
Row linear × column linear within block	7
Row quadratic within block	7
Column quadratic within block	7
Row quadratic × column quadratic within block	7
Row cubic within block	7
Column cubic within block	7
Residual or error	22

To screen 30,000 new genotypes, k would be 33 and $k=r=33$ replicates would be required. As stated earlier, the $2k=66$ checks could consist of two standard checks plus 64 new genotypes in their final stage of testing.

As an alternative design in this class, the checks could be in a lattice square experimental design. Then, each of the row–column intersections within each complete block could be enlarged to include the desired number of new genotypes.

AUGMENTED SPLIT PLOT EXPERIMENTAL DESIGNS

In order to compare the effect of environments and management procedures on new genotypes, the class of augmented split plot experimental designs has been proposed by Federer (2005b). The effects of factors such as tillage, fertilizers, insecticides, irrigation, planting density, date of planting, etc on new genotypes could be assessed. The effect of the date of planting is often confused with site-to-site effects. The new genotypes to be assessed may appear in split plot treatments or in whole plot treatments. New genotypes can be tested for several factors at a time by using split split plot, split split split plot, etc augmented designs. These designs allow for genotype-by-factor interactions and GEI, and are useful, especially in the final stages of screening genotypes. A schematic plan of a design is shown below for four whole plots, such as tillage practices, three checks (20, 21, and 22), and 19 new genotypes such as the 7 or 8 split plot treatments, and $r=4$ blocks or replicates of check genotypes.

There are seven split plot treatments in Block 4 and eight in the other three blocks. The checks are given the highest numbers because SAS software subtracts the highest numbered effect from all the others for the estimated effects, and gives a standard error of a difference between an estimated effect of a genotype and the highest numbered one, rather than a standard error of an effect as indicated. It is usually more desirable to compare all new genotypes with a check, rather than compare all entries with a new genotype. The usual randomization procedure for a split plot experimental design would be used.

Replicate 1							Replicate 2							Replicate 3							Replicate 4						
1	x	x	x	x	x	14	1	x	x	x	x	x	13	1	x	x	x	x	x	12	1	x	x	x	x	x	11
8	2	x	x	x	x	x	14	2	x	x	x	x	x	13	2	x	x	x	x	x	12	2	x	x	x	x	x
x	9	3	x	x	x	x	x	8	3	x	x	x	x	x	14	3	x	x	x	x	x	13	3	x	x	x	x
x	x	10	4	x	x	x	x	x	9	4	x	x	x	x	x	8	4	x	x	x	x	x	7	x	x	x	x
x	x	x	11	5	x	x	x	x	x	10	5	x	x	x	x	x	9	5	x	x	x	x	x	8	5	x	x
x	x	x	x	12	6	x	x	x	x	x	11	6	x	x	x	x	x	10	6	x	x	x	x	x	9	6	x
x	x	x	x	x	13	7	x	x	x	x	x	12	7	x	x	x	x	x	11	7	x	x	x	x	x	10	7

Replicate 5							Replicate 6							Replicate 7						
1	x	x	x	x	x	10	1	x	x	x	x	x	9	1	x	x	x	x	x	8
11	2	x	x	x	x	x	10	2	x	x	x	x	x	9	2	x	x	x	x	x
x	12	3	x	x	x	x	x	11	3	x	x	x	x	x	10	3	x	x	x	x
x	x	13	4	x	x	x	x	x	12	4	x	x	x	x	x	11	4	x	x	x
x	x	x	14	5	x	x	x	x	x	13	5	x	x	x	x	x	12	5	x	x
x	x	x	x	8	6	x	x	x	x	x	14	6	x	x	x	x	x	13	6	x
x	x	x	x	x	9	7	x	x	x	x	x	8	7	x	x	x	x	x	14	7

A partitioning of the degrees of freedom in an ANOVA would be:

Source of variation	Degrees of freedom
Total	124
Correction for mean	1
Block, B	3
Tillage, T	3
B × T, error T	9
Genotype	21
Check	2
New	18
Check versus new	1
T × genotype	63
T × check	6
T × new	54
T × check versus new	3
B × check within T	24

Codes for analysing data for this design and others in this class are given by Federer (2005b).

Tillage	Block 1								Block 2							
1	20	21	22	1	2	3	4	5	20	21	22	6	7	8	9	10
2	20	21	22	1	2	3	4	5	20	21	22	6	7	8	9	10
3	20	21	22	1	2	3	4	5	20	21	22	6	7	8	9	10
4	20	21	22	1	2	3	4	5	20	21	22	6	7	8	9	10
Tillage	**Block 3**								**Block 4**							
1	20	21	22	11	12	13	14	15	20	21	22	16	17	18	19	
2	20	21	22	11	12	13	14	15	20	21	22	16	17	18	19	
3	20	21	22	11	12	13	14	15	20	21	22	16	17	18	19	
4	20	21	22	11	12	13	14	15	20	21	22	16	17	18	19	

AUGMENTED SPLIT BLOCK EXPERIMENTAL DESIGNS

Augmented split block experimental designs are another class of augmented experimental design for assessing the effects of various factors on new genotypes, as described by Federer (2005a) who discussed five different examples of this class and presents a numerical example and a code for analysis of the data. New genotypes may be considered to be random or fixed effects. One of the cases considered is an intercropping example for two crops with new genotypes for both crops. Allowing for interaction of factors with genotypes is an important aspect of this class of design. To illustrate one design within this class, an augmented randomized block experimental design is used for $c = 3$ checks (A, B, C), $n = 25$ new genotypes (1–25), and $r = 4$ blocks. Then, $d = 4$ dates of planting (D1, D2, D3, D4) are strip blocked across the entries in each of the four blocks. This is illustrated in the schematic layout at the bottom of the page.

The date treatments are in an RCBD and the checks and new genotypes are in an augmented randomized block experimental design. The date experimental units are distributed across all the genotype entries in a block.

A possible partitioning of the degrees of freedom in an ANOVA table is:

Source of variation	Degrees of freedom
Total	148
Correction for the mean	1
Block, B	3
Genotype	27
Check	2
New genotype, G	24
Check versus new	1
B × check	6
Date, D	3
B × D	9
D × genotype	81
D × check	6
D × G	72
D × check versus new	3
B × D × check	18

Block 1										**Block 2**									
Date	A	B	C	1	2	3	4	5	6	Date	A	B	C	7	8	9	10	11	12
D1										D1									
D2										D2									
D3										D3									
D4										D4									
Block 3										**Block 4**									
Date	A	B	C	13	14	15	16	17	18	Date	A	B	C	19	20	21	22	23	24	25
D1										D1									
D2										D2									
D3										D3									
D4										D4									

DISCUSSION

In the early stages of a plant breeding program, expected genetic gains may be increased by screening a large number of genotypes in contrast to having more precise comparisons of a fewer number of genotypes. This makes it necessary to evaluate many entries where there may not be sufficient seed to replicate each. For this reason Federer proposed augmented designs where a set of check entries are replicated an equal (or unequal) number of times in a specified field design and an additional set of new test entries are included in the experiment only once. In this review we show different type of augmented complete and ICBD for the check treatments with the test entries being added or "augmented" to the blocks.

This approach provides a very efficient means of screening test entries and has a considerable amount of flexibility. Augmented

ICBD might be preferred over augmented CBD when the number of repeated checks is large. When soil variability runs in two directions augmented row–column designs should be a good alternative, and when the experiment is "latinized" so that entries do not occur more than once in a row or column, then the efficiency of increasing precision increases. The augmented incomplete block or/and the row-column designs can be used for association mapping and/or genomic selection where a large number of entries (usually more than 1000) are needed but cannot be planted in all possible environments. The advantages of using these augmented designs is when the soil heterogeneity increases due to limiting factors as low water, and nitrogen availability in the field.

CONCLUSIONS

There are many variations of split plot and split block experimental designs. Federer and King (2007) discuss several of these variations as well as combinations of the designs. Experimenters may find some of these variations suitable for augmenting with new genotypes that will fit the conditions for their experiment. Such designs as given in the last two sections above allow the experimenter to obtain interactions of new genotypes with a variety of factors. Instead of a single factor, a factorial combination of several factors could be used. For example, instead of date only, a factorial arrangement of date, fertilizer level, and insecticide could be used. Considerable flexibility is possible through the use of augmented experimental designs.

When it is advisable to use an augmented design, it may be used at several sites. For example, the Manglesdorf design presented by Federer et al. (1975) was used at several sites in Brazil. Methods for combining results over sites have been described by Federer et al. (2001), and they even allow for different designs at the different sites.

ACKNOWLEDGMENTS

Sadly, Professor Walter T. Federer, the lead author of this chapter, passed away in April 2008. He was one of the greatest statisticians on the theme of experimental design for plant breeding, agronomy, and agriculture in general. Professor Federer was a unique, enthusiastic human being who was always ready to discuss serious scientific issues without losing his unique character of extreme kindness and gentlemanliness. I have the privilege to say that he was my friend.

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Plant response to environmental conditions: assessing potential production, water demand, and negative effects of water deficit

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This paper reviews methods for analyzing plant performance and its genetic variability under a range of environmental conditions. Biomass accumulation is linked every day to available light in the photosynthetically active radiation (PAR) domain, multiplied by the proportion of light intercepted by plants and by the radiation use efficiency. Total biomass is cumulated over the duration of the considered phase (e.g., plant cycle or vegetative phase). These durations are essentially constant for a given genotype provided that time is corrected for temperature (thermal time). Several ways of expressing thermal time are reviewed. Two alternative equations are presented, based either on the effect of transpiration, or on yield components. Their comparative interests and drawbacks are discussed. The genetic variability of each term of considered equations affects yield under water deficit, via mechanisms at different scales of plant organization and time. The effect of any physiological mechanism on yield of stressed plants acts via one of these terms, although the link is not always straightforward. Finally, I propose practical ways to compare the productivity of genotypes in field environments, and a "minimum dataset" of environmental data and traits that should be recorded for that.

Keywords: stress, drought, temperature, intercepted light, plant performance

INTRODUCTION

Plants transform light and CO_2 into biomass. This occurs during a given period of time, the duration of which depends essentially on air temperature and on the earliness of the considered genotype. During the same period of time, the plant requires an amount of water that depends on environmental conditions (light, air humidity, and wind) and on plant traits such as stomatal conductance and leaf area. It follows that the biomass accumulated by a plant primarily depends on environmental conditions, but also depends on plants traits with their genetic variability. The objective of this paper is to provide a basis for analyzing yield from environmental conditions, thereby enabling characterization of the differences in behavior between genotypes. This basis is the common ground of most existing crop models (Sinclair et al., 1976; Brisson et al., 2003; Hammer et al., 2010), and of global analyses of the effects of climate change on plant performance (Brisson et al., 2010; Lobell et al., 2011).

VARIABILITY OF YIELD DEPENDING ON LIGHT AND WATER AVAILABILITY

THE MAXIMUM YIELD THAT CAN BE OBTAINED IN A GIVEN FIELD DEPENDS ON THE AMOUNT OF INTERCEPTED LIGHT

Biomass accumulation is proportional to the amount of light in the photosynthetically active radiation (PAR) domain that the plant intercepts over a period of time (Monteith, 1977). Why is biomass accumulation proportional to light while photosynthesis is not? Photosynthesis depends on light intensity, with a relationship that is approximately linear for low light intensities (about 0–700 μmol m^{-2} s^{-1}), but curvilinear at higher intensities (Farquhar et al., 1980). The linear relationship between biomass and light is due first to lowest leaves of the canopy being shaded, so they receive light in the range where photosynthesis is nearly proportional to light. Second, the light intensity only exceeds 700 μmol m^{-2} s^{-1} during the late morning and early afternoon, and is below this value during the rest of the day. Hence, the resulting relationship between photosynthesis and light is linear at the field level and during the entire day.

The biomass accumulation by a crop on a given day (Bio_i) depends on:

- The amount of light on the considered day in the range of wavelengths used by photosynthesis [photosynthetic photon flux density ($PPFD_i$)]. Most light sensors directly record the amount of light in this range.
- The proportion of light that is intercepted by plant leaves. The light that reaches the soil is not used for photosynthesis. The proportion of intercepted light depends on leaf area on the considered day and is characterized by the leaf area index (LAI), which is the number of layers of leaves per unit soil area. For instance, an LAI of 1 corresponds to a plant canopy with 1 m^2 of leaves per m^2 of soil. The proportion of light intercepted by plants increases with LAI (**Figure 1**) until an LAI of 4 or 5 depending on the species. At a higher LAI, nearly all the available light is intercepted (there are no spots of unused light on the soil). The relationship between LAI and the proportion of intercepted light differs among species (**Figure 1**).

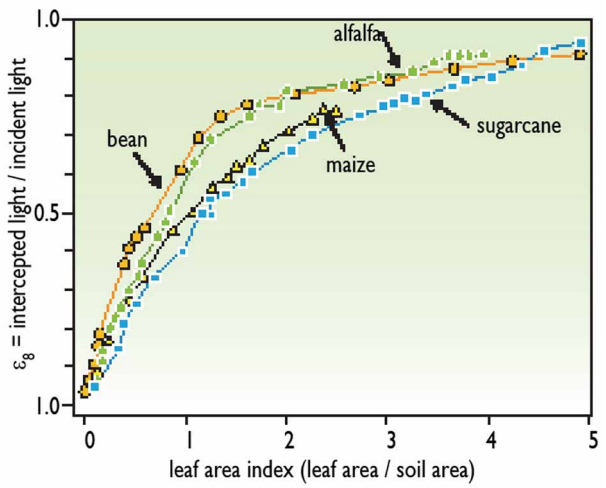

FIGURE 1 | Relationship between leaf area and light interception (redrawn from Gosse et al., 1986).

Plants intercept more light per unit leaf area in species with sub-horizontal leaves, such as clover or cassava, than in species with erect leaves, such as cereals.

- The efficiency of transformation of intercepted light into biomass, which depends essentially on the photosynthesis rate of leaves (Gosse et al., 1986; Brisson et al., 2003; Hammer et al., 2010). This efficiency differs between species; it is maximal in C_4 species such as maize, sorghum, or millet, which have a very efficient photosynthetic apparatus. It is roughly similar in all C_3 species that are neither legumes nor oil-rich seeds, such as wheat or rice. It is lower in species that have a special metabolism such as legumes, which use part of the photosynthesized sugars for nitrogen fixation (Gosse et al., 1986). It is also lower in species with oil-producing seeds, which have high energy content per unit biomass of seeds.

These three effects can be summarized in a simple equation:

$$\text{Bio}_i = \text{PPFD}_i \cdot \varepsilon_a \cdot \varepsilon_b \quad (1)$$

where Bio_i is the biomass accumulated on a given day, PPFD is the photosynthetic photon flux density, also called light intensity (in W m^{-2} or μmol m^{-2} s^{-1}), ε_a is the proportion of intercepted light (in percentage), and ε_b is the efficiency of transformation (in g per unit light intensity). The biomass accumulated during the whole season (Bio_{tot}) is the sum of biomasses accumulated each day:

$$\text{Bio}_{\text{tot}} = \sum (\text{PPFD}_i \cdot \varepsilon_a \cdot \varepsilon_b) \quad (2)$$

The yield is the fraction of Bio_{tot} that is transferred to harvested organs, such a grains or tubers. The proportion of harvested biomass divided by the total biomass is usually termed the "harvest index" (HI), expressed as a percentage:

$$\text{Yield} = \sum (\text{PPFD}_i \varepsilon_a \cdot \varepsilon_b)\,\text{HI} \quad (3)$$

This expression (Monteith, 1977) is very useful for analyzing the yield performance of a given genotype and to compare genotypes. In particular:

- The best genotypes are those that give a high priority to the harvested organs in the biomass partitioning. A large part of the genetic progress of several species has consisted of increasing HI (Lopez Pereira et al., 1999; Duvick, 2005).
- In contrast, the efficiency of conversion of intercepted light into biomass is less variable between genotypes of a given species. In particular, the genetic progress of photosynthesis has been slow, if not negligible (Bolanos and Edmeades, 1993a; Lopez Pereira et al., 1999; Lee and Tollenaar, 2007). Some genetic programs have nevertheless increased RUE (Rebetzke et al., 2002).
- Another large source of genetic variation is the proportion of light that is intercepted by plants. The genetic variation in leaf area then translates into a change in accumulated biomass and, in turn, into yield in the range of LAI from 1 to 5 (Gosse et al., 1986; Hammer et al., 2010). However, confounding effects, as analyzed below, may obscure this relationship.
- Finally, yield largely depends on the number of days during which biomass accumulates (term Σ in Equation 3). It is intuitive that the longer the crop cycle the higher the maximum potential yield. This number of days depends on the temperature sensed by plants during the crop cycle. Increasing temperature tends to cause shorter crop cycles, thereby decreasing the potential yield. It also depends on the genotype via two traits: first the earliness of the considered genotype, which defines the time for flowering and the duration of the period between flowering and maturity; and second the degree of maintenance of this period under stressing conditions.

AN ALTERNATIVE WAY OF EXPRESSING YIELD AS A FUNCTION OF WATER AVAILABILITY AND WATER-USE EFFICIENCY

Water availability does not appear directly in the analysis presented above, because water is not involved *per se* in the process of biomass accumulation. In contrast to light which has a nearly proportional effect on the accumulation of biomass, water "only" serves to allow biomass accumulation to occur in good conditions by favoring stomatal opening, organ growth, and plant metabolism. In order to express yield as a function of water-use, an alternative expression of yield has been proposed by Passioura (1977). This states that the biomass accumulation on 1 day depends on the transpiration rate multiplied by the water-use efficiency (WUE) i.e., the ratio of biomass accumulation to transpiration. As in Equation (2), the biomass accumulated over the plant cycle is the sum of that accumulated every day of the cycle. The yield is the fraction of the accumulated biomass that is transferred to harvested organs, such a grains or tubers (i.e., the HI):

$$\text{Yield} = \sum (T_i \cdot \text{WUE}_i)\,\text{HI} \quad (4)$$

where T_i is the transpiration on day i, WUE_i is the WUE on day i and Σ indicates that the biomass is accumulated over the whole crop cycle.

Transpiration rate

T_i changes every day depending on evaporative demand and on leaf area. Evaporative demand depends essentially on light, on the degree of water saturation of the air, measured as vapor pressure deficit (VPD), and on wind speed (Sinclair et al., 1976; Brisson et al., 2003). Most weather stations provide the evaporative demand, termed "potential evapotranspiration" or "reference evapotranspiration." Otherwise, evaporative demand can be calculated using a spreadsheet by using Penman's formula.

Leaf area affects the transpiration rate in the same way as it affects the intercepted light (**Figure 1**). Thus, transpiration is nearly proportional to leaf area for low LAI, and saturates for LAI higher than 3 or 4.

$$T = ET_r \cdot \varepsilon_a \quad (5)$$

where ET_r is the reference evapotranspiration, as provided by a weather station or calculated using Penman's formula, and ε_a is the proportion of transpiration of the studied field to the reference evapotranspiration, which has the same value as that in Equation (1).

The root system also affects the transpiration rate, via several traits such as total root length, rooting depth, or the hydraulic conductivity of roots. It should be noted that this is the case only if roots have access to a large volume of soil. In contrast, an increase in root length has virtually no effect in a shallow soil. Two breeding programs for drought have resulted in the surprising result that the root length was reduced in drought-tolerant genotypes compared with drought-sensitive ones (Bolanos et al., 1993; Bruce et al., 2002).

Water-use efficiency

WUE is defined here as the ratio of the biomass accumulated on 1 day to the transpiration rate on the same day. Defined in this way, it is difficult and tedious to measure. A surrogate measurement consists of the ratio between the photosynthetic rate and the transpiration rate, or between photosynthesis and stomatal conductance as measured using gas exchange equipment. The latter can be measured indirectly via the ratio of two natural isotopes of carbon in leaves or grains (carbon isotope discrimination, often called $D^{13}C$), providing rapid estimates with a high throughput.

Environmental conditions greatly affect WUE (defined as in Equation 6). In particular, WUE decreases when evaporative demand increases, because transpiration is higher at high evaporative demands for a given photosynthesis. It follows that WUE is higher in regions with wet air, and that crops that are grown during winter or during rainy seasons have a higher WUE than those grown during summer or during dry seasons (**Figure 2**). Large differences in WUE exist between species. WUE is higher in C_4 species, such as maize, sorghum, or millet, than in C_3 species. It is noteworthy that the method based on carbon isotope discrimination cannot be used in C_4 species.

Finally, it should be noted that the concept of WUE, and therefore Equation (4), can be misleading, depending on the definition that is taken for WUE and on the time scale (Blum, 2005, 2009).

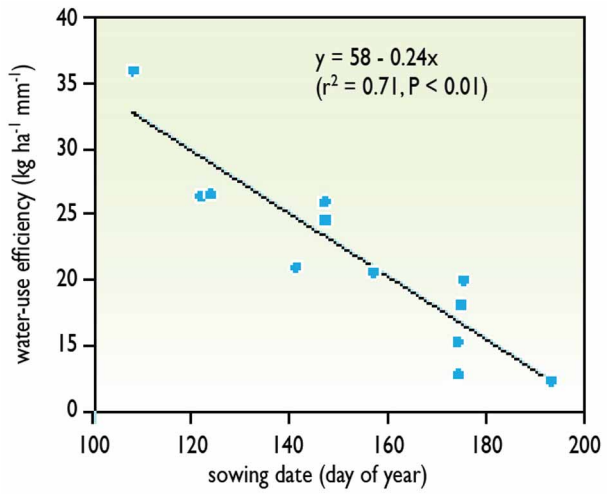

FIGURE 2 | An example of variation of WUE during the crop cycle. The later in the growing period, the higher is the evaporative demand. Because the accumulated biomass does not increase in the same proportion, the WUE is lower during periods with high evaporative demand. Data obtained with wheat lines sown at different times of the year in Australia (redrawn from Richards et al., 2002).

Depending on the study or paper, WUE is defined in different ways that are not equivalent, have different genetic variabilities, and respond differently to environmental conditions. Defined as the ratio of photosynthesis to transpiration, WUE has a lower genotype-by-environment interaction (GEI), but it cannot be used directly as the efficiency of transforming transpiration into biomass. At the other extreme, WUE can be defined at the scale of the crop cycle as the ratio of the total biomass (or yield) to the total transpiration. It should be noted that, in this case, WUE is not a direct consequence of stomatal and photosynthetic functioning, and is affected by growth conditions. For instance, a short stress that causes abortion of reproductive organs affects total biomass accumulation, with a lesser effect on transpiration. Therefore, it affects WUE at the whole cycle scale, although it has a small effect on gas exchange.

A THIRD EXPRESSION OF YIELD AS A SERIES OF YIELD COMPONENTS: ROLES OF INDIVIDUAL PHASES OF THE CROP CYCLE

Agronomists have long expressed yield by a multiplicative series of yield components: the number of plants per m^2; the number of immature reproductive organs per plant (e.g., the number of seeds per tiller multiplied by the number of tillers per plant, or the number of tubers per stem multiplied by the number of stems per plant); the proportion of non-aborted reproductive organs; and the individual weight of seeds or tubers. Thus:

$$\text{Yield} = N(1 - \text{A})W_r \quad (6)$$

where N is the number of immature reproductive organs (e.g., ovules) per unit area, A is the proportion of aborted reproductive organs, and W_r is the mean weight of individual reproductive organs.

This expression has the advantage of breaking down the yield into several phase of the crop cycle. The setting of reproductive organs occurs during the pre-flowering time, the proportion of non-aborted reproductive organs is determined during a phase around flowering, and the individual weight of seeds or tubers is determined between flowering and maturity. Therefore, it is possible to express the result of each phase as a function of environmental conditions during that phase. For instance, one can relate the abortion rate to the water availability during the same period (Claassen and Shaw, 1970) or to the biomass accumulation during that period (Vega et al., 2001) (**Figure 3**). In the same way, seed number usually correlates well to the intercepted light during the pre-flowering period. These relationships help to identify the behaviors of genotypes, which can either have common behaviors (common relationships) or different behaviors (different relationships).

HOW DOES EACH TERM OF EQUATIONS 1–6 VARY WITH WATER AVAILABILITY AND GENETIC DIVERSITY?

Each of the three approaches of yield provided by Equations (3), (4), and (6) has its own interests, and represents a view of the yield setting. The first equation is more mechanistic and is the one used in all crop models, but the effect of water deficit does not appear explicitly. The second is perhaps more intuitive for understanding the effects of water deficit but can be misleading, depending on the definition that is taken for WUE. The third is the most intuitive, but cannot be related to physiological functions of plants. These three views should be considered as frameworks of analysis to help identify where a given trait is involved in yield formation and what the effects are of a water deficit. The following analyses the contribution of different processes and traits to yield under water deficit. It should be stressed here that none of these traits or function is beneficial or detrimental *per se*. Each trait can be positive, negative, or with negligible effect on the drought scenario (Tardieu, 2012). Hence, tolerance to water deficit and the contribution of traits cannot be considered without considering the drought scenario.

Growth reduction of expanding tissues

The first effect of a water shortage is to drastically reduce the growth of expanding tissues, with effects on terms ε_a, T and N, and indirect effects on other terms. Expansive growth is one of the processes most sensitive to water deficit in leaves, internodes (e.g., the peduncle in cereals) or reproductive organs (e.g., the silks in maize or tubers in potatoes) (Boyer, 1970; Saab and Sharp, 1989; Muller et al., 2011; Tardieu et al., 2011). This occurs because turgor—the driving force for cell expansion—is reduced in the case of water deficit, but also because of other indirect processes such as a reduction in cell division rate or in the extensibility of cell walls (Cosgrove, 2005). A water deficit during the vegetative stages affects leaf growth and hence light interception but, in most species, it also affects the growth of immature storage organs (seeds or tubers).

Via this mechanism of reduced growth, a water deficit can affect the term ε_a of Equation (3), because it reduces LAI and, therefore, both light interception (Equation 3) and transpiration (Equation 4). The reduction in growth also affects the number of reproductive organs and their abortion ratio (N and A, respectively in Equation 6) via a reduction in biomass accumulation (Gambin and Borras, 2007), but also because vegetative and reproductive growth can depend on common mechanisms and common genetic determinists (Welcker et al., 2007). Other terms can also be affected by a reduction in growth, in particular HI if young reproductive organs or young tubers abort. In this case, biomass cannot accumulate in harvested organs in later stages of the crop cycle, and is stored in stems or roots.

There is a very large genetic variability of the sensitivity of growth to water deficit. For instance, quantitative trait loci (QTLs) have been identified for the degree of maintenance of growth under water deficit of leaves (Welcker et al., 2011), silks (Ribaut et al., 1997), or the peduncle (Maccaferri et al., 2008). The sensitivity of leaf growth to evaporative demand and to soil water deficit, which can be determined in controlled conditions, translates into differences in leaf area and into biomass accumulation observed in the field (Chenu et al., 2008). Several QTLs for

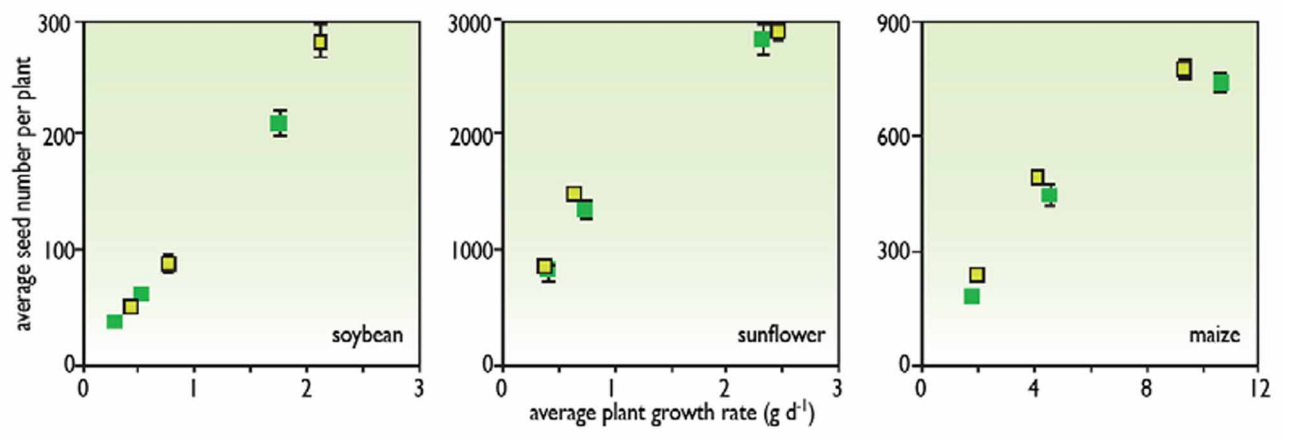

FIGURE 3 | The relationship between seed number per plant at maturity and average plant growth rate at flowering time in soybeans, sunflowers, and maize (redrawn from Vega et al., 2001).

growth maintenance have been shown to translate into QTLs for yield (Ribaut et al., 1997).

The beneficial effect of growth reduction is to decrease transpiration rate, thereby saving water for the end of the crop cycle. This is favorable under severe terminal water deficit, but is detrimental under mild deficit because of the decrease in cumulated photosynthesis (Tardieu, 2012).

Stomatal closure

A second effect of water shortage is to close stomata, thereby affecting the terms ε_b, WUE, N, and W_r. Plants subjected to water deficit close their stomata, with the involvement of hydraulic and chemical messages such as the plant hormone abscisic acid (ABA) (Tardieu and Simonneau, 1998). This reduces the loss of water by the plant, thereby saving soil water and improving leaf water status, but also reducing the rate of photosynthesis and increasing leaf temperature. These effects can be measured via gas exchange equipment, but measurement of leaf temperature can provide a convenient surrogate for gas exchange if used carefully (Jones, 2007; Guilioni et al., 2008).

There is some genetic variability for stomatal sensitivity to water deficit, but probably less marked than that for growth maintenance. In contrast, there is an interesting genetic variability for WUE, when defined as the ratio of photosynthesis to transpiration rate. Genetic analyses and breeding programmes have been carried out on WUE via its relation to carbon isotope discrimination, resulting in appreciable gains in yield in wheat grown in dry conditions (Condon et al., 2004).

A water deficit decreases the term ε_b of Equation (3), because the plant accumulates less biomass per unit leaf area than a well-watered plant. This is reversible, because stomata can reopen when more water is available after a rain or watering. In contrast, the heat stress caused by stomatal closure can result in permanent damage to the photosynthetic apparatus, thereby decreasing ε_b for the rest of the crop cycle. Conversely, WUE usually increases with stomatal closure, because photosynthesis and stomatal conductance are linked with a non-linear relationship. The reduction in photosynthesis affects kernel weight and also the proportion of reproductive organs that develop into seeds. In a number of species, the latter is related to sugar metabolism (Zinselmeier et al., 1999).

The advantages and drawback of an early stomatal closure are similar to those of a reduction in leaf area. This reduces transpiration rate and soil water depletion, but also cumulated photosynthesis. It is therefore advantageous only in case of severe deficit. However, an early stomatal closure has an additional drawback, namely leaf heating. Transpiration contributes to maintain leaves at temperatures compatible with their metabolism, so stomatal closure causes an increase in leaf temperature. One can therefore avoid a water shortage at the cost of a heat stress (Tardieu, 2012).

Duration of the crop cycle

A third effect of water deficit is to affect the duration of the crop cycle, thereby affecting the terms Σ, T, and W. In most species, water deficit affects the duration of the crop cycle by accelerating senescence. This is due to an early expression of genes associated with remobilization of proteins, which are redirected from leaves to reproductive organs (Pic et al., 2002). This reduction in the duration of the crop cycle is an adaptive mechanism, since it allows the plant to complete its cycle earlier while there is still water in the soil, and redirects assimilates to the reproductive organs. This reduces the total intercepted light and, therefore, the biomass accumulation in Equation (3), and the total transpiration in Equation (4). It may also affect the seed weight (Equation 6) if the seed number is not reduced in the same proportion as the reduction in biomass accumulation.

Genetic variability exists in the degree of maintenance of the green leaf area (Borrell et al., 2000), and a breeding strategy has been developed, aimed at maintaining photosynthesis in leaves for a longer duration ("stay-green"). This strategy is adequate for soils with an appreciable water reserve, and may otherwise cause severe stress at the end of the crop cycle through increased transpiration (Hammer et al., 2006).

GENETIC STRATEGIES FOR YIELD MAINTENANCE UNDER WATER DEFICIT

The above paragraphs provide an understanding of possible strategies for improving yield under water deficit. They also suggest that the maintenance of biomass accumulation under water deficit should be considered as an optimization process between transpiration, biomass accumulation, and its partitioning between root and shoot, rather than as a tolerance process *per se*. It follows that a given trait can have positive, null, or negative consequences, depending on the drought (Chapman et al., 2003; Hammer et al., 2006; Vargas et al., 2006; Tardieu, 2012).

Escape strategy

The escape strategy consists of adapting the crop cycle to water availability and evaporative demand, usually by reducing its duration, thereby reducing the total demand for water and avoiding severe terminal stresses. It leads farmers to choose species and genotypes according to local environmental conditions. It is also a strategy adopted by some desert plants that have a very rapid cycle after rain, and finish this cycle before the occurrence of water deficit. For a given genotype, it also consists of reducing the duration of the cycle, thereby reducing the total demand for water and avoiding severe terminal stresses. This strategy saves water but also reduces the accumulated photosynthesis during the crop cycle (Equation 3). Therefore, it consists of a trade-off between a lower risk of terminal stress against a reduced potential yield.

Avoidance strategy

The avoidance strategy consists either of the maintenance of transpiration rate under water deficit achieved by improving the size, architecture, or hydraulic conductance of the root system (de Dorlodot et al., 2007) or a reduction in the demand for transpiration by stomatal closure or reduction in leaf area.

Maintenance of transpiration rate under water deficit via the root system. This strategy is observed when the improvement of the root system increases access to soil moisture, i.e., in deep soils. In contrast, when roots grow in a limited volume of soil because of physical barriers (e.g., a hard layer due to compaction) or chemical barriers (e.g., acid soil), improvement of in ability of the root system to rapidly take up water can be detrimental.

This is because soil depletion occurs more rapidly, thereby causing severe stress at the end of the season (Tardieu et al., 1992), and because the assimilates invested in roots would be better invested in other organs. Accordingly, while a number of genetic studies of root systems have shown a positive association between yield and root features (Tuberosa et al., 2002), some programs to improve yield under water deficit have resulted in a reduced root biomass (Bolanos et al., 1993; Bruce et al., 2002), or decreased conductivity of the root system (Richards and Passioura, 1989). Therefore, this strategy is a trade-off between a greater carbon investment in roots against an expectation of higher water uptake, which only occurs if soil properties allow the higher uptake.

Reduction in transpiration by stomatal closure or reduction in leaf area. Stomatal closure and reduction of leaf growth rate under water deficit has been selected by evolution to reduce the risk of failure at the end of the growing season, because they both reduce the plant demand for water. However, they intrinsically reduce the yield expectation by decreasing the proportion of light intercepted by leaves (ε_a, Equation 3), and/or the efficiency of the transformation of light into biomass, which follows stomatal conductance (ε_b, Equation 3). It is noteworthy that many experiments in pots that identify "drought-tolerant" plants, in fact, use this strategy (e.g., Iuchi et al., 2001) Therefore, this strategy trades off a lower risk of plant failure against lower potential biomass production.

Growth maintenance

Unlike in the other strategies described so far, that of growth maintenance consists of continued growth of the most important organs, thereby maintaining yield. However, the maintained transpiration may cause a crop failure at the end of the crop season. Therefore, this strategy exchanges the maintenance of yield potential for a high risk of crop failure.

Maintenance of leaf growth. The maintenance of leaf growth under water deficit allows better light interception, thereby increasing photosynthesis but also increasing the transpiration rate and soil water depletion. Therefore, it is appropriate in many cases, although not for severe, terminal water deficits. It is noteworthy that, in one mapping population, half of the QTLs for sensitivity of leaf growth overlapped with those of silk growth (Welcker et al., 2007), suggesting that mechanisms favoring expansive growth may also favor reproductive development.

Maintenance of reproductive growth. The maintenance of reproductive growth around the time of flowering allows the maintenance of capacity for storage of photoassimilates later in the crop cycle, thereby increasing HI (Equations 3, 4) and decreasing A, the proportion of aborted reproductive organs (Equation 6). This strategy has been successful in several species, in particular maize, via the assessment of the anthesis-silking interval (ASI), which is typically increased by water deficit and negatively correlated with yield. Phenotypic selection under well-managed stress environments for low ASI has produced large genetic gains and resulted in significant impacts (Bolanos and Edmeades, 1993b; Ribaut et al., 2004).

Increase in water-use efficiency

An increase in WUE may seem to be the ideal candidate mechanism for drought-prone environments. In crops, WUE has been regarded as a "conservative strategy" involving reduced transpiration, such that the positive influence of a higher WUE on yield may be reduced under moderately favorable environments and become a penalty under the most favorable conditions (Rebetzke et al., 2002; Richards et al., 2002). This strategy has been used in wheat for Australian environments, where water must be used conservatively to allow the crop to complete its life cycle. It has led to the release of two cultivars (Condon et al., 2004).

Increase in harvest index

Finally, an increase in HI (Equations 3, 4) has been a major way of increasing yield, even under water deficit (Turner, 1997). Furthermore, a change in biomass allocation between stem, roots, and seeds has been a clear route to progress.

THE PROGRESSION OF DEVELOPMENTAL STAGES OF A PLANT CAN BE PREDICTED BY USING THERMAL TIME

WHY USE THERMAL TIME AND PLANT DEVELOPMENT MODELS?

The above paragraphs show that environmental stress has different consequences depending on the phenological stage at which it occurs in the plant. In particular, some stages such as flowering present a higher sensitivity to stresses, while others such as grain filling present a lower sensitivity. It follows that a genetic comparison can be biased if the stress occurs at different stages in each genotype, because some genotypes will encounter the stress at a sensitive stage while others will encounter it at a stage with lower sensitivity. This results in non-reproducible experiments.

Therefore, it is essential that the main phenological stages of each genotype are precisely recorded. This raises two problems. The first is that, because a key stage such as flowering can occur over one or more weeks in a population of genotypes, it is usually impossible to visit each day to obtain the flowering date of every individual genotype. The second is that some key stages are difficult and lengthy to determine. While emergence, leaf number or flowering time can be obtained in a straightforward way, determining other stages such as flower initiation requires a detailed analysis. However, these stages can often be determined from other phenological stages such as the number of leaves.

When an experiment is repeated in naturally fluctuating conditions, phenological stages occur at different dates in each experiment. The number of days after emergence cannot, therefore, provide a good prediction of the stages. For instance, the progression of leaf initiation on the stem generally differs between different experiments in the field or in the greenhouse (Granier and Tardieu, 1998; Granier et al., 2002). Therefore, we need a tool that can: (1) simulate the exact date of a given stage from several datapoints obtained at different dates; (2) compare the behavior of a given genotype in different experiments; and (3) estimate the dates of "hidden" stages, e.g., flower initiation or the beginning of stem elongation, from other stages that are easier to determine.

WHAT IS THERMAL TIME?

Rates are related to organ temperature with stable relationships

Biological processes have a rate that follows temperature, with a non-linear relation that resembles the enzymatic responses to

temperature (**Figure 4**) (Parent et al., 2010a; Parent and Tardieu, 2012). However, in a restricted range of temperature, this relationship can be considered as linear in pea (Turc and Lecoeur, 1997) and sunflower (Granier and Tardieu, 1998). In the latter, the same relationship held for plants grown in the growth chamber, in the greenhouse or in the field. In a study of the relationship between meristem temperature and maize leaf elongation rate over 15 field experiments, three growth chamber experiments and three greenhouse experiments at night (i.e., in the absence of evaporative demand), using a single genotype (hybrid Dea), the same relationship was found to apply to all three conditions (Tardieu, 2003). Marked differences in slopes between inbred lines were observed consistently over successive experiments. The slope is therefore a stable characteristic of a genotype (Reymond et al., 2003). These relationships only apply during the night. Elongation rates at a given temperature are lower during the day owing to the effect of evaporative demand, which is taken into account by a second relationship.

Several conclusions can be drawn:

- If the same relationship holds for experiments in different places, years, and experimental conditions, this means that the temperature dependence of rates is a stable characteristic of a genotype. One can therefore calculate a common thermal time for all genotypes of a mapping population.
- The relationships presented in **Figure 4** imply that rates can be deduced from the temperature. For example, in **Figure 4**, twice as many leaves will have been initiated at 26°C than at 16°C. Alternatively, it can be stated that the time sensed by the plant elapses twice as rapidly at 26°C as at 16°C. This is the intuitive basis for thermal time: thus, plants "sense" thermal time rather than calendar time, and thermal time depends on temperature. The x-intercept of this relationship is termed the "threshold temperature." If the relationship were linear across the whole range, this threshold temperature would be the temperature at which the rate is zero. This is not usually the case, since the response tends to be curvilinear at low temperatures, hence the threshold temperature has rather a statistical definition.
- Several processes such as leaf appearance rate, cell division rate, or leaf growth rate have a common relationship with temperature over the whole range 6–35°C (Parent and Tardieu, 2012). This means that thermal time as sensed by several processes or organs is the same. It is, therefore, the "physiological age" of the plant.

Calculation of thermal time

Thermal time depends on the existence of the linear relationships described above. The first relates temperature to the rates of processes involved in leaf growth:

$$dP/dt = a(T - T_0) \tag{7}$$

where P is the studied process (e.g., expansion, cell division, or leaf initiation), T is the current temperature, a and T_0 are the slope, and the x-intercept, respectively, of the relationship between dP/dt and T. The second relationship involves the reciprocal of the duration of the studied process:

$$1/d = b(T - T_0) \tag{8}$$

where d is the time during which expansion (or any other developmental process) occurs in a given leaf, or the time during

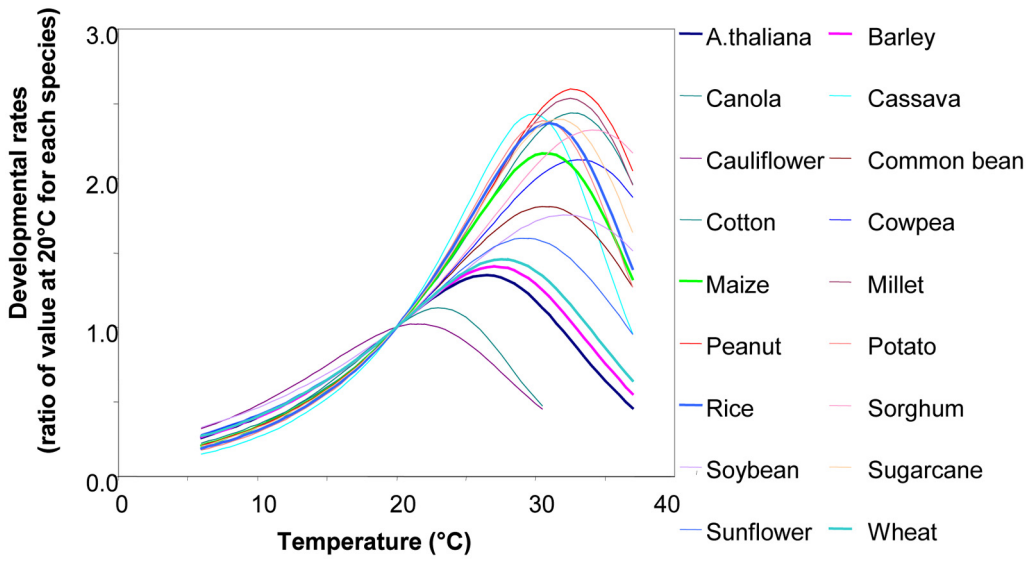

FIGURE 4 | The relationship between temperature and developmental rates for 18 species, e.g., leaf appearance rate, leaf or root elongation rates, germination rates, and reciprocal of the duration of cycle phase (but *not* photosynthetic rate and enzymatic rates involved in carbon metabolism). Rates are presented as a ratio of their value at 20°C, so thermal time can be expressed as equivalent days at 20C°. Redrawn from Parent and Tardieu (2012). Data and equations can be found in Parent and Tardieu (2012) (SI). See also Parent et al. (2010a,b).

which leaf initiation occurs on the apex. It follows that, at time d:

$$P = a \sum_{0}^{d} (T - T_0) dt \quad (9)$$

$\sum_0^d (T - T_0)\ dt$ is commonly named thermal time (unit of °Cd, when calculated with a daily timestep).

This calculation can easily be carried out using a spreadsheet, where each line represents a date. First, the mean temperature for each day must be calculated. An efficient way for that is to consider the average of the maximum and minimum temperatures, which are usually available in weather stations. The thermal time elapsed during a given day is the difference between the mean temperature of the day and threshold temperature of the considered species. This is available in the literature (e.g., 10°C for maize, 11°C for sorghum, 13°C for rice, etc.). The thermal time for a given period of time is the sum of the thermal times of all days in question.

However, when the temperature sensed by plants decreases below 15°C or reaches temperatures higher than the optimum temperature (see optimum temperatures of main crop species in Parent and Tardieu, 2012), the calculation presented above can cause serious bias. In this case, and in the general case for some species-like rice, another calculation of thermal time should be preferred, which takes into account the plant response in the whole range of temperature. This alternative method is presented in Parent and Tardieu (2012) and Parent et al. (2010b), with associated spreadsheets. Crop models such as APSIM use a series of linear relations that approximate the general relation (Hammer et al., 2010).

THE DEVELOPMENT OF PLANTS FOLLOWS A PROGRAMME THAT IS STABLE FOR A GIVEN GENOTYPE

A model of plant development can be built at the whole plant level, using the method presented previously. The occurrence of several phenological stages of the plant can be predicted, depending on thermal time. For example, **Figure 5** (Chenu et al., 2008) presents the number of leaves that have been initiated, the number that are visible, and the number that have stopped growth as a function of thermal time after emergence. Presented relations summarize different experiments and different plants in each experiment. For instance, leaf 10 was initiated at 90°Cd, was visible at 320°Cd, and ceased elongation at 490°Cd. If we consider all experimental points, three regression lines appear which allow prediction of the phenological stages. It can therefore be assumed that, in any experiment in any place in the world, leaf 10 of this genotype stops elongation 490°Cd after emergence. As an example, it has been checked that a common development model for sorghum was valid in both Mali and in Montpellier, France (Lafarge and Tardieu, 2002).

The model of plant development summarized in **Figure 5** can be read in two ways. First, if considered vertically, for instance at 400°Cd, it indicates the number of initiated leaves and leaves that have ceased expansion on that day. Thus, it is a "snapshot" of the plant on a given day. Then, if considered horizontally, it indicates the development of each organ. If a phenological stage has not been recorded exactly when it occurred, it can be inferred from measurements carried out before and after the date in question. For instance, if the date of plant emergence occurs between two visits to the experiment, it can be reconstructed by recording the leaf number at 2 or 3 dates, and calculating the date at which the leaf number would be zero.

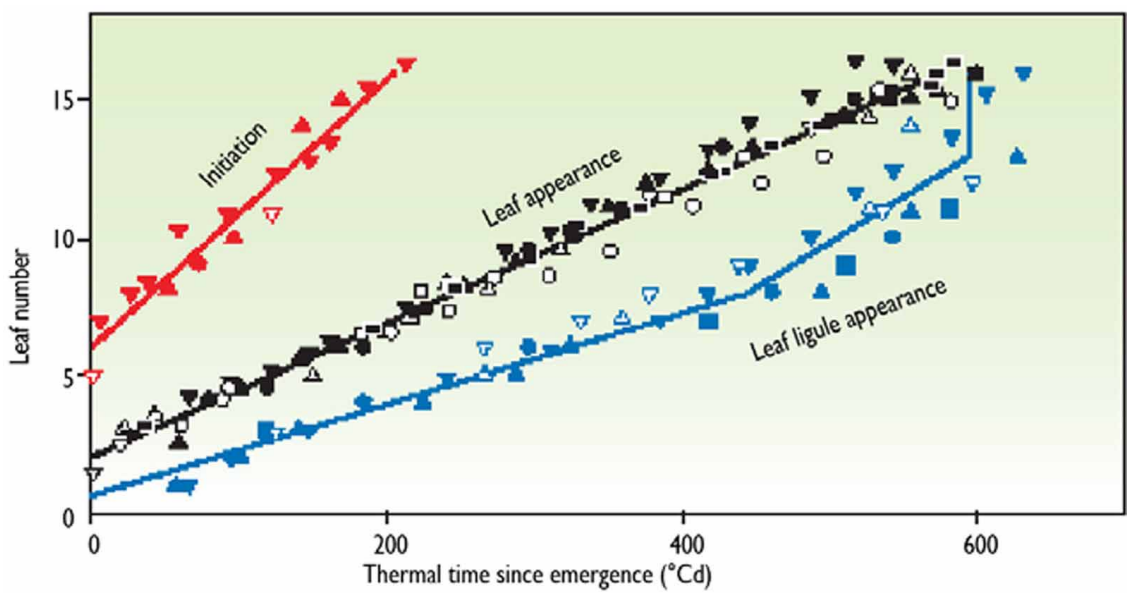

FIGURE 5 | Example of a model of plant development in maize. Initiation, leaf appearance, and ligule appearance in maize are expressed as a function of thermal time. The y axis represents each position of the leaf on the stem. For each leaf, the beginning and end of linear elongation occurred at a common thermal time in all experiments (redrawn from Chenu et al., 2008).

Several experiments can be analyzed jointly, and the timing of stresses of each experiment, and each genotype can be placed on a single scale of development. This is of considerable help in the interpretation of a network of experiments, which would otherwise have apparently erratic behavior.

A MINIMUM DATASET FOR USING METHODS DESCRIBED HERE

The above methods allow comparison between experiments, genotypes, and treatments, provided that a minimum set of measurements is collected. This dataset is currently used in several consortium, e.g., http://www.drops-project.eu. These are described below.

KEY DATES OF THE CROP CYCLE

Although this information is relatively straightforward, it is frequently missing, meaning that none of the methods presented above can be used. The most important dates are sowing, emergence, flowering, and physiological maturity (harvest). It is useful to record intermediate stages such as leaf number, which allow recalculation of missing stages by using thermal-time-based interpolation as described above.

DAILY IRRADIANCE OR PHOTOSYNTHETIC PHOTON FLUX DENSITY

This information about available light is essential because: (1) it is an input for calculating the soil water balance; and (2) it allows estimation of the potential biomass accumulation in the environment in question. Irradiance (I_r, measured in W m^{-2}) is better suited for the first use and is provided by pyranometers, while PPFD (mol m^{-2} s^{-1}) is better suited to the second use, and is provided by PAR sensors. Because either variable can be translated into to the other under field conditions, both are acceptable.

Light intensity has a relatively low site specificity. It is acceptable to record data from a weather station located at several kilometer distance provided that: (1) the weather station is in the same geographical situation as the experimental field (altitude etc.); and (2) there can be reasonable confidence in the data (especially if missing data are not too frequent, if the sensors are of satisfactory quality, etc.). In contrast, special care has to be taken in greenhouse and growth chamber experiments because of the high spatial variability (both horizontal and vertical) of light in these environments. A map of light intensity, or at least the use of several sensors, is recommended.

AIR TEMPERATURE

Together with irradiance, information on the air temperature (T) is necessary for calculating the soil water balance. It also allows estimation of thermal time if plant temperature is close to air temperature. This is usually acceptable for well-watered adult plants, but is prone to large errors during early phases in monocot species and in plants subjected to water deficit. It allows estimation of the occurrence of high temperature stresses (e.g., at $T > 40°C$), of risks of oxidative stresses (e.g., at $T < 3°C$ and PPFD $>$ 1000 mol m^{-2} s^{-1}), and of phenological stages, with the use of thermal time. This information must be recorded close to the experimental field using a local weather station or a data logger with thermocouples. Data can be recorded at daily intervals as minimum and maximum temperatures. The data need to be measured at plant height in greenhouse or growth chamber experiments.

AIR RELATIVE HUMIDITY, VAPOR PRESSURE DEFICIT, AND REFERENCE EVAPOTRANSPIRATION

These three variables quantify the evaporative demand, which is essential for estimating stress levels, for characterization and for calculation of the soil water balance. They provide essentially the same information, but with different time scales and usefulness. Relative humidity (RH), expressed as a percentage and VPD (in kPa) are calculated on short timescales (minute to hour), and ET_0 (in mm per day) is on a daily timescale. The variable recorded in the database would be ET_0, either calculated from other climatic data (I_r, VPD, and T wind speed) recorded in a datalogger (see above), or directly calculated by the weather station. ET_0 is species-independent and calculated by energy balance.

RH and wind speed have relatively low site specificities. As in the case of air temperature, it is acceptable to record these data from a weather station located at several kilometer distance. RH in greenhouse and growth chamber experiments should be recorded with replications, because of the large spatial variability. A method for calculating ET_0 is available at: http://www.fao.org/docrep/X0490E/x0490e04.htm#reference%20crop%20evapotranspiration%20(eto).

It might be useful here to emphasis on two frequent errors:

- RH should not be interpreted *per se*, because it does not characterize the evaporative demand when the air temperature is fluctuating. The use of both RH and air temperature allow a very simple calculation of VPD, which is the driving force for transpiration. Extreme events such as the sirocco should be recorded as daily maximum VPD over a period of 3 or 4 h.
- Mean daily air VPD or RH recordings are not acceptable for characterizing the daily evaporative demand; ET_0 should be used.

RAINFALL AND IRRIGATION

Recordings should be made near the field (<300 m distant) because of very high spatial variability. Simple rain gauges are efficient and inexpensive but require frequent visits, while automatic rain gauges connected to a datalogger are more expensive but are useful in distantly located experiments.

INITIAL SOIL WATER CONTENT IN THE FIELD

The water balance data begin at a given date (e.g., emergence), at which time the soil water content must be recorded. This can be done with augers over a depth similar to the final rooting depth, with particular attention to spatial variability in the field. This measurement is important especially in experiments where the rainfall is zero or negligible. Some "shortcuts" can be acceptable, especially when either the rainfall or irrigation before the experiment is sufficient to guarantee that the soil is at retention (or field) capacity.

SOIL HYDRAULIC PROPERTIES

These are essentially the variables that allow calculation of the limits of soil water reserves, namely the field capacity and the limit

of water extraction. These should be measured in experimental fields where drought experiments often take place, using equipment that measures soil water content, e.g., neutron probes or time domain reflectometry (TDR). These properties can also be inferred from the soil texture (e.g., loamy sand, clay loam, etc.) and the estimated rooting depth.

LIGHT INTERCEPTION

With current techniques, it is usually not feasible to measure LAI of all genotypes in an experiment. LAI can be measured by collecting all leaves on a sample soil area and measuring their area. It can also be measured indirectly and non-destructively using sensors that directly measure the proportion of intercepted light. Finally, novel imaging technique with NDVI remote sensing will shortly allow one to measure leaf area of all genotypes in an experiment.

CONCLUDING REMARKS

It is not possible to present in detail here how to use each method for each species However, it can be stressed that the tools presented here help in the interpretation of data gathered from networks of experiments, and that they are the base of all existing plant models.

The potential production of each site can be calculated from the development model, which provides an estimate of leaf area, and the available light. For instance, the biomass accumulation in cloudy years is lower than that of bright years, if water is not seriously limiting yield. In the same way, a hot year reduces yield even in the absence of heat stress or water stress, by reducing the duration of the crop cycle. It is particularly useful to compare the potential productivity of experimental sites and years, in order to distinguish the natural variability of yield linked to light availability from the effects of stressing events.

The soil water balance can be calculated for each genotype, provided that a minimum dataset has been collected. This requires estimation of the change with time in leaf area of each genotype. The latter information can be inferred from measurements of "probe genotypes" having approximately the same cycle duration as a class of genotypes under examination. Once leaf area has been estimated, it is possible to calculate the proportion of evapotranspiration needed by the genotype in question in comparison with the reference level of evapotranspiration.

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The statistical analysis of multi-environment data: modeling genotype-by-environment interaction and its genetic basis

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Genotype-by-environment interaction (GEI) is an important phenomenon in plant breeding. This paper presents a series of models for describing, exploring, understanding, and predicting GEI. All models depart from a two-way table of genotype by environment means. First, a series of descriptive and explorative models/approaches are presented: Finlay–Wilkinson model, AMMI model, GGE biplot. All of these approaches have in common that they merely try to group genotypes and environments and do not use other information than the two-way table of means. Next, factorial regression is introduced as an approach to explicitly introduce genotypic and environmental covariates for describing and explaining GEI. Finally, QTL modeling is presented as a natural extension of factorial regression, where marker information is translated into genetic predictors. Tests for regression coefficients corresponding to these genetic predictors are tests for main effect QTL expression and QTL by environment interaction (QEI). QTL models for which QEI depends on environmental covariables form an interesting model class for predicting GEI for new genotypes and new environments. For realistic modeling of genotypic differences across multiple environments, sophisticated mixed models are necessary to allow for heterogeneity of genetic variances and correlations across environments. The use and interpretation of all models is illustrated by an example data set from the CIMMYT maize breeding program, containing environments differing in drought and nitrogen stress. To help readers to carry out the statistical analyses, GenStat® programs, 15th Edition and Discovery® version, are presented as "Appendix."

Keywords: adaptation, genotype by environment interaction, multi-environment trials, QTL by environment interaction, QTL mapping methodology, REML

INTRODUCTION: PHENOTYPE, GENOTYPE, AND ENVIRONMENT

The success of a plant breeding program depends on its ability to provide farmers with genotypes with guaranteed superior performance (phenotype) in terms of yield and/or quality across a range of environmental conditions. To achieve this aim, it is necessary to have an understanding of the factors leading to a good phenotype.

Usually the phenotype is the value for a trait at the end of the growing season. The reason is that we are primarily interested in phenotypes like yield or grain weight at maturity and not, or less, in yield or grain weight at earlier stages. The final state of a trait is the cumulative result of a number of causal interactions between the genetic make-up of the plant (the genotype) and the conditions in which that plant developed (the environment). Plants differ in the efficiency and adequacy with which they capture and convert environmental inputs and stimuli into the biomass and organs that constitute a final product. The capture and conversion abilities of a plant are determined by its particular ensemble of genes. Environments differ in the amount and quality of inputs and stimuli that they convey to plants including, e.g., the amount of water, nutrients or incoming radiation. A primary objective in plant breeding is to match genotypes and environments in such a way that improved phenotypes are obtained. For example, a breeder might be interested in selecting genotypes that do well under water stress conditions.

While there can be genotypes that do well across a wide range of conditions (widely adapted genotypes), there are also genotypes that do relatively better than others exclusively under a restricted set of conditions (specifically adapted genotypes). Specific adaptation of genotypes is closely related to the phenomenon of genotype-by-environment interaction (GEI). GEI exists whenever the relative phenotypic performance of genotypes depends on the environment, or in other words, when the difference in reactions of genotypes varies in dependence on the environment.

To illustrate the phenomenon of GEI, we can consider two different genotypes that differ in the genetic machinery involved in tolerance to water-limited conditions, while being equal for all other characteristics. If these two genotypes are exposed to a poorly watered environment, their performance will differ depending on the genetic properties related to tolerance for

water-limited conditions. However, this genotypic difference will disappear in an environment that provides the right amount of water. So, the difference in performance between the two genotypes depends on the environment, through the amount of water that it provides.

Some scenarios that can occur when comparing the performances of pairs of genotypes across environments are presented in **Figure 1**. The function describing the phenotypic performance of a genotype in relation to an environmental characterization is called the "norm of reaction" (Griffiths et al., 1996). **Figure 1A** shows the case where there is no GEI, the genotype and the environment behave additively (this will be developed later) and the reaction norms are parallel. The remaining plots show different situations in which GEI occurs: divergence (**Figure 1B**), convergence (**Figure 1C**), and the most critical one, crossover interaction (**Figure 1D**). Crossover interactions are the most important for breeders as they imply that the choice of the best genotype is determined by the environment.

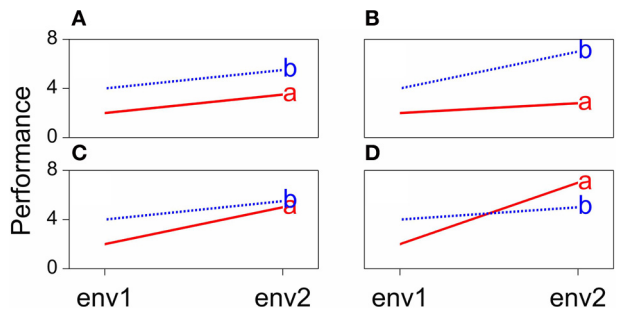

FIGURE 1 | Genotype-by-environment interaction in terms of changing mean performances across environments: (A) additive model, (B) divergence, (C) convergence, (D) cross-over interaction.

GEI was introduced in terms of the relative difference between genotypic means. GEI can also be regarded in terms of heterogeneity of genetic variance and covariance, or correlation. As a consequence of GEI, the magnitude of the genetic variance as observed within individual environments will change from one environment to the next. Often, the genetic variance tends to be larger in better environments than in poorer environments, although the opposite can be observed as well (Przystalski et al., 2008). **Figure 2A** illustrates the phenomenon of heterogeneity of genetic variance across environments, showing box plots for a series of maize trials, where the range of variation in the poor environments LN96a and LN96b is smaller than that in the good environments HN96b and NS92a.

GEI has also consequences for the correlations between genotypic performances in different environments. When GEI is large, the observed performance of a set of genotypes in one environment may not be very informative for the performance of the same genotypes in another environment. Environments with similar characteristics will induce corresponding responses in plants and will lead to strong genetic correlations. **Figure 2B** shows that the correlation between the similar environments IS92a and IS94a is larger than the correlation between the dissimilar environments NS92a and HN96b.

In conclusion, given the complexity of the mechanisms and processes underlying the phenotypic response across diverse and changing environmental conditions—frequently in an unpredictable way—it is necessary to develop analytical tools to help breeders understand GEI. The use of adequate strategies to analyze GEI is a first and important step toward more informed breeding decisions. Good analytical methods are a prerequisite for predicting the performance of genotypes as accurately as

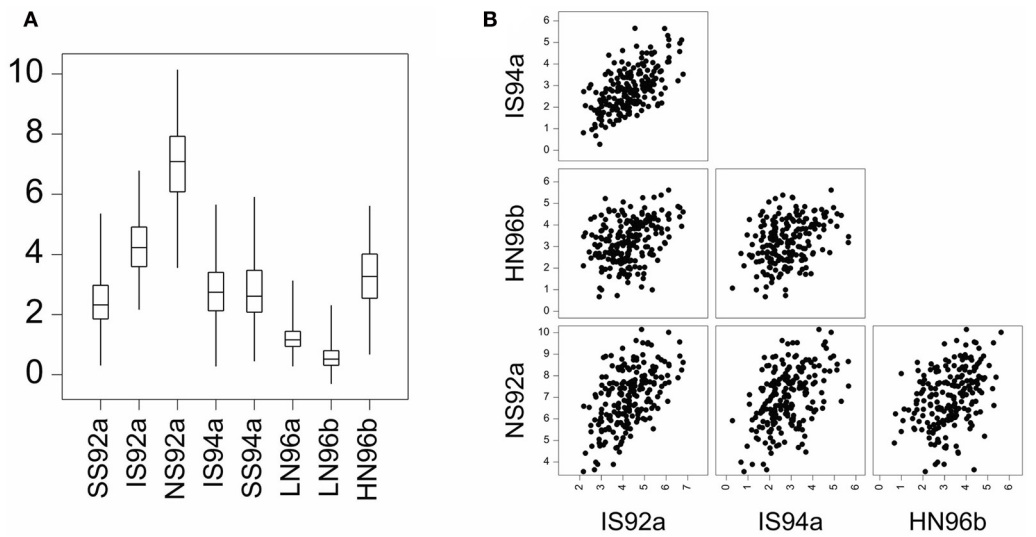

FIGURE 2 | (A) Boxplot for yield of a maize F2 population in eight environments displaying total range, interquartile range (box) and median (line). Environment names are coded as: LN, low nitrogen; HN, high Nitrogen; SS, severe water stress; IS, intermediate water stress; NS, no water stress. The two digits indicate the year of the trial, and the letters a and b the cropping season: a, winter; b, summer. **(B)** Scatter plot matrix for two stress environments (IS92a, and IS94a) and two non-stress environments (HN96b and NS92a).

possible. This paper explores several strategies to model GEI, starting with simple methods that have been historically popular within the plant breeding community. It then moves to more elaborate models in which additional information is used in the form of explicit environmental characterization to model GEI. A final section is devoted to the integration of molecular marker information into GEI models, leading to the detection of quantitative trait loci (QTLs) and more specifically, to the modeling of QTL by environment interaction (QEI). The statistical methodology is illustrated using a maize data set obtained from a series of drought and nitrogen stress trials from the maize breeding program at Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT; the International Maize and Wheat Improvement Center; Ribaut et al., 1996, 1997). To encourage readers to carry out these statistical analyses themselves, GenStat® programs for the 15th Edition (VSN International, 2012) and the Discovery® version of this statistical package (Payne et al., 2007) are presented as "Appendix."

GENERATING DATA TO STUDY GENOTYPE-BY-ENVIRONMENT INTERACTION

An obvious first step to investigate GEI is to obtain phenotypic observations on a set of genotypes exposed to a range of environmental conditions. The set of genotypes can include advanced lines of a breeding program, cultivars, and segregating offspring from a specific cross such as F_2, a backcross, or a recombinant inbred line (RIL) population.

Genotypes can be tested under different management regimes that represent increasing levels of a particular stress, or a combination of stresses. This type of experiment is called a "managed stress trial" and is appropriate when the researcher wishes to focus on a particular type of stress. When performing managed stress trials, it is important to control the system in such a way that all other factors influencing the phenotype are as homogenous as possible.

Managed stress trials are not a default option in plant breeding, because stress type and level can be difficult to implement and because the relationship between phenotype and stress is complex, with genes and environmental stress(es) interacting throughout the various developmental phases. In those situations, a common way for plant breeders to screen for genotypic reactions to environmental factors is by "multi-environment trials" (METs). In a MET, a number of genotypes are evaluated at a number of geographical locations for a number of years in the hope that the pattern of stresses that the genotypes experience is representative of future growing environments.

A convenient way to summarize data from managed stress trials and METs is in the form of two-way tables of means, with genotypes in the rows and environments in the columns. Each cell of such a table contains an estimate of the performance (adjusted mean) of a particular genotype in a specific environment. To identify genotypes and environments unequivocally, we use indices, the letter i for genotypes ($i = 1 \ldots I$), and the letter j for environments ($j = 1 \ldots J$).

The models in the following sections will assume as a starting point a genotype-by-environment table of means. These models are used in a so-called two-stage strategy for analyzing MET data. In the first stage, individual trials are analyzed with models including terms for design features and spatial variation. From these individual trial analyses, adjusted means and weights, usually reciprocals of the variances of the means, are carried forward to the second stage, where a model is fitted to the genotype by environment means, using either no weights or weights estimated in the first stage. Various choices can be made for the weights in a two stage analysis (Mohring and Piepho, 2009; Welham et al., 2010), and a good choice of weights will lead to a two-stage analysis with results very close to those of a so-called single stage analysis, in which plot data are analyzed instead of means. Single stage analyses have certain theoretical advantages over two-stage analyses, but two-stage analyses are logistically and computationally easier to handle. This paper focuses on two-stage analyses, because of the small differences with single stage analyses and the aforementioned larger handling ease. Still, good descriptions of single stage analyses are offered by Cullis et al. (1996a,b), Gilmour et al. (1997), and Smith et al. (2005). In principle, the QTL mapping approach outlined later in this paper could also be embedded in a single stage analysis strategy.

CIMMYT MAIZE DROUGHT STRESS TRIALS: EXAMPLE DATA

The models to be presented here are illustrated using data produced by the maize drought stress breeding program of CIMMYT. A brief description of the data is given here, a more detailed description is available in the original publications (Ribaut et al., 1996, 1997). A maize F_2 population was generated by crossing a drought tolerant parent (P_1) with a drought susceptible one (P_2). Seeds harvested from each of 211 F_2 plants formed F_3 families, which were stored for further evaluation. The F_3 families were evaluated in managed stress trials in 1992, 1994, and 1996. In the winter of 1992, a managed water stress trial was conducted in Mexico, including no stress (NS), intermediate stress (IS), and severe stress (SS). In the winter of 1994, a similar trial was conducted, but it only included the IS and SS treatments. In the summer of 1996, the families were tested in a nitrogen stress trial with two levels: low (LN) and high nitrogen (HN). An extra LN trial was conducted in the winter of the same year. In total, the families were evaluated in eight different environments, each environment characterized by year, stress type and intensity, and management factors. DNA was extracted from each of the 211 F_2 plants to produce a total of 132 restriction fragment length polymorphism (RFLP) markers covering the 10 maize chromosomes.

MODELS FOR GENOTYPE-BY-ENVIRONMENT INTERACTION: MODELING THE MEAN

THE ADDITIVE MODEL AS A BENCHMARK

The phenomenon of GEI is of primary interest in plant breeding, and has resulted in a large body of literature on models and strategies for analysis of GEI [see, for example, the reviews in Cooper and Hammer (1996), Kang and Gauch (1996), van Eeuwijk et al. (1996), van Eeuwijk (2006)]. A dominant feature of strategies used to describe and understand GEI is a heavy reliance on parameters that are statistical rather than biological. This is no coincidence, since historically, a large part of quantitative

genetics has relied on simple, yet very useful, statistical models. A notorious example is the well-known model: $P = G + E$, where P stands for phenotype, G for genotype and E for environment (Falconer and Mackay, 1996; Lynch and Walsh, 1998). A statistical formulation of this model for a two-way table of means can be written as:

$$\underline{\mu}_{ij} = \mu + G_i + E_j + \underline{\varepsilon}_{ij}. \quad (1)$$

From here onwards, in the model formulations, random terms are underlined to emphasize the fact that their effects are assumed to follow a normal distribution. Model 1 describes the response variable, that is, the mean of genotype i in environment j, $\underline{\mu}_{ij}$, as the result of the common fixed intercept term μ, a fixed genotypic main effect corresponding to genotype i, G_i, plus a fixed environmental main effect corresponding to environment j, E_j, and finally the random term, $\underline{\varepsilon}_{ij}$, representing the error term, typically assumed normally distributed, with a mean of zero and constant variance, σ^2; $\underline{\varepsilon}_{ij} \sim N(0, \sigma^2)$.

Model 1 predicts that for any genotype the difference means between any two environments j and j^* will be equal to the difference in the environmental main effects: $E_j - E_{j^*}$. Consequently, the norms of reaction of genotypes will be parallel (**Figure 1A**). Another important aspect is that, although the parameters in the model suggest that something intrinsically genetic and something intrinsically environmental is determining the trait, the genotypic and environmental effects purely follow from a convenient way of partitioning phenotypic variation from a statistical point of view. In a balanced data set, the genotypic main effects can be estimated from the average performance of the genotypes across environments. Rather than being something inherently genotypic, this is dependent on the set of environments used in the experiment. If a few environments are dropped, the genotypic effects will change. The same argument applies to the environmental main effects, which depend on the set of genotypes used in the experiment.

The results of the fit of an additive model to the maize data set are presented in **Table 1**. The results show that, according to the *F*-test, there is a significant environmental and genotypic main effect (the *F* statistic for environments equals 1466.5, and for genotypes 5.3, both of which are highly significant: $P < 0.001$). As just mentioned, environments are characterized by the average performance of the genotypes in the particular environment, and the results indicate that the environments differ significantly in their quality. In general, differences between environmental main effects are significant, and from the breeder's point of view, this is not a major concern. Breeders want to concentrate on differences between genotypes. A significant genotypic main effect indicates that genotypes differ in their average performance across environments, something certainly more interesting to breeders. Finally, it should be mentioned that the residual $\underline{\varepsilon}$ in **Table 1** corresponds to the discrepancy between the predicted genotype-by-environment means from an additive model and the observed means.

Table 1 | ANOVA table for the additive model (model 1), as applied to CIMMYT maize stress trials.

Term	Degrees of freedom	Sum of squares	Mean squares	*F*	Probability
E	7	5679	811.2	1466.5	<0.001
G	210	614	2.9	5.3	<0.001
$\underline{\varepsilon}$	1470	813	0.6		
Total	1687	7106	4.2		

There are two reasons for the disagreement between the predicted values from an additive model and the observed means for environment-specific genotypic performances: (1) an effect proper to the particular combination of genotype and environment; and (2) experimental error. Model 1 can be extended with an effect that is specific for genotype-by-environment combinations, GEI, or a double-indexed term GEI_{ij}:

$$\underline{\mu}_{ij} = \mu + G_i + E_j + GEI_{ij} + \underline{\varepsilon}_{ij} \quad (2)$$

When we are working on a two-way table of means, we cannot straightforwardly separate GEI from error. For that, we would need to develop a model based on plot observations. Use of model 2 implies estimation of as many parameters as there are genotype-by-environment combinations, something that is not desirable in the interest of parsimony. Another limitation of the model is that it is not possible to estimate the genotypic performance in environments that are not included in the trial. Accordingly, fitting model 2 could tell us something about the amount of variation due to genotypic main effects in relation to GEI, by comparing sums of squares or mean squares, but it does not bring much progress toward understanding GEI.

THE REGRESSION ON THE MEAN MODEL

A more attractive alternative is to extend the additive model (model 1) by incorporating terms that explain as much as possible of the GEI. A popular strategy in plant breeding is that proposed by Finlay and Wilkinson (1963), which describes GEI as a regression line on the environmental quality. In the absence of explicit environmental information, the biological quality of an environment can be reflected in the average performance of all genotypes in that environment. Good environments will have a high average genotypic performance, and bad environments will have a low average genotypic performance. The GEI part is then described by genotype-specific regression slopes on the environmental quality, and the model can be written in the following equivalent ways:

$$\underline{\mu}_{ij} = \mu + G_i + E_j + b_i E_j + \underline{\varepsilon}_{ij} \quad (3a)$$

$$\underline{\mu}_{ij} = G'_i + b'_i E_j + \underline{\varepsilon}_{ij} \quad (3b)$$

Model 3b follows from model 3a by taking $\mu + G_i = G'_i$ and $E_j + b_i E_j = (1 + b_i)E_j = b'_i E_j$. Model 3b is easier to interpret because it looks as a set of regression lines; each genotype has a linear reaction norm with intercept G'_i and slope b'_i. The explanatory environmental variable in these reaction norms is simply the environmental main effect E_j. Model 3a shows more clearly how GEI is captured by a regression on the environmental main effect,

with the hope that as much as possible of the GEI signal will be retained by the term $b_i E_j$.

In the regression on the mean model, GEI is explained in terms of differential sensitivities to the improvement of the environment, with some genotypes (the ones with larger values of b_i) benefiting more than others from an increase in environmental quality. Note that in model 3a, $\Sigma b_i = 0$, so that the average slope value is zero, while in model 3b the average value of b' is 1, meaning that $b' > 1$ for genotypes with a higher than average sensitivity, and $b' < 1$ for genotypes that are less sensitive than average.

Table 2 gives the fit of model 3a to the maize example data. The first two rows of the table, corresponding to the genotypic and environmental main effects, are identical to **Table 1**. The third row corresponds to the GEI effect in terms of the regression on environmental quality, where quality is represented by the environmental mean. This regression is highly significant, according to the *F*-tests ($F = 2.4$, $P < 0.001$). The residual sum of squares in **Table 1** ($SS_\varepsilon = 813$) has been divided into a part explained by genotypic sensitivities to environmental quality ($SS_b = 230$), and a residual ($SS_\varepsilon = 583$).

By way of example, the fitted reaction norms of five genotypes (out of the full set of 211 genotypes) are given in **Figure 3**, together with the parameters estimated according to the parameterization in model 3b (G' and b'). **Figure 3** shows that, in the average environment, genotypes G025 and G045 are better than G008, G012, and G016. The estimates for the parameters G' can be read-off from the plot as the fitted values at the null value of the *x*-axis, i.e., the average environment indicated by the dashed vertical line. Although G045 does slightly better than G025 in the average environment, G025 is superior to G045 in the high-quality environments. This is because G025 has a better ability to exploit improved environmental conditions, which is reflected in its higher genotypic sensitivity ($b'_{G025} = 1.27 > b'_{G045} = 0.99$). A similar observation can be made for G008 vs. G012 and G016. While G008 does relatively better in low quality environments, it is clearly surpassed by G012 and G016 in the best environments, since it is not capable of profiting from the better environmental conditions ($b'_{G008} = 0.65$, which is the lowest sensitivity among the five genotypes).

In summary, the regression on the mean model describes GEI in terms of parameters that can be given some biological meaning. In addition, and in contrast with the full interaction model (model 2), model 3 can be used to predict the performance of genotypes in environments that were not present in the MET, as long as the environment for which predictions are required can reasonably be placed within the range of environments used in the original MET. Nevertheless, the regression on the mean model suffers from the fact that the environmental characterization is based on a single dimension. Environmental quality can be hard to summarize within a single explanatory variable. Therefore, a substantial amount of GEI can remain unexplained. In the next section, the regression on the mean model will be extended by including multidimensional environmental characterizations in the statistical model for the genotype-by-environment data.

Table 2 | ANOVA table for the regression on the mean model (model 3), as applied to CIMMYT maize stress trials.

Term	Degrees of freedom	Sum of squares	Mean squares	*F*	Probability
E	7	5679	811.2	1752.3	<0.001
G	210	614	2.9	6.3	<0.001
Heterogeneity of slopes	210	230	1.1	2.4	<0.001
$\underline{\varepsilon}$	1260	583	0.5		
Total	1687	7106	4.2		

THE ADDITIVE MAIN EFFECTS AND MULTIPLICATIVE INTERACTIONS MODEL

The limitation of a single dimension in environmental characterization can be removed by employing a more flexible model, in which more than one environmental quality variable is allowed. A popular model of this type is the additive main effects and multiplicative interaction (AMMI) model (Gollob, 1968; Mandel, 1969; Gabriel, 1978; Gauch, 1988; van Eeuwijk, 1995). To emphasize the similarities with model 3a, we write the AMMI model as:

$$\underline{\mu}_{ij} = \mu + G_i + E_j + \sum_{k=1}^{K} b_{ik} z_{jk} + \underline{\varepsilon}_{ij} \qquad (4)$$

where the GEI is now explained by K multiplicative terms ($k = 1 \ldots K$), each multiplicative term formed by the product of a genotypic sensitivity b_{ik} (genotypic score) and a hypothetical environmental characterization z_{jk} (environmental score). Although genotypic and environmental scores are deemed to represent genetic and environmental qualities, they come from a

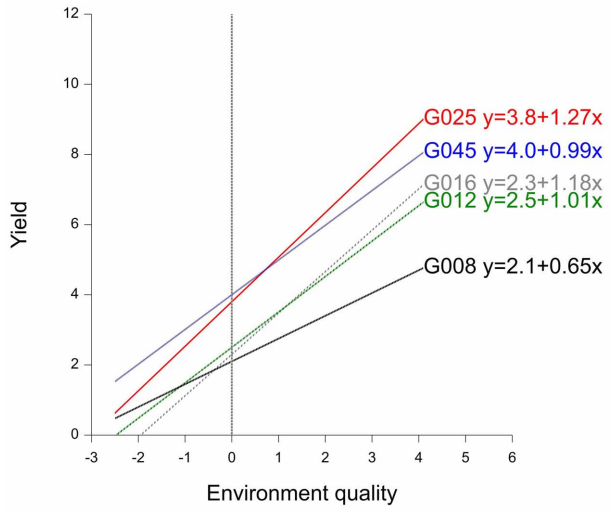

FIGURE 3 | Finlay–Wilkinson regression curves of five maize genotypes. The vertical line indicates the average environment. Next to genotype labels, the corresponding Finlay-Wilkinson regression equation is given.

mathematical procedure, a principal components analysis on the GEI (Gabriel, 1978; Gauch, 1988) that maximizes the variation explained by the products of the genotypic and environmental scores. The first product term is the one that explains most of the variation, followed by the second one, and so on. This is reflected in **Table 3**, which shows the results from the AMMI model to the maize example data. In the AMMI model, GEI is explained by two axes (principal component 1, PCA1, and principal component 2, PCA2) that are highly significant ($F = 2.8$ and 2.0 respectively, both with an associated $P < 0.001$). The first axis (PCA1) explains the largest part ($SS_{PCA1} = 242$), the second one explains a little less ($SS_{PCA2} = 173$), with a total explained sum of squares for GEI of $242 + 173 = 415$, an improvement over the explained sum of squares in the regression on the mean model ($SS_b = 230$).

Table 3 | ANOVA table corresponding to application of AMMI2 model (model 4) to CIMMYT maize stress trials.

Term	Degrees of freedom	Sum of squares	Mean squares	*F*	Probability
E	7	5679	811.2	1752.3	<0.001
G	210	614	2.9	6.3	<0.001
PCA1	216	242	1.1	2.8	<0.001
PCA2	214	173	0.8	2.0	<0.001
ε	1040	398	0.4		
Total	1687	7106	4.2		

PCA1 and PCA2 are the principal component axes 1 and 2, respectively.

A desirable property of the AMMI model is that the genotypic and environmental scores can be used to construct powerful graphical representations called biplots (Gabriel, 1978) that help to interpret the GEI. **Figure 4A** presents a biplot for the maize data. A first thing to recognize is that both genotypes and environments are present in the same plot; genotypes are represented by gray circles and environments by filled triangles (red, blue, and black). The environments are typically represented as axes intersecting at their origins. The origins represent the averages for the trait in the corresponding environments. The triangles point in the direction of increasing trait values. By projecting genotypes on environmental axes, GEI for individual genotypes is approximated. To help interpretation, environmental axes can be enriched by including a scale (Graffelman and van Eeuwijk, 2005).

Biplots facilitate the exploration of relationships between genotypes and/or environments. Genotypes that are more similar to each other are closer to each other in the plot than genotypes that are less similar. The same is true for environments. Genotypes/environments that are alike tend to cluster together. The angle between environmental axes is related to the correlation between the environments. An acute angle indicates positive correlation (e.g., between LN96a and LN96b), a right angle indicates no correlation (e.g., between HN96b and NS92a), and an obtuse angle indicates negative correlation (e.g., NS92a and LN96a). The projection of a genotype onto an environmental axis reflects the performance of that genotype in that environment (for GEI). For example, genotype G091 projects on the NS92a axis above the origin, indicating a positive interaction with that environment i.e., the relative performance (GEI part) of G091 in NS92a is above the

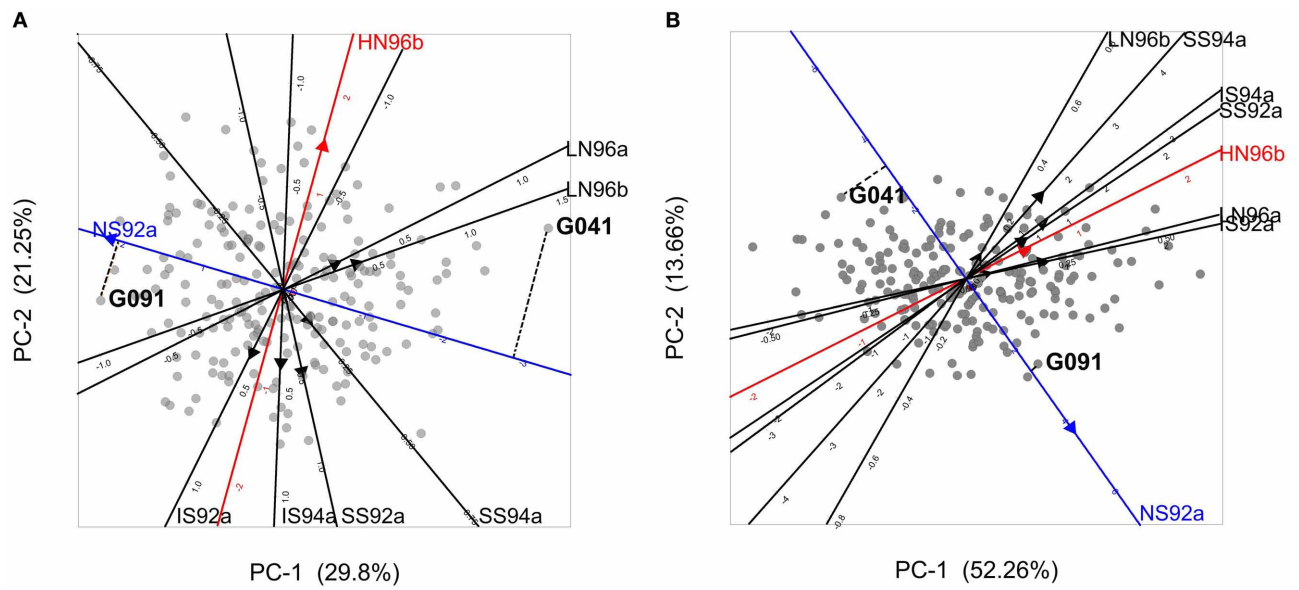

FIGURE 4 | (A) Biplot from the AMMI model used to describe GEI in the maize example data. Gray circles represent genotypes, and filled triangles environments, with triangles pointing in the direction of increasing GEI (at origin GEI = 0). The projection of two genotypes (G041 and G091) on the NS92a axis is shown by a dashed line. **(B)** GGE biplot for the maize data set, with same characteristics as of the AMMI biplot, except that triangles point in the direction of increasing overall performance (G + GEI), so the origin corresponds to the average performance of all genotypes in the particular environment. Projections for genotypes G041 and G091 are given.

average of all genotypes in NS92a. Conversely, genotype G041 (on the right hand side of the plot) projects below the origin on the same axis, which points to a negative interaction with environment NS92a (i.e., G041 performs worse than average). Following a similar procedure it is possible to conclude that while genotype G091 showed positive adaptation to environment NS92a, it is not well adapted to environments LN96a and LN96b (the projection of G091 on the LN96a and LN96b axes falls below the origin). Biplots are useful tools to investigate patterns in GEI, because they can help to identify interesting genotypes that are adapted to particular environments, and to classify environments in groups.

Plant breeders are interested in the total genetic variation and not exclusively in the GEI part. For that reason, it is useful to have a modification of model 4 that considers the joint effects of the genotypic main effect and the GEI as a sum of multiplicative terms. Effectively, the two-way table of genotype-by-environment means is exposed to a standard principal components analysis, with genotypes as objects and environments as variables (Yan et al., 2000). For this model, closely the same estimation and interpretation procedures hold as for model 4. Because genotypic scores now describe genotypic main effects G and GEI together, this type of model is also known as the "Genotype main effects and GEI model," or "GGE model" and the biplots are called "GGE biplots" (Yan et al., 2000). The model reads:

$$\underline{\mu}_{ij} = \mu + \mathrm{E}_j + \sum_{k=1}^{\mathrm{K}} \mathrm{b}_{ik}\mathrm{z}_{jk} + \underline{\varepsilon}_{ij} \tag{5}$$

The results of model 5 fitted to the maize data are presented in the form of a biplot in **Figure 4B**. GGE biplots approximate overall performance (G + GEI). This is in contrast to AMMI biplots, **Figure 4A**, that approximate only the GEI part of the phenotype. **Figure 4B** shows the high yielding genotypes concentrated on the right hand side of the biplot, with their projections on environmental axes covering the above average range (for example, G091 projects above the origin in NS92a, whereas G041 is found below the origin). In contrast, low yielding genotypes (as G041) are concentrated on the left hand side of the biplot (projects below origin in most of the environments).

FACTORIAL REGRESSION MODELS

The models discussed so far assumed that we do not have explicit information about the environments. While such models can be useful to explain GEI, the biological interpretation of their results is not always obvious. What do hypothetical environmental variables, as in AMMI, mean in terms of quantifiable environmental characteristics such as temperature, water, nutrients etc? A straightforward approach is to correlate environmental scores with environmental covariables. However, if we do have explicit information about the environment, the information can be used directly in the model by including it in the form of explanatory variables. GEI is then described as differential genotypic sensitivity to explicit environmental factors such as temperature, precipitation, water availability etc. Such models are known as factorial regression models (Denis, 1988; van Eeuwijk et al., 1996). Two examples of factorial regression models are given here. Model 6a includes a single environmental covariable, while model 6b includes multiple environmental covariables:

$$\underline{\mu}_{ij} = \mu + \mathrm{G}_i + \mathrm{E}_j + \mathrm{b}_i\mathrm{Z}_j + \underline{\varepsilon}_{ij} \tag{6a}$$

$$\underline{\mu}_{ij} = \mu + \mathrm{G}_i + \mathrm{E}_j + \sum_{k=1}^{\mathrm{K}} \mathrm{b}_{ik}\mathrm{Z}_{jk} + \underline{\varepsilon}_{ij} \tag{6b}$$

Models 6a and 6b look very similar to models 3a and 4, but there is a substantial difference between them. In models 6a and 6b, Z_j represents an explicit environmental covariable and not a hypothetical environmental covariable as in models 3a and 4 (note that Z is capitalized to highlight this difference). This distinction is critical since the interpretation of the GEI in models 6a and 6b is automatically placed into a biological context. Instead of describing GEI as differential reactions to hypothetical environmental covariables, factorial regression models help to identify genotypes that are differentially sensitive to changes in identified environmental quality components, for example, in a particular nutrient, or in water availability.

Table 4 shows the results of a factorial regression model fitted to the maize example data, in which GEI is explained by differential genotypic sensitivities to the minimum temperature during flowering (minTF, $F = 1.7$, $P < 0.001$) and to the amount of radiation during grain filling (radiationGF, $F = 1.2$, $P \leq 0.038$). In many cases, different combinations of explanatory variables could produce closely similar models in terms of the amount of explained GEI. Therefore, to arrive at biologically meaningful models, it is crucial to combine statistical criteria for model selection with physiological knowledge about the trait that is involved (Voltas et al., 1999a,b, 2002).

MIXED MODELS FOR GENOTYPE-BY-ENVIRONMENT INTERACTION: MODELING GENETIC VARIANCES AND COVARIANCES

In the introduction, it was mentioned that GEI can be regarded both in terms of differential mean responses across environments and in terms of heterogeneity of genetic variation and covariation between environments. While the models considered so far focus on modeling the mean response, the models in this section focus on the modeling of GEI in terms of heterogeneity of variances and covariances. This section switches to the framework of so-called mixed models. We concentrate on the main characteristics of a few, relatively simple yet powerful, mixed models that

Table 4 | ANOVA table corresponding to application of a factorial regression model (model 6) to CIMMYT maize stress trials.

Term	Degrees of freedom	Sum of squares	Mean squares	*F*	Probability
E	7	5679	811.2	1752.3	<0.001
G	210	614	2.9	6.3	<0.001
G.minTF	210	172	0.8	1.7	<0.001
G.radiationGF	210	124	0.6	1.2	≤0.038
$\underline{\varepsilon}$	1050	517	0.5		
Total	1687	7106	4.2		

can be used to model GEI in terms of heterogeneity of variance and covariance. A more detailed description of mixed models can be found in the literature elsewhere (Verbeke and Molenberghs, 2000; Galwey, 2006).

The models discussed in the previous sections were all examples of fixed effects models, with all terms except the residual term fixed. However, genotypes can be regarded as a random sample from a larger population (especially easy when the number of genotypes is large, say more than 10), in which case genotypes are an extra source of random variation. This situation calls for a mixed model, with genotypes taken as random term. A review of the use of mixed models to analyse complex data sets in plant breeding can be found in Smith et al. (2005). For the maize example data set, there are 211 genotypes. When the genotypic main effects are taken as random, the following mixed model equivalent of the additive model can be defined as:

$$\underline{\mu}_{ij} = \mu + \underline{G}_i + E_j + \underline{\varepsilon}_{ij} \qquad (7)$$

$$\underline{G}_i \sim N(0, \sigma^2_G) \quad \underline{\varepsilon}_{ij} \sim N(0, \sigma^2_\varepsilon)$$

The term $\underline{G}_i$ is underlined to indicate that it is a random term; its distribution needs to be specified, and usually is taken to be normal, with zero mean and a variance specific to the term. Model 7 contains two variance components, one corresponds to the random genotypic main effects, σ^2_G, and a second one, σ^2_ε, corresponds to the residual (which includes true GEI and error). An important consequence of including genotypes as random is that automatically genetic covariances and correlations between performances in different environments are imposed. The total variance for individual genotypic observations in a particular environment j, σ^2_j, is the sum of two sources of variation: $\sigma^2_j = \sigma^2_G + \sigma^2_\varepsilon$. The covariance between observations for a particular genotype in environments j and j^*, σ_{jj^*}, following from model 7 is: $\sigma_{jj^*} = \sigma^2_G$. For observations on different genotypes $\sigma_{jj^*} = 0$. In model 7, similarities (or covariation, and therefore correlation) between observations made on the same genotype in different environments are assumed to be positive, but covariation between observations on different genotypes (regardless whether the observation is done in the same or in different environments) is assumed to be zero. Model 7 is referred as the compound symmetry model (Verbeke and Molenberghs, 2000).

The general definition for a correlation between two traits, or two environments, x and y is:

$$r_{(x;\ y)} = \frac{\text{covariance}(x; y)}{\sqrt{\text{var}(x)}\sqrt{\text{var}(y)}}$$

Model 7 imposes a constant correlation between environments, with the correlation between any pair of environments j and j^* (for clarity, we write Env_j and Env_{j^*} when referring to those environments), being equal to:

$$r_{(\text{Env}_j;\ \text{Env}_{j^*})} = \frac{\sigma_{jj^*}}{\sqrt{\sigma^2_j}\sqrt{\sigma^2_{j^*}}} = \frac{\sigma^2_G}{\sqrt{\sigma^2_G + \sigma^2_\varepsilon}\sqrt{\sigma^2_G + \sigma^2_\varepsilon}} = \frac{\sigma^2_G}{\sigma^2_G + \sigma^2_\varepsilon}$$

Although mixed models can be fitted by standard least squares procedures in the case of balanced data, a more general method of inference to fit mixed models is by residual maximum likelihood, or REML (Patterson and Thompson, 1971). Results of analyses based on REML are presented in another way than the familiar ANOVA tables. **Table 5** shows the results obtained by fitting mixed models to the maize example data.

Table 5 does not contain sums of squares, nor mean squares. Instead, there is a table with three main sections. For model 7, the compound symmetry model, one section contains the results for testing fixed model terms (header Fixed terms). A second section shows the estimates for the variances of the random terms (header Random terms), and a third section a goodness-of-fit statistic, the deviance, that can be used to compare mixed models with equal fixed terms and differing random terms (header Deviance). For the fixed effects (environments in this case), **Table 5** shows a Wald test statistic, the corresponding degrees of freedom (DF), and a P value. The Wald test statistic is used to assess the significance of fixed effects in the REML mixed model framework. Under the null hypothesis of no fixed effects, the Wald test has a distribution that is approximately a Chi-square with DF equal to the number of independent effects for the particular fixed term. In the maize example, the Wald test statistic for environments is 10,265.3 and it has $8 - 1 = 7$ degrees of freedom. This Wald statistic has a very low tail probability in the Chi-square distribution under the null hypothesis of no environmental effects ($P < 0.001$). So, it is concluded that there is a significant difference between environments. Some statistical packages, including GenStat®, can provide an F-distributed approximation to the Wald statistic.

The estimates of the two parameters associated to the random terms in the model: $\sigma^2_G = 0.297$ and $\sigma^2_\varepsilon = 0.553$ are given in the second part of **Table 5**. The magnitude of the variance components can be compared to have an impression of the relative importance of genotypic main effects (σ^2_G) in relation to the sum of GEI and error (σ^2_ε). The genetic correlation between any two environments is estimated as:

$$r_{(\text{Env}_j;\ \text{Env}_{j^*})} = \frac{0.297}{0.297 + 0.553} = 0.349$$

The last row in **Table 5** presents the deviance (equal to −2 times the restricted loglikelihood), which is a measure of how well the model fitted to the data. The better the model, the lower the deviance is. As will be seen later, the deviance can be used to compare different models to select the best model for the data, provided that the fixed part of the model remains unchanged.

Model 7 assumes a constant genetic variance and correlation between pairs of environments. For METs, the assumption of constant genetic variance and genetic correlation across environments is unrealistic (**Figure 2A**). In the presence of GEI, a more realistic model would allow the total genetic variance to change from environment to environment, which will in turn, cause heterogeneous genetic correlations between environments:

$$\underline{\mu}_{ij} = \mu + \underline{G}_i + E_j + \underline{\varepsilon}_{ij} \qquad (8)$$

$$\underline{G}_i \sim N(0, \sigma^2_G) \quad \underline{\varepsilon}_{ij} \sim N(0, \sigma^2_{\varepsilon_j})$$

In model 8, there is still a single genetic variance component for genotypes, and therefore, a constant genetic covariance between

Table 5 | REML output of the fit of different mixed models to the CIMMYT maize stress trials.

Model 7			Model 8			Model 9		
Fixed	**Wald (*DF*)**	***P***	**Fixed**	**Wald (*DF*)**	***P***	**Fixed**	**Wald (*DF*)**	***P***
E	10265.3 (7)	<0.001	*E*	9759.4 (7)	<0.001	*E*	6268.8 (7)	<0.001
Random	**Estimate**	***SE***	**Random**	**Estimate**	***SE***	**Random**	**Estimate**	***SE***
σ^2_G	0.297	0.036	σ^2_G	0.125	0.017	σ^2_{C1}	0.439	0.053
σ^2_ε	0.553	0.020	$\sigma^2_{\varepsilon 1}$	0.551	0.057	σ^2_{C2}	1	–
			$\sigma^2_{\varepsilon 2}$	0.692	0.071	σ^2_{C3}	0.042	0.013
			$\sigma^2_{\varepsilon 3}$	1.399	0.140	σ_{C1C2}	0.551	0.077
			$\sigma^2_{\varepsilon 4}$	0.672	0.069	σ_{C1C3}	0.109	0.019
			$\sigma^2_{\varepsilon 5}$	0.704	0.072	σ_{C2C3}	0.115	0.032
			$\sigma^2_{\varepsilon 6}$	0.135	0.018	$\sigma^2_{\varepsilon 1}$	0.446	0.051
			$\sigma^2_{\varepsilon 7}$	0.152	0.019	$\sigma^2_{\varepsilon 2}$	0.445	0.052
			$\sigma^2_{\varepsilon 8}$	0.761	0.078	$\sigma^2_{\varepsilon 3}$	0.736	0.169
						$\sigma^2_{\varepsilon 4}$	0.428	0.050
						$\sigma^2_{\varepsilon 5}$	0.508	0.057
						$\sigma^2_{\varepsilon 6}$	0.145	0.018
						$\sigma^2_{\varepsilon 7}$	0.138	0.017
						$\sigma^2_{\varepsilon 8}$	0.740	0.080
Deviance (*DF*)	**1077.9 (1678)**		**Deviance (*DF*)**	**838.4 (1671)**		**Deviance (*DF*)**	**619.9 (1667)**	

Model 7 assumes compound symmetry, model 8: assumes heterogeneity of genetic variance across environments, and model 9 assumes heterogeneity of genetic covariance between groups of environments and heterogeneity of genetic variance across individual environments. Environments are indexed as: 1 = SS92a, 2 = IS92a, 3 = NS92a, 4 = IS94a, 5 = SS94a, 6 = LN96a, 7 = LN96b, 8 = HN96b. Groups of environments are indexed as: C1 = SS92a, IS92a, IS94a, SS94a, HN96b; C2 = NS92a; C3 = LN96a, LN96b.

environments. However, the variance for the term $\underline{\varepsilon}_{ij}$ that includes GEI and error, is assumed to depend on the environment (i.e., the variance component $\sigma^2_{\varepsilon j}$ is indexed by j). **Table 5** presents the results of fitting model 8 to the maize data. Instead of two variance components, there are now nine, one corresponding to the variance component for genotypes ($\sigma^2_G = 0.125$), and eight corresponding to a form of GEI for each of the eight environments (for convenience, we assume constant errors). The heterogeneity of variance for $\underline{\varepsilon}_{ij}$ reflects that in some environments there is a larger variation (e.g., in environment 3, which is the high-yielding NS92a) than in other environments (e.g., in environments 6 and 7, which are low-yielding, LN96a and LN96b). The heterogeneity of variance leads to heterogeneous genetic correlations between environments. For example, the correlation between environments 6 and 7 is:

$$r_{(\mathrm{Env}_6;\ \mathrm{Env}_7)} = \frac{0.125}{\sqrt{0.125 + 0.135}\sqrt{0.125 + 0.152}} = 0.466$$

and between environments 3 and 6 is:

$$r_{(\mathrm{Env}_3;\ \mathrm{Env}_6)} = \frac{0.125}{\sqrt{0.125 + 1.399}\sqrt{0.125 + 0.135}} = 0.199$$

In conclusion, model 8 accommodates heterogeneity of variance between environments and, with it, allows for heterogeneous correlations between environments, which can be desirable when analyzing environments that strongly differ (e.g., with strong stress and without stress).

The deviance for model 8 is 838.4 with 1671 DF, which is much lower than the one for model 7 (deviance 1077.9 with 1678 DF). The deviance has dropped, but at the expense of having to estimate more parameters (nine instead of two parameters). Is the decrease in deviance large enough to consider model 8 a significant improvement over model 7? Because model 7 and 8 are nested models (model 7 is a special case of model 8 when the $\sigma^2_{\varepsilon j}$ are equal for all j), a deviance test can be used to answer this question. Under the null hypothesis of no difference in quality of the fits, the difference in deviance between the two models is Chi-square distributed with the number of DF equal to the difference in the number of parameters between the models. In the example, the difference in deviance is $1077.9 - 838.4 = 239.5$, and the models differ by seven parameters. The P value associated to 239.5 in a Chi-square distribution with 7 DF is very small ($P < 0.001$), so it is concluded that model 8 provides a significant improvement over model 7.

In cases where the models are not nested, the comparison can be done by the Akaike Information Criterion (AIC) (Akaike, 1974). For model 7, AIC = 4170, and for model 8 AIC = 3944. The model that has the lowest AIC value is the one that is chosen. Model 8 has the lowest AIC value, which agrees with the conclusion based on the deviance test.

Model 8 assumes heterogeneous variances across environments, in combination with a constant covariance between environments. This latter assumption can be relaxed by also allowing the genetic covariance between environments to be heterogeneous. A possibility is to estimate a covariance parameter for each pair of environments, producing a variance-covariance model that is referred to as the "unstructured model" (Verbeke and Molenberghs, 2000). A somewhat simpler strategy consists of estimating covariances between groups of environments instead of between individual environments, in which the environments are first grouped in a number of clusters and then fitting the following model:

$$\underline{\mu}_{i(c)j} = \mu + \underline{G}_{i(c)} + E_j + \underline{\varepsilon}_{i(c)j} \quad (9)$$

$$\underline{G}_{i(c)} \sim N(0, \Sigma_c) \quad \underline{\varepsilon}_{i(c)j} \sim N(0, \sigma^2_{\varepsilon_j})$$

In model 9 a random genetic main effect is fitted that changes between groups of environments and that has a covariance matrix Σ_c that consists of group specific genetic variances, with σ^2_{cj} for group j, on the diagonals, and pairwise-specific genetic covariances, with σ_{cjj^*} between groups j and j^*, on the off-diagonals. Model 9 retains the residual heterogeneity of model 8, which means that environment specific genotypic effects are added to group specific genotypic effects. To illustrate model 9, using the maize example, and based on **Figure 4**, the environments were clustered in three groups: group 1 = (SS92a, SS94a, IS92a, IS94a, HN96b), group 2 = (NS92a), and group 3 = (LN96a, LN96b). Therefore, the covariance matrix Σ_C will contain on the diagonal the genetic variances for groups 1, 2, and 3 (σ^2_{c1}, σ^2_{c2}, and σ^2_{c3} respectively), and on the off-diagonals the covariances between the groups (σ_{c12}, σ_{c13}, and σ_{c23}). The full covariance matrix can be written as:

$$\Sigma_C = \begin{pmatrix} \sigma^2_{c1} & & \\ \sigma_{c12} & \sigma^2_{c2} & \\ \sigma_{c13} & \sigma_{c23} & \sigma^2_{c3} \end{pmatrix}$$

The results of fitting model 9 to the maize data are presented in **Table 5**, where the estimates of the parameters in the covariance matrix Σ_C can be found.

The diagonals of Σ_C show that, on average, the genetic variation is lower in group 1 (the group of nitrogen stress environments) than in group 2. It should be noted that because group 3 is composed of a single environment, the genetic variation cannot be partitioned into a component due to the group and a residual, so σ^2_{c3} is not estimated but arbitrarily fixed to 1. The total variance in each of the environments is equal to the sum of the group's variance plus the environment-specific variance. For example, the variance in environment 1 is equal to 0.885, which is the sum of the variance of group 1, i.e., $\sigma^2_{c1} = 0.439$, and $\sigma^2_{\varepsilon 1} = 0.446$. Recalling that the covariance between environments within the same group is given by σ^2_{c1}, σ^2_{c2} and σ^2_{c3}, and the covariance between environments in different groups by σ_{c1c2}, σ_{c1c3}, and σ_{c2c3}, the correlation between any pair of environments can be estimated. For example, the correlation between environments 1 and 2 is:

$$r_{(Env_1;\ Env_2)} = \frac{0.439}{\sqrt{0.439 + 0.446}\sqrt{0.439 + 0.445}} = 0.496$$

and between environments 1 and 7 is:

$$r_{(Env_1;\ Env_7)} = \frac{0.109}{\sqrt{0.439 + 0.446}\sqrt{0.042 + 0.138}} = 0.273$$

Finally, the deviance can be used to evaluate whether the allowance for heterogeneity of covariance between environments improved the quality of the model or not.

The deviance for model 9 is 619.9 with 1667 DF, and the difference in deviance with model 8 is 218.5, with four extra parameters. The associated P value for 218.5 in a Chi-square distribution with 4 DF is very low ($P < 0.001$), so it can be concluded that model 9 is a significant improvement over model 8. For model 9 AIC = 3736, which is smaller than for model 8 (AIC = 3944), and confirms this conclusion.

We have presented different mixed model formulations to model GEI in terms of heterogeneity of variance and covariance between environments. The compound symmetry model, which is the commonly used default model when fitting a mixed model to a two–way table of means, forces variances and covariances to be constant across environments. Two alternative models accommodated either heterogeneity of genetic variances across environments, or heterogeneity of genetic variances and covariances across environments. There are other useful variance-covariance models such as the factor analytic (Malosetti et al., 2004; Boer et al., 2007) that combines flexibility with parsimony (reduced number of parameters), but their discussion is outside the scope of this paper.

The analysis of a data set is an iterative process consisting of fitting and comparing alternative models to identify a good model for the data under study. That process has been illustrated with a maize data set. The next section goes one step further in the modeling process by including molecular marker information, with the ultimate objective of identifying genomic regions, QTLs, that underlie genetic variation of quantitative traits. Within the context of METs, the use of such models is a powerful tool to identify and understand the genetic basis of GEI, that is, QEI.

QTL MAPPING IN THE CONTEXT OF MULTI-ENVIRONMENT TRIALS: MODELING MAIN EFFECT QTLs AND QTL-BY-ENVIRONMENT INTERACTION

So far, we discussed models that use either implicit or explicit environmental characterizations to understand GEI. We switch in this section to the use of explicit *genotypic* information in the models describing GEI. Use of such information in statistical models for GEI can help understand the basis of GEI in terms of the action of genome regions, QTLs, in their dependence on the environment, i.e., QEI. Molecular marker systems (RFLP, AFLP, DArT, SSR, SNP) provide information about variation at the DNA level that can be employed in statistical models. For example, within the framework of factorial regression models, markers can serve as explanatory variables, which is at the core of regression–based approaches for

QTL mapping (Haley and Knott, 1992; Martínez and Curnow, 1992).

Elaborating upon factorial regression ideas, the following section presents mixed models that can accommodate explicit genotypic information to describe GEI in terms of QTL and QEI effects (Malosetti et al., 2004; Boer et al., 2007; van Eeuwijk et al., 2007, 2010). The genotypic information stemming from markers is introduced in the statistical models in the form of so-called genetic predictors. Applications of mixed model QTL by environment detection as the one described here, can be found in wheat (Mathews et al., 2008), sugar cane (Pastina et al., 2012), and sorghum (Sabadin et al., 2012). We should emphasize, that although we focus on QTL models applied to standard biparental populations, these models can be adapted rather easily to multiparental populations (van Eeuwijk et al., 2010; Huang et al., 2011), or association mapping panels (Malosetti et al., 2007; van Eeuwijk et al., 2010).

While here we focus in this paper on mixed model QTL detection, this is certainly not the only method for multi-environment QTL mapping. A well known and common alternative is to use mixture model approaches (Jiang and Zeng, 1995), for which various user-friendly QTL software packages exist (e.g., QTL Cartographer, Basten et al., 2002). However, such QTL software packages typically provide little or no opportunity to intervene with the statistical model, nor do they allow for applying different model building strategies. For example, in the mixture model context, it is hard to switch between different models for representing the dependencies between environments or add explicit information on the environments, something that is relatively easy in the mixed model context.

EXPLANATORY VARIABLES FOR DIFFERENCES BETWEEN GENOTYPES: GENETIC PREDICTORS

Most populations in QTL mapping originate from crosses between pairs of inbred lines. A segregating offspring population can be produced from an F_1 hybrid after one generation of selfing (F_2), after several generations of self-pollination (recombinant inbred lines or RIL), or after crossing the F_1 with one of the parental lines (backcross). In addition, by chromosome doubling of F_1 gametes, a population of doubled haploid lines can be generated. In all of these cases, two alleles at most will segregate at each locus. For a locus M_1, individuals can have the genotypes M_1M_1, M_1m_1, or m_1m_1, with M_1 the allele that comes from the paternal line, and m_1 the allele that comes from the maternal line. By convention the locus names are given in italics (so for example M_1 refers to locus 1, and M_1 and m_1 refer to the paternal and maternal alleles at locus 1, respectively). The relative frequency of the genotypes in the offspring population depend on the type of population; for example, in an F_2 the expected frequencies are ¼, ½, and ¼ for M_1M_1, M_1m_1, and m_1m_1, respectively.

With the help of molecular markers, it can be revealed whether a particular individual is of the M_1M_1, M_1m_1, or m_1m_1 type. To detect QTLs and estimate their effects, it is necessary to translate the marker information into explanatory variables or genetic predictors. A straightforward way of constructing genetic predictors is to create an explanatory variable that contains the number of copies of one of the alleles, for example, the M_1 allele. The genetic predictor will then take the value 2 whenever an individual has two paternal alleles (M_1M_1), the value 1 when the offspring individual is M_1m_1, and 0 when it is m_1m_1. Using a simple regression model, the slope for the regression of the genotypic means on a genetic predictor defined by the number of M_1 alleles corresponds to the effect of a substitution of an m_1 allele by an M_1 allele at the given locus (Lynch and Walsh, 1998; Bernardo, 2002). This effect is also known as the additive genetic substitution effect of the QTL allele. By analogy, a dominance genetic predictor can be constructed by creating an explanatory variable with values 0, when the offspring individual is M_1M_1 or m_1m_1, and value 1 whenever it is M_1m_1.

With complete information on the marker genotypes, i.e., codominant markers without missing values, the construction of genetic predictors at marker positions consists of simply counting the number of alleles coming from a particular parent. For genomic positions in between marker loci (putative QTL positions), for dominant markers, and for markers with missing values, the construction of genetic predictors requires more effort. In a general formulation, the value for the additive genetic predictor, X^{add}, for an offspring individual can be defined as the expected number of alleles coming from the paternal line, the number of M_1 alleles:

$$X^{add} = \Pr(M_1M_1|\text{all markers}) \times 2 + \Pr(M_1m_1|\text{all markers}) \times 1 + \Pr(m_1m_1|\text{all markers}) \times 0, \quad (10a)$$

with $\Pr(M_1M_1|\text{all markers})$, $\Pr(M_1m_1|\text{all markers})$, and $\Pr(m_1m_1|\text{all markers})$ the conditional probabilities of the individual being of the M_1M_1, M_1m_1, or m_1m_1 type, respectively given the observed marker information. Note that in the case of complete information, the individual's genotype is known, so one of $\Pr(M_1M_1|\text{markers})$, $\Pr(M_1m_1|\text{markers})$ and $\Pr(m_1m_1|\text{markers})$ will be equal to 1, while the others will be 0.

In the case of incomplete information, although the genotype for a locus of an individual may not be known with certainty, information can be obtained from nearby markers to estimate the probability of the offspring individual being of a particular genotype. This probability is a function of the observed genotypes at neighboring markers and the expected recombination occurring between those marker loci and the locus under evaluation (Lynch and Walsh, 1998). Efficient methods to calculate conditional genetic probabilities for the different types of population commonly used for plants have been proposed in the literature; see Jiang and Zeng (1997) for an exhaustive overview. The calculation of genotypic probabilities conditional on marker information provides the basis for all QTL mapping strategies; QTL mapping packages calculate these probabilities behind the scenes. In GenStat® (see "Appendix"), a very general Hidden Markov Model algorithm has been programmed to calculate those condtional probabilities. Other packages that calculate those probabilities and that are free are *Grafgen* (Servin et al., 2002) and r/qtl (Broman et al., 2003).

With the estimated conditional probabilities, the genetic predictors at positions where no or partial marker information is available can be calculated by using the conditional probabilities

in expression 10a. An analogous reasoning holds for the estimation of dominance genetic predictors:

$$X^{dom} = Pr(M_1M_1|\text{all markers}) \times 0 + Pr(M_1m_1|\text{all markers}) \times 1 + Pr(m_1m_1|\text{all markers}) \times 0. \quad (10b)$$

MODELING GENOTYPE-BY-ENVIRONMENT INTERACTION IN TERMS OF QTL EFFECTS

The inclusion of genetic predictors in a GEI model allows testing the hypothesis that the DNA at a particular genome position has an effect on a phenotypic trait, and whether that effect is environment dependent or not. A basic GEI phenotypic model, as the one discussed in the previous sections, can be extended to accommodate two new terms, one for the additive genetic effect of a possible QTL ($X_i^{add}\alpha_j$), and a second for the dominance effect of the same locus ($X_i^{dom}\delta_j$):

$$\underline{\mu}_{ij} = \mu + E_j + X_i^{add}\alpha_j + X_i^{dom}\delta_j + \underline{G}_i + \underline{\varepsilon}_{ij}, \quad (11)$$

where X_i^{add}, and X_i^{dom} stand for the values of the additive and dominance genetic predictors of individual i at the position at which a QTL is postulated and tested for. The parameters α_j and δ_j represent the additive and dominance effects of this QTL. In model 11, both types of QTL effects are indexed by j, because environment-specific effects are allowed. Residual genetic main effects (i.e., genetic effects not explained by the QTL) contribute to the random genetic effect, $\underline{G}_i$, and residual GEI (residual QEI) contributes to $\underline{\varepsilon}_{ij}$. The conclusion about the presence of a QTL at a particular position is based on a Wald test (Verbeke and Molenberghs, 2000) that assess the null hypothesis of the environment-specific additive and dominance genetic effects being zero across all environments: Ho: $\alpha_j = 0$, and Ho: $\delta_j = 0$, $j = 1 \ldots J$. Note that as by definition, dominance effects are deviations from additivity, so dominance effects should be tested conditional on the additive effects present in the model. In practice, and to assure that the proper test is used, it is adviced to include the term for additive genetic effects in the model before the term for the dominance effects, and use the sequential Wald test (e.g., in GenStat® output, the test under the heading "Sequentially adding terms to fixed model").

For the maize data, **Table 6** shows an example of the application of model 11 to a particular genomic position. The table indicates that the dominance effect at this genome position was not significant (Wald statistic = 13.5 on 8 DF, $P \leq 0.097$), and, therefore, the null hypothesis of no dominance effects is not rejected. However, the Wald statistic for the additive genetic effects was highly significant (Wald = 100.9, on 8 DF, $P < 0.001$), indicating the existence of additive QTL effects. It is still necessary to find out whether they are environment specific, i.e., whether a QEI term is needed, or whether a model with just main effect QTL expression would suffice. To this purpose, the environment–specific QTL effects (α_j) are partitioned into an additive main effect (α^Q) and QEI effects (α_j^{QEI}), leading to the following model:

$$\underline{\mu}_{ij} = \mu + E_j + X_i^{add}\alpha^Q + X_i^{add}\alpha_j^{QEI} + X_i^{dom}\delta_j + \underline{G}_i + \underline{\varepsilon}_{ij} \quad (12)$$

Table 6 | Results of the test for fixed effects in a mixed model including a fixed environment–specific additive (α_j) and dominance (δ_j) QTL effect.

Fixed terms	Wald	*DF*	*P*
E	10875.5	7	<0.001
Additive effect (α_j)	100.9	8	<0.001
α^Q	12.8	1	<0.001
α_j^{QEI}	88	7	<0.001
Dominance effect (δ_j)	13.5	8	≤0.097

The additive QTL effect is partitioned into a QTL main effect (α^Q), and a QEI effect (α_j^{QEI}).

If required, a similar partitioning of the QTL effects may be carried out for the dominance effects. As a result of the partitioning of the environment-specific QTL effects, there is a Wald test for QTL main effect and a Wald test for QEI (**Table 6**). The QEI effects should be tested, conditional on the main effect being fitted into the model, i.e., the QTL main effect should always precede the term for QEI. In the example, it is observed that the QEI interaction effect is highly significant (Wald = 88.0 on 7 DF, $P < 0.001$), so it is concluded that QTL effects are dependent on the environment. Since there is significant QEI, no attempt will be made to interpret the QTL main effect. When QEI is not significant, the model can be simplified by omitting the QEI term, as the QTL main effect will suffice to describe the QTL effect.

A QTL MAPPING STRATEGY FOR MULTI-ENVIRONMENT TRIALS BASED ON MIXED MODELS

The preceding section presented a number of models that can be useful in the detection of QTLs for MET data. The present section discusses a strategy for a genome-wide scan for QTLs. QTL mapping can be regarded as a model selection process aiming to identify a model that describes the phenotypic response in terms of QTL effects. Since a priori neither the number of QTLs nor their effects are known, we need a strategy that allows to explore the vast range of possible models. There is no unique way of performing this search, but an effective strategy is presented here consisting of the following steps: (1) find a good model for the phenotypic data; (2) perform a genome–wide scan for QTLs by simple interval mapping (SIM); (3) perform one or more rounds of composite interval mapping (CIM) starting with cofactors selected from the SIM step; and (4) fit a final multi–QTL model to estimate QTL effects. Each step is illustrated using the maize example data. An example code that performs the different steps in GenStat® (VSN International, 2012) and in GenStat Discovery® (Payne et al., 2007) is given in the "Appendix."

STEP 1: IDENTIFY THE BEST VARIANCE-COVARIANCE MODEL FOR THE PHENOTYPIC DATA

A number of models can be fitted (for example models 7 to 9 plus the unstructured model), and compared based on the AIC values. The selected mixed model will be the starting point from which to develop a QTL model. **Table 7** gives the AIC for four candidate models for the maize example data, and shows that

Table 7 | Comparison of the goodness of fit for four different mixed models (models 7 to 9 and the unstructured model), as fitted to CIMMYT maize stress trials.

Model	Deviance	*DF*	Δ Deviance	Δ *DF*	*P*	AIC
Model 7	1077.9	1678	–	–	–	4170
Model 8	838.4	1671	239.5	7	<0.001	3944
Model 9	619.9	1667	218.5	4	<0.001	3736
Unstructured	548.7	1644	71.2	23	<0.001	3708

The columns "Δ deviance" and "Δ DF" indicate the differences in deviance and number of degrees of freedom between the current and the preceding model in the list. The associated P values correspond to a Chi-square distribution with Δ DF degrees of freedom.

the unstructured model is the best (lowest AIC) and is, therefore, chosen as the basic phenotypic model.

STEP 2: GENOME-WIDE QTL SCAN, SIMPLE INTERVAL MAPPING

After choosing the phenotypic model, a genome-wide scan is performed by fitting single QTL models across the genome at marker and in between marker positions, i.e., SIM. To perform SIM, we need to estimate genetic predictors that cover the genome. For most population types and population sizes of a few hundred individuals, calculating the genetic predictors every 5–10 cM is sufficient. The genetic predictors are used to test for QTL effect at the predictor location. The unstructured model was selected for the maize data set, so the SIM scan can be done by fitting the following model at every genetic predictor position (only additive effects are tested as a previous analysis showed little dominance):

$$\underline{\mu}_{ij} = \mu + E_j + X_i^{add}\alpha_j + \underline{G}_i + \underline{\varepsilon}_{ij} \qquad (13)$$

The results of a genome-wide SIM scan are plotted in **Figure 5**. The upper plot displays the *P* value of the Wald test (on a $-\log_{10}$ scale) for the effect of a QTL along the chromosomes. The horizontal line indicates a threshold value, above which the null hypothesis of no QTL is rejected. The profile shows evidence of QTLs on chromosomes 1, 3, 4, 6, and 10. The two largest QTLs are the ones on chromosome 1 and on chromosome 10. The lower panel shows an indication of the magnitude of the QTL effects in each of the environments at a particular chromosome position. The type of color points to the parent that contributes the high value allele (blue = maternal line, red = paternal line), and the color intensity to the magnitude of the effect. QEI is reflected in this plot by changes in color at a particular chromosome position (cross-over interaction) or by changes in intensity of the color (convergence-divergence). For example, the large QTL on chromosome 1 not only shows changes in magnitude of the effects between environments (different color intensities), but also shows change of colors. For example, while in HN96b the allele increasing yield comes from the mother (blue), in IS92a, IS94a, NS92a, SS92a, and SS94a the allele increasing yield comes from the father (red). This is an example of cross-over interaction. The large QTL on chromosome 10 shows only differences in magnitude of the QTL effect (from largest in HN96b to no effect in LN96a, LN96b,

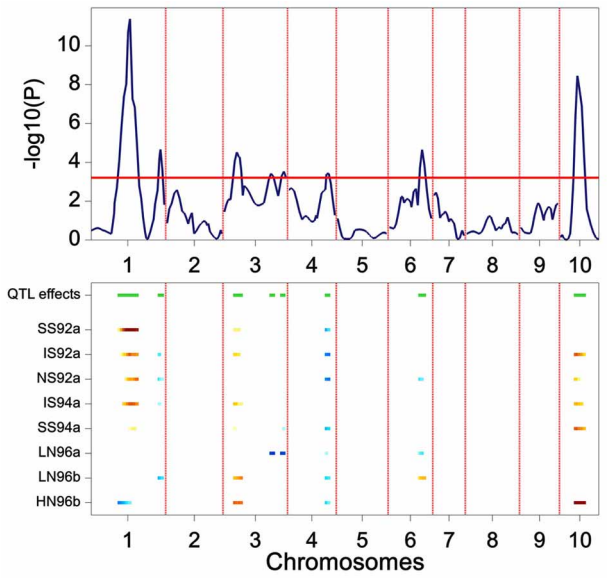

FIGURE 5 | Plot produced by a SIM QTL scan in a maize F2 population. The upper panel shows the *P* value of the Wald test (on a –log10 scale) for the effect of a QTL along the chromosomes (solid line). The horizontal line indicates a threshold value for significance. The lower panel gives an indication of magnitude of QTL effects (higher intensity, larger effect), and parental line contributing the superior allele (blue, maternal; red, parental line).

and SS92a), but always with the allele from the father contributing to higher yield.

Scanning the results across the full set of chromosomes produces a list of putative QTL positions that can be used as cofactors at the following stage of the QTL mapping.

SIM implies performing multiple tests along the genome, one test at each putative QTL position. For example, for the maize data genetic predictors were calculated at 246 chromosome positions, which means that model 13 was fitted 246 times. When performing multiple tests, the probability of at least one false positive (i.e., falsely rejecting the null hypothesis) increases according to the expression $1-(1-\alpha)^n$,with α the test level for a single test and n the number of tests. A simple correction method is the Bonferroni correction that uses α/n instead of α to test individual null hypotheses, assuring that the proportion of false rejections among n tests will be at most equal to α. For example, to accept a maximum of 5% of false rejections in the whole of the experiment (genome–wide), one should use a threshold equal to $0.05/n$. A disadvantage of the Bonferroni correction is that it is very conservative risking that some QTLs may go undetected, especially when not all tests are independent, which is the case in QTL mapping where nearby positions are correlated.

Modifications to the Bonferroni correction in the context of QTL mapping have been proposed by Cheverud (2001), and further modifications proposed by Li and Ji (2005). Both approaches essentially compensate for the fact that, in QTL mapping, tests are correlated by using an estimated effective number of tests (n^*) instead of the actual number of tests (n) to set the significance threshold. For the maize data, the Li and Ji (2005) approach

produced a value of $n^* = 81$, which gives a larger threshold P value than the Bonferroni correction (divide 0.05 by 81, instead of dividing by 246). By default, GenStat estimates n^* and uses it to set the corresponding significance threshold.

STEP 3: COMPOSITE INTERVAL MAPPING

The power of QTL detection can be improved by reducing the background noise caused by QTLs outside the region under test. This is the principle of the CIM approach, simultaneously proposed by Jansen and Stam (1994) and Zeng (1994). What makes the difference between SIM and CIM, is that when performing CIM the model includes a number of cofactors that corrects for the effects of the genetic background:

$$\underline{\mu}_{ij} = \mu + E_j + \sum X_{if} c_{jf} + X_i^{add} \alpha_j + \underline{G}_i + \underline{\varepsilon}_{ij} \qquad (14)$$

In model 14 the term $\sum X_{if} c_{jf}$ accounts for the effects of QTLs outside the region that is being tested (X_i^{add}), reducing the error variation and thereby improving the power for QTL detection. Various strategies exist for the selection of a set of cofactors, but a pragmatic approach is to use the results from the SIM scan, including the positions indicative of QTLs by SIM as cofactors.

Another issue that needs to be addressed is that when testing in a region close to a cofactor, it is necessary to exclude the particular cofactor from the model to avoid colinearity with the tested position. A popular solution is to choose a window around an evaluation position such that if a cofactor falls inside that window, then the cofactor is excluded from the model. Window size affects the results of a CIM scan, and there are no clear–cut recommendations about which window size to use. For the present example, all cofactors that are on the chromosome being evaluated are excluded, a strategy known as restricted CIM.

The results of the restricted CIM scan for the maize data are presented in **Figure 6**. The profiles point to QTLs on chromosomes 1, 2, 3, 4, 6, 9, and 10. In comparison with the results from SIM, the CIM profile reveals the same QTLs (the two major QTLs on chromosome 1 and 10, and the ones on chromosome 3, 4, and 6), but in addition it shows indications of QTLs on chromosomes 2 and 9.

STEP 4: ESTABLISHING A FINAL QTL MODEL

In a subsequent modeling step, the QTLs for all positions that were found significant in the restricted CIM scan are included simultaneously in the mixed model:

$$\underline{\mu}_{ij} = \mu + E_j + \sum X_{iq}^{add} \alpha_{jq} + \underline{G}_i + \underline{\varepsilon}_{ij} \qquad (15)$$

Model 15 is a multi–QTL model constructed by inclusion of the full set of QTLs identified in the previous CIM scan. QTLs with non-significant effects will be removed using Wald tests (conditional on all other QTLs) to arrive at a final model. The final model for our example data showed that nine out of the ten QTLs from the CIM scan were significant in the multi-QTL model. Further, by breaking down the QTL effects into QTL main effects (α_q^Q) and QEI effects (α_q^{QEI}), it was possible to investigate whether QTL effects were consistent across environments or not. All QTLs

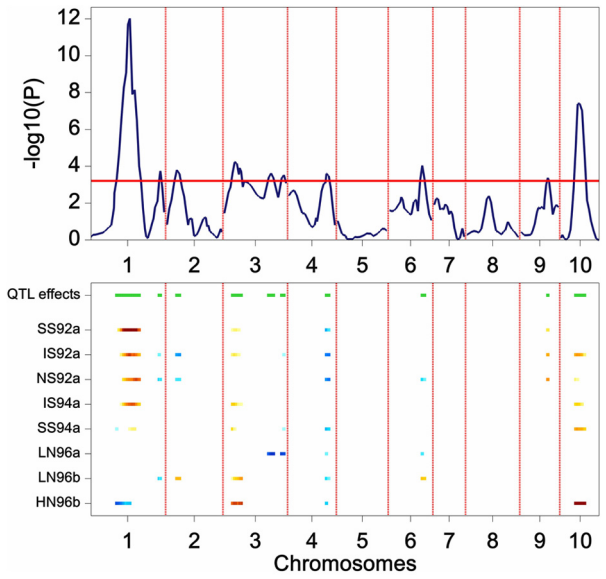

FIGURE 6 | Plot produced by a CIM QTL scan in a maize F2 population. The upper panel shows the P value of the Wald test (on a –log10 scale) for the effect of a QTL along the chromosomes (solid line). The horizontal line indicates a threshold value for significance. The lower panel gives an indication of magnitude of QTL effects (higher intensity, larger effect), and parental line contributing the superior allele (blue, maternal; red, parental line).

but the one at the end of chromosome 3, had significant QEI ($P < 0.01$).

The estimated QTL effects are given in **Table 8**. The effect of a QTL in a particular environment is declared significant when zero is outside the confidence interval of the estimated effect (CI = estimate ± 2*s.e., with s.e. the average standard error obtained from the REML analysis). Results for the large QTL on chromosome 1 ($QTL_{1,141}$) showed that the QTL had a significant effect of 0.469 ton·ha^{-1} in environment SS92a, which means that for each replacement of the maternal allele by a paternal allele, a yield increase of about half a ton is expected. The effect of the same QTL in environment HN96b had a negative sign (−0.232 ton·ha^{-1}), which means that rather than an increase, a decrease in yield is expected for the same allele substitution. The effects of $QTL_{1,141}$ are inconsistent across environments not only in terms of the size of the effects, but also in terms of the sign of the effect. Inconsistency in size and sign of QTL effects underlies crossover interactions, the most important case of GEI (recall **Figure 1D**). From the breeder's point of view, the crossover QEI means that, while the maternal allele has to be selected when breeding for environment HN96b, the paternal allele will be the choice when selecting for all the other environments. The other large QTL, which is on chromosome 10 ($QTL_{10,67}$) showed changes of the sizes of the effects but not of their signs, indicating that the favorable allele came always from the paternal line. The size of the QTL effect was largest in HN96b (0.564 ton·ha^{-1}), around 0.300 ton·ha^{-1} in IS92a, IS94a, NS92a, and SS94a, and not significant in LN96a, LN96b, and SS92a. Despite changes in effect sizes,

Table 8 | QTL effect estimates (ton·ha^{-1}) for individual environments.

	SS92a	IS92a	NS92a	IS94a	SS94a	LN96a	LN96b	HN96b
$QTL_{1,141}$	0.469*	0.351*	0.370*	0.370*	0.214*	−0.005	−0.002	−0.232*
$QTL_{1,252}$	−0.026	−0.078	−0.292*	−0.061	0.182	−0.05	−0.106*	0.093
$QTL_{2,36}$	−0.123	−0.304*	−0.329*	−0.026	−0.091	−0.003	0.131*	0.106
$QTL_{3,38}$	0.224*	0.236*	0.035	0.323*	0.241*	−0.007	0.152*	0.480*
$QTL_{3,217}$	−0.129*	−0.129*	−0.129*	−0.129*	−0.129*	−0.129*	−0.129*	−0.129*
$QTL_{4,136}$	−0.272*	−0.344*	−0.456*	−0.147	−0.293*	−0.093*	−0.107*	−0.262*
$QTL_{6,125}$	−0.006	0.015	−0.332*	0.061	0.004	−0.096*	0.116*	−0.155
$QTL_{9,97}$	0.187*	0.251*	0.386*	0.016	0.023	0.026	−0.018	0.021
$QTL_{10,67}$	0.056	0.324*	0.258*	0.251*	0.322*	0.072	0.054	0.564*

A positive sign indicates that the superior allele comes from the parental line, and a negative sign indicates the superior allele comes from the maternal line. QTL effects significantly different from zero are indicated with an asterisk.

in this case, selection will always be for the paternal allele. In contrast to these two QTLs, the QTL at 217 cM on chromosome 3 ($QTL_{3,217}$) showed a consistent effect across all environments (−0.129 ton·ha^{-1}) with the maternal allele as the yield increasing allele. The other QTLs showed different degrees of interaction with the environment, involving crossovers ($QTL_{2,36}$ and $QTL_{6,125}$) or only differences in magnitude of effects ($QTL_{1,252}$, $QTL_{3,38}$, $QTL_{4,136}$, and $QTL_{9,97}$). The QTL effect information is useful at the moment of selecting complementary lines that combine in future crosses the favorable alleles coming from the maternal and paternal line.

MODELING QTL EFFECTS IN RELATION TO ENVIRONMENTAL INFORMATION

An interesting possibility with the QTL models presented here is that they allow the inclusion of environmental information to explain QTL effects in terms of sensitivities to environmental factors. Similarly to GEI models in which environmental information can be integrated to describe GEI effects, QEI models can integrate environmental information to describe QEI effects. Expressing QTL effects in terms of sensitivities to a particular environmental factor allows prediction of the effect of the QTL under any condition within the range of the original experiments. In addition, the inclusion of environmental information can help unravel the physiological mechanisms that are behind the action of a particular QTL.

The final QTL model for the maize example data consisted of nine QTLs. It can now be investigated as to whether the variation in effects of those QTLs is related to changes in one or more external environmental variables (There exists a strong analogy with the factorial regression models discussed for GEI, models 6a and 6b). **Figure 7** presents a scatter plot of the $QTL_{1,141}$ effects across environments vs. the minimum temperature during flowering time. The plot shows a negative relationship between the QTL effect and temperature.

Assuming a simple linear relationship between the effect of a QTL and a given environmental covariable, it is possible to test for that relationship using the following model:

$$\underline{\mu}_{ij} = \mu + E_j + \sum X_{iq}^{add}\alpha_{jq} + X_i(\alpha_{q^*} + \beta_{q^*}Z_j + \underline{a}_{jq^*}) + G_i + \underline{\varepsilon}_{ij} \quad (16)$$

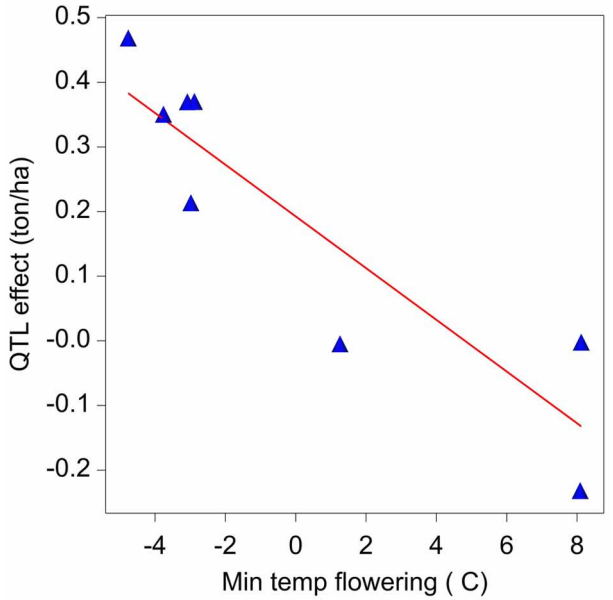

FIGURE 7 | Effect on yield (ton ha-1) of the QTL on chromosome 1 at 141 cM in relation to the minimum temperature (°C) during flowering time.

For simplicity, in model 16, the regression of environment-specific QTL effects on environmental covariables is developed for one QTL (q^*). However, the procedure can be applied equally well to other QTLs with environment–specific effects. In model 16, the effect of the QTL is expressed in relation to an environmental covariable (Z), where the effect of the QTL is equal to: $\alpha_{jq^*} = \alpha_{q^*} + \beta_{q^*}Z_j + \underline{a}_{jq^*}$. Z_j represents the value of the covariable Z for environment j. When Z_j is centered around zero, the parameters of the QTL effects can be interpreted as follows: α_{q^*} corresponds to the effect of QTL in the average environment (that is, when Z = 0); β_{q^*} corresponds to the change of the QTL effect per unit of change of the covariable's value; and the random term $\underline{a}_{jq^*}$ corresponds to the residual (unexplained) QTL effect, with $\underline{a}_{jq^*} \sim N(0, \sigma^2_{aq^*})$. For example applying model 16 to $QTL_{1,141}$, and with minimum temperature during flowering time as covariable,

showed a significant reaction of $QTL_{1,141}$ to changes in the minimum temperature during flowering, with β estimate equal to -0.040 ton ha^{-1} $°C^{-1}$. We can interpret this result saying that when the maternal allele is replaced by the paternal allele, we expect a yield decrease of 0.040 ton ha^{-1} for each degree Celsius of increase in the minimum temperature during flowering.

The example assumed a simple linear relationship between the QTL effect and a single environmental covariable, but more complex explanatory models can be constructed. For example, it is possible to include higher order terms to model the response curve (e.g., a quadratic term), to use spline formulations, or to include more than one environmental covariable in the model. It is important to mention that a close interaction with physiologists is crucial to explore and select biologically sound models.

CONCLUSION

We have discussed a suite of statistical models that are useful to plant breeding practitioners who are dealing with GEI. What all models have in common is that they make an attempt to replace the ANOVA GEI_{ij} term by product terms of genotypic parameters/covariates and environmental parameters/covariates, with as examples $b_i z_j$ (FW, AMMI, and GGE), $b_i Z_j$ (factorial regression), and $X_i \alpha_j$ (QTL mapping). For some models no other information than the two-way table of means is required (FW, AMMI, and GGE), others require explicit environmental (factorial regression) and/or genotypic information (QTL models). For exploring patterns of GEI, FW, AMMI, and GGE are very useful. For prediction and understanding, factorial regression and QTL models are more appropriate.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Physiology/10.3389/fphys.2013.00044/abstract

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Analysis of constituents for phenotyping drought tolerance in crop improvement

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Investigators now have a wide range of analytical tools to use in measuring metabolites, proteins and transcripts in plant tissues. These tools have the potential to assist genetic studies that seek to phenotype genetic lines for heritable traits that contribute to drought tolerance. To be useful for crop breeding, hundreds or thousands of genetic lines must be assessed. This review considers the utility of assaying certain constituents with roles in drought tolerance for phenotyping genotypes. Abscisic acid (ABA), organic and inorganic osmolytes, compatible solutes, and late embryogenesis abundant proteins, are considered. Confounding effects that require appropriate tissue and timing specificity, and the need for high-throughput and analytical cost efficiency are discussed. With future advances in analytical methods and the value of analyzing constituents that provide information on the underlying mechanisms of drought tolerance, these approaches are expected to contribute to development crops with improved drought tolerance.

Keywords: water stress, compatible solutes, metabolites, osmotic adjustment, abscisic acid, plant breeding

INTRODUCTION

Phenotyping involves measurement of observable attributes that reflect the biological functioning of gene variants (alleles) as affected by the environment. In general, phenotyping for crop improvement via breeding requires that hundreds or thousands of genetic lines be assessed. To date, most phenotyping of secondary traits (i.e., those traits in addition to yield, which is often the primary trait) has involved field assessments of easily scored morphological attributes such as plant height, leaf number, flowering date, and leaf senescence. However, investigators have recognized that drought tolerance involves metabolic and regulatory functions, for which measurements of targeted processes are likely to provide valuable information on the underlying biology, and suggest approaches by which it could be modified. Moreover, excellent methods have been developed for assay of such traits, and they have been used in controlled-environment studies to determine the mechanistic basis of drought response. Notwithstanding their positive aspects, many of these methods are too time-consuming, expensive, or technically demanding to be used in large-scale phenotyping. The challenge, then, is to identify those attributes that provide the most meaningful phenotypic information to design sampling methods suitable for use in the field, and design analytical methods that can be efficiently scaled up to the number of samples required in phenotyping projects.

An important prerequisite for the successful phenotyping of secondary traits is to identify key functional attributes that contribute to drought tolerance. Ideally, such identification is based on evidence that there is genetic variation for the trait in the crop of interest, on the trait being correlated with crop performance in drought environments, and on its having sufficient heritability to be used to make progress in a breeding program. Another criterion is that a trait has a clear-cut and rational explanation for its physiological or molecular function in drought tolerance. Some key traits that satisfy this latter criterion include: (i) favorable stomatal behavior; (ii) rooting depth; (iii) osmotic adjustment (OA) and other processes that sustain cell integrity and function; (iv) carbohydrate storage and remobilization; and (v) sustained development (as opposed to abortion) of harvested organs. This article will discuss methods that can contribute to phenotyping of these five traits by analyzing the presence and level of substances that can serve as diagnostic tests to evaluate them. While methods for field-based assessment of these traits are described in other articles in this special issue, the focus of the current article is on laboratory-based analyses of tissue constituents in samples obtained from field-grown plants.

CASE STUDY: ABSCISIC ACID

Although it is valuable to focus phenotyping efforts on metabolic traits that have a clear-cut rational connection with stress tolerance mechanisms, this goal is often difficult to achieve in practice. A particular constituent may be involved in several metabolic and signaling systems, and its involvement may differ in various tissues and developmental stages. To illustrate the difficulty, the prospects for phenotyping abscisic acid levels as an indicator of stress response will be considered below.

WHY PHENOTYPE ABSCISIC ACID?

Abscisic acid (ABA) plays a central role in plant response to water deficit, and the extent to which a genotype synthesizes and accumulates ABA is a possible indicator of that genotype's adaptation to drought. It has been demonstrated that ABA has regulatory roles in all five of the traits listed above. While this argues for its importance, it also presents difficulty with respect to the use of

ABA analysis as a phenotyping tool, because the multiple effects can confound interpretation.

A situation where higher leaf ABA could be advantageous is in environments where water-use efficiency (WUE) or postponement of dehydration is desirable. A recent example related to this idea involved production of transgenic canola (*Brassica napus*) with an enhanced response to ABA signaling (Wang et al., 2005). Investigators anti-sensed farnesyl transferase, a negative regulator of ABA signaling. They used a drought-responsive promoter (RD29A), thereby confining the expression to drought episodes. Studies showed a slightly earlier stomatal closure response during the onset of water deficit and, in field trials, yields appeared to be slightly higher in drought environments. In addition to ABA sensitivity, genotypes that generally have higher activity for ABA synthesis in leaves might maintain lower stomatal conductance, closing their stomata at an earlier stage of soil water depletion, or closing them sooner after daybreak, thereby keeping the tissues at a high water potential and avoiding damage from dehydration. Maintaining a low stomatal conductance can also increase WUE, because it keeps the CO_2 concentration in the leaf internal airspace (Ci) below the CO_2-saturating (asymptote) portion of the photosynthesis versus Ci function. Elevated ABA in the afternoon can restrict stomatal opening when temperature and leaf-air vapor pressure deficit (VPD) are highest, and when a genotype couples this behavior with low ABA and partial stomatal opening in the morning, WUE is enhanced. While analysis of ABA is a way to predict stomatal status, other methods, might be better suited to this purpose. Notably, stable isotope assay of ^{13}C discrimination, or field sampling of stomatal diffusive conductance is more direct and, in the case of ^{13}C, are more integrative of Ci throughout the season (Condon et al., 2004, 2007).

However, the basis for a genotype's tendency to accumulate leaf ABA may not be as straightforward as a genetic tendency for high ABA synthetic activity. A genotype with a shallow root system or low hydraulic conductance, which restricts the supply of water to the leaves, can also cause high ABA accumulation. Evidence that measured ABA levels can be related to root architecture is provided by presence of a quantitative trait locus (QTL) in maize, Root-ABA1, which was initially identified as a QTL that affected leaf ABA (Lebreton et al., 1995; Giuliani et al., 2005; Landi et al., 2007). The QTL was identified in two different maize populations, and in several different environments and locations. Yet, in both cases, root architecture was related to leaf ABA accumulation. This QTL, in Bin 2.04 of chromosome 2, was initially identified by QTL mapping of leaf ABA in populations scored under water limited environments. The QTL allele for high ABA also confers decreased stomatal conductance, as expected, given the role of ABA in stomatal closure (Lebreton et al., 1995; Giuliani et al., 2005). In addition, greenhouse studies of backcross-derived lines have shown that the high ABA QTL allele is associated with increased root dry weight, root diameter, and root:shoot ratio, and with decreased root lodging (Giuliani et al., 2005). While it is not yet known whether the observed phenomenon is related to the effect of ABA in stimulating seedling root growth (Sharp et al., 1994; Ober and Sharp, 2007), an alternative interpretation is that the QTL allele alters root architecture to a shallower root system that rapidly depletes surface moisture. This would be consistent with the observation in these studies that the QTL allele for high ABA is associated with lower grain yield (Landi et al., 2007) and with other genetic studies that have shown a correlation between high leaf ABA and lower grain yield in water deficit environments (Mugo et al., 1998; Setter et al., 2011). Thus, to gain valuable phenotypic information, data on leaf ABA levels need to be interpreted in relation to other information, such as the depth of soil water availability and stomatal behavior. An ideal genotype may be one with relatively low levels of leaf ABA, indicating good root depth and water transport properties, but with desirable stomatal behavior, indicating high stomatal sensitivity to ABA.

INTERPRETING ABA PHENOTYPIC DATA IS TISSUE-DEPENDENT

ABA levels in organs other than leaves also require interpretation to be valuable for phenotyping. For example, as a stress-signaling hormone, ABA increases the expression of numerous gene products that have putative roles in stabilizing proteins and membrane systems so that they are better able to tolerate desiccation stress. However, ABA is also associated with the arrested development of sink organs, including soybean seeds (Liu et al., 2004), rice spikelets (Ji et al., 2011), maize kernels (Ober et al., 1991; Setter et al., 2001), and wheat grains (Westgate et al., 1996; Ji et al., 2011). Consistent with this, among lines of maize, a negative correlation has been found between ear ABA levels and prepollination ear growth rate (**Table 1**; Setter et al., 2011). This relationship suggests a role of ABA in the tendency of some genotypes to limit partitioning to ear and silk growth during water deficit, which increases the anthesis-silking interval (ASI), and is associated with decreased grain yield in drought environments (Ribaut et al., 2009). Analogous relationships between water deficit and arresting of reproductive organ growth are found in rice, sorghum, and other crops (Matthews et al., 1990; Liu et al., 2004, 2006; Leport et al., 2006).

For genes that are regulated by ABA, the role of these gene products in crop performance is yet to be well established. For example, there is abundant evidence that, among the genes regulated by ABA signaling, those encoding members of the late embryogenesis abundant (LEA) family are among the most rapidly and

Table 1 | Genotypic correlations between ABA levels and growth rates in ears and silks in a recombinant inbred line (RIL) population (Cimmyt P1 X P2) in two trials (2001 and 2002) under severe water deficit at flowering at the Tlaltizapan, Mexico field station.

	Silk ABA 0 DAA in 2001	Silk ABA 7 DAA in 2001	Silk ABA 0 DAA in 2002	Ear ABA 0 DAA in 2002
Ear growth rate 0 to 7 day after anthesis	−0.90	−0.62	−0.82	−0.57
Silk growth rate 0 to 7 day after anthesis	−0.51	−0.56	−0.42	−0.87

Tissues were harvested at the indicated days after anthesis (DAA). (Source: Setter and Ribault, unpublished data).

substantially upregulated by ABA (Quatrano et al., 1997). LEA proteins were first identified as abundant proteins that accumulate during the late stages of seed formation when desiccation is about to begin. They were among the first ABA-regulated proteins for which promoter ABA response elements (ABRE) and associated elements in upstream DNA sequence were determined. Nevertheless, the mechanism(s) and value of LEA proteins and specialized compatible solutes in drought tolerance of tissues have not been established except for tissues undergoing extreme desiccation (Wise, 2003; Wise and Tunnacliffe, 2004; Goyal et al., 2005; Tunnacliffe and Wise, 2007; Bies-Etheve et al., 2008). However, even when a crop is subjected to a severe drought stress that diminishes yield by 70% or more, its organs usually do not reach water potentials and levels of desiccation in the range experienced by drying seeds where these stabilizing agents are effective. This argues against the idea that more ABA accumulation would be favorable as a stimulus for upregulating LEA and other agents for macromolecular stabilization. Moreover, as discussed above, studies have indicated that there is a negative correlation between ABA accumulation in floral tissues and kernel set. Therefore, in contrast to the leaves, in the case of developing flowers and young sinks, genotypes with high levels of ABA are less likely to perform well in drought environments.

TIMING OF TISSUE SAMPLING FOR ABA

A further consideration in the use of constituent quantification for phenotyping is the dependence on timing of tissue sampling. Again referring to the ABA case study, leaf ABA levels depend on environmental and tissue development conditions. For phenotyping, a further consideration is that the levels of ABA measured in a tissue reflect the steady state of dynamically changing rates of ABA synthesis and catabolism (Ren et al., 2007). Quantification of free ABA provides an instantaneous measure of the system. Such an estimate is inherently dependent on the environment, with a likelihood of strong influence from genotype-by-environment interaction (GEI). Careful control of time of day and stage of soil water dry-down may help to control these influences. Alternatively, to obtain phenotypic values that have a relatively strong genetic component (and high heritability), it can be beneficial to average out the instantaneous environmental effects. A more time-averaged or integrative measure of ABA status might be obtained with a composite estimate of the sum of ABA plus its catabolites. The primary routes of ABA catabolism are 8′-hydroxylation to form phaseic acid and downstream products, and glucosyl esterification of the carboxyl group to form ABA-glucose ester (ABA-GE; Nambara and Marion-Poll, 2005; Priest et al., 2006; Yang and Zeevaart, 2006). The products of hydroxylation (phaseic acid and dihydrophaseic acid) also form glucose esters and other conjugates. To the extent that these catabolites are biologically inactive and accumulate into vacuoles or other compartments, their collective level could represent a valuable estimate of long-term flux through the ABA synthesis pathway. Following this logic, Setter et al. (2011) phenotyped ABA, ABA-GE, and phaseic acid in a diverse panel of maize genotypes that was used for association genetic analysis. While the phaseic acid data revealed a new marker-trait association unrelated to ABA, in this situation the addition of these ABA catabolites to the analysis did not strengthen any of the trait-marker associations that were obtained from ABA data alone. Development of cost-effective methods for a broader suite of catabolites has the potential to further improve this strategy.

METHODS FOR ABSCISIC ACID QUANTIFICATION

The prospects for an expanded range of substances that can be phenotyped have been steadily improving. In the case of ABA, methods for determining the levels of and the hormone and its metabolites can be classified into two general categories: (i) physical-chemical methods; and (ii) immunochemical methods. Advantages of physical-chemical methods include the fact that they are based on fundamental properties of the compounds and, in the case of the more advanced methods that employ mass spectrometry (MS), their chemical specificity. Advantages of immunochemical methods include their low cost and ease of scale-up for high-throughput projects. The levels of ABA are usually much too low (typically a few nmol g^{-1} dry weight) relative to other substances in an extract for methods based solely on high performance liquid chromatography (HPLC) with ultraviolet (or photodiode array) detection to have sufficient selectivity. Such methods are in common use for carotenoids and other substances which are chemically related to ABA but are much more abundant (Harjes et al., 2008). However, they do not in themselves provide sufficient separation and selectivity for ABA and other plant hormone work. Good selectivity can be achieved with gas chromatography-mass spectrometry and, when used with selected ion monitoring (GC-MS/SIM) and robotics, throughput can be satisfactory for large-scale projects. However, some metabolites, notably ABA-GE, are not stable at GC temperatures, and the method is not well suited to profiling a wide range of hormone metabolites in a single pass. A method for plant hormone profiling has been developed that is capable of analyzing in a single run ABA and its metabolites, as well as hormones in the cytokinin, gibberellin, and auxin families (Chiwocha et al., 2003; Ross et al., 2004). This method utilizes reverse-phase HPLC separation, coupled to electrospray ionization and MS with multiple reaction monitoring (MRM). Although chromatography only partially separates compounds from one another, a high level of specificity is achieved by MRM, wherein each ionized compound in a plant sample gives a distinct precursor-to-product ion transition that is diagnostic of that particular compound. An important attribute of the system is the development and use of deuterated internal standards to correct for workup variability. The system is capable of quantifying ABA levels in the 10^{-12} g range, which permits a tissue sample of about 50 mg dry weight to be analyzed for the whole profile of hormones and their conjugates. Cost is a limitation for phenotyping projects that involve large numbers of samples. The system utilizes elaborate instrumentation and requires about 40 min per sample, leading to a rather high cost per sample (Zaharia and Abrams, 2011).

A second category of methods for ABA analysis involves immunochemical procedures with labeling from either a radioisotope (radioimmunoassay, RIA) or an enzyme (enzyme-linked immunosorbent assay, ELISA). These methods take advantage of the high level of binding specificity of antibodies to discriminate between substances in a complex mixture. In RIA, a radioactively

labeled form of the analyte (e.g., ABA) is mixed with the sample to provide quantification, while in ELISA an enzyme reporter system is used to generate a measurable signal indicating presence of the analyte. Both immunochemical systems have been widely used for ABA quantification (Mertens et al., 1983; Walker-Simmons, 1987; Quarrie et al., 1988; Vernieri et al., 1989; Perata et al., 1990; Philosoph-Hadas et al., 1993; Banowetz et al., 1994; Xie et al., 1996; Kalantari et al., 2001; Hradecka et al., 2007).

An advantage of immunochemical analyses over physical-chemical systems is the low detection limit. The mid-point of the assay range for ABA ELISA is about 0.2×10^{-12} mol (Setter and Parra, 2010). This means that tissue samples as small as 1 mg dry weight are sufficient, permitting field sampling of small, but representative, portions of leaves or floral parts whose removal does not significantly affect plant growth in the field plot for yield and other phenotyping data. In practice, samples are usually composites of several plants per plot and of sufficient size for them to be used for analysis of multiple constituents in addition to ABA. In addition to analyzing ABA, it is also possible to use ABA immunoassay to analyze ABA catabolites in plant samples. ABA-GE can be assayed by chromatographically separating ABA from ABA-GE, then using alkaline hydrolysis to cleave the ester linkage between ABA and glucose, and then imunoassaying ABA as usual. Phaseic acid (PA) and its glucose ester can be analyzed with immunoassay as well, using a monoclonal antibody directed against the conjugated form of PA (Gergs et al., 1993; Setter et al., 2011). To date, there are no reports of the development of antibodies directed against the other major ABA catabolite, dihydrophaseic acid (DPA). If an anti-DPA antibody were to be produced, investigators would have a set of antibodies that could be used to immunoassay a full profile of ABA and its most abundant catabolites.

OSMOTIC SOLUTE ACCUMULATION AND OSMOTIC ADJUSTMENT

Osmotic adjustment refers to the accumulation of osmotically active solutes in response to the imposition of stress. It is a potential contributor to drought tolerance, and a sizable body of literature about it has developed. The potential benefit OA provides to a plant is that it helps cells retain water in the face of decreasing water potential that would otherwise result in cell shrinkage, distortion, and plasmolysis (**Figure 1**).

Although currently available tools to assess OA have enabled progress to be made in developing our understanding of the physiological role of OA and in identifying genotypic differences, studies that have involved large-scale phenotyping of populations have had mixed results. As discussed below, this may be due, in part, to a lack of easy-to-use sampling and analytical methods. Nevertheless, several studies have reported identification of QTLs for OA and related traits in crops (**Table 2**).

A major difficulty of current methods of measuring OA is that it is necessary to harvest fresh tissue and immediately perform several operations on it while keeping it alive, keeping cell membranes intact, and not disturbing metabolism. For field studies, this generally requires care to prevent overheating of specimens during handling, and the availability on-site of apparatus such as a balance to weigh fresh tissue and an osmometer to measure solute potential. Also, since samples for all plots should be taken at a defined number of days after water deficit, the need to sample and analyze fresh tissue means the investigator must perform the analyses on-the-spot and cannot put the samples in storage for analysis at a later, more convenient time.

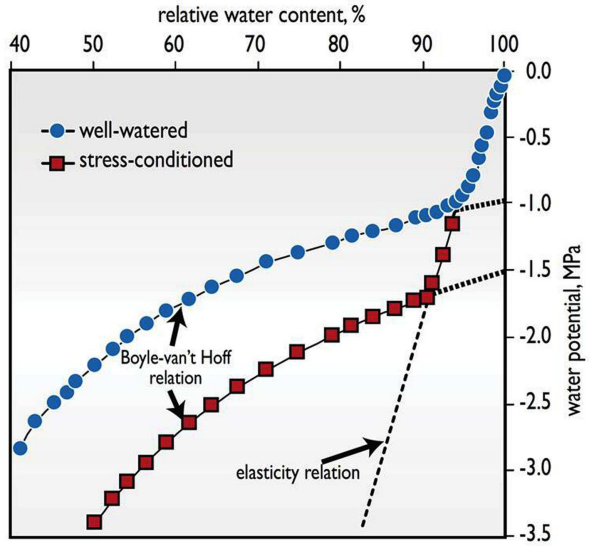

FIGURE 1 | The advantages of accumulating osmotically active solutes is illustrated with the response of total water potential (Ψ_w) to a lowering of relative water content (RWC) in wheat leaves, comparing a plant that was well-watered with another that was stress-conditioned by several cycles of water deficit and rewatering over a number of weeks, causing it to accumulate additional osmotically active solutes (Adapted from Melkonian et al., 1982). Two phases are identified. In tissue equilibrated with free water ($\Psi_w \approx 0$ MPa), a large positive turgor pressure balances the osmotic component of water potential. As water is lost, turgor decreases steeply, reflecting the rather stiff cell walls in mature organs, in accordance with the elasticity relation. At RWCs below the point of zero turgor (the inflection point), the relationship is dictated by the less steep Boyle-van't Hoff relation between osmotic solute activity and water potential, $\Psi_w \approx -RTC$, where R is the ideal gas constant, T is the absolute temperature, and C is the concentration in moles of osmotically active solutes per kg of water. Tissues that accumulate more solutes via the process of osmotic adjustment, such as the stress-conditioned leaf shown here, shift the Boyle-van't Hoff curve to lower water potentials. As a consequence, the tissue can maintain positive turgor over a wider Ψ_w range and incur less shrinkage at still lower levels of Ψ_w below the turgor loss point. Such shrinkage and low Ψ_w can damage the integrity of cell membranes and other cell constituents, and lead to cell death as in stress-induced leaf necrosis.

Several studies have assessed the adequacy of various methods for quantifying OA. In a comprehensive comparison of four methods for evaluating OA, Babu et al. (1999) considered two of them feasible for phenotyping work. In the first of these methods, the tissue relative water content (RWC) and osmotic potential are measured, then the osmotic potential is extrapolated to an RWC of 100% using an assumed RWC-solute potential relationship. In the second, the plants (or portions thereof) are fully rehydrated overnight. Then the osmotic potential is measured on samples. It is assumed that the rehydration does not alter solute concentrations significantly, even though it takes several hours and sometimes involves floating specimens on water, allowing the possibility of

Table 2 | Studies in which QTLs were identified for osmotic adjustment (OA) and related traits.

Crop	QTLs identified			Citation
	OA	Solute potential	Others	
Barley	x	x	$\Delta^{13}C$, soluble carbohydrate	Diab et al. (2004)
Barley	x	x	RWC	Teulat et al. (1998)
Barley	x	x	Carbohydrate, RWC	Teulat et al. (2001)
Cotton	x		$\Delta^{13}C$, yield, canopy temperature, chlorophyll	Saranga et al. (2004)
Cotton	x			Saranga et al. (2001)
Rice	x	x	Lethal osmotic potential	Lilley et al. (1996)
Rice	x			Robin et al. (2003)
Rice	x		Root traits	Zhang et al. (2001)
Sunflower	x	x	Turgor, RWC	Kiani et al. (2007)

RWC, relative water content.

solute efflux. Measurement of osmotic potential involves several steps that must be carried out immediately and with care to avoid evaporative losses. Sap is obtained either by pressing it out of fresh tissue or releasing it from freeze-thawed tissue, then transferring it to a dew point or freezing point osmometer. Measurement of RWC is also a substantial undertaking under field conditions. Usually, tissue specimens are placed into preweighed vials and the vials are weighed soon after collection to obtain the initial fresh weight. Samples are then incubated in water for several hours to rehydrate fully, are blotted to remove unabsorbed surface water, weighed again, dried in an oven, and weighed a final time. With so many manipulations and measurements, the final calculated value for RWC inevitably incurs some loss of precision.

Another potential weakness of OA phenotyping is that it is a composite property that has many contributing components. A wide range of osmotically active solutes can contribute to osmotic potential and to OA. This can confound identification of OA QTLs and the genes responsible for OA. An alternative approach that is becoming ever more realistic with the advent of high-throughput analytical methods for large numbers of metabolites ("metabolomics") is to analyze the main constituents that contribute to OA. There have only been a few studies where the substances contributing to the osmotic component of water potential have been determined (**Table 3**). The main categories of osmotic solute are sugars, mineral ions such as potassium, and nitrate, organic acids, and amino acids. In most cases, potassium along with its anion partners is the main class of osmotically active solute. In certain cases, such as the leaves of sorghum and maize, sucrose and other sugars are important contributors to OA, while the contributions of proline and other amino acids are relatively small on a bulk tissue basis. Hence, it is feasible to quantify each of the major contributors to OA in phenotyping projects.

MEASUREMENT OF OSMOTICALLY ACTIVE SOLUTES

Determination of the component compounds which contribute to OA can be straightforward and done on a large scale. For example, in studies of maize, a population of over 200 recombinant inbred lines (RILs) was sampled at three stages of stress in 2 years with replication such that over 10,000 samples were analyzed (T. Setter and J.-M. Ribaut, unpublished data). Sampling involved cutting disks from mature leaves of several plants representative of each plot and immersing them in tubes containing ice-chilled 80% methanol. In this solvent, metabolic changes are halted by the cold and by the enzyme denaturing action of methanol. Samples for determination of osmolyte can be stored safely in the solvent for weeks, permitting the research team to schedule further work on them at a convenient time. In mature leaves with living cells (Ψ_w above the lethal point), the cell walls are elastic but sufficiently rigid to prevent significant shrinkage at the prevailing RWCs, so that passive concentration of solutes on a leaf area basis is not a significant contributor to the measured content of solutes.

SUGARS

Several methods are available for analyzing organic osmolytes. If there is likelihood that a particular compound or related group of compounds are of primary interest, it is possible to use targeted assays. The analysis of sugars can be done using coupled enzyme reagents that use glucose oxidase/peroxidase, or hexokinase/glucose-6-phosphate-dehydrogenase, and invertase, to develop a chromogen whose color is determined in a 96-well colorimeter (Cairns, 1987; Setter et al., 2001; Alves and Setter, 2004). These steps are easy to scale up to the desired number of samples by using 12- or 96-channel pipetting and automated data transfer from colorimeter to computer. Three QTLs for leaf sugar accumulation were identified using this approach (**Table 4**). In support of the hypothesis that sugar accumulation contributed to leaf stability during water deficit, it was observed that the accumulation of leaf sugar had significant genetic correlation with the maintenance of leaf chlorophyll at 3 weeks into drought, as expected for a protective osmolyte effect (**Table 4**, lower panel).

METABOLOMIC APPROACHES

An alternative approach to targeted assays is use methods that are capable of assaying a wide range of compounds in a single step. Such metabolomic approaches are capable of quantifying hundreds of metabolites in a single chromatographic operation, though they require a substantial budget and justification for using a broad exploration of constituent composition. Recent reviews describe a wide variety of powerful analytical systems that have been developed for this purpose (Stitt and Fernie, 2003; Sumner

Table 3 | Contributions to total solute potential by sugars, potassium (K) salts, and proline in mature leaves of plants subjected to water deficit.

Family	Species	Common name	Osmotic adjustment (MPa)	Contribution to solute potential (MPa)			Citation
				Sugars	K-salts	Proline	
Asteracea	*Helianthus annuus*	Sunflower	0.17	−0.04	−0.84	nd	Jones et al. (1980)
Asteracea	*Heteropogon contortus*	Spear grass	0.39	−0.09	−0.30	−0.02	Ford and Wilson (1981)
Brassicacea	*Brassica napus, B. junea*	Canola, juncea	0.39	−0.24	−0.64	−0.03	Ma et al. (2004), Ma and Turner (2006)
Euphorbeacea	*Manihot esculenta*	cassava	0.27	−0.14	−0.85	0.00	Alves and Setter (2004)
Leguminacea	*Cicer arietinum*	Chickpea	0.76	0.02	nd	nd	Basu et al. (2007)
Leguminacea	*Macroptilium atropurpuueum*	Siratro	0.34	−0.09	−0.41	0.00	Ford and Wilson (1981)
Leguminacea	*Trifolium alexandrinum*	Berseem clover	0.41	−0.23	−0.88	−0.12	Iannucci et al. (2002)
Leguminacea	*Trifolium incarnatum*	Crimson clover	0.24	−0.20	−0.71	−0.09	Iannucci et al. (2002)
Leguminacea	*Trifolium resupinatum*	Persion clover	0.20	−0.20	−0.70	−0.08	Iannucci et al. (2002)
Leguminacea	*Trifolium squarrosum*	Squarrosum clover	0.52	−0.30	−1.11	−0.11	Iannucci et al. (2002)
Malvacea	*Gossypium hirsutum*	Cotton	0.30	−0.02	−0.41	nd	Cutler and Rains (1978)
Poacea	*Cenchuus cillaris*	Buffell grass	0.71	−0.05	−0.68	−0.02	Ford and Wilson (1981)
Poacea	*Panicum maximum*	Green panic	0.55	−0.06	−0.38	−0.04	Ford and Wilson (1981)
Poacea	*Pennisetum glaucum*	Pearl millet	0.40	−0.11	−0.88	−0.06	Kusaka et al. (2005)
Poacea	*Sorghum bicolor*	Sorghum	0.49	−0.25	−0.66	nd	Jones et al. (1980)
Poacea	*Triticum durum*	Durum wheat	0.02	−0.16	−0.86	−0.06	Bajji et al. (2001)
Poacea	*Triticum durum*	Durum wheat	0.39	−0.22	−0.24	−0.05	Rascio et al. (1994)
Poacea	*Triticum durum*	Durum wheat	0.08	−0.16	−0.74	−0.18	Kameli and Lösel (1995)
Rhamnacea	*Ziziphus mauritiana*	Indian Jujube	none	−0.13	−0.23	−0.02	Arndt et al. (2000)
Rosacea	*Prunus persica*	Peach	0.15	−0.05	−0.43	0.00	Arndt et al. (2000)
Vitaceae	*Vitus vinifera*	Grape	0.41	−0.84	−0.61	nd	Patakas et al. (2002)

Values are referenced to RWC=100%. Potassium is assumed to have an equivalent concentration of monovalent anion partner. (nd, not determined).

et al., 2003; Schauer and Fernie, 2006; Dixon et al., 2007; Shulaev et al., 2008; Saito and Matsuda, 2010; Hall, 2011). For the analysis of organic osmotic solutes, GC-MS with either quadrupole or time-of-flight (TOF) mode is capable of quantifying several hundred compounds of diverse classes including sugars, sugar alcohols, organic acids, amino acids, and fatty acids. However, samples require derivatization to make them volatile for GC analysis. HPLC-MS is a favored alternative not requiring derivatization and more suitable for unstable compounds (Tohge et al., 2011). For phenotyping a narrower range of compounds, nuclear magnetic resonance (NMR) has the advantage of not requiring separation and providing high-throughput.

Apart from sugars and sugar alcohols, the main contributors to the organic osmolyte pool are organic acids (Hummel et al., 2010), amino acids, and, in some species, quaternary ammonium compounds (QACs). High-throughput analysis of organic acids can also be performed using coupled enzyme procedures, as described above for sugars. For example, malate and citrate dehydrogenase and associated colorimetric reagents can be used. However, these assays operate in parallel mode. Thus, as more analytes are added to a project, assay of a full profile of them on a large set of samples involves splitting samples into aliquots and running numerous separate assays, which becomes cumbersome and costly. With appropriate choice of analytics, metabolomics methods are able to include these additional analytes.

There are a few published examples of metabolomics used in plants. Among those studies involving stress responses, Gagneul et al. (2007) studied the effect of salt treatment in the halophyte *Limonium latifolium* using a combination of GC-MS for organic substances, HPLC-fluorescence detection for amino acids, proton NMR for QACs, flame photometry for sodium and potassium, and salicylate-colorimetry for nitrate. They concluded that, contrary to expectations, organic solute accumulation is predominantly constitutive and only slightly modulated by salinity. The major contributors to osmolarity were inorganic solutes and, although present, the compatible solutes proline, QACs, and inositols were rather minor. Sanchez et al. (2008), studying *Lotus japonicus*, also used GC-MS to assess salinity effects on organic solutes, whereas they used ICP to analyze inorganic ions. They found that *L. japonicus* had broad shifts in metabolism in response to salinity, with decreases in potassium and organic acids, and increases in many amino acids, sugars, and polyols. Schauer et al. (2006) reported a QTL study of tomato fruit morphology and metabolites. By using GC-MS to quantify 74 metabolites, they identified 889 QTLs for metabolite levels, offering the possibility that this approach could be used to modify fruit composition and quality.

A further advantage of the metabolomics approach is that clusters of correlated metabolites may be identified that are either part of a common metabolic pathway or whose accumulation

Table 4 | Upper panel: QTLs identified for leaf sugar accumulation in a maize RIL population (Cimmyt P1 X P2) subjected to severe water deficit during flowering. Lower panel: genetic correlations between leaf sucrose and leaf chlorophyll.

Trait	Year of trial	Chromosome bin of QTL
Leaf suc 3W	2001	8.04
Leaf suc 4W	2001	8.04
Leaf glc 2W	2001	8.04
Leaf suc 2W	2001	8.04
Leaf glc 3W	2002	8.04
Leaf glc 4W	2002	8.04
Leaf suc 3W	2002	8.04
Leaf total sugar 3W	2002	8.04
Leaf total sugar 2W	2001	9.04
Leaf total sugar 4W	2001	9.04
Leaf suc 4W	2001	9.04
Leaf suc 2W	2002	9.04
Leaf total sugar 2W	2002	9.04
Leaf total sugar 3W	2002	9.04
Leaf suc 3W	2002	9.04
Leaf suc 4W	2001	10.03
Leaf total sugar 4W	2001	10.03
Leaf glc 2W	2002	10.03
Leaf suc 2W	2002	10.03
Leaf suc 4W	2002	10.03
Leaf total sugar 2W	2002	10.03
Leaf total sugar 4W	2002	10.03
Genetic correlations	**Leaf suc 3W**	
Leaf chlorophyll 2W	0.80	
Leaf chlorophyll 3W	0.53	
Leaf chlorophyll 4W	0.98	

Trials were in 2001 and 2002 at Tlaltizapan field station, Mexico. Leaf disks were sampled at 2, 3, and 4 weeks after withholding irrigation (2W, 3W, 4W) and analyzed with coupled enzyme procedures for sucrose (suc), glucose (glc), and total sugar, expressed per unit leaf area. (Source: Setter and Ribaut, unpublished data).

is coordinately regulated. For instance, in comparisons of barley cultivars differing in salt tolerance, Widodo et al. (2009) found that the tolerant cultivar responded to salinity by accumulating a broad range sugars, polyols, and organic acids and maintaining relatively low levels of amino acids. Using MS systems to quantify over 500 metabolites in *Arabidopsis* leaves subjected to water deficit, Urano et al. (2009) found that amino acid levels responded coordinately and involved ABA signaling, whereas raffinose oligosaccharide accumulation responded differently. Co-regulated clusters may provide a more reliable and robust phenotypic trait than any individual compound, thus providing more utility for phenotyping.

POTASSIUM

The single most abundant osmotic solute in water stressed plant tissue is usually potassium. Flame photometry, atomic absorption spectrometry and inductively coupled plasma-atomic emission spectrometry (known as ICP) are usually the methods of choice (Munns et al., 2010).

For profiling the levels of potassium and other mineral ions, ICP spectroscopy is the most commonly used method. Although usually performed in relatively small numbers of samples, it can be automated to increase the rate of throughput, as has been done on large "ionomics" projects (Lahner et al., 2003; Salt et al., 2008).

A further consideration in the analysis of metabolites is that metabolite concentrations tend to be variable with respect to time of sampling, age of an organ, and environmental conditions. To characterize a genotype, investigators might be able to use full-scale metabolomics to identify a smaller set of key diagnostic compounds such that it is cost-effective to determine levels of a small number of key compounds in several organs and at several time-points rather than to determine the whole metabolic profile for just one sample. For phenotyping projects that involve hundreds or thousands of samples, it may be valuable to use more targeted, less expensive methods for all samples, and reserve full-scale metabolomics for a subset.

ANTIOXIDANTS

In leaves, water deficit can result in excess electron flow to the production of reactive oxygen species (ROS) which in turn damages leaf membranes and proteins (Demmig-Adams and Adams, 2002). While high-throughput luminol chemiluminescence methods are available to assay the composite ROS levels in tissues subjected to stress, the functions of ROS are multifaceted and include signaling (Mittler et al., 2011), thereby complicating their interpretation. It is plausible that metabolomics may provide valuable phenotypic information on the spectrum of antioxidants and photoprotectants in a genotype. For example, studies indicate that the xanthophyll carotenoids perform a critical photoprotectant role (Demmig-Adams and Adams, 2002), and methods for metabolite profiling of carotenoids are now available (Fraser et al., 2007).

COMPATIBLE SOLUTES

Compatible solutes are small molecular weight osmolytes which are highly soluble in the cell solution and do not interfere with cellular metabolism, even at high concentrations. They differ in this regard from inorganic solutes, which can disrupt protein and membrane structure at high concentrations. Examples of compatible solutes include proline, QACs (glycine betaine), and sugar alcohols (mannitol, pinitol). Given their putative properties, they have been of interest with respect to genetic improvement of crops for a long time. The possibility of manipulating the levels of compatible solutes through transgenic metabolic engineering has been considered to be within grasp because the genes encoding their synthesis and catabolism have been cloned. While such overexpression transgenics have had limited success in improving drought tolerance (Abebe et al., 2003; Su et al., 2006; Chen and Murata, 2008; Szabados and Savoure, 2010; Sanchez et al., 2012), it might still be of interest to phenotype populations to identify alleles that contribute to compatible solute synthesis. In addition to the metabolite and protein assays described above, another strategy might be to analyze the activities of key enzymes in compatible solute synthesis or catabolism. Recently, methods have been developed to vastly increase the throughput of such assays, so that it is

now feasible to use this approach in phenotyping. For example, Gibon et al. (2004) reported the development of a robot-based system for assay of the activity of 23 enzymes that are involved in central carbon and nitrogen metabolism.

LATE EMBRYOGENESIS ABUNDANT PROTEINS

Studies of plant organs that undergo extreme desiccation, such as dry seeds and resurrection plants, have led to the discovery of a broad class of proteins called "LEA proteins." LEAs are highly expressed during desiccation and are thought to play a role in desiccation tolerance, although the mechanisms for this are not yet known. LEAs are unstructured, unfolded proteins that are highly hydrophilic and remain water soluble even when heated to 80°C. This is due to their high content of glycine, glutamic acid, glutamine and lysine the near absence of cysteine, and their unique peptide profiles (Wise and Tunnacliffe, 2004; Tunnacliffe and Wise, 2007). But there is evidence that during stress, LEAs can develop a secondary structure and interact with proteins and membranes, and this may explain their function in drying tissue. Under such conditions, they might serve a chaperone function to form three-dimensional structures that could provide stability to cellular protein and membrane systems, to prevent enzymes and other structures from aggregating and denaturing (Goyal et al., 2005). In the highly dehydrated state, they might also serve as a molecular shield to prevent excessively close proximity of proteins, or they might serve a water-binding role that helps create a more favorable environment for protein stability during desiccation. Studies of transgenic plants in which LEA polypeptides have been overexpressed have been reported to contribute to tolerance of water loss (Sivamani et al., 2000; Cheng et al., 2002; Xiao et al., 2007). Much of this work involves severe water loss, such as in desiccation. Hence it is possible that their roles only come into play when a tissue experiences severe dehydration, such as during advanced stages of a drought episode, where soil water is depleted to a severely low water potential and leaves desiccate to an even lower water potential. In these cases, LEA proteins may act together with compatible solutes to prevent tissue death, and permit subsequent rehydration to a viable state, if and when deeper rooting or precipitation occurs. Hence, with respect to crop growth during the vegetative stage, the situations in which they might be most important are where more tolerant genotypes use LEA polypeptides to help avoid lethal desiccation and ensuing cell death, such as in conditions where susceptible genotypes suffer from leaf firing and tassel blasting.

Late embryogenesis abundant levels have been measured using immunochemical approaches. By taking advantage of the fact that members of each LEA family of polypeptides have a few conserved domains, antibodies developed against a conserved domain are useful in detecting several members of the family (Close and Chandler, 1990). Assays using such antibodies have been developed for protein gel blots and ELISA (Jayaprakash et al., 1998; Volaire, 2003; Yang et al., 2007; Pinheiro et al., 2008). Recently, proteomic methods wherein polypeptides are separated by two-dimensional polyacrylamide gel electrophoresis followed by identification by liquid chromatography-mass spectrometry (LC-MS) have been used to analyze the levels of LEA polypeptides in plant tissues (March et al., 2007). As with other "omics" methods, proteomics provides the capability to survey the levels of the whole family of LEA polypeptides, which are numerous; for example, there are 50 members in *Arabidopsis* LEA family (Bies-Etheve et al., 2008).

REPRODUCTIVE AND STORAGE ORGAN DEVELOPMENT

Many important traits that contribute to superior crop performance in stress environments involve better developmental regulatory systems whereby deep root growth is stimulated and lateral rooting decreased, or development of sink organs that will contribute to yield is sustained rather than aborted. These traits can be especially challenging to phenotype using analytic methods due to their complex regulatory networks and the large number of component factors. While quantification of easily scored morphological traits such as root length and plant height are straightforward, there may be value in quantifying processes that underpin these growth processes and the impact of stress on them. Methods to phenotype processes that contribute to seed-set, fruit-set and tuber-set are of interest for many crops, as are similar developmental attributes that are often diminished in stress, and genetic selection for these traits has been successful in improving crop yield in drought stress Ribaut et al. (2009). While morphological measures of these traits can be effective (e.g., ASI in maize, spikelet fertility in rice, and components of yield in most crops), it is possibly of interest to have diagnostic tests that would involve sampling tissue at the moment it is engaged in developmental decision-making, so that genotypes could be evaluated more directly for important stress tolerance behavior. For example, in maize and rice, a common response to water deficit at flowering is decreased cell wall invertase (INCW) activity (Ji et al., 2005; Boyer and McLaughlin, 2007). Given that expression of INCW represents one of many genes that are part of the growth-arrest syndrome, measurement of INCW levels can be considered a diagnostic test for this growth-arrest phenotype. Many other diagnostic test candidates can be considered, including other growth-specific proteins such as cyclins (for cell division), expansins (for cell expansion growth), and starch synthase (for starch accumulation).

In addition to direct participants in growth, the level of expression of key signaling or transcription factor proteins could also be considered valuable in this phenotyping strategy. Candidates for diagnostic tests might be identified from transcript profiling studies that are being carried out on reproductive tissue exposed to drought. For example, studies by Yu and Setter (2003) in maize and Agarwal et al. (2007) in rice have profiled transcription in pedicels, endosperms, and panicles during their early stage of development, and have found sets of transcripts that are consistently up- or downregulated in response to stress and studies by Fujita et al. (2010) have identified transcripts expressed at specific stages of reproductive organ development. In a review of over 100 transcriptomic studies of plant response to drought and salinity, Deyholos (2010) concluded that with future improvements, transcriptomics could be valuable as a screening tool for candidate gene discovery. This approach is consistent with the strategy of identifying a small number of key transcripts (e.g., Rabbani et al., 2003; Boyer and McLaughlin, 2007) which could be used to construct a diagnostic test of stress tolerance attributes for use in phenotyping large populations. Tools for handling hundreds of RNA samples are available. For example, real-time polymerase chain

reaction (RT-PCR) can be run on 384 plates fairly economically and reliably.

Other methods are under development to extend transcriptomic assays from being highly focused on just a few samples (profiling tens of thousands of genes) toward a capability of efficiently handling thousands of samples. An advantage of such an approach is that it might reveal genetic variability for processes underlying the decision-making involved in reproductive set versus abortion. This approach could be particularly effective for developmental traits such as flower and seed-set, which involve complex signaling and gene expression networks rather than clear-cut metabolic pathways. Methods for morphological measurement of growth or components of yield at final harvest as currently used might miss some of this information. As the cost of transcript profiling by RNA sequencing (RNAseq) and other genomics methods continue to decrease, the prospects of using these methods for phenotyping will be enhanced. This may enable clustering of RNAseq (or proteomic) data points to identify higher order patterns in profiles of gene expression that correlate with superior stress tolerance. Such systems approaches might capture trait information on a complex process such as sustained floral development that could not be explained by quantifying a small set of transcripts.

CONCLUSION

Investigators now have a wide range of analytical tools to use in measuring metabolites, proteins and transcripts in plant tissues. The tools range from inexpensive to costly, from single-purpose to broad based profiling in "omic" mode. The most appropriate choice for phenotyping will depend on a project's goals, the relative merit of each analytical approach, the cost, and any trade-offs between phenotyping a large number of entries at low cost per analysis versus a smaller number in great detail.

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Bridging the phenotypic and genetic data useful for integrated breeding through a data annotation using the Crop Ontology developed by the crop communities of practice

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The Crop Ontology (CO) of the Generation Challenge Program (GCP) (http://cropontology.org/) is developed for the Integrated Breeding Platform (IBP) (https://www.integratedbreeding.net/) by several centers of The Consultative Group on International Agricultural Research (CGIAR): bioversity, CIMMYT, CIP, ICRISAT, IITA, and IRRI. Integrated breeding necessitates that breeders access genotypic and phenotypic data related to a given trait. The CO provides validated trait names used by the crop communities of practice (CoP) for harmonizing the annotation of phenotypic and genotypic data and thus supporting data accessibility and discovery through web queries. The trait information is completed by the description of the measurement methods and scales, and images. The trait dictionaries used to produce the Integrated Breeding (IB) fieldbooks are synchronized with the CO terms for an automatic annotation of the phenotypic data measured in the field. The IB fieldbook provides breeders with direct access to the CO to get additional descriptive information on the traits. Ontologies and trait dictionaries are online for cassava, chickpea, common bean, groundnut, maize, Musa, potato, rice, sorghum, and wheat. Online curation and annotation tools facilitate (http://cropontology.org) direct maintenance of the trait information and production of trait dictionaries by the crop communities. An important feature is the cross referencing of CO terms with the Crop database trait ID and with their synonyms in Plant Ontology (PO) and Trait Ontology (TO). Web links between cross referenced terms in CO provide online access to data annotated with similar ontological terms, particularly the genetic data in Gramene (University of Cornell) or the evaluation and climatic data in the Global Repository of evaluation trials of the Climate Change, Agriculture and Food Security programme (CCAFS). Cross-referencing and annotation will be further applied in the IBP.

Keywords: Crop Ontology, breeding trait, plant phenotype, trait dictionaries, breeding fieldbook, data annotation, integrated breeding platform, crop community of practice

INTRODUCTION

In recent years, sequence information has become readily available for a variety of crop species. However, a gap is emerging between the physical genome information and the quantitative information regarding phenotypes. It is becoming clear that the application of quantitative genetic information by researchers and breeders is limited by a lack of standard nomenclature used to describe both crop development and agronomic traits. Without either a nomenclature or information, which provides the equivalence links between trait descriptions, it is hard to compare information from Quantitative Trait Loci (QTL) and association studies in a way that permits systematic transfer of knowledge about genotype-phenotype relationships among crops or between crops.

In the case of crop breeding programs, plant breeders repeatedly measure a large number of traits in order to understand the crop phenotype, based on variation in genotype and environment. Some traits are common across crops whereas some other traits are crop specific such as anthesis silking interval (ASI) for maize. Common traits across crops can be measured with different methods and scales. Likewise, one trait could be measured under several environmental conditions at different growth stages within a crop. Therefore, the management of crop characterization and evaluation data in databases at the global level is always complex and critical. The situation is more complex for traits like resistance to disease or to abiotic stresses such as drought and salinity tolerance. For example a plant pathologist could score stem rust disease in the greenhouse at seedling stage or in the

field (adult plants for severity and incidence) by artificial inoculation of pathogen or via natural infestation using different scoring rating scales. To enable comparison of these different types of measurements related to a single trait, and to support future modeling of the correlation among several traits the following are required: (1) that a nomenclature and controlled vocabularies in the form of ontologies are applied in databases and knowledge bases and (2) the data generated by the trials/experiments are properly annotated by crop communities practiced in using validated trait names, and adjusted to the recommended methods of measurement and scales. Data annotation is the addition of metadata (i.e., ontological terms) that describe the data file and possibly the data point. Phenotype and genotype data annotation enable researchers to attach information and data to a botanical term, a development stage and a trait name. It can also be used to specify the process through which trait data has been obtained and its provenance. Although annotation of genetic data is commonplace, data produced via phenotyping studies are usually not annotated using a controlled vocabulary to facilitate their integration into multi-crop platforms.

APPLICATION OF THE INTEGRATED BREEDING CROP ONTOLOGY IN CROP RESEARCH

The fundamental scientific question underlying research on diverse genotypes of any plant species is "What is the causal relationship between genotype and phenotype?" DNA is transcribed into RNA, which is either bioactive itself (as non-coding RNA gene products) or is translated into peptides that form part of protein gene products. Ultimately, these products act as structural elements, genetic regulatory control factors, or modulators of the biochemical fluxes within metabolic and physiological pathways, at the sub-cellular, tissue, organ, and whole organism level. This sum total of molecular expression integrates the overall structural and behavioral features of the plant—its "phenotype." The unfolding of this story also has an essential environmental context, including biotic (ecosystem) and abiotic (geophysical) factors modulating expression in a variety of ways via diverse sensory and regulatory mechanisms in the plant. Various classes of experimental data associated with this tapestry of germplasm function are summarized in **Figure 1**.

Phenotypes and genotypes can be characterized at various levels of abstraction and resolution (Bruskiewich et al., 2006). In the case of plant phenotypes, it includes measurements of traits at different growth stages, in various environments and treatment conditions. Genotypes include laboratory measurements of DNA and simple observations of visible phenotypes. The molecular variation measured by genotyping can be neutral or biologically significant. Neutral molecular variation generally involves markers that simply exhibit DNA structural polymorphism that is usefully applied to answer basic questions on the extent of similarity between germplasm samples (i.e., "fingerprinting" experiments) or on the chromosome location of a marker (i.e., "mapping" experiments). Answering such questions will often lead to deeper exploration of germplasm, such as evolutionary studies, practical management of plant crosses, and genetic resource management. Whatever the nature of phenotype and genotype measurements, the primary task is to completely capture and accurately codify the raw and derived phenotype

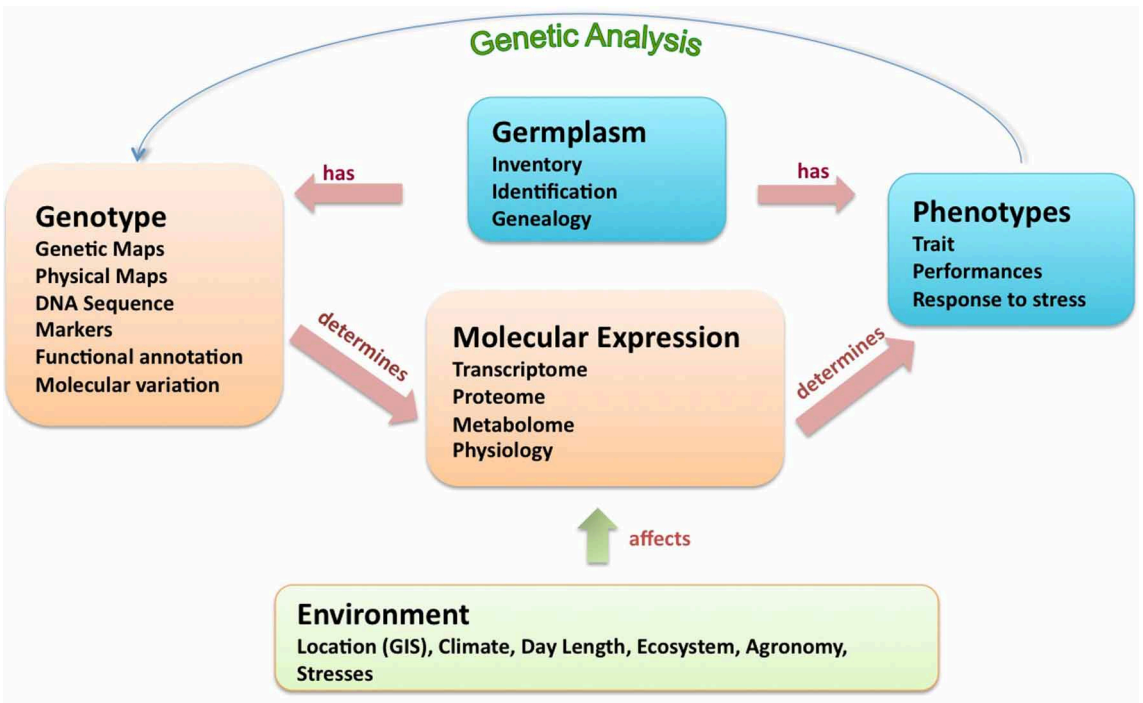

FIGURE 1 | Biological relationships in germplasm research adapted from Bruskiewich et al. (2006).

and genotype data. The role of the ontology is precisely to support the description of all the pathways between the gene and the expression of the trait, enabling data interpretation (Shrestha et al., 2011). The Crop Ontology (CO) provides additional terms and descriptions of traits, along with methods and scales that complement the Gene Ontology (GO; http://geneontology.org), Plant Ontology (PO; http://plantontology.org) and Trait Ontology (TO; http://www.gramene.org/) for bridging a wider set of annotated genetic, genomic, and phenotypic data with formalized phenotype descriptions and leading to data discovery. Documentation of protocols related to phenotypic data is very important for enabling comparison across crops, environments and plant growth stages and the CO aims to provide comprehensive information about the trait and the measurement of the trait.

THE CROP ONTOLOGY (CO) AND THE TRAIT DICTIONARIES IN THE INTEGRATED BREEDING FIELDBOOK

The Integrated Breeding Platform (IBP; https://integratedbreeding.net/) is developed by the Generation Challenge Programme (GCP; http://www.generationcp.org/) for crop breeders. The objective of the IBP is to provide access to modern breeding technologies, breeding material, and related information and services, in a centralized and functional manner. This should improve plant breeding efficiency in developing countries and facilitate the adoption of molecular breeding approaches (Delannay et al., 2011). The Integrated breeding fieldbook (referred to in the text as the IB Fieldbook, **Figure 2**) supports the harmonized capture of trait measurements in the evaluation sites and their integration in the crop databases. The fieldbook's trait template is based on the trait dictionary and includes a link to the corresponding trait name in the IB CO.

The objectives of the integrated workflow between the IB Fieldbook, the Trait Dictionary and the CO are (1) for breeders and data managers to define a standard list of traits; (2) for breeders to access more information on the trait and the protocols used for measurement when defining their evaluation experiment; (3) to provide an automatic annotation of the data captured by breeders via the CO terms. The CO, in combination with the crop trait dictionaries, provides a tool to foster the phenotypic and genotypic data curation and annotation by the communities of practice (CoP) of several crops using validated common trait names, particularly breeders' traits, protocols, and scales.

CREATING TRAIT DICTIONARIES FOR THE CROP DATABASES AND THE FIELDBOOKS

The IB Fieldbook and the crop databases based on the International Crop Information System (ICIS) contain the trait dictionaries to support the harmonization of the trait measurements across the phenotyping sites and the data annotation across databases. The trait dictionaries and the ontology are embedded into the crop databases for cassava, chickpea, rice, maize, wheat, and soon for banana, groundnut, cowpea, common beans, pigeon pea, and sorghum. Each crop-specific trait ontology and dictionary will be maintained by acrop lead center and/or a crop research community.

To assist breeders an Excel spread sheet template was developed to simplify the process of submitting traits, trait descriptions, allocation of categories or valid ranges and measurement

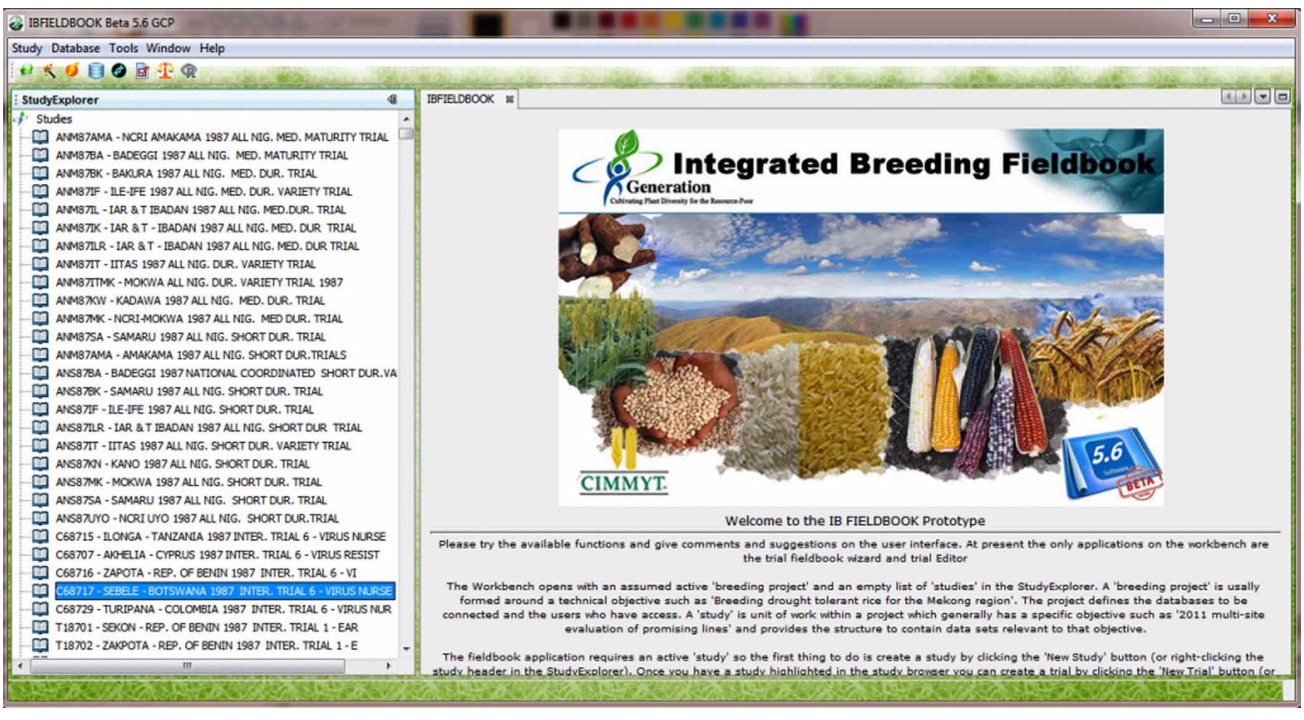

FIGURE 2 | Integrated Breeding Fieldbook for capturing trait measurement with mobile devices.

protocols. Utilization of the trait template was very helpful to obtain extended trait information and manage the quality control of trait names within the databases. Multi-location evaluation programs have been conducted in several countries to ensure that trait names are stored in the fieldbooks and databases in several languages. An indicator of the language has also been added to the online trait dictionaries so that crop communities can send trait names in different languages via the basic trait template. The same term identifier will be used for the same trait in different languages, so that different versions of the same trait are referred to as synonyms to facilitate the search of data across languages.

Recently, the trait dictionaries were used to prioritize the traits according to the frequency of use by breeders in their research programs and importance for the crop. The objective was to provide a core standard set of crop specific traits that will appear by default in the crop fieldbook wherever the crop is evaluated. A list of optional traits is also available and can be added by the breeder according to the evaluation objective. All existing trait dictionaries have been uploaded in the CO and are also available for download on each crop page of the IBP website. The harmonization between the CO and the trait dictionaries will be continuously performed by the CoP and the use of the online ontology will be prioritized to avoid deviation from a single reference list of traits, methods and scales.

DEPLOYING THE TRAIT DICTIONARIES ANNOTATED WITH THE CROP ONTOLOGY TERMS

The schema of the GCP crop database, along with the trait dictionaries, is being deployed within each CoP through the installation of a central database managed by the crop lead center and several local databases installed in the research stations and partners institutions. The trait dictionaries that include the CO terms are embedded into the central database and are maintained by crop data curators. The curator manages the validation and synchronization of trait dictionaries with the online CO curation tool. The local crop databases contain the reference trait dictionaries inherited from the central database that is used to design the field book template for the handheld or the printed form. This data flow (**Figure 3**) ensures that traits measured in the field are harmonized across sites and are captured within the template format. The CO terms and their identifiers, which are embedded into the fieldbook template, ensure that data are already annotated

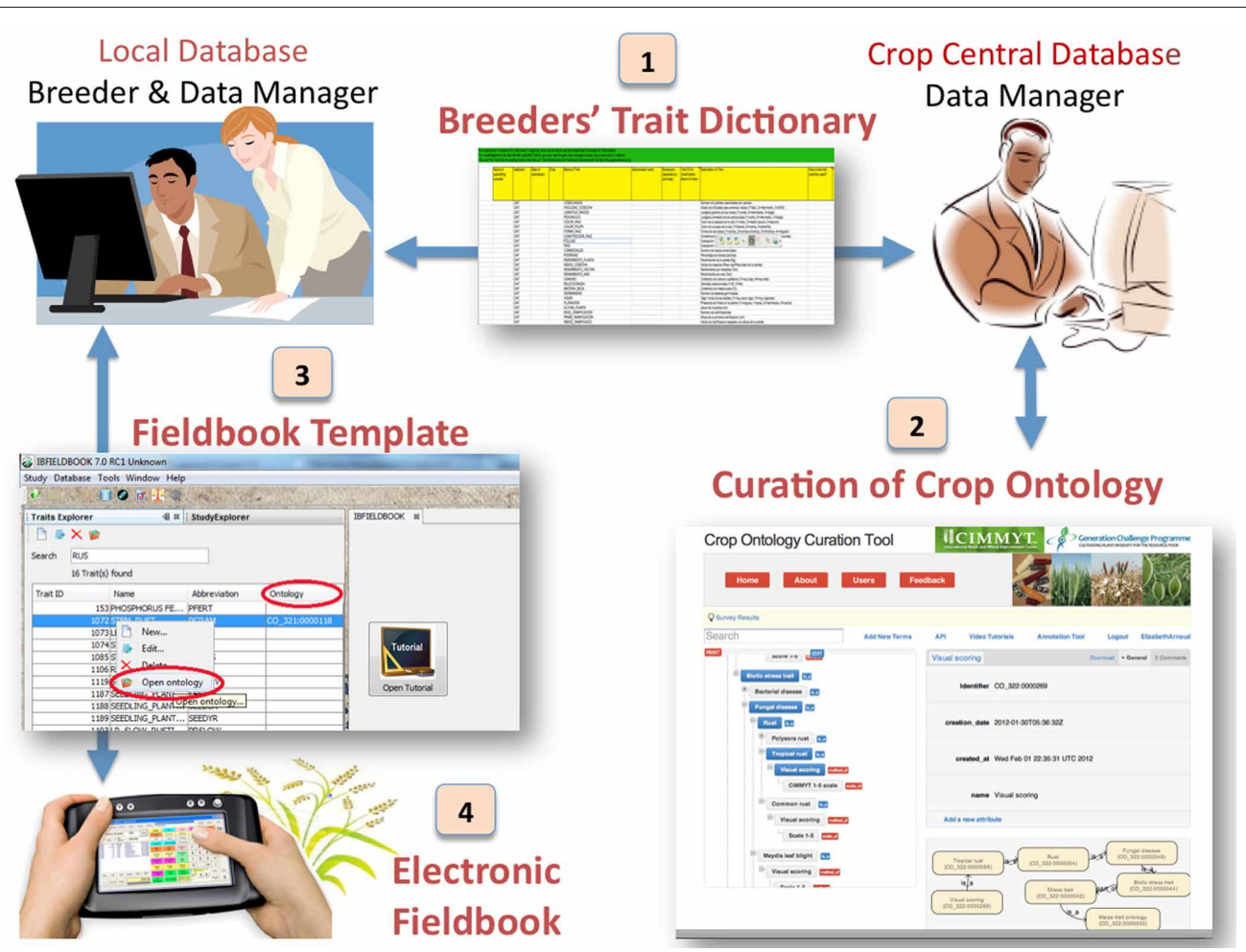

FIGURE 3 | Trait data flow between the ontology, the crop databases and the field book.

without any additional effort from the database curator. The annotated data could therefore easily be synchronized from the hand held data capture devise to the local database and then to the central crop database.

DEVELOPMENT OF THE CROP-SPECIFIC TRAIT ONTOLOGIES

At present, the CO provides crop-specific trait ontologies for cassava, chickpea, maize, musa, potato, sorghum, rice, wheat, as well as online trait dictionaries for common bean, cowpea, and groundnut developed by the crop lead centers of the GCP challenge initiatives. These simple trait lists built in the form of controlled vocabularies with short descriptions do not fulfill all the requirements for ontology-based access to data. Therefore, the trait dictionaries will be upgraded into ontologies by adding multiple relationships and cross referencing to other major ontologies. Since 2007, the crop-specific ontologies were developed in the crop lead centers, by teams of breeders, biometricians and data managers using the OBO-Edit software promoted by the Open Biomedical Ontology (OBO) communities such as GO (Ashburner and Lewis, 2002; Day-Richter et al., 2007), PO and TO (Jaiswal et al., 2002). By using OBO-Edit, ontology curators are able to construct the ontology from lists of traits, create the necessary multi-relationships between terms, and simultaneously create cross-references with the terms in TO and PO. Multi-relationships between biological terms provide the semantic framework, which is necessary to model the biological pathways, describing the expression of the traits in plants, in various tissues, at different development stages and different environments.

The CO describes agronomic, morphological, physiological, quality, and abiotic and biotic stresses related traits of several crops using most common "is_a" and "part_of" relations assigned by OBO-foundry (Shrestha et al., 2010). The methodology, which was applied for developing the PO and TO, was also used for developing the CO. In order to embed methods and scales in the Crop specific ontologies, new ontological relations were created such as "method_ of," "scale_of," and "derived_from" for meaningfully describe the traits and their relations to methods and scales (**Figure 4**).

THE ONLINE CROP ONTOLOGY SITE FOR A COMMUNITY-BASED CURATION AND ANNOTATION

In 2011, the new CO website (www.cropontology.org) was released providing a tool for participatory ontology development, curation, and annotation by the crop database curators (**Figure 5**). Users can browse crop-specific ontologies, access trait definition with the bibliographic reference, synonyms, images, term abbreviation, as well as online cross references to PO, TO and the GCP crop databases. The tool provides features for posting comments and printing trait information. Only crop specific curators are allowed to upload ontologies, add new terms and attributes of traits and edit text to control quality. Video tutorials are available in the website. The code used for the development is hosted on Google App Engine and the versioned code is hosted on GitHub.

Trait measurement methods are displayed as derived terms of the related trait name with newly created relationship "method_of" and scales are derived terms of their related method with relationship "scale_of" (**Figure 6**). Providing protocols related to traits facilitates the selection of appropriate terms for data annotation and data exchange across databases.

The prototype of the online annotation tool was inspired by Terminizer, developed by David Hancock (University of Manchester, http://terminizer.org/). This tool allows the user to associate the ontology terms with existing trait names extracted from the database or text and overcome the heterogeneous manner of naming the traits (**Figure 7**).

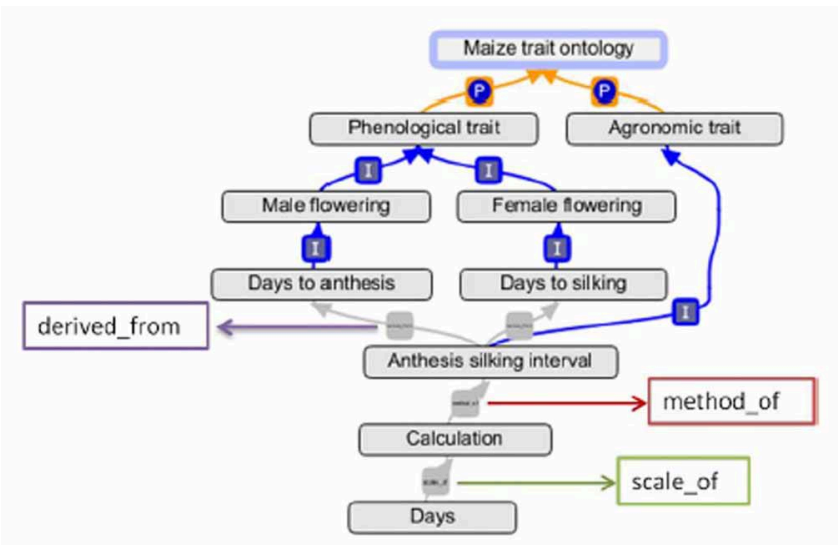

FIGURE 4 | Representation of the multi-relationships of "Anthesis silking interval" in OBO-Edit.

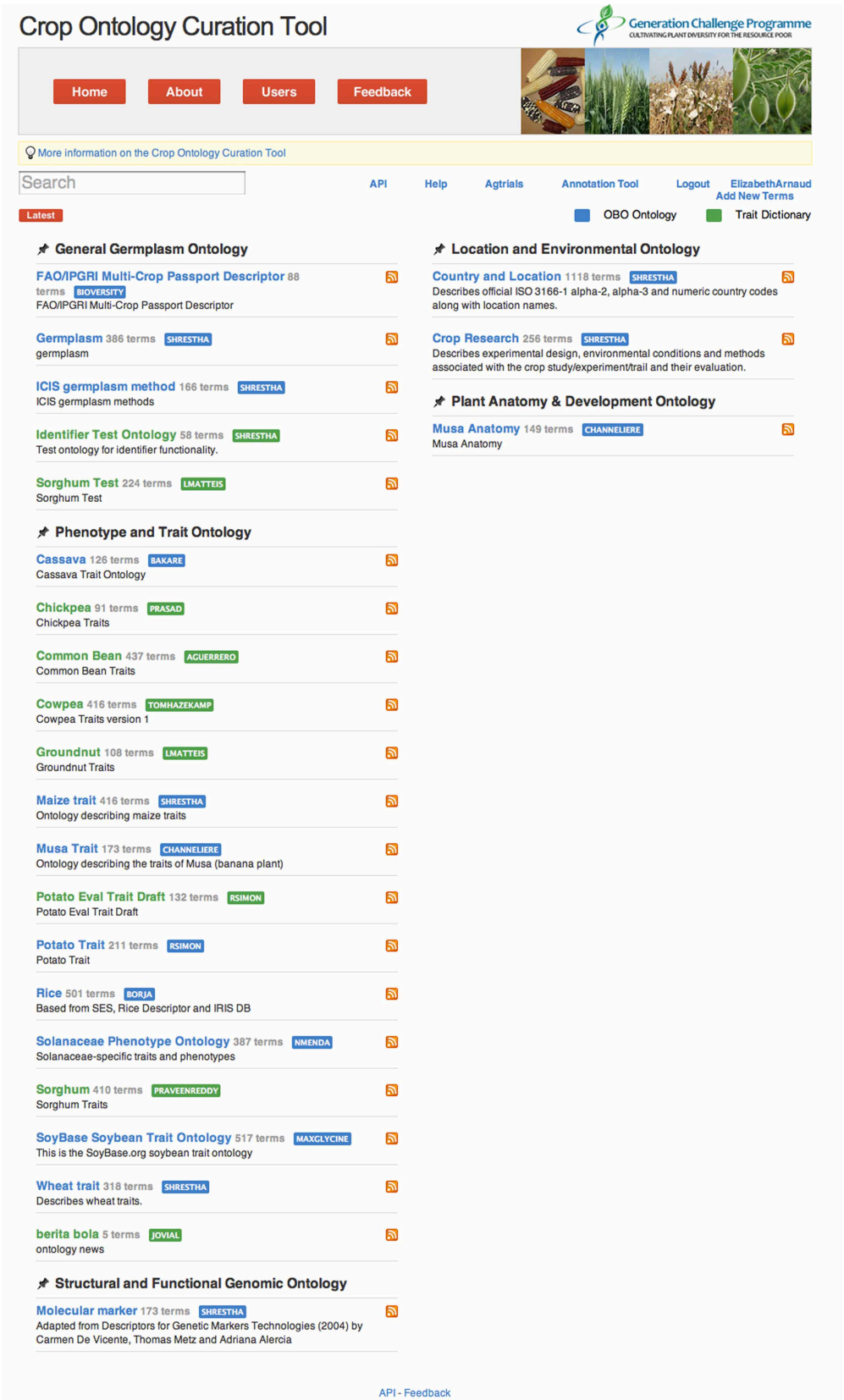

FIGURE 5 | Crop Ontology homepage (http://www.cropontology.org).

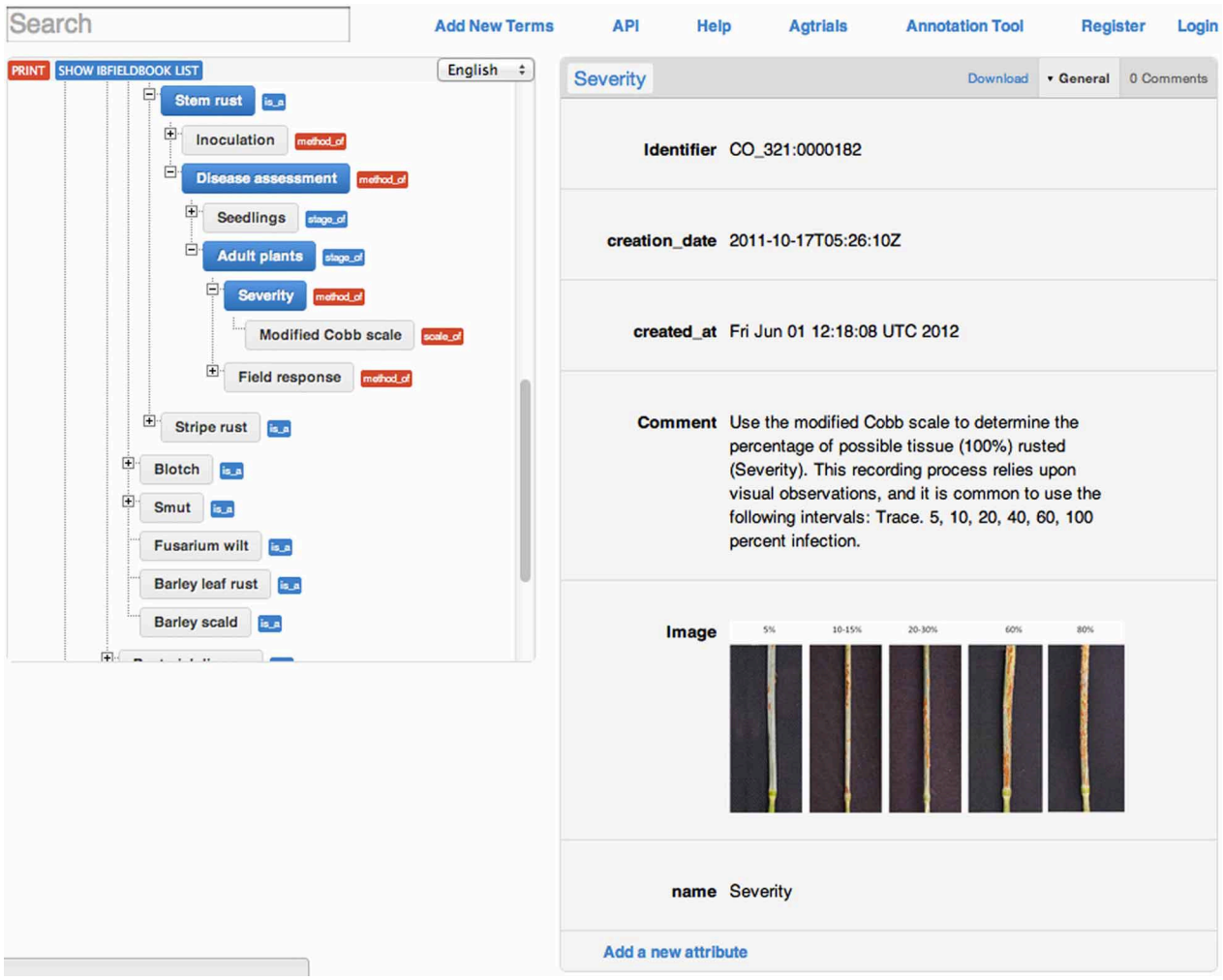

FIGURE 6 | Online display of new relationships "method_of" and "scale_of" for "stem rust" along with information and images about the scale used for measurement.

EXPANDING THE USE OF THE CROP ONTOLOGY INTO THE INTERNATIONAL COMMUNITY FOR DATA DISCOVERY

New CO terms were submitted for addition to PO and TO. The collaboration will continue through the cross-referencing of PO, TO and CO in order to develop internationally shared crop trait ontology. To extend the access to genetic information, CO curators have cross-referenced most of the traits with synonyms in PO and TO. An important online feature is the active web linkages of these cross-referenced terms that direct users to the corresponding term-specific page on Gramene (Cornell) or on PO and the annotated genetic data (e.g., QTL) associated with the trait (if available) (**Figure 8**).

The United State Department of Agriculture (USDA) and the Solanaceae Genomics Network (SGN)—who are presently the most interested to cross reference their respective ontology and data with the GCP CO to enable data integration—have uploaded their respective ontology on the online curation tool: the Soybean ontology for Soybase and the Solanaceae ontology.

AN OPEN SOURCE SERVER OF CROSS-REFERENCED TRAIT NAMES FOR DATA INTEGRATION

The online Integrated Breeding CO is a freely available resource that acts as open-source server for names of traits thanks to an Application Programming Interface (API). The API enables programmatic access to the CO by web sites, web services or data template wizards that can dynamically synchronize their lists of traits with the CO. This synchronization supports the harmonization of data annotation and then enables the discovery of annotated data through web queries based on the ontology terms. The first site to use the API is the Global Agricultural Trial Repository of the CGIAR program on Climate Change for Food and Agriculture Security (CCAFS; http://www.agtrials.org:8080/). The CCAFS initiative dynamically links the names of

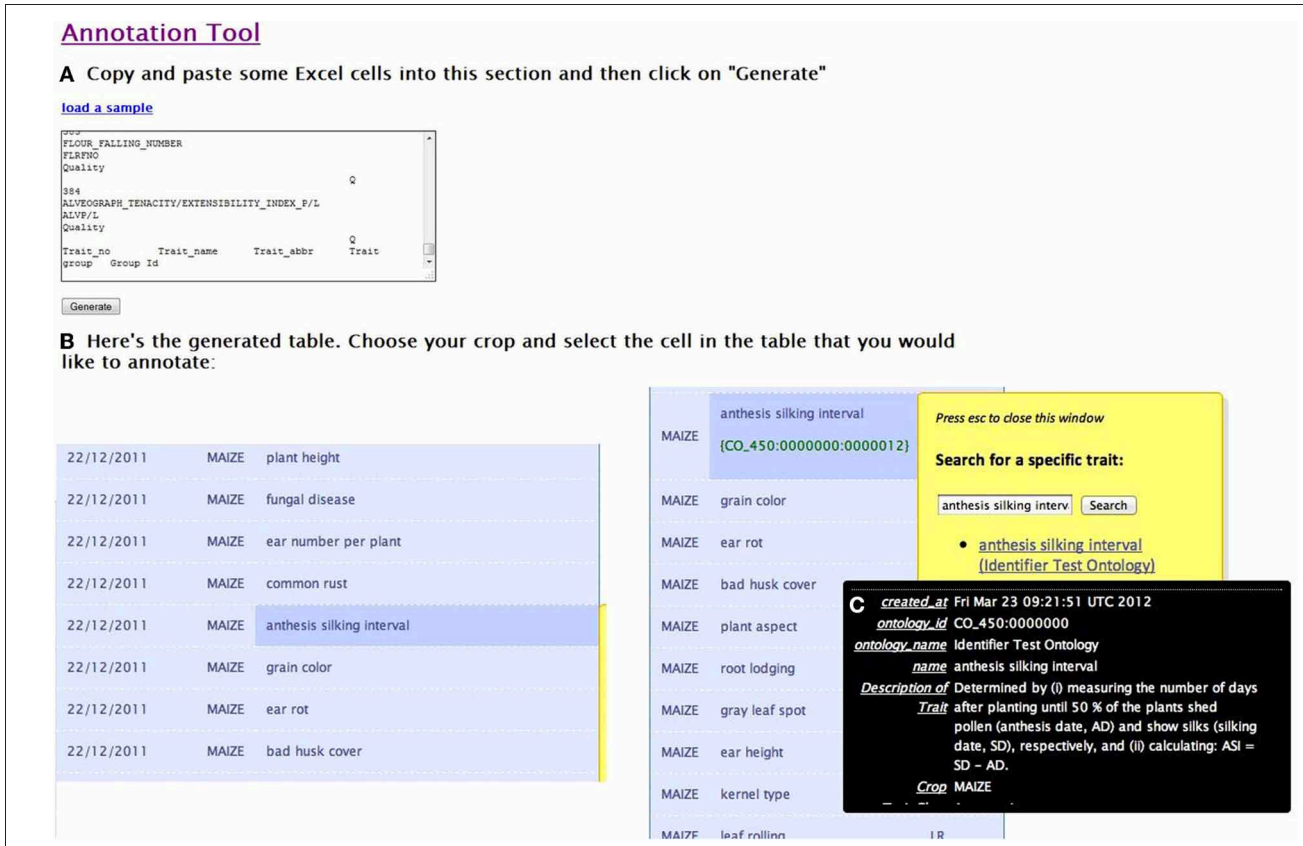

FIGURE 7 | Screenshot of the online annotation tool showing steps in the annotation process: (A) paste data or metadata to annotate (B) the tool generates a table and user can select one ontology (e.g., maize trait) before annotation (C) information and images about the corresponding ontological term are displayed below the term selected for annotation (e.g., anthesis silking interval). Users can check and validate or reject the proposition.

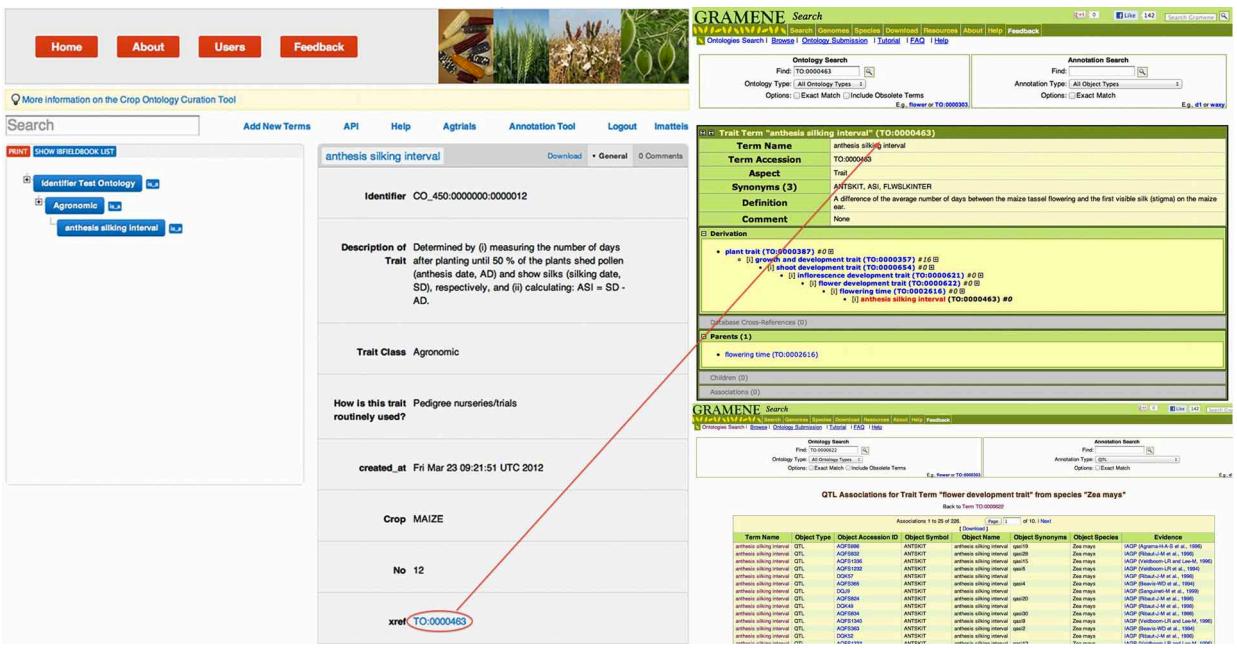

FIGURE 8 | Direct access to the QTL information associated with the trait "anthesis silking interval" on the Gramene website through the cross referencing link placed in the Crop Ontology.

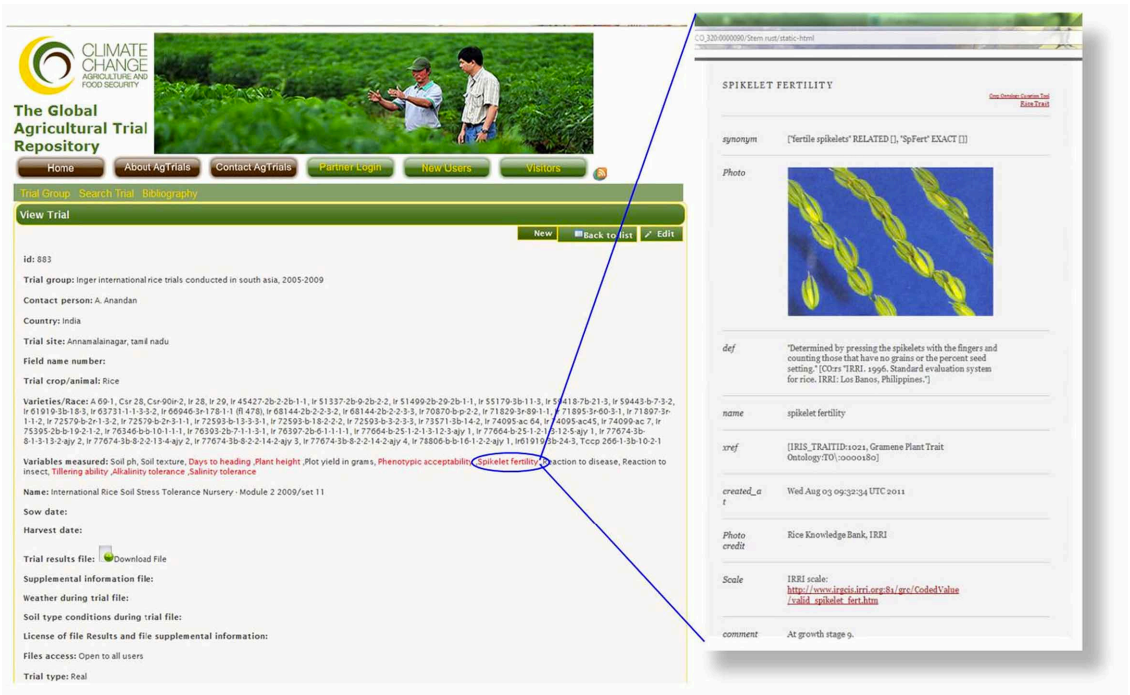

FIGURE 9 | Screenshot showing the dynamic link from the variable "spikelet fertility" on Agtrials to additional information in the online Rice Ontology.

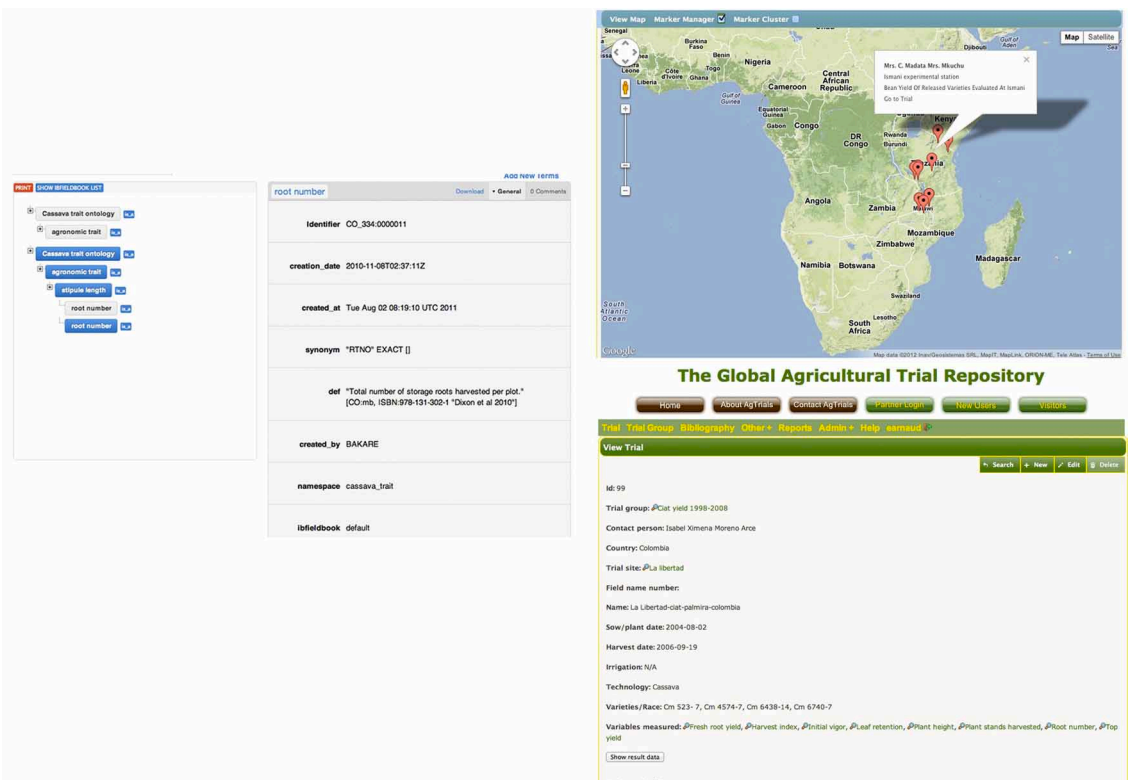

FIGURE 10 | Mockup of an ontological trait based access to the map of trials on Agtrials.

variables measured during the evaluation of varieties with the CO terms. The objectives are (1) to facilitate the annotation of the data files by users with harmonized trait names; and (2) to provide users with access to detailed information on the variables (**Figure 9**).

This cross-referencing prepares the ground for integration of online data into a single site, and the objective is to integrate this further within the IBP. Integrating the Agtrials website with the CO would provide, for any given trait and crop, access to the phenotypic data combined with geographical and environmental data (**Figure 10**).

CONCLUSIONS

The development of a GCP CO for breeders' traits is a pioneering activity that was acknowledged by major partners in the agronomic research and in the landscape of phenotype ontology development such as the USDA, the Solanaceae Genomics Consortium, Cornell University, the PO Consortium, the National Center for Biotechnology Information and the NSF Research Coordination Network on Phenotype. The CO development is currently based on Trait dictionaries defined by teams of breeders and data managers for direct use in the IB Fieldbook. This initiative facilitates direct annotation of breeders' data captured in the field and will enable the integration of phenotypic and genetic data sets. It will also help the breeders, when evaluating traits in the field, to access the correct trait information they need, including detailed standard protocols and scales. Thanks to the new online curation and annotation tool, the curators of crop specific ontologies can interactively modify existing trait names or add new ones along with images, methods and scales. A full ontology can easily be uploaded or created online, which encourages partnership for the cross-referencing of terms. Once published online, the cross reference of traits are converted into a web link to directly access related data in other websites like Gramene (University of Cornell) or Agtrials (CCAFS-CIAT). This is the premise of the integration of phenotypic, genotypic and environmental data associated with a given trait. The IBP will further utilize the CO to integrate as much as possible of the genetic data in the genomic data management system with the phenotypic data collected in the GCP phenotyping sites. This online access of the CO provides a useful mechanism for bridging a wider set of annotated genetic, genomic and phenotypic data with formalized phenotype descriptions that will lead to new data discovery.

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Spatial analysis to support geographic targeting of genotypes to environments

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Crop improvement efforts have benefited greatly from advances in available data, computing technology, and methods for targeting genotypes to environments. These advances support the analysis of genotype by environment interactions (GEI) to understand how well a genotype adapts to environmental conditions. This paper reviews the use of spatial analysis to support crop improvement research aimed at matching genotypes to their most appropriate environmental niches. Better data sets are now available on soils, weather and climate, elevation, vegetation, crop distribution, and local conditions where genotypes are tested in experimental trial sites. The improved data are now combined with spatial analysis methods to compare environmental conditions across sites, create agro-ecological region maps, and assess environment change. Climate, elevation, and vegetation data sets are now widely available, supporting analyses that were much more difficult even 5 or 10 years ago. While detailed soil data for many parts of the world remains difficult to acquire for crop improvement studies, new advances in digital soil mapping are likely to improve our capacity. Site analysis and matching and regional targeting methods have advanced in parallel to data and technology improvements. All these developments have increased our capacity to link genotype to phenotype and point to a vast potential to improve crop adaptation efforts.

Keywords: spatial analysis, genotype-by-environment interaction, geographic targeting

INTRODUCTION

From the 1960s to the 1980s, the Centers of the Consultative Group on International Agricultural Research (CGIAR) pursued research on genotype by environment interactions (GEI) research with relatively unfettered budgets. Their mandate was to produce new crop varieties, train people to use them, and get the seeds to the world's farmers. To this end, the Centers produced great networks of testing sites all over the world (see, e.g., Peterson and Pfeiffer, 1989; CIAT, 2001; Magorokosho et al., 2007). Collaborators came from national government breeding programmes and universities, wherever there was an interest. Many of the results are archived, and can be very useful in studies of GEI and in suggesting where a new genotype might fit. The collapse of the Berlin Wall saw a new era in funding for international agricultural research and development (Pardey et al., 2006), with developed nations reducing their contributions. The world's food supply problems were no longer of geopolitical importance. Accordingly, the international cultivar testing programmes have declined, hindering our capacity to supply farmers with improved varieties adapted to their environments.

While crop improvement programmes are faced with reduced funding for agricultural research, the use of models, maps and computer tools can help boost efficiency in their development and testing of cultivars for dissemination to farmers. Cultivar testing and dissemination programmes need spatial analysis to help target genotypes to environments. By supporting GEI assessments, maps and models can predict how well cultivars will respond to particular environments. Ultimately, spatial analysis can help in the dissemination of varieties to the farmers that need them.

How can spatial analysis be used to help breeders decide where to test and disseminate varieties? Systematic sampling of sites can help ensure coverage of a diverse range of environments where farmers may take up the cultivar. A site testing design focusing on one or several environments but leaving out many others could miss areas where the cultivar might produce high yields. A breeding programme with a fixed budget for testing may also want to avoid duplication of sites in similar environments.

Several advances over the last few decades have improved the capacity to apply spatial analysis to phenotyping and GEI analysis. Vital to all these advances has been the development of computer hardware and software that has allowed many types of analysis that were impossible to carry out before. Advances in weather and soil monitoring instruments have improved data collection, and a key resource for spatial analysis in agriculture has been the availability of climate data in digital formats. Global soil mapping efforts have been slow to develop—with little attention prior to 1950. New soil mapping methods have improved on the standard Food and Agriculture Organization (FAO) global soil base map at 1:5 million scale (FAO, 1996, 2008b). Agricultural censuses and surveys have also added to the set of data resources available. Related to these baseline datasets are derived data such as climate maps, crop distribution surfaces, and socioeconomic

information. These advances have led to agro-ecological zoning maps (Bunting, 1987), weather generators (Hartkamp et al., 2003), and sophisticated statistical analysis of GEI (e.g., Crossa et al., 2004; Setimela et al., 2005). More recently, combinations of crop simulation models and geographic information systems (GIS) have improved our understanding of spatial and temporal aspects of GEI (e.g., Loffler et al., 2005).

This paper reviews and discusses the development of spatial analysis for crop improvement and how it can be used to increase the efficiency of testing and deployment of genotypes. First, advances in the development of spatial data for agricultural applications are discussed, followed by how spatial analysis, and GIS can be used to support geographic targeting of genotypes to environments. The discussion includes the development of agro-ecological maps and environmental change considerations in crop improvement efforts. The paper concludes with a discussion of trends in the use of spatial databases and GIS in crop improvement programmes. Throughout the paper, references are made to data, tools, and resources for applying spatial analysis to crop improvement.

ADVANCES IN SPATIAL DATA

Several types of spatial analysis for crop improvement as conducted today would have been difficult to carry out even 5 or 10 years ago. Perhaps the greatest advances have come in mapping climate, although information on soils and other environmental parameters is now much more widely available than in the past. Advances in data availability have substantially increased the potential for spatial analysis to support the planning and assessment of phenotyping and variety trials. Assessments should ensure that a sufficient range of environments is tested, so as to adequately study GEI. Improvements in data have more than kept pace with advances in the methodology of spatial analysis for phenotyping. This section surveys data development for spatial analysis, and serves as a guide to spatial data acquisition for the agricultural scientist using GIS for phenotyping. **Table 1** lists some key spatial data sets that are publicly available and can be used in crop improvement efforts.

SOILS

Data on soil properties are a key category of information for agro-ecological assessments. However, advances in the development of soil datasets are hindered by the difficulty of mapping the entire world. The main problem is that soils can be highly variable even across short distances. Moreover, not all countries use the same soil classification systems. The concept of the likelihood or probability of finding a given soil property has been used to reflect data uncertainty at a particular point when using maps like the FAO 1:5 million soil map of the World (FAO, 1996, 2008b). This map remains the most widely used soil map for continental and global applications. Sanchez et al. (2003) derived soil constraint data in the context of the Fertility Capability Classification, based on this FAO map. The International Soils Reference and Information Center (ISRIC) also used the FAO soil map, adding soil profile information to develop the World Inventory of Soil Emission Potentials (WISE) database of derived soil parameters (e.g., pH, drainage, organic carbon content) for the world at 5 arc minute resolution (Batjes et al., 2007; Batjes, 2009). However, an initiative is underway to develop the Harmonized World Soil Database (FAO et al., 2008). The project aims to merge different soil maps and produce a new global map at a 1:1 million scale. To date, the effort includes FAO's regional Soil Terrain Database studies (SOTER; FAO, 1995), the European Soil Database and the Soil Map of China. The main gaps that need to be filled

Table 1 | Key spatial data sets that are publicly available.

Source[a]	Application	Resolution	URL
FAO SOIL	Soil analysis	1:5m	http://www.fao.org/nr/land/soils/digital-soil-map-of-the-world/en/
ISRIC	Soil analysis	n/a	http://www.isric.org/UK/About+Soils/Soil+data/
HWSD	Soil analysis	n/a	http://www.iiasa.ac.at/Research/LUC/luc07/External-World-soil-database/HTML/
WISE	Soil profile analysis	n/a	http://www.isric.org/UK/About+ISRIC/Projects/Track+Record/WISE.htm
CRU	Climate	0.5°	http://www.cgiar-csi.org/data/climate
IWMI World Water Atlas	Climate; hydrology	Various	http://www.iwmi.cgiar.org/WAtlas/
NOAA	GSOD[b]	Point data	http://www.ncdc.noaa.gov/oa/gsod.html
Worldclim	Climate	1 km	http://www.worldclim.org
NASA POWER	Climate	1°	http://power.larc.nasa.gov/
TRMM	Tropical rainfall	0.25°	http://trmm.gsfc.nasa.gov/
SRTM	Elevation	90 m	http://srtm.csi.cgiar.org/
AgroMaps	Crop distribution	n/a	http://www.fao.org/landandwater/agll/agromaps/interactive/page.jspx
Globcover	Land cover	300 m	http://ionia1.esrin.esa.int/
Biogeomancer	Gazetteer	n/a	http://www.biogeomancer.org/

[a] ISRIC, International Soils Reference and Information Center; HWSD, Harmonized World Soil Database; WISE, World Inventory of Soil Emission Potentials; CRU, Climate Research Unit of the University of East Anglia; IWMI, International Water Management Institute; NOAA, National Oceanic and Atmospheric Administration; NASA POWER, National Aeronautics and Space Administration Prediction of World Energy Resource; TRMM, Tropical Rainfall Measuring Mission; SRTM, Shuttle Radar Topography Mission.

[b] GSOD, Global Surface Summary of the Day.

include Central and West Africa, the Middle East and South Asia. This data set should be updated in the coming years as new data become available. Finally, CIAT (2009) has initiated the production of a digital soil map of Africa. This new effort aims to create high-resolution maps of better quality, based on innovations in the remote sensing of soil properties and the management of geographic information.

CLIMATE

Phenotyping programmes and GEI assessments can benefit from broad-scale climate analysis to assess to what extent sites represent target environments. An important recent advance in climatic analysis is the availability of ready-to-use climate data available over the Internet or in software applications. Acquiring climate data depended in the past on contacts between researchers who developed climate datasets. The overall quantity of weather station data has dropped compared to past decades (Ramirez-Villegas and Challinor, 2012). More recently, software tools such as CIAT's FloraMap®, Homologue and MarkSim® provide climate data associated with specialized applications (Jones and Thornton, 1993, 2000; Jones et al., 2002, 2007a). Other climate tools include some of FAO's standard data CD-ROMs and applications, such as their Local Climate Estimator (LocClim), and datasets on CD-ROM from the International Water Management Institute (IWMI) (FAO, 2005; IWMI, 2008). While some of these tools lack the capability to extract global or regional climate surfaces, they were the first to provide broad-scale climate data for agricultural science applications.

Two relatively new sources of data on the Internet have broadened the capacity to incorporate climate information in spatial analysis applications for agriculture. The University of East Anglia's Climate Research Unit data include key variables needed for climate analysis, such as rainfall, temperature, relative humidity, wind direction and speed, among others (New et al., 2002; CGIAR-Consortium for Spatial Information (CGIAR-CSI), 2006). Another important data source is Worldclim (Hijmans et al., 2005), which includes precipitation and temperature data available at spatial resolutions of 1 km and coarser. Worldclim has also derived some data sets from precipitation and temperature variables, including information on seasonality, temperature ranges, and climate conditions in the wettest, driest, coldest, and warmest months and quarters (Busby, 1991). Both of these datasets draw on spatial interpolation methods to estimate climate parameters between weather stations.

Climate datasets derived from remote sensing hold some promise for use in agro-ecological assessment. The Moderate Resolution Imaging Spectroradiometer (MODIS) satellite platform includes surface temperature data (NASA, 2008a). Rainfall estimates (RFE) from satellite-based datasets are now widely available. The Tropical Rainfall Measuring Mission (TRMM) provides RFE for 3-h time periods for much of the world (NASA, 2008b). The Climate Prediction Center MORPHing technique (CMORPH) dataset (Joyce et al., 2004) provides 3-hourly RFE globally at a spatial resolution of 0.25°. The RFE dataset from the National Oceanic and Atmospheric Administration (NOAA)/Climate Prediction Center (CPC) provides daily data at a spatial resolution of 8 km (Herman et al., 1997; Xie et al., 2002). These data have yet to be verified and validated to the point that they are widely used for agro-ecological assessments, although it should be noted that the RFE data form the basis of several famine early-warning products[1] and FAO routinely uses the CMORPH data in monitoring Desert Locusts[2]. Combining ground weather data with remotely sensed information will be a key area of research in the future.

ELEVATION

Elevation is another important data set for spatial analysis in agriculture and can be used to help establish the ecological niche of a genotype. It can be used as an auxiliary variable in assessing climate or in analysing the role of topography in agriculture. Until recently, global digital elevation models were derived from 1:1 million mapping efforts, such as the Digital Chart of the World (ESRI, 1992). The now widely available Shuttle Radar Topography Mission data set has 90 m spatial resolution, the best available from coverage of the whole land surface (Jarvis et al., 2004; CGIAR-CSI, 2008).

VEGETATION AND CROP GEOGRAPHY

Vegetation and crop geography assessments can be made from remote sensing data, censuses, and surveys, and from combinations of these. Remote sensing platforms provide vegetation data as an additional dataset for agro-ecological characterization, even though it has rarely been used in classification to date. The Advanced Very High Resolution Radiometer (AVHRR) and MODIS satellite platforms provide 1-km resolution data sets going back to 1980. Satellite data at finer resolutions can also produce vegetation data. The most common variables are the normalized difference vegetation index (NDVI) and the enhanced vegetation index (EVI).

Land cover maps derived from remotely sensed data can be used to match potential crop environments with areas classified as croplands. Several broad-area assessments have been conducted. These include GeoCover, GLC2000 and Globcover (Bartholome and Belward, 2005; Bicheron et al., 2006; Arino et al., 2008). Wood et al. (2000) developed a map of cropland intensity for the year 2000, showing the percentage of a grid cell with cropland. The Globcover dataset, a 2005 snapshot of land cover at 300 m resolution, is the most recent global land cover product. While global land cover datasets all have their shortcomings with respect to accuracy and discrimination of land cover types, their increasing availability will lead to their increased use for agricultural applications.

Research and development efforts have produced several important datasets on the geography of key staple crops, including cassava (Carter, 1987; Carter et al., 1992), sweet potatoes [International Potato Center (CIP), 2006a,b, Hijmans, 2001; Hijmans et al., 2001], beans (Wortman et al., 1998), maize (Hodson et al., 1999), rice (Huke and Huke, 1997; Robison et al., 1984), and wheat (Lantican et al., 2005; Hodson and White, 2007), among others. Unfortunately, these crop-specific mapping

[1] http://earlywarning.usgs.gov/adds/index.php?img1=rf&extent=af
[2] http://ingrid.ldeo.columbia.edu/maproom/.Food_Security/.Locusts/index.html

efforts often lack comparability between crops. They may have widely different temporal and spatial frameworks, as well as using different methods to produce the datasets. While this lack of standardization does not necessarily affect genotype targeting efforts, standardized initiatives would go a long way toward improving the quality of data for analysis.

Mapping programmes that include multiple crops could take advantage of a common set of standards in data development. Drawing on the support of United Nations (UN) member countries, FAO's AgroMaps programme aims to map sub-national agricultural production data from agricultural censuses and surveys (FAO, 2008a). FAO plans to link the effort with national level statistics from FAOSTAT—something that could improve the quality of both datasets. Other efforts map sub-national agricultural production at global, regional, and local levels but AgroMaps is the only one that makes its data freely available on the Internet. An inspection of the number of crops and the resolution of administrative districts points out some substantial limitations of AgroMaps—problems that will be difficult to overcome without greater international efforts to promote agricultural census-taking.

A recent trend in crop mapping is the combination of survey and census data with remote sensing information. Crop production data can be converted to grid cell maps to more precisely characterize the spatial distribution of the crop (e.g., Leemans and Van Den Born, 1994; Ramankutty and Foley, 1998; Leff et al., 2004; Ramankutty, 2004; You and Wood, 2006; You et al., 2009). The conversion allocates production to small grid cells where the likelihood of the presence of the crop is greatest, eliminating forest, urban, pasture and other types of land cover where we would not find the crop. While the conversion of production data to a grid cell framework raises concerns with ecological fallacy and the modifiable areal unit problem (MAUP), the coarseness of most production datasets requires grid maps (Openshaw, 1984; Freedman, 1999). Improving these grid maps requires, first and foremost, better input data. Researchers need greater spatial and temporal resolution of crops statistics and remotely sensed data. Even so, there is great scope for improving allocation algorithms used in making grid cell maps.

TRIAL SITES DATA

Efforts to target genotypes to environments may also take advantage of locating genetic resources data in terms of their respective development and testing sites or the location of pedigree accessions, including wild relatives of food crops (Jarvis et al., 2005). Many genebanks lack well-documented information on the spatial location of the materials they manage (Hijmans et al., 2000). When genetic resources data do have coordinate information, it is often incorrect, requiring an effort to georeference the data (Hijmans et al., 1999; Biogeomancer, 2007). Several efforts are now underway to address these issues and provide improved access to georeferenced genebank data. The Focused Identification of Germplasm Strategy (FIGS) system for Bread Wheat accessions is one example[3].

Maps of variety trial sites are essential for linking phenotyping to spatial analysis. International yield trials networks, such as the bean, *Musa*, maize and wheat initiatives (Peterson and Pfeiffer, 1989; Jones and Tezenas du Montcel, 1994; CIAT, 2001), have tended to develop reasonably good maps of their trial networks. Usually, the locations of trial sites are held outside of the public domain. In many cases, information on these trial sites is outdated or poorly documented. Another problem is that the location information is often imprecise, leading to the generation of errors in spatial analysis.

The greatest deficiency with respect to trial site data is the lack of weather and soil information. In some cases, these data simply were not collected. In other cases, they remain unpublished, either in journal publications or in gray literature. One solution to acquire these data is to find them through international climate and soil databases, either in GIS formats or by locating the nearest point location. For example, the WISE database may include some soil profiles taken from experiment stations where trials are conducted. Station climate data from NOAA's Global Surface Summary of the Day (GSOD)[4] can be used to match a site to the nearest site to a weather station. Weather information for any site could also be acquired from the National Aeronautics and Space Administration (NASA) Prediction of World Energy Resource (POWER) dataset, which provides daily rainfall and temperature data for the last 12 years (NASA, 2009). However, using secondary data from GIS databases—data that was not actually derived at the trial site—may increase errors substantially. Whenever these secondary data are used, the researcher should mention its' reliability and should include any available error estimates.

A whole series of other socioeconomic data sets could be used to target genotypes to environments. These might include human population data sets (Center for International Earth Science Information Network (CIESIN) et al., 2004), accessibility and transportation infrastructure data (Nelson, 2008), and human welfare data (e.g., CIAT, 2006). However, these would be useful more for logistics planning of germplasm deployment, rather than for testing. Sites with high rural populations and accessibility and with substantial poverty may be attractive relative to isolated sites outside of areas that would be likely targets for variety dissemination. These types of data could be used when a breeding programme is near the end of the variety development cycle, to search for sites that can be used to support germplasm deployment. Building on efforts since the 1980s to collect this type of information, dedicated programmes aimed at global mapping have improved the availability of these socio-economic and agricultural production data.

SITING AND REGIONAL TARGETING OF GENOTYPES

Targeting genotypes to environments has developed substantially since the middle of the last century. Early breeding efforts led scientists to use their knowledge of a crop to speculate on how well their varieties might perform in new locations, and they could experiment in a range of sites to test GEI. Eventually, international trial networks were set up to provide scientifically rigorous testing regimes (e.g., Peterson and Pfeiffer, 1989; CIAT, 2001).

[3]http://www.figstraitmine.org/index.php?dpage=11

[4]ftp://ftp.ncdc.noaa.gov/pub/data/gsod

Most early breeding efforts sought to develop cultivars with wide adaptation (e.g., Braun et al., 1996). Later efforts aimed to target niche environments with unique abiotic or biotic stresses (e.g., Wilhelmi et al., 2002; Annicchiarico et al., 2005). The latter approach led to greater demand for mapping the agro-ecology of a crop, supporting the breeders' targeting of a genotype to specific conditions.

An understanding of the target environment and the extent of GEI are essential elements of all breeding programmes. GEI take several forms but of major concern are the crossover interactions, where the GEI result in a change in the rank of the genotypes between environments and hence influence the nature, magnitude, and predictability of the selection response achieved by any breeding programme (e.g., Cooper, 1999).

Using multienvironmental trials, breeders draw on statistical techniques developed to measure GEI (Finlay and Wilkinson, 1963). The statistical tools developed have centered on the use of 88 linear–bilinear models and mixed models (Crossa et al., 2004), and have permitted a better understanding of crossover GEI. These tools permit the identification of clusters of sites or genotypes that show little or no crossover GEI. As a result, a smaller number of globally representative key locations can be identified that assist breeders in the selection of widely adapted germplasm. Ultimately, these statistical methods and GIS can be used to recommend cultivars for specific locations (Annicchiarico et al., 2005, 2006).

For wheat, analyses of several major international trial nurseries of CIMMYT (Centro Internacional de Mejoramiento de Maiz y Trigo; International Maize and Wheat Improvement Center) have been undertaken using these statistical approaches (e.g., Trethowan et al., 2001, 2002, 2003; Lillemo et al., 2005). Analysis of sites and variety performance builds on an extensive literature related to multienvironment trial networks (DeLacy et al., 1996; van Eeuwijk et al., 2001).

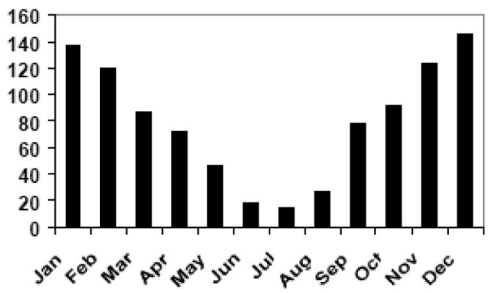
Plant breeders can use soil and climate information of the trial sites to classify these point locations into more or less homogenous environment types (DeLacy et al., 1994; Mgonja et al., 2002; Setimela et al., 2003, 2005; Maideni, 2006; Roozeboom et al., 2008). Grouping trial sites can be useful in designing field testing plans for plant breeding programmes, but may not tell us ultimately where genotypes can perform well because the sites only represent a limited number of point locations. Therefore, linking individual trials sites to larger regions for which they are representative opens up numerous possibilities for phenotyping work and, ultimately, for introducing varieties into environments where they are expected to perform well (DeLacy et al., 1994; Gauch and Zobel, 1997). The following sections discuss environment-matching methods and crop-specific agro-ecological mapping, and their use for targeting genotypes to environments.

SITE ANALYSIS AND MATCHING

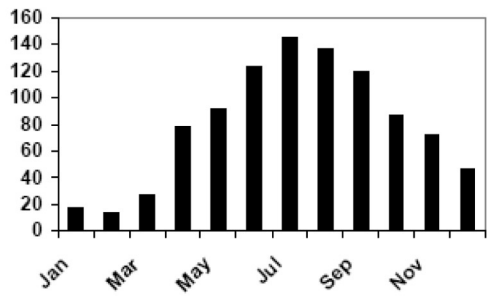
Environmental data on the sites of variety trials or potential future trials can give us key information for targeting genotypes to environments. Any number of sites can be compared to each other to determine their similarity in terms of climate and soils. Researchers may use a number of different methods to make these comparisons. A few examples are given here to illustrate some of the issues in comparing sites.

Measuring site similarity requires methods to be able to compare climate data at different locations. Since climates vary with latitude and season, similar levels of rainfall or temperature can occur at different times of the year. One way to account for these differences is to express climate data in terms of their relationship to climate extremes, removing reference to the date of the data. For example, the BIOCLIM method uses data on rainfall and temperatures in the wettest, driest, warmest, and coldest months (Busby, 1991). A more common method is to transform the data to "standard" time scale. **Figure 1** illustrates rainfall of a hypothetical climate in the northern hemisphere and an identifical one in the southern hemisphere. In order to standardize these climate patterns, Jones and Thornton (1993) describe a 12-point Fourier

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Northville	137	120	87	72	46	18	14	27	78	92	123	145
Southville	18	14	27	78	92	123	145	137	120	87	72	46



Northville monthly rainfall



Southville monthly rainfall

FIGURE 1 | Two hypothetical pluviographs exhibiting identical rainfall patterns (Source: http://gisweb.ciat.cgiar.org/marksim/).

transform to rotate the data to a standard season. Other methods are variations on the same process of standardizing the seasons so that climates at different latitudes can be compared.

Once climate data have been standardized, comparisons can be made to evaluate the degree of similarity between any set of stations. The use of the climate-matching software CLIMEX illustrates this concept[5]. The software utilizes a large database of climate stations with 30 years of weekly data. CLIMEX displaces data to standardize them according to latitude. Difference equations are applied to create indices of similarity for maximum, minimum, and average temperature, rainfall, and rainfall pattern, humidity and soil moisture. **Table 2** shows the results of a climate similarity analysis between Valparaiso, Chile, and four other stations in Mediterranean climates. Included here are stations with some of the highest similarity indices in the United States of America (USA), Australia, and the northern and southern extremes of Africa. Temperature and rainfall values are shown together with the similarity indices calculated by CLIMEX. The composite match index (CMI) combines the six climate parameters mentioned above. The corresponding map (**Figure 2**) shows the CMIs for over 2000 weather stations throughout the world. Higher CMI values indicate greater similarity.

Table 2 | The similarity of locations to Valparaiso, Chile: Temperature, rainfall, and similarity indices[a].

Location	T_{min}	T_{max}	R_{tot}	$I\text{-}T_{min}$	$I\text{-}T_{max}$	$I\text{-}R_{tot}$	*CMI*
Valparaiso, Chile	8.3	22.2	506	1.00	1.00	1.00	1.00
Kingscote, Australia	8.2	24.8	485	0.87	0.86	0.97	0.88
San Francisco, USA	7.2	20.6	463	0.90	0.75	0.92	0.87
Wingfield, South Africa	7.2	26.1	509	0.82	0.68	0.99	0.86
Shahhat, Libya	4.4	28.3	608	0.69	0.59	0.87	0.79

[a] *T_{min}, minimum temperature; T_{max}, maximum temperature; R_{tot}, total rainfall; $I\text{-}T_{min}$, $I\text{-}T_{max}$, and $I\text{-}R_{tot}$ are the similarity indices. The CMI is a combination of similarity indices. For a description of the method, see Sutherst and Maywald (1991).*

[5]http://www.hearne.com.au/products/climex/

Similarity analyses can be extended from weather station data to cover a continuous surface through spatial interpolation of climate data. For example, **Figure 3** shows the result of the Homologue model for Bambey, Senegal. Homologue eliminates the need for input weather station data by interpolating climate data between stations[6]. The mapped results cover a continuous surface. The Bambey, Senegal station is similar to environments across the Sahel region of sub-Saharan Africa, and has been important in French efforts in agricultural research throughout West Africa. The map shows many areas that are right at the edge of very dry areas marginal for agriculture, such as northeast Brazil and the southern African area bordering the Kalahari desert.

The tools described above can be used for planning variety trials but lack information on the crop of interest. As discuss below, linking locations to the ecological niches of the crop of interest provides a more reliable basis for considering where a genotype could be targeted.

AGRO-ECOLOGICAL MAPPING

Maps of the systems characteristics, production, and ecology of crops can support the task of targeting genotypes to environments

[6]Contact article author Glenn Hyman (g.hyman@cgiar.org) to request, Homologue software.

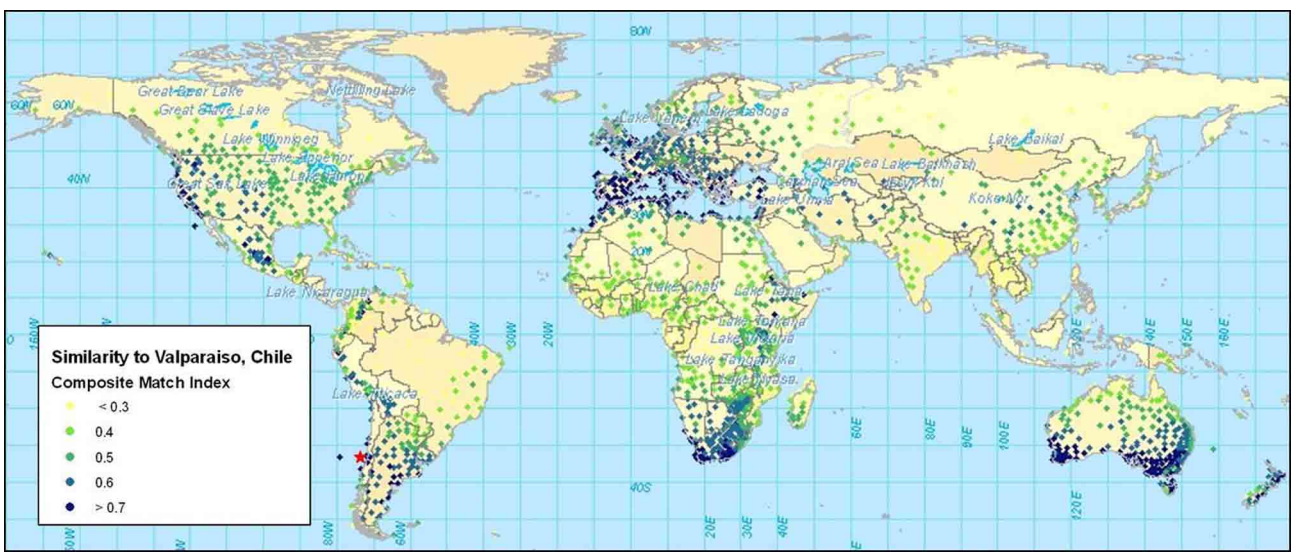


FIGURE 2 | The composite match index (CMI) showing the similarity of locations to Valparaiso, Chile.

for both testing and deployment. Agricultural system maps draw on qualitative and quantitative information to depict regions of similar farming characteristics (Whittlesey, 1936; van Lanen et al., 1992; Pollack and Corbett, 1993; Dixon et al., 2001). We have already described the development of crop production maps. Maps of the ecology and environment of a particular crop are especially useful in targeting genotypes to environments.

In the latter part of the 1980s, an FAO workshop and resultant publication indicated a growing interest in environmental and agro-ecological mapping by the international agricultural research and development community (Bunting, 1987). Examples of this type of mapping work include CIAT's agro-ecological maps of cassava (CIAT, 2003) and rice (Jones, 1984). Such work often focused on regions instead of crops, and CIAT used these maps to define its research domain in Latin America (Jones et al., 1990). They were also used to assess the geographical distribution of environments that were the target of research in the Brazilian Cerrados (Jones et al., 1992).

Methods for making these maps vary with respect to the type of data used and the statistical analyses employed. A cassava agro-ecology map is based on key precipitation, soil and elevation thresholds that define regions according to moisture conditions, soil acidity, and altitude (**Figures 4** and **5**; Carter, 1987; Carter et al., 1992; CIAT, 2003). For this classification system, cassava specialists identified key environmental thresholds for distinguishing between seven cassava agro-ecological regions. In a different approach, the Brazilian Cerrados was mapped using climatic and soils data in a cluster analysis (Jones et al., 1992). Clusters were mapped directly from the data and then generalized into homogenous regions within the Cerrados. A similar approach was carried out to map wheat agro-ecologies in Algeria

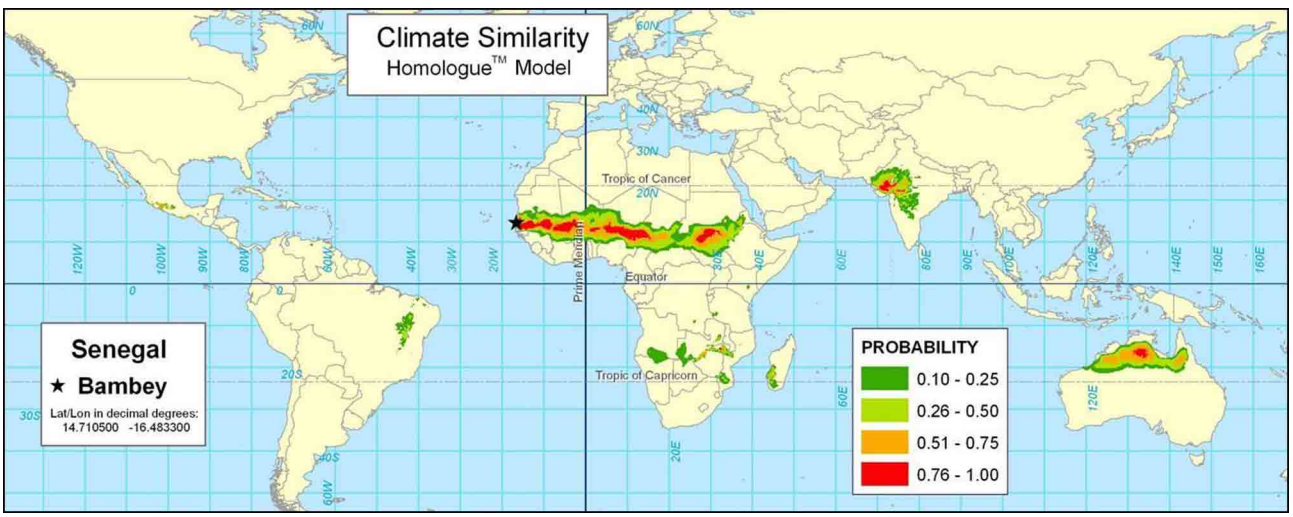


FIGURE 3 | The Homologue model showing areas similar in climate to Bambey, Senegal.

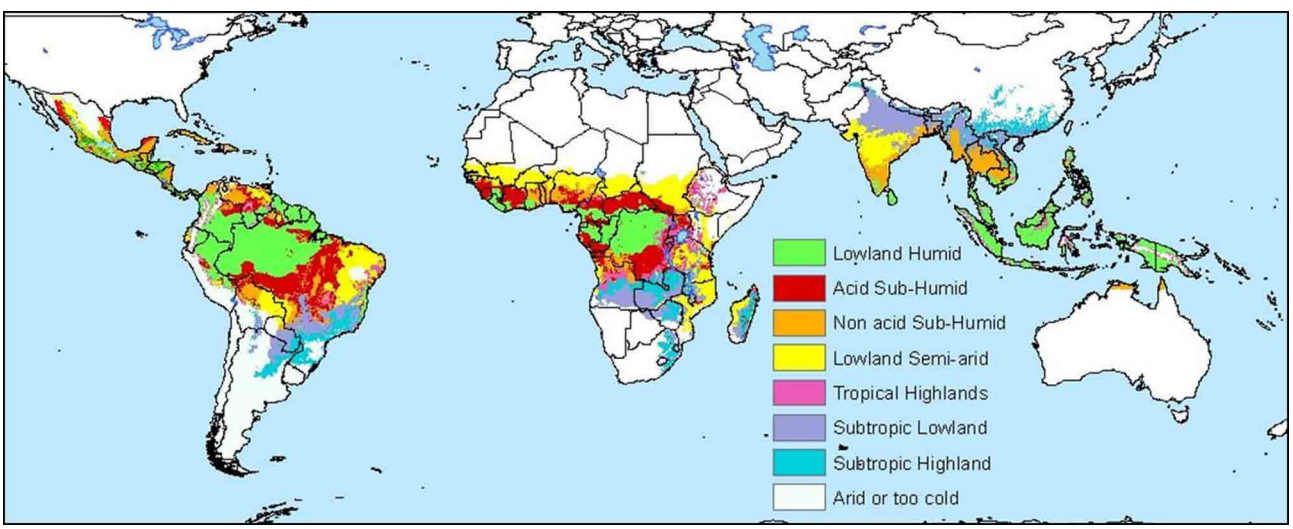


FIGURE 4 | Edapho-climatic map of cassava (CIAT, 2003).

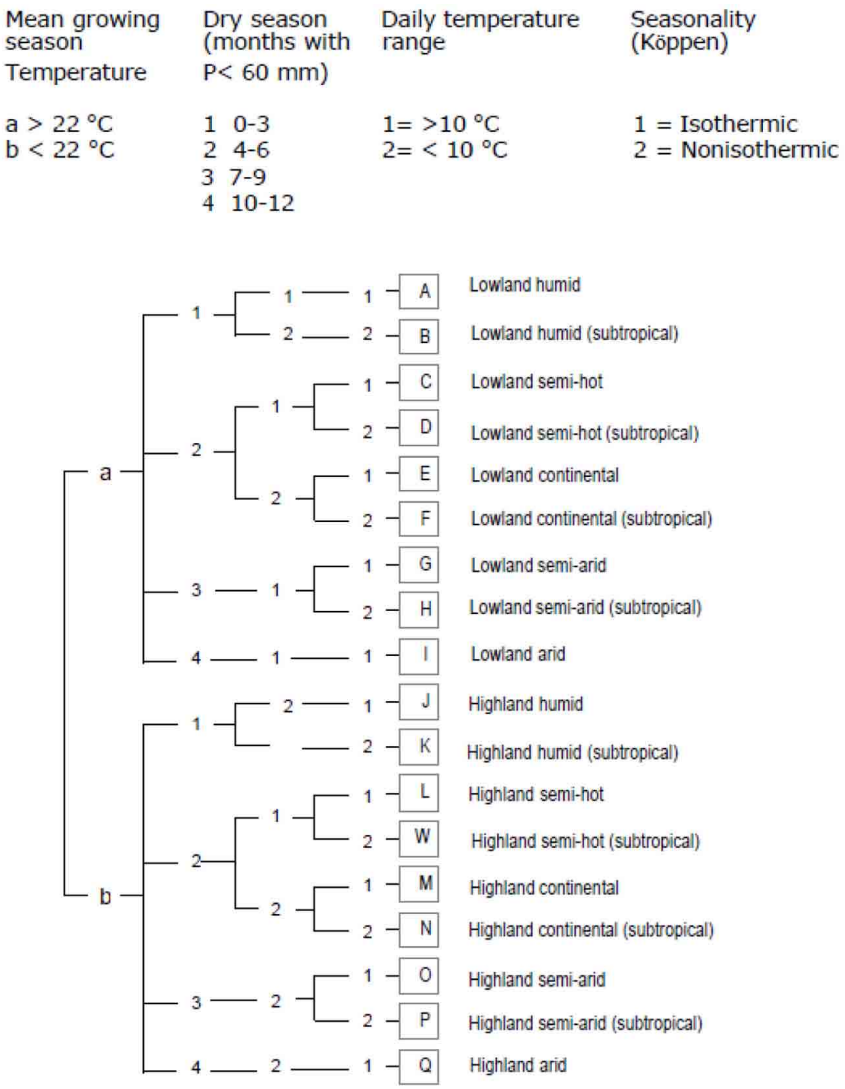


FIGURE 5 | Diagram showing the classification scheme for CIAT's cassava agro-ecology map (CIAT, 2003).

using an unsupervised classification of GIS data layers (Delli et al., 2002).

The agro-ecological maps described above include knowledge of the crop but are not based on actual trial data. The discussion below turns to the CIMMYT methodology for mapping mega-environments, an approach that starts with the results of cultivar trials.

CIMMYT's MEGA-ENVIRONMENT APPROACH FOR MAIZE AND WHEAT

For maize, and specifically in the highly variable drought-prone environments of southern Africa, similar statistical techniques to multilocation yield trial data were applied, combined with environmental factors derived from GIS (Setimela et al., 2003, 2005; Maideni, 2006). Cluster analysis grouped the regional trials into seven groups with seasonal maximum temperature, precipitation, soil pH, and nitrogen stress identified as the factors accounting for repeatable GEI. Six final mega-environment zones were derived based on seasonal maximum temperature and precipitation, because available soil pH data were considered too unreliable for inclusion. Hence, maize germplasm in any mega-environment would have a requirement for evaluation under both low and high nitrogen and low and neutral pH. This combination of approaches has resulted in a better understanding of target environments in southern Africa (Bänziger et al., 2004) and has assisted in the identification of breeding strategies and key locations for regional variety testing. The stress factors responsible for GEI at the global scale were extrapolated and fine-tuned for southern Africa through feedback from experts (**Figure 6**).

For wheat, CIMMYT has developed mega-environments that have as a foundation the extensive network of international wheat testing sites, comprising over 800 unique sites. Wheat experts classified trial sites according to the predominant mega-environment and, subsequently, GIS was used to extract the underlying climatic and edaphic factors, resulting in quantitative criteria for mapping

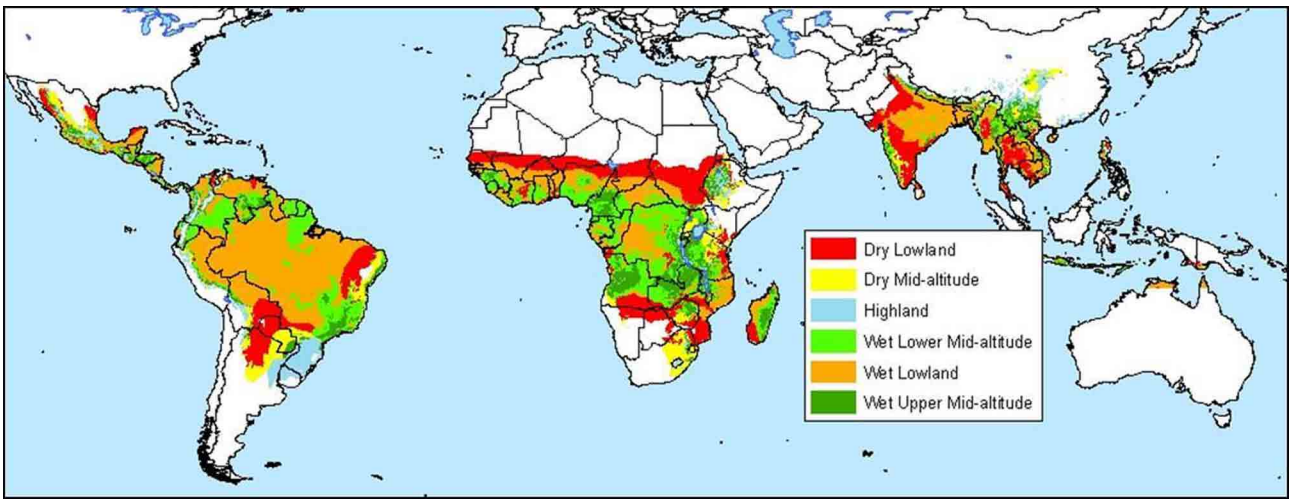


FIGURE 6 | Maize mega-environments.

the mega-environments (Hodson and White, 2007). Long-term mean minimum temperature in the coolest quarter (i.e., three consecutive coolest months of the year) proved effective in distinguishing among the winter-grown spring, winter/facultative, and summer-grown spring wheat types. This temperature criterion was also useful for separating favorable, irrigated spring wheat environments from environments that are similar but where heat tolerance is required.

The climatic basis of both the maize and wheat mega-environments, and other agro-ecological mapping efforts, relies on long-term normal data such as Worldclim, described in the introduction (Hijmans et al., 2005). While the approaches have improved the understanding of general crop agro-ecologies, they ignore temporal variation due to year-to-year variation in climatic conditions. Trethowan et al. (2005) showed how specific locations may fluctuate between high or low rainfall wheat mega-environments depending on seasonal conditions. Such limitations are now being addressed by work on frequencies of environment types.

In practical terms, the real nature of the problem from the point of view of GEI is that testing environments may represent the wrong balance of stress intensity or timing, so selection will not address optimally the needs of the target population of environments (TPE). In highly variable environments, the degree of mismatch between the sample from multienvironment trials and the TPE is likely to be high, and could lead to decreased or even reversed genetic gain (Cooper et al., 1996).

Considerable advances are being made in the area of improved characterization of TPE, environment types, and frequencies of environment types. These advances are largely due to the coupling of crop simulation models with long-term weather records in order to generate seasonal sequences of stress that can subsequently be used to determine frequencies of stress environment types (Chapman and Barreto, 1996; Hartkamp et al., 1999, 2001; Chapman et al., 2000a, 2002, 2003; Loffler et al., 2005; Putto et al., 2009). This type of information, in combination with multienvironment trial data, can be used to weight data from different trials according to how representative they are of the TPE and so improve selection, especially in variable environments (Chapman et al., 2000b).

Loffler et al. (2005) used the crop simulation and GIS approach to classify the major maize environments in the Corn Belt of the USA. Even in this highly productive maize environment, the spatial and temporal dimensions of environmental variation in the TPE were highly significant. For each of the six major environment types identified, relative frequencies of each of the environments varied greatly from year to year and significant hybrid by environment interaction variance was observed. Stratification of environments sampled by the multienvironment trials by the temporally specific environment type explained a significant portion of the GEI for observed grain yield. This methodology is therefore likely to improve the predictability of cultivar performance in the TPE. These new approaches have only been reported from the USA or Australia but future application to highly variable environments such as Africa have the potential to produce significant breeding gains.

SPATIAL ANALYSIS OF ENVIRONMENTAL CHANGE

Projections of environmental change are motivating greater emphasis on future constraints to agricultural production. The pace of population, climatic, and environmental change has compelled the crop improvement community to consider those stresses that are likely to result in significant yield declines (Cassel-Gintz et al., 1997). Spatial analysis is already playing a role in assisting breeding programmes to respond to environmental change. The rapid changes in soils and climate will likely increase this role in the coming decades.

Intensive land use and agricultural development erode, leach, and degrade our soils. In the absence of improved agronomic practices and land management, cultivars of the future will probably need to be tolerant of aluminium toxicity, low nutrient status and other chemical changes that make soils less fertile.

Salinisation of soils will demand salt-tolerant cultivars. New varieties will have to survive in poorly structured soils with low water-holding capacity. Crop improvement to overcome abiotic soil constraints will focus on these difficult soil environments. Crop improvement specialists can map accessions of wild relatives overlaid on environmental stresses to provide clues about which accessions may be adapted to a given stress. More cultivar testing needs to be carried out in those soil environments where a particular production constraint is representative of the growing soil problems we shall face in the future. However, improved agronomic practices will play a vital mitigating role and these need to be an integrated part of crop improvement.

Of more immediate concern for crop improvement are the effects of climate change (Jones and Thornton, 2003; Lobell et al., 2008). Improved cultivars have a product life cycle (research, development, testing and use) of 46 years on average (Jones et al., 2007b). Therefore, the development of new cultivars should aim for adaptation in the climate we will find in 30–50 years from now. For example, an analysis of testing sites for biofortification programmes found that many of the current maize testing sites in Africa do not represent the likely environments for maize in 2055 (Jones et al., 2007b). Another important consideration for crop improvement is the conservation of wild relatives and landraces that may otherwise become extinct due to climate change. Jarvis et al. (2003) found that of 17 wild *Arachis* species in South America, 12 could be extinct in 50 years time due to climate change. If we do not conserve these genetic resources now, future efforts may lack valuable material needed for crop improvement.

New data and tools are facilitating spatial analysis of climate change. Downscaled weather data from General Circulation Models are often used in modeling climate change impacts on agriculture (Jones and Thornton, 2013). The Worldclim data set now includes downscaled projections of future climate for three popular climate models from the Intergovernmental Panel on Climate Change (IPCC) family of climate change scenarios[7]. These data can be used directly in GIS software packages such as DIVA (Hijmans et al., 2001). Initial efforts have been made to incorporate future climate projections in CIAT's Homologue and Marksim tools. Researchers of the CGIAR have downscaled the 21 IPCC model scenarios for climate change to 1-km climate surfaces, some of which have not been publicly released to date. These recent developments suggest that the prospects for using spatial analysis for studies of genetic resources and climate change are improving.

[7]http://www.worldclim.org/futdown.htm

CONCLUSION

Methods to target genotypes to environments are evolving. Plant breeders used a "hit-or-miss" approach for many years, simply testing their cultivars in as many environments as they could. The development of agro-ecological mapping (as per Bunting, 1987) gave them a better idea about the target environments. Developing maps from large international yield trials, as in CIMMYT's mega-environment approach, improved on agro-ecological mapping. Spatially explicit crop modeling has improved targeting studies over the last decades. Recent efforts to account for changes in year-to-year environmental conditions have further improved our understanding of how to more efficiently reach our goal of getting the right genotype to farmers.

Geographic information science and technology has played a valuable role in the evolution of genotype targeting approaches. It has provided high-resolution spatial and temporal data to help breeders unravel GEI. Spatial synthesis of model and statistical outputs has improved our capacity to map out target environments and the frequencies of environments, an effort that ultimately leads to a more effective deployment of germplasm. Greater collaboration between breeders, crop improvement specialists, and the climate change modeling community are needed now more than ever.

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Field phenotyping strategies and breeding for adaptation of rice to drought†

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This paper is a section of the book "Drought phenotyping in crops: from theory to practice" (Monneveux Philippe and Ribaut Jean-Marcel eds, published by CGIAR Generation Challenge Programme. Texcoco, Mexico). The section describes recent experience in drought phenotyping in rice which is one of the most drought-susceptible crops. The section contains genetic and genomic resources for drought adaptation and methods for selection of drought-resistant varieties in rice. In appendix, there is experience from Thailand on integration of direct selection for grain yield and physiological traits to confer drought resistance.

Keywords: drought adaptation, drought resistance traits, phenotyping, target population of environments, Water stress

CHALLENGES AND GENERAL INFORMATION

IMPORTANCE OF RICE IN THE HUMAN DIET

Rice is the staple food for approximately 340 million poor people in South Asia and 140 million each in Southeast Asia and sub-Saharan Africa (International Rice Research Institute; IRRI, 2006). It is the basic food crop of Asia, providing over 30% of the calories consumed in the region. Overall, there is an estimated global need for an additional 116 million tons of rice by 2035 as compared to 439 million tons production in 2010 (Seck et al., 2012). The estimated annual increase is expected to be 13% for the first 10 years and 12% in the next 15 years as population growth drops and people diversify from rice to other crops (Seck et al., 2012).

CULTIVATED AREA AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

Irrigated rice accounts for almost 75% of total world rice production. It was the source of the large increases of productivity leading to the Green Revolution. However, technological progress in rice cultivation has slowed down substantially since the early 1990s from the 2.5% per year during the first two decades of the Green Revolution to about 1.1% per year since the late 1980s. The stagnation in yield growth is because yields are approaching the practical potential of the rice crop growing under favorable environments (IRRI, 2006). Further increases will have to come from new breakthroughs in increasing the yield potential under favorable conditions and from increased performance of rice growing under less favorable conditions. In both scenarios it is likely that there will be less water and probably less available labor. Thus, research needs to increase the productivity of water for both irrigated and rain-fed systems.

IMPORTANCE OF DROUGHT IN RICE FARMING

Rain-fed rice ecosystems are home to 80 million farmers on 60 million ha. Progress has been slow in improving productivity, and drought is a major constraint affecting rice production, especially in rain-fed areas across Asia and sub-Saharan Africa. Pandey et al. (2007) estimate that at least 23 million ha of rain-fed rice area (20% of the total rice area) in Asia are drought-prone. Even in

Abbreviations: ACIAR, Australian Center for International Agricultural Research; CIAT, Centro Internacional de Agriculture; CSSL, chromosomal segment substitution lines; CV, coefficient of variation; DH, double haploid (lines); DM, dry matter; DRI, drought response index; EMS, ethyl methane sulfonate; FST, flanking sequence tags; GCP, Generation Challenge Programme; GEI, genotype-by-environment-interaction; GLD, green leaf duration; H, broad based heritability; HI, harvest index; HIF's, heterogeneous inbred families; IRD, Institut de Recherche pour le Development (France); IRFGC, International Rice Functional Genomics Consortium; IRRI, The International Rice Research Institute; LWP, leaf water potential; LSD, least significant difference; MAGIC, multiple advanced generation intercross; MAS, marker aided selection; MET's, multi environment trials; NIAS, National Institute of Agroecological Sciences (Japan); NERICA, new rice for Africa; OA, osmotic adjustment; PNHI, panicle harvest index; QTL's, quantitative trait loci; RCB, randomized complete block; RGA, rapid generation advance; RIL's, recombinant inbred lines; RNAi, RNA interference; RWC, relative water content (leaves); SAG's, stress-associated genes; SE, selection environment; SNP, single nucleotide polymorphism; SSD, single-seed descent; T-DNA, transfer DNA-based vectors; TPE, target population of environments; WARDA, Africa Rice Center; WUE, water-use efficiency.

traditionally irrigated areas, which account for almost 75% of total rice production, drought is becoming an increasing problem because of water scarcity resulting from rising demand for water for competing uses. Drought imposes a serious economic burden on society and has been historically associated with food shortages of varying intensities, including those that have resulted in major famines in different parts of Asia and Africa. For example, Pandey et al. (2007) estimate production losses of 36% of the average value of production in eastern India in drought years. This represents a massive loss of US$856 million and, on a yearly basis, a loss of 6.8% of the average value of output in India.[1] In addition to the direct effects on production, there are indirect effects of drought which may be felt over several years. Its impact can even span generations as, e.g., when children fail to recoup lost educational opportunities (Pandey et al., 2007).

OPPORTUNITIES TO IMPROVE DROUGHT TOLERANCE IN RICE

O'Toole (2004) suggests that the probability of success in developing drought-tolerant varieties of rice[2] is enhanced because of scientific progress in: (i) understanding the physiological mechanisms that impart tolerance of drought in rice (Fukai and Cooper, 1995); (ii) new molecular tools; and (iii) the practical application of this knowledge and tools for screening selection and improvement of rice germplasm for drought (Atlin, 2003; Jongdee et al., 2006; Lafitte et al., 2006; Bernier et al., 2007; Venuprasad et al., 2007a). Also several international workshops and training courses have dealt with the theory and practice of science-based screening of rice for drought tolerance (Ito et al., 1999; IRRI, 2002; Saxena and O'Toole, 2002; also see: www.plantstress.com). Bennett (2003) has provided an overview of the opportunities for increasing water productivity of major food crops through plant breeding and molecular biology, and Fischer et al. (2003a) have provided a practical manual with updated information for rice breeders regarding the theory and practice of breeding for drought tolerance in rice.

In recent years, rice research programs in India (Babu et al., 2003), China (Zheng et al., 2004, Thailand (Jongdee et al., 2006), Laos and Cambodia (Report to Rockefeller Foundation, 2006), The Philippines (Atlin, 2003; Lafitte et al., 2006; Bernier et al., 2009), and Brazil (da Silveira Pinheiro, 2003) are now selecting for drought tolerance as a specific trait to improve performance under rain-fed conditions. O'Toole (2004) identifies two innovations that characterize this new and successful approach. First, the work of physiologists, geneticists, and breeders led to more reliable control of water-stress severity and duration at the critical yield determining growth stages, and this gave rise to the development and utilization of effective selection measures. Second, by employing farmers' participatory selection groups as the final evaluators (Witcombe et al., 2002), real and lasting progress is now within reach. While end-user evaluations are important to any breeding program, they are particularly critical across drought-prone regions, where local variation in soils and landscape result in strong genotype-by-environment interactions (GEI). O'Toole (2004) further suggests that these innovations, when taken together, bode well for the large-scale dissemination of new drought-tolerant rice varieties across Asia in the very near future.

RELEVANT RESEARCH AVAILABLE

GENETIC AND GENOMIC RESOURCES

Since genetic resources are being produced continuously in breeding and genetic research programs around the world, it is not possible to provide an exhaustive list of current genetic stocks. Instead, it is more meaningful to indicate the principles and approaches behind the development and use of genetic resources relevant to drought breeding, and illustrate each principle with specific examples.

Due to the complexity of genetic control, genetic stocks for drought research require unique features that enable detection of not only individual genes but also possibly complex genetic loci (e.g., gene clusters or interacting loci). **Table 1** summarizes the categories of publicly accessible genetic resources useful for drought research and breeding.

Specialized genetic stocks can be classified broadly as those derived from natural genetic variation, and those induced by artificial means. This distinction is useful in a practical sense because different genetic and molecular approaches are required to analyze the materials. A wide range of natural variation is harbored in the deep genepool of rice germplasm comprising domesticated and wild species (Leung et al., 2007). This genepool represents genetic diversity resulting from thousands of years of natural selection and more recent selection through breeding. Thus, the genetic variation present in germplasm is likely to be agronomically relevant. On the other hand, artificially induced variation is generated by randomly mutating the genome to increase the probability of detecting novel variation, or by over-expressing or silencing specific genes. Mutants offer the advantage of carrying precise genetic alterations in the genome, and are, therefore, ideal for investigating genes with major phenotypic effects. Being essentially isogenic, mutants are useful for examining genetic loci with quantitative effects, provided that fixed lines are evaluated in replicated trials. A limitation of mutation analysis is that it is confined to analysis of two alternate alleles in a fixed genetic background. Thus, for phenotypes that are conditioned by large gene blocks or complex genetic interactions, mutation analysis alone is not adequate and should be complemented with analysis of natural diversity present in the germplasm.

RANDOM AND TARGETED INDUCED VARIATION

Randomly induced mutations

To take advantage of rice genome sequence information, a large collection of rice mutants has been produced in the scientific community (Hirochika et al., 2004). These mutants can be classified broadly as transgenic and non-transgenic. The transgenic mutants are produced by transformation vectors, primarily transfer DNA (T-DNA)-based vectors. Depending on the features of the vectors, insertion events can cause knockout or activation mutations. Activation mutations are unique in that a normally "dormant" gene can be activated to unleash novel variation (Leung and An, 2004).

[1] In the same study Pandey et al. (2007) showed that for northeast Thailand and southern China, the losses were smaller, averaging less than US$20 million per year (or less than 1.5% of the value of output).

[2] The term "drought-tolerant variety" as used here refers to a variety that produces a high grain yield relative to other cultivars under drought stress. This definition is as given by Atlin (2003).

Table 1 | Overview of rice genetic resources useful for drought research and breeding.

Specialized genetic stock	Feature	Produced and accessible at
Chromosomal segment substitution lines (CSSL)	Two *sativa* × *glabberima* libraries Four wild interspecific libraries using different wild rice relatives Four *japonica* × *indica* libraries	Centro Internacional de Agricultura Tropical; International Center for Tropical Agriculture (CIAT) National Institute of Agrobiological Sciences (NIAS), Japan
Recombinant inbred lines [tolerant × sensitive, including doubled haploid (DH) lines]	Approximately six commonly used populations	IRRI, Institut de recherche pour le développement (IRD), France
Breeding populations	Advanced lowland and upland breeding lines, new rice for Africa (NERICA) series	IRRI, WARDA (Africa Rice Center)
Introgression lines	*glabberima* × *sativa*	CIAT, IRRI, WARDA
Near-isogenic lines	Derived from breeding populations evaluated under field conditions	IRRI
Mutants	Insertions/activations/deletions	Multiple institutions with different degrees of accessibility. Materials can be requested from individual institutions through Standard Material Transfer Agreements

Source: Information assembled from discussion sessions at the Third Annual Research Meeting of the Generation Challenge Programme (GCP), 16 September 2007, Johannesburg, South Africa.

A main advantage of insertion mutants is that the insertion sites can be sequenced, producing a large dataset of flanking sequence tags (FST; for articles on such specialized mutant populations produced in Asia see An et al., 2007). With the current FST databases, there is more than a 60% probability of finding a mutation in a given gene, providing a reverse genetics tool to search for knockout or activation mutants in genes suspected to play a role in response to water stress. An ongoing project of the Generation Challenge Programme (GCP) is to exploit this FST database to identify and phenotype mutants with insertions in stress-associated genes (SAGs; A. Pereira, personal communication).

Non-transgenic mutants include those produced by conventional chemical and irradiation mutagenesis. Wu et al. (2005) described a large collection of *indica* rice mutants produced by fast neutron, gamma ray, ethyl methanesulfonate (EMS), and diepoxybutane. [3]For forward genetics screening of drought tolerance, non-transgenic mutants are advantageous because they can be freely distributed and tested under field conditions. With very few exceptions, it remains difficult to conduct extensive field screening of transgenic mutants. Not all insertion mutants are transgenic. For example, the Tos17 population caused by insertions of a retrotransposon element can be evaluated under field conditions.

Targeted silencing and activation of specific genes

For genes with hypothesized function in drought tolerance, there is the option of creating "down-expression" mutants by silencing the gene by the RNA interference (RNAi) technique or more recently by artificial micro-RNA (Warthmann et al., 2008). By combining over- and under-expression, the function of the gene can be inferred conclusively if side effects of the introduced RNAi construct can be excluded. Recent examples include the functional characterization of the SHINE and HARDY genes (Karaba et al., 2007). Expression of SHINE and HARDY are reported to confer water-use efficiency (WUE) in rice, although their phenotypic effects have not been evaluated under field conditions.

It is hypothesized that ERECTA is a "master" gene regulating transpiration efficiency in *Arabidopsis* (Masle et al., 2003). Mutations in ERECTA have been found in the Tos17 population and in Pohang collection (Hirochika et al., 2004). However, attempts to phenotype the ERECTA mutants have proved difficult because of the extensive somaclonal variation expressed by the mutants derived from tissue culture. Most of the transgenic mutants are maintained in early generations (T_1 or at most T_2) and some of them continue to segregate in characters unrelated to the disrupted gene. It is important that mutants be backcrossed to the wild type to clean up the background mutations before extensive phenotyping (Dworkin et al., 2009).

CAPTURING NATURAL VARIATION THROUGH SPECIALIZED GENETIC STOCKS

Mapping populations

Mapping populations can broadly be defined as genetic populations that can be used to demonstrate inheritance of traits. In general, such a population is derived from a cross between two genetically distinct parents. Taking this broad definition, a large collection of rice genetic stocks are available for defining inheritance of drought response (see **Table 2**).

In rice, the most common mapping populations are recombinant inbred lines (RILs) derived from two parental lines with high and low traits for drought tolerance. A key advantage of 's is that they can be "immortalized" (Collard et al., 2005) as advanced F_7

[3]See: www.iris.irri.org

Table 2 | Segregating populations generated by drought breeding programs for genetic analysis and breeding for drought tolerance; genetic stocks maintained at IRRI (Source: information provided by Arvind Kumar, IRRI).

Population name	Parent A	Parent B	Type	Population size
IR78875	Apo	IR64	RIL	200
IR78877	Apo	IR72	RIL	200
IR78908	Vandana	IR64	RIL	200
IR78910	Vandana	IR72	RIL	200
IR79971	Vandana	Way Rarem	RIL	500
IR78937	IR 47701-6-B-1	IR55435-05	RIL	500
IR79913	IR 55419-04	Way Rarem	RIL	500
IR79915	IRRI 132	IR55419-04	RIL	500
IR72757	Bala	IR64	RIL	400
IR79971	Vandana	Way Rarem	RIL	500
IR80508	IRRI 132	AUS 257	RIL	500
IR81023	IRRI 143	CT 6510-24-1-2	RIL	500
IR81027	IRRI 143	UPLRI 7	RIL	500
IR81047	IR 01A102	CT 6510-24-1-2	RIL	500
IR81063	NOK	IR74371-46-1-1	RIL	500
IR81896	Apo	Swarna*2	BC[a]	500
IR81895	Apo	Mahsuri*2	BC	200
IR84179	IR 78877-208-B-1-2	IR72*2	BC	500
IR84182	IR 78878-53-2-2-2	IR 72875-94-3-3-2*2	BC	400
IR83632	IR 78910-34-B-2-2	IR72	RIL	500
IR84184	IR 78908-63-B-1	IR64*2	RIL	300
IR83614	IR78875-131-B-1-2	IR64	RIL	800
IR84148	IR79971-B-55-B-B	Way Rarem	RIL	500
IR83575	IR 79913-B-102-B-5	Way Rarem	RIL	200
IR81024	IR77298-5-6	IR71525-19-1-1	RIL	500
IR84129	IR77298-5-6	IR77298-14-1-2	RIL	500
IR83641	IR77298-14-1-2	IR64	RIL	300

[a] *BC, backcross.*

generations or beyond. The population can be evaluated repeatedly over time and over locations to generate a large amount of phenotype data. Historically, RIL mapping populations are made to map component or secondary traits contributing to drought response. For example, the well-studied IR64 × Azucena RIL or double haploid populations have been used for mapping osmotic adjustment among other traits. However, using these quantitative trait loci (QTLs) to reconstitute drought-tolerant varieties has had limited success (Venuprasad et al., 2009).

More recently, considerable effort has been devoted to extracting segregating materials directly from breeding programs and converting them into advanced genetic stocks that can serve the dual purposes of QTL/gene identification and breeding. A main advantage of these materials is that they are selected for yield under stress in field conditions. Hence, the traits or QTLs under investigation have a high probability of being relevant agronomically. Examples of advanced breeding populations for detecting QTLs for yield under drought stress are shown in **Table 3**.

Near-isogenic lines

Near-isogenic lines (NILs) have a special place in genetic analysis and breeding. A pair of NILs with and without the target trait provides the best genetic materials to define unique chromosomal regions conditioning phenotypes, and eventually leads to gene cloning. Compared to disease resistance, NILs for drought tolerance are neither common nor well developed in rice. To fill this gap, advanced backcross lines have been developed using breeding lines with demonstrated field performance against drought stress (see **Table 3**). Their development can be facilitated through the use of heterogeneous inbred families (HIFs) resulting in an NIL that carries a heterozygous region for the target QTL. Such a line can be selfed to produce a pair of lines homozygous at the target region. Several pairs of NILs are now available for detecting the chromosomal regions conferring large effect for drought tolerance.

Multiparent advanced generation intercross populations

The multiparent advanced generation intercross (MAGIC) approach originally developed in animal genetics is now being explored in plants (Cavanagh et al., 2008). In this approach, recombinant populations are generated by intercrossing a number of selected founder lines (between 8 and 16 genotypes) that are genetically distant from each other and carry unique genetic attributes. The resulting populations are subjected to multiple cycles of intercrossing to maximize recombination between chromosomes. At an advanced stage, a large (>2,000) RIL population is established. This recombinagenic population is expected to exhibit novel variation and to provide a permanent resource for high-resolution mapping.

Table 3 | Rice breeding populations for detecting large-effect QTLs for yield under drought stress under upland and rain-fed lowland production systems; genetic stocks maintained at IRRI (Source: information provided by Arvind Kumar, IRRI).

Population	Generation	Rice production system	Reference
Vandana × Way Rarem	F_3 derived, BC_1F_3, BC_3F_3	Upland	Bernier et al. (2007)
IR55419-04/Way Rarem	F_3 derived, BC_1F_3, BC_3F_3	Upland	IRRI, unpublished
Aday Sel/IR64	BC_3F_5, approaching NIL	Rain-fed lowland	Venuprasad et al. (2007b)
Apo × Swarna	F_3 derived, BC_1F_3	Rain-fed lowland	IRRI, unpublished
CT9993-5-10-1-M/IR62266-42-6-2	DH	Rain-fed lowland	Kumar et al. (2007)

The International Rice Research Institute (IRRI) has initiated the development of MAGIC populations for rice. Two populations will be developed: one will be targeted at irrigated and one at rain-fed ecosystems that are relevant to both Asia and Africa, recognizing that the utility of the two populations will overlap. Each population will have eight founders, selected either as elite, well-adapted varieties for the respective environment, or as potential donors of useful germplasm not found within the current elite pool. Within 3 years, it is expected to have sufficient seeds from the MAGIC populations for a first round of phenotypic evaluation.

Diverse germplasm panel for association genetics

Genetic association analysis makes use of the fact that, within an unstructured genepool, blocks of chromosome can be found associated with certain phenotypes. Unlike conventional linkage analysis, association analysis exploits the large number of historical meioses (genetic recombination events) in the germplasm. The resolution of this association depends on the levels of linkage disequilibrium (LD).

Rice is particularly suitable for developing an association genetics platform for determining the relationship between chromosomal blocks and traits of interest. Under the OryzaSNP project coordinated by the International Rice Functional Genomics Consortium (IRFGC), there is now an extensive single nucleotide polymorphism (SNP) database consisting of over 150,000 SNPs across 20 diverse rice genotypes (McNally et al., 2006; OryzaSNP website[4]). This OryzaSNP dataset, together with other SNP data from the rice research community, provides the tools for high-resolution genotyping. The OryzaSNP consortium is mobilizing the community to conduct a comprehensive survey of genome-wide SNP variation in more than 2,000 diverse rice genotypes selected based on diversity, utility in breeding, and geographical representation. If successfully implemented, the SNP haplotype and phenotype database of a large collection of rice germplasm and breeding lines will provide a powerful platform for relating phenotypes to specific regions of chromosomes in rice.

In summary, genetic variation for conditioning drought tolerance exists in rice but such variation must be captured and displayed in a suitable genetic background amenable to genetic analysis and breeding manipulation. To understand and use this genetic variation for breeding, it is necessary to continue to invest in producing and maintaining well-managed, publicly accessible, high-quality genetic stocks relevant to drought research. Such genetic stocks should enable QTL mapping for drought tolerance at 1 cM (0.5–1 Mb) resolution and they should be useful donors in prebreeding. Learning from the experience of breeding for disease resistance, developing breeding-ready NILs with sequence-indexed chromosomes and known phenotypic contribution to drought tolerance should prove highly valuable to breeding for drought tolerance.

BREEDING STRATEGY

Generally, breeding methods for rain-fed rice have been strongly influenced by experiences in irrigated rice, where the crop is usually grown under stress-free conditions and where yields in farmers' fields approach those on experiment stations. Most conventional plant breeders in rain-fed systems use the early screening phase to select for traits such as height, maturity, plant type, pest tolerance, and grain quality, often under well-watered conditions on research stations. Only at the advanced testing stage, when relatively few genotypes remain, are entries evaluated under the stress conditions of farmers' fields. The outcome is often a variety that performs well under well-watered conditions but poorly under stress.

In contrast to this conventional approach, growing evidence indicates that varieties developed for improved yield under drought stress will respond to well-watered conditions if there is early selection in both environments. There are several reasons for plant breeders' apprehension about selection under drought stress. Uppermost among them is that the target environment where selection and testing work are done is often spatially variable in terms of rainfall. Because of the variability in the rain-fed environment, breeders are searching for more reliable phenotyping protocols that can accelerate progress. However, breeders must be aware that there is a "chain of correlation" between performance in a screening environment and performance in farmers' fields. Thus, before embarking on a phenotyping protocol, the breeder must test the assumption that the performance in a given drought protocol is predictive of performance on-farm under farmer management.

Rain-fed rice is grown in two major ecosystems, rain-fed lowland where the rainwater is stored through "bunding" of the fields such that the crop is exposed to anaerobic and aerobic conditions, and upland rice where the crop grows under aerobic conditions. There is another emerging ecosystem of interest and that is the traditional irrigated rice system where there is increasing pressure on water availability. Rice researchers are developing "aerobic rice" for this emerging ecosystem. Of these, the former is by far the dominant and accounts for around half of the rice area worldwide. It is the main focus of this case study in breeding for drought resistance in rice.

[4]http://www.oryzasnp.org/

PLANT WATER STRATEGY

Background and simple model for yield under drought

Numerous workers have studied the complex processes, mechanisms, and traits that determine rice yield under moisture-limiting conditions. Fukai and Cooper (2001) have summarized this complexity, and focus on three broad mechanisms that influence yield depending on the severity and predictability of the drought in the TPE where the crop is grown (**Figure 1**). The contribution of phenology to escape from predictable drought is well understood. Its role in unpredictable drought occurring around flowering is still under investigation. There is considerable evidence that yield potential contributes to yield under drought, with recent evidence from the work of Kumar et al. (2007) showing a genetic correlation of 0.8 between yield under stress and non-stress. This indicates that much of the yield under drought is accounted for by yield potential. Plant breeders have improved yield potential, mainly by increasing harvest index (HI) through shorter plants and earlier flowering with more tillers and greater spikelet number, and, to a lesser extent, green leaf duration (GLD), by maintaining a larger leaf area for a longer period.

The main approach for breeding for drought-prone environments is to: (i) improve yield potential and, depending on the type of drought, select for the appropriate combination of maturity to avoid stress during the reproductive stage; and (ii) select for tolerance to drought stress during the reproductive period, and avoid plant types that use a lot of water prior to flowering (i.e., produce large amounts of dry matter (DM) and run out of water at the critical stage of flowering. In upland rice, as in other aerobic crops, there may also be opportunities to increase the amount of water transpired through more vigorous root systems.

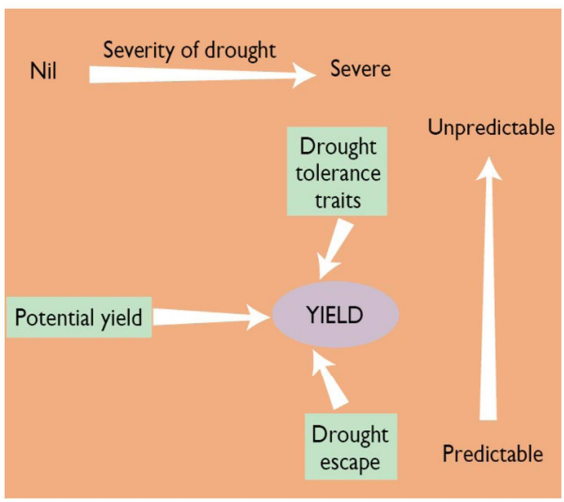

FIGURE 1 | Schematic diagram of three components of yield under drought-prone environments (potential yield, phenology, and drought-tolerance traits) and yield relationship in different types of drought in rain-fed rice. Note that when drought is not present, yield potential determines grain production. Moving to the right in the figure, drought becomes more severe and drought escape or drought tolerance becomes more important. The vertical axis represents the predictability of drought. If drought is very predictable (bottom), drought escape through changing phenology or planting date is a good option. As drought becomes more unpredictable (moving up on the axis), drought-tolerance traits become more necessary (Redrawn from: Fukai and Cooper, 2001).

Putative traits for drought tolerance

There are many putative traits that have been studied for their use in breeding for drought tolerance in rice, as listed in **Table 4**. However only a few can be recommended for use in a practical breeding program at this time. They are described in detail later. Research continues on some of the putative traits but at this stage they are not recommended for application in a breeding program.

PHENOTYPING TRAITS[5]

Why use secondary traits?

Grain yield under stress is the primary trait for selection in breeding programs for drought-prone environments. However, it is sometimes useful to screen for secondary traits as well. These traits are plant characteristics that are associated with yield under stress, and they can provide additional information for breeders to use when they make selections. Breeders who select for disease scores, plant height, and flowering date are all using secondary traits. For a secondary trait to be useful in a breeding program, it has to pass five tests:

- It must be genetically correlated with grain yield in the predominant stress situations that occur in the target environment.
- It should not be affected very much by environment; that is, it should be highly heritable in the screening system used.
- There must be variation among lines for the trait.
- It should not be associated with poor yields in the unstressed environment.
- It must be possible to measure the trait rapidly and economically.

When to use secondary traits?

Secondary traits can improve the selection response if they contribute in one of the following ways:

- They improve precision *if* the heritability of yield is reduced by stress and the heritability of the secondary trait is not reduced by stress.
- They facilitate manipulation of the drought environment. It may be easier to reveal variation in the secondary trait than to reveal variation in yield. For example, the timing of stress has a very large effect on the extent to which yield is reduced, so it is hard to compare lines with different flowering dates. If a secondary trait is less sensitive to the growth stage of the crop, this makes it easier to compare lines of different maturity.
- They focus the selection on a specific type of drought, yield being the summation of all stresses, including those not directly associated with water.
- They are cheaper and easier to measure than grain yield under stress. Frequently, experiments are lost because of pests or weather damage before the final yield can be recorded. In such cases, a good secondary trait allows useful data to be collected from the experiment.

[5]Reprinted from Lafitte et al. (2003).

Table 4 | Putative traits for drought tolerance.

Trait	Proposed function	Comments	Reference
Leaf-rolling score	To reduce transpiration	Used during vegetative stress; high heritability (ca. 0.8), but low/no association with yield. Good as an indicator of stress in an experiment	Courtois et al. (2000)
Osmotic adjustment (OA)	To allow turgor maintenance at low plant water potential	*Indica* types have high OA, *japonica* types have low OA. This trait has been associated with a yield advantage in wheat, especially in terminal stress environments	Lilley et al. (1996)
Deeper, thicker roots	To explore a greater soil volume	There is evidence from MAS that increasing root mass below 30 cm results in greater yield under stress. No evidence on root thickness *per se*. Large-scale screening is difficult	Yadav et al. (1997)
Root-pulling resistance	For root penetration into deeper soil layers	Is correlated with larger root system	Pantuwan et al. (2002b)
Greater root penetration ability	To explore a larger soil volume	Most studies use artificial barriers with known mechanical resistance. There is some controversy regarding how well this mimics the soil situation	Ali et al. (2000), Clark et al. (2000)
Membrane stability	To allow leaves to continue functioning at high temperature	Genotypic differences are clear. Has been linked to heat tolerance in several species. Link to drought tolerance is less evident	Tripathy et al. (2000)
Leaf relative water content (RWC)	Indicates maintenance of favorable plant water status	Trait has rather low heritability; QTLs not repeatable	Courtois et al. (2000)
Water-use efficiency (WUE)	Indicates greater carbon gain per unit of water lost by transpiration	Carbon isotope discrimination ($\Delta^{13}C$) provides an integrated measure of WUE over the season. It has been used successfully for crops in more arid climates but has not been applied to rice	Specht et al. (2001)

Note that QTLs have been identified for these secondary traits. Now they need to be tested for their relationship with performance under drought stress, and suitable high-throughput screening strategies must be developed (Source: Lafitte et al., 2003).

METHODOLOGY AND FIELD TRIALS

The following provides some practical advice for breeding rice for drought-prone environments with a focus on practical protocols for phenotyping. The focus is on rain-fed lowland rice. The material is taken from Fischer et al. (2003a).

TRIAL PLANNING

Definition of the target environment[6]

There is no one environment, even on the same farm, for which a breeding program is targeting improvement toward. Rather, there are several environments that will change from year-to-year and from field-to-field. These are referred as "the target population of environments" (TPE; Cooper et al., 1997). Each breeding program must clearly define the TPE for which it is developing varieties. Thus, a TPE is the set of all environments, fields, and seasons in which improved varieties are expected to do well. However, the environments must be sufficiently similar for one genotype to perform well in all of them.

[6]Reprinted from Fischer et al. (2003b).

How to determine the target population of environments for rain-fed lowland rice

Start with spatial information on water availability at the sub-ecosystem level. A commonly used system for characterizing rain-fed lowland systems is that of sub-ecosystems defined by Khush (1984), and later modified by Mackill et al. (1996). Three of these sub-ecosystems are relevant to breeding for drought tolerance:

- rain-fed, shallow, favorable sub-ecosystem, where rainfall and water control are generally adequate for crop growth, and only short periods of drought stress or mild submergence occur
- rain-fed, shallow, drought-prone sub-ecosystem, with either a short rainy season or a long and bimodal rainy period
- rain-fed, shallow, drought- and submergence-prone sub-ecosystem, where drought and submergence may occur within the same growing season or in different seasons.

Use the knowledge and experience of farmers and breeders to characterize local environments

Farmers, agronomists, and breeders who are familiar with a field and have observed rice crops grown in it over several years can

usually determine the type of drought risk it is subject to quickly and accurately. This is largely a function of toposequence position and soil texture. Upper terraces, particularly those with light soils, are most subject to drought risk. Using the knowledge of experienced farmers and researchers is the most accurate and simple approach for assigning fields to a particular TPE. As a general rule, drought risk is most severe in entirely rain-fed upper fields, in which standing water rarely accumulates, and in which farmers grow short-duration, photoperiod-insensitive varieties.

Use the performance of known varieties to define the target population of environments

Most breeding programs routinely collect data from variety trials grown over all environments, called multienvironment trials (METs). These historical data can be reanalyzed using the statistical package CROPSTAT[8] to determine the clustering or grouping of environments, based on the correlation of variety means across trials. The results can be used to define the TPE. There is a simple way to group locations and fields into the TPE, using the correlation of variety means from trials testing the same set of varieties. The repeatability (also known as the broad-sense heritability or H) of a three- or four-replicate trial usually ranges from 0.3 to 0.4. This is also the expected correlation of variety means in trials conducted in different fields if there is not much GEI between them. Thus, if the correlation between cultivar means in trials conducted at two different sites is consistently 0.3 or greater, they can be safely included in the same TPE. This method of grouping environments in the TPE should only be used if data from trials containing 20 or more varieties are available over several years.[7]

Be cautious in using this approach

First, it is necessary to make sure that the trials/locations are representative of the TPE (i.e., the farmers' fields), and that crops are not grown only at the experiment station (often with water). Second, do not exclude trials that did poorly because of drought. Our experience from several analyses of METs shows that there is a large non-predictable component of GEI (associated with year-to-year variation), as well as a large error component. This makes it difficult to define consistent patterns for the grouping on the basis of locations (Cooper et al., 1999a) and requires large datasets to estimate frequencies of environmental types based largely on variable water conditions. Since our aim is to develop varieties with adaptation to these water conditions, we need to know more about the patterns of water supply and the types of drought. The GEI analysis needs to be supplemented with measurements of the water supply at the local level.

The process of defining the target population of environments is an ongoing one

Since most breeding programs conduct METs, a few modifications can improve the data for the continuing process of the TPE definition:

- Select "probe" varieties with contrasting differences in important traits (i.e., early or late, photosensitive or insensitive) as reference lines.
- Test these varieties under representative conditions, including farmers' fields.
- Measure the water environment of the MET.

Monitoring water levels to characterize drought

Water supply can be monitored during crop growth to determine the timing and severity of drought to further define the TPE. The pattern of water level recorded over the season can be used to characterize three different types of drought:

- An early drought that occurs during vegetative growth.
- An intermittent mid-season drought that occurs between tillering and mid-grain filling.
- A late drought that occurs during flowering and grain filling.

In addition to knowing the frequency, it is also important to know the severity. For this, it is necessary to compare the yields under the drought and irrigated conditions or, if irrigation is not possible, choose a well-watered site such as the bottom of the toposequence.

Modeling the availability of water and the use of geographic information systems in the rain-fed lowlands to define the target population of environments

In the rain-fed lowland rice ecosystem, the TPEs are often defined by their position in the toposequence (hydrology). For example in Thailand, farmers' estimates of yield reduction because of late-season drought were 45–50 and 15–20%, respectively, for the upper and middle levels on the toposequence.[8] The national breeding program now uses the different positions on the toposequence to represent differences in the severity of drought in their testing program. A water balance model to predict available water has been developed and linked with geographic information systems (GIS) to characterize environments based on water availability (Inthavong et al., 2008).

How to determine the target population of environments for the upland rain-fed system

In upland rice, water availability for crop growth depends largely on rainfall patterns, rather than on total rainfall, and on land and soil properties that influence infiltration. The upland system is generally poorly buffered against variation in rainfall because it cannot store as much water as the lowland system. Short periods without rainfall (around 7 days) are most damaging if they occur just after sowing, when roots are poorly developed. Periods without rain can also cause spikelet sterility during the critical period from about 10 days before anthesis to 5 days after anthesis. As a general guideline for tropical areas:

- Flowering-stage stress will generally be significant after 7 days without significant (>5 mm) rainfall.

[7] Rajatasereekul et al. (1997) used this approach to define three broad domains for the rainfed systems of Thailand and Lao PDR and, from that, the duration of preferred varieties.

[8] Boonrat Jongdee (see The Thailand Experience of Integration of Direct Selection for Grain Yield and Physiological Traits to Confer Drought Resistance).

- For each additional day without rainfall during this critical stage, yield will decrease by about 10%.[9] The water supply during crop growth can be estimated using a simple water balance model based on weather data and knowledge of soil texture and depth at a site. Starting from a soil at field capacity, use the following as a guide to make an estimate of water use:
- Water content at field capacity can range from about 10 mm (sandy soil) to 20 mm (heavy soil) per 100 mm of soil.
- Rice grows well until about 30% of the available water is extracted. This means that the crop will have 3–6 mm of water available per 100 mm of rooting depth.
- Rice roots of many *indica* varieties below 600 mm seem mostly ineffective in water uptake, so their maximum rooting depth is probably 600 mm.
- In soils with high acidity, plow pans, or other conditions that encourage surface rooting, rooting depth will be much less. Therefore the depth of effective rooting needs to be measured for the site.

  If the roots extend to 600 mm, the crop can extract 18–36 mm of water, which is enough for 6–11 days of transpiration in the humid tropics during the vegetative and grain-filling stages, or 4–7 days of transpiration during the critical flowering stage. If the rooting depth is only 300 mm, a crop starting at field capacity can grow for only half this long before it begins to experience water stress.

It is recommended to use the rainfall and estimate of water use to develop a simple water balance for the crop to define the frequency and type of drought.

Choice and characterization of the testing environment

The TPE has to be used to define the breeding strategy; once the TPEs have been defined, a breeding strategy can then be developed for each TPE based on adaptation to the prevalent water supply and type of drought. In broad terms, that strategy includes selection for:

- Yield potential for favorable conditions.
- Drought escape (early maturing) for terminal stress.
- Drought tolerance for all stress conditions, but particularly intermittent stress.

However, when large year-to-year variation occurs in the type of drought, no one drought type can define the TPE. Under these conditions, breeders need to balance selection criteria to reflect the likelihood of each drought type in the TPE. The important point is to know which drought type occurred in each nursery and make sure that material that is well adapted to other frequently occurring drought types is retained among the selected lines. Otherwise, a cyclic pattern of genotypes adapted to different drought types can limit progress in selection.

Evaluation of the GEI helps to decide on the number of TPEs for the breeding program. In rain-fed environments, GEI or the tendency for genotypes to rank differently in different environments may be large. Under these circumstances, several TPEs, each served by different varieties, may be optimal. This is very different from irrigated rice, where the TPE can be very large, as in the example of IR36 grown on a large area. However, since each new TPE served will need additional breeding and testing resources, there will be a practical limit to the number of TPEs served by a breeding program. In some TPEs, the size of the target area will be inadequate to justify the resources required for a separate effort, and breeders must rely on the "spillover" of a variety from another TPE.

There is a trade-off between precisely defining the TPE and achieving enough replication within it. Thus, even when the TPE has been precisely defined, there will be random rank changes in variety means from site to site and from year-to-year, that cannot be explained by differences in water status. This is because many factors, such as pest damage, disease, and measurement error, routinely affect yield data collected in field trials. These "noise" factors are known to be very large in rain-fed lowland rice, and they can be overcome only through adequate replication within and across environments. If the TPE served by a breeding program is too narrowly defined, budget considerations will allow only one or a few trials to be conducted within each TPE. When genotype means are estimated from only one or two trials, least significant difference (LSD) values are very large, preventing accurate evaluations from being made and reducing progress from selection. In general, the TPE must be large enough to support three to five testing sites.

FIELD EXPERIMENTAL DESIGN[10]

The design should be precisely defined. A major departure from conventional (irrigated) rice breeding that is required in rain-fed systems is the need for early generation yield testing in selection environments (SE) that represent the TPEs and the large GEI within them. Replicate check lines must be used in early screening nurseries: in early generation screening trials, we are usually limited to very few environments. In fact, in some cases, the number of replications (r), locations (l), and years (y) may be only one. Even when all test lines cannot be replicated, one or more check lines should be replicated. Check lines in screening trials fall into two categories: probe lines that have well-known responses to specific stresses, and replicated checks that may be less well known but represent the test material as accurately as possible. Some guidelines for using replicated checks are:

- Lay out probe lines in a systematic way. The objective of these checks is to verify that the appropriate stress was in fact applied. For drought screening, a check line that is susceptible to the particular form of drought being tested should be used, and this might actually die under the applied stress.
- Identify plots for replicate check entries at regularly spaced positions in the field or screen layout. These positions must themselves be representative of the experimental space. In statistical terms, they represent a stratification of this space. They should not be selected to be at edges or along pathways, or in other non-representative areas. Border rows, plots, or pots as appropriate and necessary should in any case, protect them.

[9] Courtois and Lafitte (1999) have used this approach for a regional characterization of the uplands.

[10] Reprinted from McLaren (2003).

- The replicated checks should be allocated to the check plots according to a standard experimental design such as a randomized complete block (RCB) design or a Latin square design (see CROPSTAT tutorial[11] on "Randomization and layout of experimental designs"). The resulting nursery is then described as being laid out in an "augmented RCB" or "augmented Latin square" design as described below. If the field contains a single identifiable gradient, then an RCB with blocks perpendicular to the gradient is appropriate. For spatial control in two directions, the Latin square is better.

The main objective of the replicated checks is to quantify spatial variability in the test environment and adjust the measurements of the test lines accordingly. A desirable byproduct of using replicated checks is an estimate of measurement error and, indeed, if the checks themselves are interesting test material, extra valuable information is obtained on those particular lines [see CROPSTAT tutorial (see text footnote 12) on "Single-site analysis for variety trials"]. We recommend the use of augmented designs that have been developed to overcome the serious drawbacks of unreplicated trials, such as a lack of control of field variability, and no estimate of error for comparing entries (Federer and Raghavarao, 1975).

In advanced yield trials and METs, the main objective is to increase the number of environments where lines are evaluated. With limited resources it is preferable to increase the number of sites rather than the number of replications in any one trial. To do this, we use designs that are more efficient than the RCB designs such as modern alpha lattice designs. However they require specialist computer programs for their design and analysis. Some guidelines for effective METs are:

- Increase the locations rather than the replications to maximize the chance of testing under drought conditions.
- Choose locations that are likely to experience the relevant drought stress.
- Use a lattice design with only two replications and small blocks (<less than 10 plots per block) at each location (see CROPSTAT tutorial[8] on "Randomization and layout of experimental designs" and on "Single-site analysis of variety trials" for examples of how to use classical simple lattice designs).
- Use data from drought trials even if coefficients of variation (CVs) are high (provided that the trials were well-conducted).
- Do not use yield data from locations that do not experience the target drought stress for the TPE, unless the wish is to use them as an estimate of yield potential.[12]

BREEDING TO IMPROVE YIELDS UNDER DROUGHT: FROM THE SE TO THE FARMER'S FIELDS AND HOW TO INCREASE RESPONSE TO DIRECT SELECTION FOR YIELDS

The SE must be representative of the TPE. Performance in the TPE and the SE can be thought of as correlated traits expressed by a single genotype in separate environments. This relationship is measured as the genetic correlation (r_G). Thus, the r_G is an indicator of the accuracy with which performance in the TPE can be predicted in the SE. An r_G value of 0 indicates that there is no association between performance in the selection and target environments. An r_G value of 1 indicates that the SE is perfectly predictive of performance in the TPE. Therefore, before embarking on a controlled-drought screening program, the breeder needs to test the assumption that the performance in the controlled experiments is predictive of performance in the research station field (r_{G1}) and that performance in the research station field is predictive of performance on-farm under farmer management (r_{G2}). To maximize r_G between the SE and the TPE:

- Ensure that conditions at the research station (nursery and trials) are similar to those in farmers' fields. Note that selection is often conducted at research stations under management regimes that are not representative of those used by farmers. This type of selection may be justified in terms of selecting for yield potential or maximizing the precision of yield trials, but breeders must ensure that performance on-station is predictive of performance on the farm.
- Use two kinds of screening trial, one that predicts performance in drought years and one that predicts performance in favorable years. For the design of the managed-drought screening trial see the section "Water-stress management and characterization" below. Note that nurseries in which managed levels of stress are purposefully applied are useful in ensuring that r_G is maximized for stresses, such as drought, that occur sporadically in the TPE. It is important to verify that the results of managed-stress trials really are predictive of performance on-farm.
- Select directly in the target environment, that is, on-farm. For on-farm screening, the correlation between performance in the selection and target environment is necessarily 1, assuming that representative farmer-cooperators have been chosen. Therefore, on-farm screening should be a component of all breeding programs in which any uncertainty exists about the predictive power of on-station screening. Note that on-farm trials can be expensive and imprecise because of variability caused by weeds and low fertility, and are subject to a high risk of failure. Consequently, on-farm testing programs must be carefully designed and conducted to avoid wasting money and time, and to maximize the reliability of the data obtained. Use the robust experimental designs discussed earlier.
- Irrigate only if the objective is to measure yield potential.
- Use data from trials affected by drought even when the coefficient of variation (CV) is large; the inherent variability of stressful environments is often high (Atlin and Frey, 1989). This has important implications for the use of data from METs and on-farm trials in selecting drought-tolerant materials. Often, trials with high CVs are omitted from the analysis. However, these are frequently the trials in which stress was most severe. Omitting high-CV trials almost always introduces bias into the sampling of the TPE toward more favorable environments. This bias can be avoided by not using an arbitrary CV value as a criterion for accepting or rejecting a certain on-farm or off-station trial. If no obvious errors have been made in layout or data collection, results from low-yield, high-CV trials should be retained. These are often precisely the trials that are the most informative about cultivar performance in stressful environments.
- Select genotypes that perform well under both drought and well-watered conditions. Varieties that perform well in both types of

[11] http://www.riceworld.org/science/software/cropstat.asp
[12] Reprinted from Atlin (2003).

SE can generally be identified because r_G across drought stress levels is usually positive in other crops (Atlin and Frey, 1989; Bänziger et al., 1997) and there is evidence that r_G is also usually positive (sometimes with a low value) in rice grown under a range of water-stress environments (Lafitte and Courtois, 2002; Pantuwan, personal communication). Selection intensity must be high. Drought-tolerance breeding programs must be large to make progress. In most rain-fed rice breeding programs, only a few lines (usually fewer than 50) are tested in the replicated MET at several locations, although this is the selection phase most responsible for making gains in stress environments. If little selection pressure for yield under drought stress is applied, little progress will be made. For a small rain-fed rice breeding program focusing on drought tolerance and producing 1,000 new F_6 or F_7 lines per year from its pedigree breeding program, an appropriate distribution of effort might look something like the scheme below:

- preliminary managed-stress screening: $N = 1{,}000$
- preliminary replicated yield testing under stress: $N = 200$
- METs – advanced lines: $N = 100$
- participatory on-farm testing: $N = 20$.

The following techniques can increase the number of plots and, therefore, the number of entries using the same resources:

- Use augmented experimental designs that maximize the number of entries for given resources.
- Use micro-plots and visual rating scales judiciously (see later section).
- Use screening methods that are inexpensive and able to handle large numbers.

Broad-sense heritability (H) must be maximized through careful management of drought screening nurseries and by high levels of replication within trials and across sites and years. There are several ways to increase H:

- Increase the number of replicates per trial.
- Increase the number of trial locations.
- Increase the number of years of testing.

It is important to reduce the error (σ^2_E) variance to detect real differences between lines. In our experience, the genotype-by-location-by-year (σ^2_{GLY})and the error (σ^2_E) variance are the largest contributors to random noise in field trials. The contribution of (σ^2_E) can be reduced by choosing uniform test sites, increasing within-site replication, adopting improved methods of controlling within-block error (for example, lattice designs or neighbor analysis), or increasing the number of locations or years of testing. The contribution of (σ^2_{GLY}) can only be reduced by increasing the number of tests across locations or years. This is expensive and must involve:

- cooperation among research centers in collaborative networks for the early stages of yield testing, rather than extensive testing at a single center until advanced stages (Cooper et al., 1999b)
- increasing the number of test locations rather than the number of replications at each site.[13]

Increasing the number of replicates (without increasing the number of trials) is less expensive but also less effective in increasing heritability!

WATER-STRESS MANAGEMENT AND CHARACTERIZATION[14]

One of the major limitations to the improvement of rice for drought-prone areas has been the lack of appropriate methods to impose drought routinely and reliably in order to select more tolerant lines. Many methods have been used to impose drought in order to have a better understanding of the mechanisms that lead to higher yields and the traits that can be selected for drought tolerance. However, few have been evaluated to assess their predictability of performance in farmer's fields (see more on this later). Each has a strength and weakness as outline in **Table 5**. Therefore, care is needed in deciding which approach to use. We advocate more studies to validate that the testing environment predicts performance in farmer's fields.

Start with a uniform field and apply all inputs uniformly

When fields are well irrigated, they often appear uniform. However, as drought develops, differences in topography, slope, soil texture and field history can have a large effect on plant growth. Choose a level field with minimum variation in soil depth or texture. Not all the variation in a field can be seen from the surface; observations of weed or crop growth in a previous season can give hints of problems. A transect of soil cores or soil impedance readings can also indicate below-ground variation. If irrigation is applied, it must be uniform in depth. Replicates or incomplete blocks should be placed inside a basin. If sprinklers are used, irrigation must be applied when there is little wind. All sprinkler heads must throw the same amount of water, so the pump pressure must be high enough to pressurize the system evenly. Sprinkler heads must be cleaned and checked, and leaks should not occur within plots. Other management practices such as the application of fertilizer and weed control should also be carried out uniformly. If it is found that uneven drying still occurs in the field, a visual score of soil drying can be given to each plot when differences are obvious, and this score can be used to adjust for field differences. Statistical designs are available that can also help deal with variability, but there is no substitute for starting with a good, uniform field.

Know what happened

Whether managing irrigation or relying on natural drought periods for stress, the essential measurements needed to characterize the environment are depth of standing water (in lowland fields), depth of the water table, and daily rainfall:

[13] In rainfed rice METs, both within-site residual variance and genotype-by-location-by-year variance tend to be large and much more important than genotype-by-location or genotype-by-year variance. Increasing either the number of trials or the number of replications per trial will usually increase selection response, but increasing the number of trials will have the greater effect.

[14] Reprinted from Lafitte (2003).

Table 5 | Evaluation of different field devices for genotype study/screening in response to drought.

Field devices for drought study	Cost	Strengths	Limitations	Suitable climate and soils	Reference
Late planting with drainage in rainy season trial	Large uniform field management	High chance of reproductive and terminal drought	Photoperiod non-sensitive	Semi-arid tropics	Pantuwan et al. (2002a)
Dry-season trial	Large uniform field management	High chance of drought, vegetative drought	Photoperiod non-sensitive, genotype-by-season interaction	Semi-arid tropics	Pantuwan et al. (2004)
Line-source sprinkler	Equipment, water source, monitoring	Different water regimes	Wind, space	Semi-arid to arid climate	Garrity and O'Toole (1994)
Rainout shelter	Construction	All types of drought	Space, cost		Lilley and Fukai (1994)
Greenhouse	Construction	All types of drought	Space, cost, rhizosphere differences (small and loose)		Yadav et al. (1997), Wade et al. (2000)
Root restriction	Rhizosphere manipulation	Evaluation of non-root traits[a]	Space	Hardpan, simulated lowland	Kato et al. (2007)
Raised bed	Rhizosphere manipulation	Dry surface soil (interrupt capillary water)	Space	Sub-humid climate	Kato et al. (2007)

(Source: Kamoshita et al., 2008).

[a]Restriction of the root zone removes the advantage of deep rooted varieties that would be expressed if no restriction; in most puddled lowland fields roots are restricted.

- The simplest measure is to record the presence or absence of standing water weekly. A late-season drought can be identified by the last date of the standing water relative to the flowering date of the variety.
- A measure of the depth of the water above and below the ground is more informative. For an accurate measure of the above ground water, use a "slant meter"; for below the ground, use a PVC tube.
- Use a minimum of three recording stations for each trial located across any perceived water gradient.
- Make some additional measurements. It is useful to know pan evaporation and this can be measured from a central station in a region. For upland experiments, it is useful to know soil moisture tension, which can be measured inexpensively using a tensiometer. For guidelines on making groundwater wells and tensiometers, see Mackill et al. (1996).
- Remember that many potentially useful datasets cannot be interpreted because no one knows whether drought affected the experiment or not. Observations of leaf rolling in check cultivars can provide good evidence of when water stress began. It is critical to know both the dates of disappearance of standing water in lowland fields and the amount of water in upland experiments. If the water table is at a depth of 1–1.5 m, it can provide an additional source of water to the crop; so check for groundwater depth.

Keep out unwanted water

To apply stress consistently, there must be a way to limit water input to the plots. This can be done by the following means:

- Sow at a time of year when a good chance of low rainfall is expected (provided that this season is representative of the regular season in the target environment).
- Use a rain exclusion shelter. Such shelters are expensive to build and maintain, so these are usually used only for small experiments. The temperature under shelters tends to be higher than the outside air temperature. This may affect crop flowering date and can, in some cases, result in high-temperature damage. Monitoring of air temperature will allow interpretation of the results.

Check for water from underground sources, especially if there is lowland rice nearby. To avoid entry of water from adjacent wet areas, between the experimental field and the source of free water, it is necessary to dig a ditch that is at least 40 cm deeper than the expected root zone. This ditch will intercept water moving into the field, and the water must then be drained away. At upland sites, lateral water movement is not usually more than about 1 m but, depending on the irrigation method, it may be necessary to have wider borders.

Remove water at the desired time

In rain-fed lowland experiments, the soil is generally saturated before stress begins, and the field is then drained to allow the development of drought. The number of days it takes for drought to develop depends on the moisture-holding characteristics of the soil, losses from seepage and percolation, and the amount of water transpired by the crop. Thus it is necessary to conduct an initial experiment to see when to remove water to induce stress at the desired time. Remove water at a developmental stage of a check variety. With experience, it is possible to estimate the number of days this will require in the experimental field. For a fully developed crop growing in a heavy clay soil at IRRI, it takes about 10 days for a field to dry from saturation to near field capacity. After about 1 week more, some leaf rolling can be observed. This means that it

takes about 20 days for stress to develop after the field is drained, and would take more time if the crop were small. In contrast, sandy soils dry much more quickly and stress can develop within 14 days or so.

In upland experiments, it will take much less time for stress to develop after rainfall or irrigation stops. If root depth is shallow (25–30 cm), the amount of water available to the crop between field capacity (about 10 kPa) and 20 kPa is only adequate for a few days of transpiration, and irrigation must be applied every 2–3 days in control plots. Stress will begin almost immediately on the withholding of the irrigation.

It is also possible to apply a mild continuous stress by simply reducing irrigation frequency. This has the advantage that it has a similar effect on genotypes with different flowering dates, and the stress treatment is not affected much by minor rainfall events. However, a mild continuous stress is not very effective in separating lines for some traits that require more severe stress, such as flowering delay and leaf drying.

How severe a drought stress?

Aim to reduce yield by almost 50%. One reason for this is that r_G for line means estimated in trials with only slightly different stress levels is likely to be very close to 1.0. Another reason is that severe stress, when skillfully and uniformly applied, can amplify genetic differences between lines. For example, if uniform and severe drought stress can be applied to rice breeding lines at flowering, some highly susceptible lines simply do not flower. This is a large, visible genetic response that can make it easy to eliminate susceptible genotypes.

Conduct a companion nursery under well-watered conditions

In addition to the controlled-drought SE, it is very useful to have a companion nursery with well-watered conditions to estimate the yield potential of the genotypes:

- Estimate the severity of the controlled environment as the mean reduction in yield between the well-watered and the drought nursery.
- Avoid water deficit in the uplands; irrigation is usually applied when the soil moisture tension at 15 cm depth reaches about 20 kPa.
- Maintain free-standing water in the well-watered rain-fed lowlands.

Correct for differences in flowering date

Rice is especially sensitive to stress around flowering. This means that a line that flowers shortly after the field has been drained will be much less affected by stress than a line that flowers later. One option is to place genotypes in early, middle, and late maturity groups, and stagger the planting dates so that all genotypes flower at the same time. This requires good information on flowering time and is difficult to manage. Another possibility is to stratify the entries based on the flowering dates of the well-watered plots, and select lines that are less affected by stress within each group. If there is a clear linear relationship between stress yield and flowering date, a drought response index (DRI) can be used (Bidinger et al., 1987).

This means regressing stress yield on flowering date in the control, and finding the predicted yield as follows:

Predicted yield $= a + b$ (flowering date) And the DRI is calculated as: (observed yield-predicted yield)/standard error of predicted yield.

OTHER POINTS TO CONSIDER

Dry-season screening is, in most parts of the world, equivalent to out-of-season screening. Fields that are sown out of season are generally much more susceptible to insect, bird and rodent attack because other food sources are unavailable. There are also climatic factors to consider, such as low temperature, high radiation and low humidity. Because of these factors, performance in a dry-season nursery may not accurately predict yield potential for a variety targeted to the wet season. The main purpose of the dry-season nursery is to obtain additional information about drought tolerance. This information can be combined with other data from wet-season screening in a selection strategy.

When rice is grown repeatedly in upland fields, yield potential often declines markedly after the first crop or two, perhaps because of nematode accumulation, micronutrient deficiencies, or other unknown factors. If a field is developed as a long-term screening site, it should be large enough to allow part of the field to be rotated with a non-rice crop each year.

PHENOTYPING (TRAITS)

Which secondary traits are useful?

There must be a relationship between the secondary trait and grain yield in the target environment. The traits expected to be of value in some drought-tolerance breeding programs are shown in **Table 6**. However, even when this relationship is found, that is not enough to show that breeders should use the secondary trait. For breeders to use the trait, the expected progress from selection using the secondary trait and yield together must be greater than the progress made using grain yield alone. Kamoshita et al. (2008) provide a review of the broad-sense heritability of the main traits proposed for use in selection for drought tolerance in rice. Based on an earlier assessment by Lafitte et al. (2003) the recommended traits are:

- *Flowering/maturity date* (useful for predictable terminal drought): Rice is extraordinarily sensitive to water deficit from about 12 days before 50% flowering to about 7 days after flowering. If the pattern of water deficit is predictable in a given region, selection for a flowering date that does not coincide with the period of water deficit is a very effective way to improve drought tolerance. The limitations to this approach are that very early varieties may suffer a yield penalty in good seasons, and that this approach works only where the timing of the water stress is quite predictable. As well as avoiding drought at critical growth stages, there may be an additional advantage to comparative earliness. Early materials sometimes tend to have a more stable HI than later ones.
- *Flowering delay* (useful for intermittent mid-season drought): When rice experiences a water deficit before flowering, a delay usually occurs in flowering date. Lines with a longer delay will tend to produce less grain, even if the water stress is relieved later. The length of the delay is partly related to the amount of

Table 6 | An assessment of selected secondary traits expected to be of value in some drought-tolerance breeding programs.

Trait	Relationship to stress yield	Growth stage for selection	Earliest generation for selection	Technical difficulty of selection	Heritability
Flowering/maturity date (Babu et al., 2003)	Depends on reliability of stress timing; effective for predictable and terminal stress	Flowering	Single plants at F_2	Easy	High heritability (ca. 0.9)
Flowering delay (Kumar et al., 2007)	High for stress at flowering	Flowering	When available, seed is sufficient for a small plot	Easy if water can be controlled to provide uniform stress	Moderate heritability (ca. 0.6)
Percent fertile spikelets (Babu et al., 2003; Kumar et al., 2007)	High for stress at flowering	At or near maturity	Single plants at F_2	Labor-intensive; error-prone; requires control of water	Moderate heritability (ca. 0.6)
Leaf-rolling score (Babu et al., 2003)	Negative and moderate	Vegetative	Single plants at F_2	Easy if water can be controlled to provide uniform stress	High heritability (ca. 0.8)
Leaf-death score (Yue et al., 2005)	Negative and moderate	All stages	Single plants at F_2	Easy if water can be controlled to provide uniform stress	Moderate heritability (ca. 0.7)
Canopy temperature (Yue et al., 2005)	Negative and fairly high if maximum stress occurs near flowering	Pre-flowering during full ground cover	When available, seed is sufficient for a small plot	Medium	Fairly low heritability (ca. 0.2) unless climate is very stable and vapor pressure deficit (VPD) is large

Source of assessment: Lafitte et al., 2003; more detail assessments of the heritability for each trait is found in the reference for each trait. Practical instructions on the measurement of these traits are provided in Measurement of Secondary Traits: Some Practical Considerations.

stress the line experienced, but there is also genetic variation in how much delay results from a given level of stress. The reason for the delay in flowering is not fully understood.

- *Percentage of fertile spikelets*: When stress occurs near flowering, i.e., the most sensitive growth stage, the main yield component affected is the percentage of fertile spikelets. The genetic correlation between yield under stress and this trait is very high, and the heritability of spikelet fertility is less affected by stress than is the heritability of grain yield. The way that spikelet fertility is affected by drought at flowering is quite specific, so it gives clearer information on genotypic response to stress than does yield, which is the integrated result of many processes that occurred over the season. However, many factors other than drought can affect spikelet sterility, and some of these, such as stem borer damage, interact with drought. Experiments should be monitored for possible confounding factors.
- *Leaf-death (desiccation or "firing") score*: Leaf water deficit can be reduced further beyond the point of turgor loss, reaching the point of tissue death. Leaf tissues may die (showing desiccation) because of extreme loss of water or because of heat stress when the leaf temperature rises as a result of inadequate transpirational cooling. Unlike leaf rolling, leaf desiccation is irreversible. All leaves in the canopy should be observed when leaf death is scored. Desiccation may not occur throughout a given leaf in a uniform fashion, unless the water deficit is acute. More typically, it begins at the tip of the leaf, which is usually under greater water deficit than the basal part closer to the stem. If the timing and severity of drought in the screening environment are similar to those of the target environment, leaf drying can be correlated well with yield under stress.

CONCLUSION

Choice of parental material

Atlin (2003) notes that choosing parents is one of the most important steps in a breeding program. No selection method can extract good cultivars if the parents used in the program are not suitable. Although breeders have different approaches to parent choice and have achieved success in different ways, many successful crosses have some common features that can be recommended:

- Use at least one locally adapted, popular cultivar as a parent. This helps ensure the recovery of a high proportion of progenies with adaptation and quality that are acceptable to farmers. If quality requirements are very important and if the local variety is highly preferred by farmers, a backcross to the local variety may be required to reach an acceptable level of quality.
- Choose each parent to complement the weaknesses of the other. For example, if both parents are susceptible to an important disease, it is highly unlikely that many offspring will be resistant. Thus, when breeding for drought tolerance, avoid parents that are highly drought-susceptible.
- Use improved modern varieties in crosses with an adapted parent. Often, elite modern varieties have high yield potential and many disease-, insect-, and abiotic stress-tolerance genes that local ones lack.
- If no drought-tolerant cultivars are known, evaluate a diverse range of cultivars and advanced lines for the characters identified

for the TPE, including the specific characters for drought tolerance. This will mean testing the potential parental material under controlled drought.

Researchers in Thailand, Cambodia, and Laos have screened local materials for drought tolerance; they used DRI to normalize the effects of yield potential and flowering date on yield under drought stress. DRI ranges from −2 to +2, and values greater than 1.4 may be considered as drought tolerance. When several experiments are considered, the mean DRI of the drought-tolerant genotype may be below 1.4, with the actual value depending on the consistency of performance across the experiments. The DRI provides a better estimate of the contribution of drought-tolerance traits to yield under drought, independent of those for yield potential and flowering. However, this estimate is prone to high errors and should be considered mainly as supporting evidence. These researchers screened a total of 1,279 rice genotypes including a large number of landraces for drought resistance in 34 experiments across the three countries. Drought was imposed (i.e., controlled drought) in 76% of the trials. The project validated the use of DRI for grain yield and spikelet fertility as important drought traits. DRI heritability ranged from 0.39 to 0.88, and from 0.31 to 0.77 for grain yield and spikelet fertility, respectively. In each country, the selected donor lines were crossed to local recipient cultivars with a high yield potential and/or good grain quality attributes. A total of 85 populations (40 for Thailand, 19 for Laos, and 26 for Cambodia) were developed that were derived from single-seed descent (SSD). In Thailand, a number of populations were backcrossed to the recipient parent to form NILs. Five RIL populations in Laos, eight in Cambodia, and six in Thailand were selected based on the performance of the putative drought lines, and are being carried forward. These, plus some of the original populations, are now part of the routine breeding program of the three countries. The progenies (F_6) will be phenotyped for drought response, and superior lines will enter the routine advanced testing trials (Report to Rockefeller Foundation, 2006).

Early generation yield testing in the target population of environments

A major departure from conventional (irrigated) rice breeding that is required in rain-fed systems is the need for early generation yield testing in SEs that represent the TPE (Atlin, 2003; Jongdee, 2003). The aim of the breeding program is then to develop fixed lines for early yield testing at a large number of sites (direct selection for yield) and under controlled-drought conditions (indirect selection). A number of strategies can be followed:

- Fix lines through SSD. The main goal is to fix the lines with minimum selection. Where facilities are available to control day length (and when using photoperiod-sensitive materials), up to three generations per year can be produced using rapid generation advance (RGA), thus reducing the time to develop fixed lines (F_5 and later) for yield testing.
- Fix lines through the normal process of single plant selection within the F_2 and later generations in the bulk method. Usually, two generations are developed each year by the use of an off-season nursery. This provides an opportunity to select for characters that are more highly heritable – selection is based on a single plant or progeny row and one observation. It also creates a danger that selection, particularly under irrigation or in the off-season nursery, will not be representative of the TPE.
- Select for traits such as maturity and height (main season) and disease resistance only in the early generations, if the desirable agronomic traits have been identified with farmers' priorities in mind. For example, breeders may select short materials because of their high yield potential, but farmers may not accept these because of various problems such as poor weed competition and low straw yield.
- Select under drought conditions in the early stages. Many plants in a segregating population may not produce any seed because of susceptibility to drought. Since the heritability of drought tolerance is usually low, it will be beneficial to practise this type of selection for more than one generation. Many breeders find that the bulk method of breeding is suitable for this type of environment, and requires fewer resources than the pedigree method.
- When fixed lines are developed (F_5 or later), seed supplies are sufficient for replicated testing. This will allow more flexibility in conducting METs in the TPE.

All breeding programs should include participatory on-farm trials

To ensure that selection has been effective and that progress made at the station will be transferable to the farm, on-farm trials managed by farmers should be part of the testing of a new cultivar (Atlin, 2003). In such trials:

- Include as many cultivars as possible in participatory testing by farmers in their fields.
- Consider the use of "mother-baby" trials (Bänziger et al., 1997) to maximize the number of genotypes tested.
- Run participatory trials concurrently with advanced METs.
- Test for grain quality, in consultation with farmers from the TPE. This is cheaper than replicated yield testing. Hence, quality screening should be done before METs to discard varieties with quality unacceptable to farmers.

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APPENDIX

THE THAILAND EXPERIENCE OF INTEGRATION OF DIRECT SELECTION FOR GRAIN YIELD AND PHYSIOLOGICAL TRAITS TO CONFER DROUGHT RESISTANCE

The rain-fed lowland is a major rice ecosystem in Thailand with an area of approximately 5.7 million ha, more than 60% of the total rice land of Thailand. Rainfall is bimodal and drought may develop early and late in the growing season. The early season drought occurs in most areas, affecting the time of transplanting of seedlings and the growth of direct-seeded rice. Late-season drought develops at the end of the monsoon season in most years in the northeast, particularly on upper part of the toposequence of the paddy, where there is more water loss from soil percolation and lateral water movement. Early season drought is more frequent than late-season drought, but yield loss is more severe in the latter. Thus, the target of our breeding program is the development of late-season drought-tolerant cultivars. The following describes our approach to screening and breeding for drought tolerance. To date nine breeding lines of traditional and improved germplasm have been selected and used as donors for drought tolerance. Single and backcross populations had been developed. Presently, 10 lines which have been selected under field screening are being tested under farmer fields in target drought-prone areas.

The breeding approach

The breeding approach has been changed to increase efficiency and shorten the selection process. The change is based on recommendations from the Australian Center for International Agricultural Research (ACIAR) project on "Plant breeding strategies for rain-fed lowland rice in northeast Thailand and Laos" (Cooper et al., 1999a,b). The previous breeding program took 12–15 years, now the cycle is completed in 10–11 years. There are three major phases of the selection cycle: intra-station (local, on-station selection); inter-station (across 13 stations, on-station selection); and on-farm selection. In the previous breeding system, selection was carried out mainly in the intra-station phase and most lines were discarded based on visual selection and on the results from yield testing at a single location (i.e., local adaptation). Only a small number of lines relative to the total generated from the crossing program were selected for subsequent inter-station (wide adaptation) and on-farm performance. This selection system made it difficult to identify high yielding lines at the farm level due to a large GEI for grain yield (Cooper et al., 1999a). One of the recommendations for the breeding system was to replace the intra-station phase with early generation inter-station yield testing of F_4 bulks in order to select for wide adaptation at an earlier stage of the selection process. However, the F_4s are still segregating for flowering date and this causes some error in estimating grain yield. We modified the recommendation to develop our new breeding system which tests large numbers of F_7/F_8 in the inter-station (multilocation) trials. We use the RGA technique at the intra-station phase to save time. Recently we have again modified the selection to incorporate on-farm testing earlier in the selection process. The details of these changes in the selection process are described by Jongdee (2003).

Definition of the target domain

The majority of the lowlands are in the northeast and north, and are classified as shallow-favorable and shallow drought-prone. We used GEI and cluster analysis of grain yield from multi-location trials to further define our TPE. However, groups of environments changed from year-to-year, resulting in a large genotype-by-year component of the GEI, and it was difficult to define genotype-by-location groupings. Recently, we changed the system of defining the TPE based on our work with farmers. We conducted a farmer participatory workshop for production improvement for rain-fed lowland rice in north and northeast Thailand and, from this, identified the target domains based on hydrology of rice paddies. Three levels of the paddy toposequence are identified – upper, middle, and lower terrace paddies – and these three water environments are included in the test locations in each region. Drought may occur at any time during the growing season as shown in **Table A1**, but our focus is on improvement for the intermittent and late-season drought. The upper terrace paddy can be defined as unfavorable conditions, in which drought can develop at any growth stage. The middle terrace paddy can be drought prone, where rainfall is variable and soils are light in texture. In other areas, the middle part of the toposequence can be considered as favorable. The lower terrace paddy can be defined as less favorable, because drought may develop in the early season followed by a sudden flood. The estimates by farmers of yield reduction due to late-season drought were 45–50 and 15–20% for the upper and middle terrace respectively. We use the different positions of the toposequence to provide differences in the severity of drought in our testing program.

The selection strategy

The different selection criteria used for developing cultivars for each of the TPE defined by the upper, middle, and lower terraces are shown in **Table A2**. Phenology, particularly flowering time, is the most important trait for avoiding the late-season drought in each of the different domains. Flowering must occur before the standing water in the paddy disappears. Thus we select three flowering groups for the different domains of the toposequence:

- early maturing: flowering around mid-September to beginning of October
- intermediate maturing: flowering around mid-October
- late maturing: flowering around late October.

We select directly for yield in the multi-site selection program (described below) and we manipulate the water environment at a few sites in order to measure the drought-tolerant traits of flowering delay, spikelet sterility and, increasingly, for leaf water potential (LWP).

Water management to simulate late-season drought (at three of the test locations)

The drought screening trials under water-managed conditions are conducted in the wet season, in which the seeding is delayed by 2–3 weeks compared to the normal planting time. This increases

Table A1 | Use of the position on the toposequence to define the types of drought occurrence and the target population of environments (TPE) for the breeding program.

Position on the toposequence	Type of drought occurrence	Yield loss in the TPE
Upper	Early, intermittent and late drought	Late drought causes 45–50% yield loss
Middle (drought-prone)	Early and late drought	Late drought causes 15–20% yield loss
Middle (favorable)	Early drought	Minimal yield loss
Lower	Early drought and sudden flood	Minimal yield loss in drought; higher risk of loss from flooding

Table A2 | Selection criteria to develop varieties for each target domain.

Target domain	Cultivar requirement	Drought traits	Selection strategy
Upper	Early maturing drought tolerance Low number of tillers	Maintenance of LWP Less delay in flowering Low spikelet sterility	Select for yield under the test location
Middle (drought-prone)	Intermediate maturing photoperiod sensitivity drought tolerance Intermediate height	Maintenance of LWP Less delay in flowering Low spikelet sterility	Select for yield under the test location
Middle (favorable)	High grain yield Intermediate height	Nil	Select for potential grain yield
Lower	Late maturing photoperiod sensitivity submergence tolerance	Nil	Select for yield under the test location

the chance of the development of a late-season drought. Also, the standing water is drained from the field 2 weeks prior to flowering time, to further induce drought stress during the targeted growth stage. The water is drained from the field when the earliest lines have reached the flag leaf stage. If necessary, irrigation water is added to ensure free-standing water prior to the flag leaf extrusion. Measurements in this trial include grain yield, spikelet sterility, and flowering date. The main measure of drought resistance used to complement direct selection for yield is spikelet sterility. We measure the percentage of sterile spikelets from panicles that are randomly harvested in each line, and which are grown in the controlled-stress trial. Variation in flowering date among the test lines causes differences in the severity of the drought stress and, thus, the spikelet fertility. To adjust for this effect, we compare spikelet sterility and grain yield among lines within the same maturity group. Because drought can occur at any time during the growing season, we record the pattern of water supply and the severity of the drought. We measure the standing water in the paddy as an indicator for drought development, and the level of underground water below the soil surface as an indicator for the severity of drought. We use a slant meter to measure the surface water and a piezometer to measure the water underground. All observations are made on a weekly basis.

Crossing and rapid development of fixed lines for yield testing

Only a few research stations are involved in the development of lines for yield testing. Photoperiod-insensitive materials are advanced for one or two generations in the same year by growing them in the dry season. In photosensitive materials, we use a dark room to induce flowering as part of the RGA methods.

In order to reduce the number of materials before RGA, selection can be conducted on the F_2 generation for characters with high heritability such as height, plant type, flowering time, and grain size. (Note that there is no selection while under RGA).

Direct selection for yield and for drought-resistant traits at the station

Thirteen research stations across the north (five stations) and northeast (eight stations) are involved in the multilocation yield-testing program. The trials are conducted under two conditions of water availability: the water regime of the normal rain-fed lowlands in 10 stations, and a water regime that is manipulated to simulate late-season drought in three stations (two in the northeast and one in the north). The objective of this selection is to evaluate families for grain yield under normal rain-fed and late-season drought conditions.

The F_7 lines developed from the intra-station selection (i.e., mainly for plant and grain type), are evaluated in two steps: an inter-station observation trial and an inter-station yield trial. The inter-station observation trial contains a large number of lines (200–300) grown in two replications and in plots of four rows, 2 m in length. In some cases, the lines are grouped on flowering time and form a separate trial, with each trial containing a set of check varieties that have been selected for their known response to different water environments. An alpha-plus experimental design is employed. The data are analyzed using REML, SAS, and GenStat. The selection in the inter-station observation trials is based on grain yield under normal and manipulated late-season drought. The first analysis is of grain yield data from the normal water regimes from each of the 10 stations. The data are analyzed by site and also in a combined analysis across the stations. The lines are grouped based on the GEI analysis for yield into different patterns by cluster analysis. The group(s) of lines that perform well at most environmental sites are selected, and the group(s) that have low grain yield in most environmental groups are discarded.

Because there is variation in flowering time among test lines and thus the timing of drought influences the yield, the second analysis is conducted for lines within the selected groups. Individual lines are selected based on spikelet sterility percentage and on

grain yield under the manipulated late-season drought, bearing in mind the variation in the flowering date. Lines with resistance to the major diseases and insects pest, and with appropriate grain chemical quality are selected at this step as well.

The inter-station yield trial is conducted across the same stations in the north and northeast, using the same experimental design as that of the inter-observation trial, but with three replications. The plot size is expanded to five rows, 5 m long. The lines may be grouped by flowering date if there are a large number of lines in each flowering group. The grouping facilitates trial management of the timing of fertilizer application and of bird control, and it allows for the adjustment of the effects of different flowering times (and therefore different levels of stress) on grain yield. The selection of lines is based on the grain yield under rain-fed conditions, and also under the manipulated late-season drought. The approach is the same as described for the observational trials. Again, there is selection for resistance to important insects and diseases, and for chemical grain-quality characters.

Selection at the farm level

Our previous on-farm trials included only four to six lines with different flowering times, and favored the selection of lines for shallow-favorable conditions which are not representative of farmer fields. More recently, Inthapanya et al. (2000) have suggested more rigorous testing in farmer fields representative of their risk of drought and of the levels of fertility. We now conduct two stages of on-farm trials, the first with a large number of lines in each of the three flowering groups of our target domain, in which 20 lines are grown with a small plot size (6–8 rows per plot). The second is conducted with a small number of lines with a large plot size (16 rows per plot). The farmers' evaluation of agronomic characters (panicle size, grain color, etc.), is conducted during grain filling. The selected lines are tested for eating quality at harvest using 15–20 farmers at each site.

Selection of parents

Now that we have modified our routine breeding program we are focusing on the selection of parental material based on more in-depth screening of sound physiological traits. We are selecting drought resistance donors based on the following criteria:

- maintenance of LWP
- drought score
- DRI
- delay in flowering
- spikelet sterility.

The trials used to phenotype the progenitors are conducted in three locations, two in the northeast and one in the north. We use two screening systems to induce drought: a line-source sprinkler and the water drainage technique applied before flowering, as described earlier. We measure LWP at midday (11.30–15.00 hours) on up to 60 plots per hour (one to three leaves per measurement) per team of five people. The flowering time and grain yield under both well-watered and stress conditions, and drought score and spikelet sterility under stress conditions are determined. These data are used to select progenitors with high drought resistance for crossing with well-adapted and accepted commercial cultivars. The progenies from these crosses are used in the routine breeding program described above.

Use of molecular markers

Recently, the number of lines derived from QTL-based selection has been increased in the rain-fed lowland rice breeding program. The QTL-based selection was done mostly for tolerance to disease (e.g., blast) and eating quality traits. Then, they are selected in the manner described earlier. The use of molecular-assisted selection has reduced the time to release varieties by 3–4 years, and is also more resource effective by selecting specific target traits.

Outcomes from the screening for drought tolerance

We have identified a number of drought-tolerant lines, e.g., three double haploid lines from a cross between CT9993 and IR62266, two lines from the rain-fed lowland rice breeding program, and seven lines from local germplasm. The double haploid lines were crossed with Surin 1 (a variety for irrigated areas), KDML105, and RD15. The latter two are popular rain-fed lowland rice varieties, and were backcrossed to BC_3 using molecular markers, and then F_2 materials have been selected under well-watered conditions. The Surin 1 backcross population is now undergoing field screening for drought tolerance. The populations from crosses between drought-tolerant lines and RD6 have also been developed with the aim of producing varieties with high grain yield, grain quality, and drought tolerance. These crosses have been backcrossed without using markers. The materials are used for breeding purpose as well as identifying QTLs for drought tolerance.

Already, there is some anecdotal evidence of the advantages of farmer participation in the selection of experimental lines. For example, RD12, an early maturing, blast resistant, good eating quality glutinous variety was released in early 2007 after farmer participatory selection. Adoption of this variety by the farmers is already high and increasing in northeast Thailand.

We are exploring two innovations to improve the selection process. We are determining spikelet sterility on a weight basis, weighing the total spikelets and then filled grain weight. The value is then adjusted for the difference in flowering time among lines tested. This is a quick and more accurate method. We are also improving the estimation of the time of flowering, so that we can accurately estimate delay in flowering. We are testing whether or not plot-based determination is sufficiently accurate.

MEASUREMENT OF SECONDARY TRAITS: SOME PRACTICAL CONSIDERATIONS

To measure flowering date, record the date when 50% of the productive tillers in a plot have emerged. This can be a difficult date to pinpoint, especially in stressed plots where flowering is delayed, and experienced scorers can differ by as much as 3 days in their estimates of when a plot reaches 50% flowering. To improve the quality of the data, the area to be rated can be restricted to a specific central, fully bordered, part of the plot. This area will be more uniform and the data will be more consistent. Alternatively, if the crop is sown in hills, flowering date can be defined as when a certain number of hills have produced panicles. Estimates of flowering should be recorded at least three times per week.

To measure flowering delay, there must be an irrigated (unstressed) control treatment sown nearby. Make regular, reliable observations of flowering date to calculate the delay:

Floweing delay (days) = days to flowering in stress treatment − days to floweing in control treatment.

Because this character is the difference between two independent measurements of flowering date, the error is generally larger for the delay than for flowering date alone. Flowering delay is best expressed when the stress is severe, so it is easily seen in fields where drying occurs over a period of weeks. In this type of stress, lines with later flowering dates will tend to be delayed more than lines that flower early, because the stress intensity increases over time. To correct for this effect, lines can be sown with similar flowering dates in separate experiments and stress applied at the appropriate time for each experiment. Another approach is to make a statistical correction for flowering date. This can be done by using flowering date in the control as a covariate in the analysis.

To measure spikelet fertility, at maturity collect a sample of representative panicles from the plot. Do not use only the tallest tillers or tillers from the main stem only; these will be strongly biased. Weigh the sample. Divide the sample randomly into two, and repeat the division until the sub-sample is small enough to process. Weigh the sub-sample. Thresh the sub-sample by hand to remove all filled and unfilled spikelets. Rolling or other threshing methods cannot usually do this because, if the sample is dry, the rachis will break off with the unfilled grains or, if the sample is wet, the unfilled spikelets will remain stuck to the rachis. Separate the filled and unfilled spikelets by blowing or by flotation. Weigh the filled grains and the unfilled spikelets. Then count out 200 filled grains and record their weight, and do the same for 200 unfilled spikelets. All samples should be at the same moisture status when weighed.

Spikelet fertility (%) = (number of filled grains/(number of filled grians + number of unfilled spikelets)) × 100.

where the number of filled grains is determined from the weight of filled grains in the sub sample/the mean filled grain weight and the mean filled grain weight is determined by the weight of the 200 grains sample/200.

And where the number of unfilled spikelets is determined in a similar manner to that of the filled grain.

If there are large differences in spikelet fertility among lines in an experiment, this character can be scored. Some people score in the field, but there is a tendency for scorers to look only at the tallest panicles. Other groups have found that representative panicles can be collected in the field, returned to the laboratory, and then a scorer can individually score the panicles representing each plot. The selection of panicles to harvest is critical. The sample will be more representative if all panicles from a hill are harvested.

The problem with measuring spikelet fertility is that it requires a lot of labor and, because of the many measurements required, it is prone to error. To avoid this problem, some researchers have made visual scores of percentage spikelet fertility. These scores can be used to group lines into classes of high, medium, and low fertility. Experienced scorers recommend that scoring be done on a sample of representative panicles, scoring each panicle individually, rather than trying to assign an overall plot score.

Another substitute for direct measurements of spikelet fertility is the change in the panicle harvest index (PNHI) with stress, where PNHI = grain weight/weight of panicle.

If stress has mostly affected spikelet fertility, the support structure of panicles from stress plots is similar to that of control plots, but only a proportion of the spikelets from stress plots form grains. This means that the PNHI will be lower in the stress plots. The correlation between percent fertility and PNHI is quite high for rice that experiences drought near flowering.

To measure leaf desiccation, make a visual integration of the symptoms in a plot, based on total leaf area lost by desiccation. A common scoring system ranges from zero (no senescence) to five (complete leaf drying). Just as for leaf rolling, it is most helpful for the final analysis if scoring is performed several times during the drought stress cycle. Because leaf desiccation is irreversible, time of day is not critical for scoring. Furthermore, since the canopy may regain turgor during the night, the morning is a good time to distinguish those parts of the canopy that are indeed desiccated and dead.

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Phenotyping for drought adaptation in wheat using physiological traits

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Wheat (*Triticum* spp) is one of the first domesticated food crops. It represents the first source of calories (after rice) and an important source of proteins in developing countries. As a result of the Green Revolution, wheat yield sharply increased due to the use of improved varieties, irrigation, pesticides, and fertilizers. The rate of increase in world wheat production, however, slowed after 1980, except in China, India, and Pakistan. Being adapted to a wide range of moisture conditions, wheat is grown on more land area worldwide than any other crop, including in drought prone areas. In these marginal rain-fed environments where at least 60 m ha of wheat is grown, amount and distribution of rainfall are the predominant factors influencing yield variability. Intensive work has been carried out in the area of drought adaptation over the last decades. Breeding strategies for drought tolerance improvement include: definition of the target environment, choice and characterization of the testing environment, water stress management and characterization, and use of phenotyping traits with high heritability. The use of integrative traits, facilitated by the development and application of new technologies (thermal imaging, spectral reflectance, stable isotopes) is facilitating high throughput phenotyping and indirect selection, consequently favoring yield improvement in drought prone environments.

Keywords: genetic resources, phenotyping traits, wheat, indirect selection, drought tolerance

GENERAL INFORMATION

IMPORTANCE OF WHEAT IN THE HUMAN DIET

Wheat (*Triticum* spp) is one of the first domesticated food crops and, for 8000 years, has been the basic staple food of major civilizations of Europe, West Asia, and North Africa. Today, wheat continues to be an important food grain source for humans, and is a close third to rice and corn in total world production. Approximately two-thirds of the wheat produced is used for human food and about one-sixth for livestock feed. Industrial uses, seed requirements, and post-harvest losses account for the remainder. Wheat is used to produce a large variety of foods including many kinds of bread, cakes, noodles, crackers, breakfast foods, biscuits, and confectionary items. The protein content of wheat is in the range 12–16 percent and lipid content 1.5–2.0 percent.

World wheat production increased at a rate of 3.3 percent per year between 1949 and 1978. Increases at the start of this period were due to both an expansion of production area and increased yields. However, starting in the 1960s, yield increases came mainly from the use of improved varieties and a greatly expanded use of irrigation, pesticides, and fertilizers. The rate of increase in world wheat production slowed to 1.5 percent per year between 1982 and 1991, one exception being China, which maintained a rate of increase in production of 2.6 percent per year and became the world's largest wheat producer. Also, wheat production increased at nearly 3 percent per year in India and Pakistan during the same period.

Today, world wheat production is 626 million tons [Food and Agriculture Organization of the United Nations (FAO), 2007]. World leaders in order of production (all of the following figures are in million tons) are China (109), India (75.8), United States of America (55.8), Russian Federation (49.4), and France (32.8). Wheat is the dominant grain of world commerce. The five top exporters are USA (32.9), Canada (17.6), the European Community (16.5), Australia (14.7), and Argentina (9.6). The five top importers in 2007 were Brazil (6.6), Italy (6.2), Egypt (5.9), Japan (5.3), and Algeria (5.8).

HISTORY OF THE CROP, CULTIVATED AREA, AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

Wheat is believed to have been domesticated in southwestern Asia (Gupta, 2004). Some of the earliest remains of the crop have been found in Syria, Jordan, and Turkey (Pasternak, 1998). Primitive relatives of present-day wheat have been discovered in some of the oldest excavations of the world in eastern Iraq, dating back 9000 years. Bread wheat is known to have been grown in the Nile valley from 5000 BC, and was later cultivated in other regions (e.g., the Indus and Euphrates valleys from 4000 BC, China from 2500 BC, and Europe from 2000 BC). It was introduced into the American continent around 1520.

Today, bread or common wheat (*T. aestivum* L) represents more than 90 percent of total wheat production. Several classes of bread wheat can be distinguished (hard red spring, hard red winter, soft red winter, hard white, and soft white) according to grain characteristics. Durum wheat or macaroni wheat (*T turgidum* subsp *durum* (Desf) Husnot) represents around 5 percent of global wheat production, i.e., 30 million tons. Durum wheat grain is hard, translucent, and large. The protein content can be as high as 18 percent. When durum is milled, the endosperm is ground into a granular product called "semolina," used for premium pastas and breads. Due to its high level of tolerance to terminal drought, most durum wheat is grown in Mediterranean environments. The remaining part of the wheat growing area is distributed among the diploid species einkorn, the tetraploid species emmer, poulard, polish and timopheevi, and the hexaploid species spelt, club, compact and macha (**Table 1**).

Being adapted to a wide range of moisture conditions from xerophytic to littoral, wheat is grown on more land area worldwide than any other crop. About three-quarters of the land area where wheat is grown receives between 375 and 875 mm of annual precipitation, but wheat can be grown in locations where precipitation ranges from 250 to 1750 mm. The optimum growing temperature is about 25°C, with minimum and maximum growth temperatures of 3–4°C and 30–32°C, respectively (Briggle and Curtis, 1987). Classification into spring or winter wheat traditionally refers to the season during which the crop is grown. For winter wheat, heading is delayed until the plant experiences a period of cold winter temperatures (0–5°C). It is planted in the autumn to germinate and develop into young plants that remain in the vegetative phase during the winter, and resume growth in early spring. This provides the advantage of using autumn moisture for germination and making effective use of early spring sunshine, warmth, and rainfall. Spring wheat, as the name implies, is usually planted in the spring and matures in late summer, but can be sown in autumn in countries that experience mild winters, such as in South Asia, North Africa, and the Middle East.

During the past 50 years, most of the yield progress in wheat has been due to the gradual replacement of traditional tall cultivars by dwarf and fertilizer-responsive varieties (Donmez et al., 2001; Brancourt-Hulmel et al., 2003; Jiang et al., 2003). Reducing height increased the proportion of carbon partitioned to grain and increased the harvest index (HI). It simultaneously reduced the risk of yield penalties caused by lodging. Most experiments analysing the effects of genetic improvement on yield found that, while selecting for higher-yielding cultivars, wheat breeders have consistently increased the number of grains per unit land area (Calderini et al., 1999). This is a consequence of a higher survival of floret primordia, the number of potential florets per spike remaining similar (Slafer and Andrade, 1993). It is likely to be due

Table 1 | Cultivated species (C) within the *Triticum* genus, and their wild relatives (W) (Source: van Slageren, 1994).

Sections and species	Common name		Regions of cultivation
Section *Monococca* Flaksb			
Triticum monococcum L			
Subsp. *monococcum*	Einkorn	C	Mountainous areas (France, Morocco, the former Yugoslavia, Turkey)
Subsp. *aegilopoides* (Link) Thell		W	
Triticum urartu Tumanian ex Gandilyan		W	
Section *Dicoccoidea* Flaksb			
Triticum turgidum			
Subsp. *turgidum*	Poulard	C	Mediterranean countries
Subsp. *carthlicum* (Nevski in Kom) Á Löve & D Löve		C	
Subsp. *dicoccum* (Schrank ex Schübler) Thell	Emmer	C	Yemen, India, Morocco, Spain, Albania, Turkey, Italy
Subsp. *durum* (Desf) Husnot	Durum	C	
Subsp. *paleocolchicum* (Menabde) Á Löve & D Löve		W	
Subsp. *polonicum* (L) Thell	Polish	C	Mediterranean countries
Subsp. *turanicum* (Jakubz) Á Löve & D Löve		W	
Subsp. *dicoccoides* (Körn ex Asch & Graebner) Thell		W	
Triticum timopheevii (Zhuk) Zhuk			
Subsp. *timopheevii*	Timopheevi	C	Georgia
Subsp. *armeniacum* (Jakubz) MacKey		W	
Section *Triticum*			
Triticum aestivum L			
Subsp. *aestivum*	Bread	C	
Subsp. *compactum* (Host) MacKey	Compact	C	Alpine countries and Southern Europe
Subsp. *macha* (Dekapr & Menabde) MacKey		C	Caucasus area
Subsp. *spelta* (L) Thell	Spelt	C	Northern and Central Europe
Subsp. *sphaerococcum* (Percival) MacKey	Club	C	India
Triticum zhukovskyi Menabde & Ericzjan			

to pleiotropic effects on spike fertility of the two most commercially important gibberellic acid (GA)-insensitive dwarfing genes *Rht-B1b* and *Rht-D1b* (Flintham et al., 1997). Although there was an increase in grain number per m^2, there was no reduction of grain weight, probably because photosynthetic capacity during grain filling together with pre-anthesis assimilate reserves exceed the demands of the growing wheat grains during post-anthesis (Borras et al., 2004).

Since the HI in most modern cultivars seems to be close to its biological maximum, i.e., 60 percent, further genetic gain in yield potential is expected to come from biomass increases (Shearman et al., 2005). Such increases have started to be reported in spring wheat (Reynolds et al., 1999) and winter bread wheat (Shearman et al., 2005). A biomass increase of about 10 percent has been reported in spring wheat, associated with the introduction of the long arm of chromosome 7D from the wheat wild relative *Lophopyrum elongatum* (Reynolds et al., 2001a; Monneveux et al., 2003). CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo; the International Maize and Wheat Improvement Center) is exploring several approaches to exploit the excess photosynthetic capacity, like the multi-ovary characteristic which causes a single floret to set up to four kernels instead of just the usual one (Reynolds et al., 2005).

This requires, however, a good knowledge of the genetic and genomic resources available.

GENETIC AND GENOMIC RESOURCES

Genetic resources

Wheat belongs to the *Triticum* genus, family *Poaceae* Barnhart, subfamily *Pooideae*, tribe *Triticeae* Dumort, subtribe *Triticinae* Griseb (van Slageren, 1994). Kihara (1919) and Sax (1922) showed that, in the genus *Triticum*, there are three different genomes, each composed of seven chromosomes. Both genera *Aegilops* and *Triticum* (6 species and 17 subspecies) belong to a complex of wild and domesticated species of which the allopolyploid members evolved via hybrid speciation (Kimber and Sears, 1987), which have the same base chromosome number ($n = 7$), and can be divided into three ploidy levels (i.e., diploid $2n = 14$, tetraploid $2n = 28$, and hexaploid $2n = 42$). Consequently, the available genepool of wheat is exceptionally wide (Zaharieva and Monneveux, 2006). As defined by Von Bothmer et al. (1992), the primary genepool consists of the cultivated and wild forms of a crop species. Gene transfer in the primary genepool is considered to be easy. The secondary genepool includes coenospecies from which gene transfer is possible but difficult, while the tertiary gene pool is composed of species from which gene transfer is very difficult.

Genetic resources have been categorized by Frankel (1975) as modern cultivars in current use, obsolete cultivars (i.e., the elite cultivars of the past, often found in the pedigrees of modern cultivars), landraces, wild relatives of the crop, genetic and cytogenetic stocks, and breeding lines. Today, CIMMYT's wheat germplasm bank holds more than 114,000 accessions, including parental and advanced breeding lines, cultivars and landraces, and more than 13,000 wild relatives from various regions of the world. Wheat genetic stocks involving translocation and substitution lines and produced by different institutions have been collected through a project supported by the Generation Challenge Programme (GCP), and are stored at CIMMYT.

Genomic resources

To address the need for general access to genetic maps, the International Triticeae Mapping Initiative (ITMI) was launched in 1989, to ensure that such maps would be available as a public good (Gustafson et al., 2004). Mapping using restriction fragment length polymorphisms (RFLPs) was conducted by scientists in several countries (McGuire and Qualset, 1997). As it became clear that resources for functional genomics, such as expressed sequence tags (ESTs), were the next critical need, ITMI created the International Triticeae EST Cooperative (ITEC). ITEC produced some 24,000 ESTs and mapped unigenes to chromosome bins defined by a set of deletion stocks. Then, a project on "The structure and function of the expressed portion of the wheat genomes" known as "wEST," funded by the USA's National Science Foundation (NSF) Plant Genome Research Program along with support from the collaborating institutions, developed several activities such as complementary DNA (cDNA) library development, EST production, deletion stock characterization (Qi et al., 2003) and mapping, and coordination of individual chromosome maps. Project investigators and members of the international scientific community are free to use the ESTs for gene discovery and utilization. The ESTs are available from the Wheat Genomics Resource Repository—a collaboration between the United States Department of Agriculture (USDA) and the University of California, Davis, USA. It is now feasible to envisage the development of single nucleotide polymorphism (SNP) markers in wheat, due to the explosion in the availability of ESTs. The hexaploid nature of the wheat genome makes such analysis more complex than it would be in species with simple genomes. As an open international consortium of institutions (public and private), ITMI is now attempting to mine the contigs in a coordinated way, pooling information on validated SNPs and avoiding duplication of effort. Information on progress in this project can be found at: http://wheat.pw.usda.gov/ITMI/WheatSNP/

RATIONALE FOR DEVELOPING DROUGHT RESEARCH IN WHEAT

There has been a significant increase in the productivity of wheat due to the application of Green Revolution technology. This has resulted in a doubling and tripling of wheat production in many environments, most notably in irrigated areas. In these locations, the high-yielding semi-dwarf statured wheat cultivars continuously replaced the older tall types at a rate of 2 m ha $year^{-1}$ in the 1980s (Byerlee and Moya, 1993). There is, however, a growing recognition that dissemination, application, and adoption of this technology have been slower in marginal environments, especially those affected by drought. Over the last three decades, many investigators have attempted to produce wheat cultivars adapted to these semi-arid environments with limited success in earlier years.

To examine the challenges facing wheat breeders more closely, Singh and Byerlee (1990) analysed wheat yield variability in 57 countries over 35 years. Yield variability was measured by calculating coefficients of variation of yield around linear climatic

trends. The amount and distribution of rainfall was the predominant factor influencing yield variability. Countries in which half the wheat was sown in dryland conditions experienced twice as much variability as countries in which wheat was mostly grown under well-watered conditions. At least 60 m ha of wheat is grown in marginal rain-fed environments in developing countries. National average yields in these regions range from 0.8 to 1.5 t ha^{-1}, which represents approximately 10–50 percent of their theoretical irrigated potential (Morris et al., 1991).

In recent years, breeders have been more successful in increasing the adaptation of wheat to dry environments. In developing countries, farmers have traditionally grown landrace cultivars that are well adapted to serious moisture stress conditions. However, these traditional cultivars generally give a poor yield in "good years" when rainfall is more plentiful. Modern cultivars now yield the same as the traditional cultivars in dry years as well as showing a better response to more favorable conditions of moisture and nutrient supply (Osmanzai et al., 1987). Due to their improved yield stability, these modern cultivars are increasingly grown in dry regions, with rates of adoption approaching those in irrigated and high rainfall areas.

Further progress in developing drought tolerant germplasm depends on the efficiency of breeding and phenotyping methodologies. Accurate drought phenotyping implies precise definition of the target environment, choice and characterization of the testing environment, and water stress management and characterization.

METHODOLOGY

BREEDING STRATEGY

Breeding work for drought-prone environments has been largely empirical to date, with grain yield being the primary trait for selection in wheat breeding programmes. However, most breeders select strongly for traits other than yield in the early segregating generations and do yield testing only at later stages, when a certain level of homozygosity has been achieved and large enough seed quantities are available. The decision to advance or reject a genotype is often complex and, in practical terms, breeders most often use a system of multiple cut-offs. In early generations, they select genotypes that, presumably, achieve the levels required for the primary traits evaluated in segregating populations (plant type, plant height, growth cycle, spike fertility, etc.).

When a breeding programme for drought adaptation is assisted by analytical selection, the conceptual model used considers yield under drought to be a function of: (1) yield potential; (2) flowering date (which indicates whether the crop will avoid drought stress); and (3) secondary traits that provide drought resistance. Physiological secondary traits can be used for the selection of parents to be included in the crossing block, as direct selection criteria for screening among a large number of genotypes (i.e., segregating populations) and/or when the amount of seed available is too small to carry out field trials with replications. Whereas intensive work is continuously being carried out by physiologists in the area of drought adaptation, few breeders routinely use physiological criteria in their mainstream breeding programmes. In the first place, the evaluation of some of the traits proposed by plant physiologists is time-consuming or expensive. This is not practical for application to the thousands of entries that comprise the segregating generations of breeding programmes. Then, the real value of a given trait may only be assessed by determining the genetic gain in segregating populations following selection, while many traits are not available in well adapted genotypes and their validation frequently requires the development of appropriate breeding material, which is again costly and time-consuming (Royo et al., 2005). Finally, selection in segregating populations requires screening at the plant level or between very small plots, thus hindering the use of traits that require large field plots for their assessment.

Gene-based markers generated from gene sequence data, i.e., "perfect markers" can be used to screen large numbers of entries for a particular trait improving the efficiency and effectiveness of conventional breeding. Gene-based markers are particularly useful for introgressing genes whose expression is highly affected by the environment, such as genes for useful physiological traits that cannot easily be screened (e.g., root architecture traits), as well as for gene pyramiding. The most common situations in which marker-assisted selection (MAS) confers an advantage are: (1) when accurate measurement of the phenotype is expensive or difficult; (2) when multiple genes conferring a similar phenotype are being combined; and (3) when there is a need for rapid removal of donor chromosome segments in a backcrossing programme (Nelson et al., 2004). Most important traits (yield, stress adaptation, etc.) are governed by multiple genes, each producing a relatively small individual effect. MAS for these "quantitative traits" is challenging because many quantitative trait loci (QTLs) identified in mapping population studies are cross-specific, subject to genotype-by-environment interaction (GEI) effects.

QTL estimation often spans several centimorgans, and hundreds of genes underlie a region of this size. The size of such a region can be reduced through a number of approaches, such as the use of high resolution crosses, or the development of near-isogenic lines (NILs) for small chromosomal segments across the putative QTL region (Nelson et al., 2004). Linkage disequilibrium (LD) mapping offers another alternative, exploiting the long history of recombination, and rich allelic diversity in germplasm collections (Remington et al., 2001; Buckler and Thornsberry, 2002). Genome sequencing for various crops would improve the quality of molecular markers used for MAS by helping breeders to target the gene of interest, rather than a nearby sequence (Dubcovsky, 2004). Continuing efforts to sequence expressed genes will provide data for SNP markers for individual alleles, making MAS more cost-efficient (Dubcovsky, 2004).

For MAS to be useful, proper phenotyping is required and the evaluation of yield and relevant physiological traits should be done in conditions similar those of the target environment. An ecophysiological understanding of the traits in question and of how to measure them is crucial (Araus et al., 2003a,b; Slafer, 2003).

TRIAL PLANNING

Definition of the target environment

Rainfall distribution patterns and evaporative demand over the crop cycle vary considerably among locations and years.

The different sets of climatic conditions under which wheat is cultivated are characterized by breeders as "wheat mega-environments" (wheat MEs). ME delineation is based on water availability, soil type, temperature regime, production system, and associated biotic and abiotic stresses. Consumer preferences for grain color and industrial and end-use quality are also considered. CIMMYT has defined 12 MEs (**Table 2**): six focus on spring wheat production areas, three on facultative wheat areas, and three on true winter wheat areas (Rajaram et al., 1995). According to the ME classification, drought environments mainly correspond to ME4. Within ME4, three distinct patterns can be distinguished: post-anthesis water stress (ME4A); pre-anthesis water stress (ME4B); and residual moisture stress (ME4C). In the first scenario, ME4A, evapotranspiration exceeds average precipitation after anthesis, causing an increasing water deficit over the grain-filling period. Conversely, in ME4B, water deficit occurs mainly before anthesis. In ME4C, there is no significant rainfall, and evaporation is always in excess of precipitation during the growing season. Consequently, the crop must survive using the water stored in the soil profile from the summer rainfall. Wheat can also face drought situations in other MEs, such as ME6B, ME9, and ME12. In all drought situations, wheat may also experience additional stresses such as heat and cold stress, soil micro-element deficiency, or toxicity, and a range of biotic stresses. For example, late frosts frequently occur in ME4A, while high temperature stress occurs in ME5A. In ME4B, resistances to leaf and stem rust, *Septoria* spp and *Fusarium* spp, and pre-harvest sprouting are highly necessary.

Choice and characterization of the testing environment

The choice of the selection environment directly determines the potential genetic gains in the target environment. Ideally, the selection environment should mimic the target environment in all aspects: water distribution, profiles and potential evapotranspiration rates, and physical and chemical soil properties. Deviations may result in significant GEI between target and selection environments, and genetic gains achieved in the selection environment may not be expressed in the target environment. Geographic information system (GIS) tools can help considerably in describing the relationships between target and selection environments and establishing "homology maps."

The crop facing water deficit simultaneously experiences a number of additional stress factors (e.g., micronutrient deficiency, soil compaction, salinity, nematodes, and fungal pathogens) that exacerbate drought stress. Such factors are hard to control and are generally not considered in field experiments. Hence, efforts should be made to remove all other constraints except drought. Soil surveys may allow the identification of selection sites or fields that avoid confounding factors. In some cases, these surveys may enable sites to be chosen where the selection pressure for these stress factors would permit the selection of genotypes targeted for regions where these stresses interact with drought. They could also identify the within-site distribution of e.g., nematodes (Nicol and Ortiz-Monasterio, 2004) or zinc deficiency (Ekiz et al., 1998). Field trials are conducted on land that may be quite variable in terms of topography, soil fertility, and soil structure. Spatial variability in the field affects the detection of treatment differences in agricultural experiments by inflating the estimated experimental error variance. In order to account for such variation and to reduce experimental error, adapted trial designs must be applied, like the augmented designs proposed by Federer (2005).

WATER STRESS MANAGEMENT AND CHARACTERIZATION

Target environments can also be mimicked if water is controlled by imposing a water regime by gravity or, better, by drip irrigation. Water stress management (timing, intensity, uniformity) and characterization (soil, plant measurements) are essential issues in drought phenotyping.

Moisture availability can itself be a complicating factor when comparing genotypes in field experiments. Although plots growing the different genotypes may receive the same quantity of water, the genotypes can vary in their water use and/or access to underground water, thereby confounding measurements associated with plant water relations. Study of water profiles (either experimentally or by using simulation models) can provide very

Table 2 | The main wheat mega-environments (Source: Rajaram et al., 1995).

ME[a]	Moisture regime	Temperature	Wheat type	Area (%)	Production (10^6 tons)
ME1 IR	IR	Temperate	Spring	36.1	83
ME2 HR	HR (>500 mm)	Temperate	Spring	8.5	25
ME3 AS	HR (>500 mm); AS	Temperate	Spring	1.9	3
ME4 SA	LR (<500 mm)	Temperate/hot	Spring	14.6	20
ME5 TE	IR, HR	Hot	Spring	7.1	12
ME6 HL	SA	Temperate	Spring	6.2	13
ME7 IR	IR	Cool	Facultative	–	–
ME8 HR	HR	Cool	Facultative	10.0	23
ME9 SA	SA	Cool	Facultative	–	–
ME10 IR	IR	Cold	Winter	–	–
ME11 HR	HR	Cold	Winter	15.0	30
ME12 SA	SA	Cold	Winter	–	–

[a] *ME, Mega-environment; where: IR, irrigated; HR, high rainfall; AS, acid soil; SA, semi-arid; TE, tropical environment; HL, high latitude.*

useful information. Trait evaluation should preferably be carried out under field conditions, avoiding experimental situations (growth chambers, greenhouses, pots) that differ significantly from the agricultural growing environment. The ability to access water deep in the soil profile, which is an important drought-adaptive mechanism, is eliminated as a variable in pot conditions. Furthermore, the relative humidity of the air, which has an important influence on stomatal conductance (Ben Haj Salah and Tardieu, 1997), is extremely difficult to simulate in controlled environments.

When possible, drought tolerance evaluation should be done out-of-season, under irrigated conditions. This option allows better management of water stress but needs a dry season sufficiently long to cover the whole growth cycle. The photoperiod and temperature should not differ too much from the growing season, as is the case in the dry tropics, to avoid genotype-by-season interactions and allow results obtained from the out-of-season experiments to be extrapolated to the growing season conditions.

PLANT WATER STRATEGY

Survival and drought escape

In the case of drought, some traits proposed by stress physiologists appear to be associated with crop survival. For example, comparison of old and new varieties has shown that, under drought, older varieties over-produce tillers many of which fail to set grain, while modern drought tolerant lines produce fewer tillers the majority of which survive (Loss and Siddique, 1994). In most circumstances, however, the main effect of drought is to reduce grain yield without killing the plant.

If the pattern of water deficit is predictable in a given region, selection for a flowering date that does not coincide with the period of water deficit is a very effective way of improving drought adaptation (Araus et al., 2002). The limitations of this approach are that very early varieties may suffer yield penalties in good seasons, while late-in-season freezing episodes may affect spike fertility. In such cases, breeding for higher yield potential plus traits conferring stress avoidance (i.e., to avoid cell dehydration) may generally be effective (Araus et al., 2003a,b).

PHENOTYPING TRAITS

Requirements

Most of the traits currently mentioned in the literature associated with drought adaptation in wheat are shown in **Table 3**. However, the potential value of each trait needs to be considered with respect to the type of drought environment in which a cultivar is to be adapted. Secondary traits may be particularly suited to improving the selection response for stress conditions if they avoid any confounding effects of stress timing on yield (e.g., drought and flowering dates), and allow the selection to be focused on a specific type of drought. For a secondary trait to be useful in a breeding programme, it has also to comply with several requirements (Edmeades et al., 1997). Thus, a secondary trait should ideally be: (1) genetically associated with grain yield under drought; (2) genetically variable; (3) highly heritable; (4) easy, inexpensive and fast to observe or measure; (5) non-destructive; (6) stable over the measurement period; and (7) not associated with yield loss under unstressed conditions.

Wheat faces different drought scenarios worldwide; consequently, the physiological traits that confer drought resistance in specific environments may be very distinct. The combination of yield data with data relating to secondary traits in multi-site field experiments ranging from well-watered to high stress levels may be useful at this stage by providing some light on GEI of traits related to drought tolerance. This is particularly the case when the heritability of the secondary traits is higher than that of yield, and the genetic correlation of these traits with yield in the target environment is high. Secondary traits can be classified according to their relationship to pre-anthesis growth, access to water, water-use efficiency (WUE), and photoprotection.

Traits related to pre-anthesis growth

Crop establishment. Vigorous crop establishment is agronomically desirable because it helps to shade the soil and suppress weeds that compete for water. It also improves radiation interception by the crop at the early stages of growth. Rapid ground cover can be achieved by breeding for: (1) large seed and embryo size which may help to achieve early vigor (Aparicio et al., 2002); and (2) thinner, wider leaves (i.e., with a relatively low specific leaf weight) and a more prostrate growth habit which help to increase ground cover, thus conserving soil moisture and potentially increasing radiation-use efficiency (RUE; Richards, 1996). These traits are especially important in Mediterranean types of drought environment (ME4A), where up to 40 percent of available water may be lost by evaporation directly from the soil (Loss and Siddique, 1994). In ME4A, the potential for vigorous growth prior to heading also provides the opportunity to take advantage of relatively good growing temperatures and moisture availability earlier in the cycle.

Total biomass. Evaluation of total biomass is only feasible in practice through indirect methods, e.g., using spectroradiometers to measure the spectra of light reflected by the canopy (Aparicio et al., 2000, 2002; Royo et al., 2003). Field spectroradiometers able to measure the spectrum of light reflected by the canopy have been expensive in the past. However, the situation is now changing with the availability of simple, less expensive, and easy-to-handle spectroradiometers such as the GreenSeeker[1]. Designed initially for nitrogen management, this has become a potentially very useful instrument in breeding. It gives the basic spectroradiometric indices of green biomass, such as the normalized difference vegetation index (NDVI), which is the most useful for routine breeding purposes. Moreover, as the GreenSeeker includes its own radiation source, it may be used independently of atmospheric conditions and deployed on both sunny and cloudy days. Alternative techniques such as the use of an affordable conventional digital camera may provide complementary information, such as the portion of the soil occupied by green biomass (Casadesús et al., 2005). Digital pictures may also provide information that is not currently acquired through spectral reflectance measurements, such as the degree of soil covered by the crop, the percentage of yellow leaves, or even yield components such as the number of spikes per unit land area (Casadesús et al., 2005).

[1] http://www.ntechindustries.com/

Table 3 | Main secondary traits that can be used to improve drought tolerance in wheat, associated mechanisms, references, ease of use, and target mega-environment of application (Adapted from Reynolds et al., 2001b).

Secondary trait	Associated with	Methodology (References)	Ease of use	Target environment
Large seed size	Emergence, early ground cover, and initial biomass	Mian and Nafziger, 1994	+++	ME4A
Long coleoptiles	Emergence from deep sowing	Radford, 1987	+++	ME4C
Early ground cover (visual)	Decrease of evaporation and increase of radiation-use efficiency (RUE)	Hafid et al., 1998; Richards, 1996	+++	ME4A
Specific leaf dry weight	Thinner, wider leaves, early ground cover	Merah et al., 2001a	++	ME4A
Growth habit (visual)	Lower soil evaporation and higher RUE	Richards et al., 2002	+++	ME4A
Tiller survival	Survival and recovery	Loss and Siddique, 1994	++	Severe stress
Long and thick stem internodes	Storage of carbon products	Loss and Siddique, 1994	+++	ME4A
Vegetation indices (normalized difference vegetation index; NDVI)	Green biomass	Royo et al., 2003	+	
Earliness	Drought escape	Blum, 1988; Monneveux et al., 2005	+++	ME4A and ME4C
Number of grain per spike around	Spike sterility	Hafsi et al., 2006	++	Drought flowering
Stomatal conductance	Extraction of water from soil	Farquhar and Sharkey, 1982	+	
Canopy temperature depression	Stomatal conductance, extraction of water from soil	Reynolds et al., 2000	++	
Carbon isotope discrimination	Stomatal conductance, extraction of water from soil	Monneveux et al., 2005	++	
Ash content	Stomatal conductance, extraction of water from soil	Misra et al., 2006	++	
Spike photosynthetic capacity	Grain filling	Evans et al., 1972	+	ME4A, hot
Leaf color (visual, SPAD)	Delayed senescence, maintenance of photosynthesis	Araus et al., 1997	+++	
Leaf waxiness	Lower transpiration rate and reduced photo-inhibition	Richards, 1996	+++	Severe stress
Leaf pubescence	Lower transpiration rate and reduced photo-inhibition	Richards, 1996	+++	Severe stress
Leaf thickness and posture	Lower transpiration rate and reduced photo-inhibition	Reynolds et al., 2000	+++	Severe stress
Leaf rolling	Lower transpiration rate and reduced photo-inhibition	Reynolds et al., 2001b	+++	Severe stress
Glume pubescence	Lower transpiration rate and reduced photo-inhibition	Trethowan et al., 1998	+++	
Delayed senescence	Higher RUE	Hafsi et al., 2006	++	
Fructanes in stem	Storage of carbon products	Rawson and Evans, 1971	++	ME4A
Solute concentration in cells	Osmotic adjustment (OA)	Morgan and Condon, 1986	+	
Accumulation of ABA	Reduced stomatal conductance and cell division	Innes et al., 1984	+	Severe stress

Remobilization of stored assimilates. Stored assimilates can be remobilized during grain filling to supplement assimilates generated in the drier post-anthesis period. Stored fructans contribute substantially to grain filling, especially when canopy photosynthesis is inhibited by post-anthesis drought (Blum, 1998). Traits that may also contribute to remobilization during grain filling in these conditions include long and thick stem internodes, perhaps with extra storage tissue in the form of solid stems. In studies where crosses were made between lines contrasting in the solid-stem trait, the solid-stem progeny contained more soluble carbohydrate per unit of stem length, although total stem carbohydrate was unaffected due to the stems being narrower and shorter (Ford et al., 1979). Conversely, where the crop grows exclusively on stored soil moisture, long coleoptiles are desirable to avoid extremely hot soil surface temperatures and rapid soil drying. Moreover, longer coleoptiles improve seedling emergence with deep sowing (Radford, 1987), improving early biomass accumulation (Rebetzke et al.,

2005). Long coleoptiles are also useful in dryland Mediterranean environments, helping to avoid a false start in early planted crops.

Traits relating to access to water

Root characteristics. A root system that can extract whatever water is available in the soil profile is clearly drought-adaptive (Hurd, 1968), but this ability is difficult to measure directly. Traits that are indicative of the water status of a plant, especially when measured during periods of peak stress, are useful indicators of the plant's capacity to match evaporative demand by exploring and extracting soil water. Instantaneous measurement of traits affected by the water relations of the plant, such as stomatal conductance (g_s) and canopy temperature depression (CTD) can give indications of water extraction patterns. The role of abscisic acid (ABA) accumulation in stomatal regulation under drought has been demonstrated (Innes et al., 1984). It also appears to preadapt plants to stress by reducing rates of cell division, reducing organ size, and increasing the rate of development. However, high ABA can also result in sterility problems since high ABA levels may abort developing florets.

Canopy temperature depression. Among the traits relating to access to water, by far the easiest to measure in the field is CTD, which shows good correlations with other water relations parameters (Blum et al., 1982), as well as with performance under drought of random sister lines (Reynolds et al., 2000). Canopy temperature can provide information on transpiration as the main contributor to reduced leaf temperature. Although canopy temperature may seem very easy to measure, in practice there are methodological problems, particularly in Mediterranean drought environments. This is mainly found when there is variation in the air temperature with wind or cloudiness (Araus et al., 2002; Royo et al., 2002), or where there is not a homogeneous canopy. In fact, screening by canopy temperature measurements under drought stress can be done only during the vegetative growth stage after full ground cover has been attained, before inflorescence emergence, at high vapour-pressure deficits in recently irrigated crops, and without the presence of wind or clouds (Royo et al., 2005).

So far, studies have only been accomplished in recombinant inbred lines (RILs). CTD showed a significant association with yield under drought when measured pre-anthesis, suggesting an advantage from higher pre-anthesis growth rates. CTD also showed some association with final yield when measured during grain filling. Because a major role of transpiration is leaf cooling, canopy temperature, and its reduction relative to ambient air temperature are an indication of how much transpiration cools the leaves under a demanding environmental load. Higher transpiration means colder leaves and higher stomatal conductance, both aspects favoring net photosynthesis and crop duration. A relatively lower canopy temperature in drought-stressed crops indicates a relatively greater capacity for taking up soil moisture or for maintaining a better plant water status. Thus, higher transpiration is a positive trait when selecting for higher yield potential or better adaptation to moderate drought stress.

Osmotic adjustment. Solute concentration in the cell is intimately tied to plant water status and, under drought, osmotic adjustment (OA) may facilitate critical growth functions such as root growth, and also meiosis and pollen development, thereby mitigating some of the most detrimental effects of plant water deficit. Genetic variation in OA is well-established in wheat (Rekika et al., 1998). A number of experiments have shown that wheat lines selected for high OA in response to the lowering of leaf water potential have higher grain yields in field experiments. In a study by Morgan and Condon (1986), high OA was strongly associated with greater soil water extraction. Nevertheless, the role of OA on yield still remains controversial (Serraj and Sinclair, 2002). It will help to maintain leaf metabolism and root growth at relatively low leaf water potentials by maintaining turgor pressure in cells. However, OA is difficult to measure in large samples under field conditions. Some research suggests that the trait can be assayed relatively easily by measuring the coleoptile growth rate of seedlings in a polyethylene glycol (PEG) solution (Morgan, 1988).

Carbon isotope discrimination. Carbon isotope discrimination ($\Delta^{13}C$), despite being a very promising trait, is probably less widely accepted because of the cost of its determination. In recent years, Commonwealth Scientific and Industrial Research Organisation (CSIRO, Australia) Plant Industry has released the first two commercial wheat varieties selected for high transpiration efficiency using $\Delta^{13}C$ ("Drysdale" in 2002, and "Rees" in 2003). These varieties are cultivated under rain-fed conditions and rely solely upon the precipitation accumulated prior to planting. They have been selected based on their low $\Delta^{13}C$ (and thus high transpiration efficiency), fitting with what has been postulated with regard to this trait. However, for Mediterranean environments, $\Delta^{13}C$ (particularly when measured in mature grains) is frequently positively correlated with grain yield (Araus et al., 1998; Villegas et al., 2000; Merah et al., 2001b; Condon et al., 2004; Monneveux et al., 2005). One of the reasons for this positive relationship is that a genotype exhibiting higher $\Delta^{13}C$ is probably able to maintain a better water status (Condon et al., 2004). Given the relatively high costs associated with carbon isotopic analysis (about €10 per sample), several surrogate approaches which are much cheaper, faster, and easier to handle have been proposed. The option most studied has been to use the mineral or ash content of leaves (Masle et al., 1992; Mayland et al., 1993; Araus et al., 1998; Merah et al., 1999) or grains (Febrero et al., 1994; Voltas et al., 1998; Monneveux et al., 2005; Misra et al., 2006). Another promising alternative relies on the estimation of $\Delta^{13}C$ through the near-infrared spectroscopy (NIRS) technique (Clark et al., 1995; Ferrio et al., 2001), which carries with it the further advantage of being non-destructive.

Traits relating to water-use efficiency

Measurement of carbon isotope discrimination or ash content of grain or other tissues can be used to estimate the WUE of the crop, since their signals are based on the integration of plant water status over a period of time (Condon et al., 1993). However, these data must be interpreted with care. Although most field

studies have shown that better performance of wheat cultivars under Mediterranean drought conditions is associated with lower WUE (Condon et al., 1993), studies in Australia (Rebetzke et al., 2002) indicated an advantage for high WUE genotypes under conditions where crops survive exclusively on stored soil moisture.

Spikes have higher WUE than leaves, and have been shown to contribute up to 40 percent of total carbon fixation under moisture stress (Evans et al., 1972). Awns contribute substantially to spike photosynthesis and longer awns are a possible selection criterion. While gas exchange measurement of spikes is time consuming and difficult to standardize, chlorophyll fluorescence should be considered as a more rapid means of screening for spike photosynthetic capacity under stress (Horton, pers. communication).

Genes that affect a greater relative partitioning of assimilates to the sink, resulting in a higher HI, would be expected to improve yield under drought, not being associated with the water cost of generating additional biomass. Plant height is usually negatively related with HI. However, there is a minimum height below which limitation on yield becomes evident (Slafer et al., 2005). Under extreme drought stress where most of the canopy may have senesced, spike photosynthesis can play a major role in grain filling, because of high WUE of the spike due to the fact than they can refix respiratory carbon (Bort et al., 1996). Moreover, they are able to maintain a better water status than leaves, through a higher OA and a more xeromorphic structure (Tambussi et al., 2005). This stay-green spike trait is currently being introgressed by CIMMYT into elite drought-tolerant backgrounds to see if it can be combined with yield responsiveness, such that the trait is facultative, responding only in drier years. Changes in leaf color can reflect a variation in partitioning of assimilates to the sink. Stress may accelerate the senescence of leaves. The stay-green trait may indicate the presence of drought avoidance mechanisms, but probably does not contribute to yield per se if there is no water left in the soil profile by the end of the cycle to support leaf gas exchange. It may be detrimental if it indicates lack of ability to remobilize stem reserves (Blum, 1998).

To check for delayed senescence of leaves, particularly flag leaves, portable chlorophyll meters such as the Minolta SPAD[2] are extensively used, due to their speed and ease of use. Delayed senescence of leaves has been proposed as a secondary trait for performance under drought by several authors (Araus et al., 1997; Rharrabti et al., 2001). However, the relationship between delayed senescence and yield has been found by other authors to be unstable and highly dependent on drought intensity (Hafsi et al., 2006). In addition, the cost of a portable chlorophyll meter makes this device unaffordable for many breeding programmes in developing countries.

Traits relating to photoprotection

Decreased stomatal conductance in response to drought leads to warmer leaf temperatures and insufficient CO_2 to dissipate incident radiation, both of which increase the accumulation of harmful oxygen radicals and photo-inhibitory damage. Photo-inhibition can be modified by some leaf adaptive traits such as waxiness, pubescence, rolling, thickness, or posture (Richards, 1996). These traits decrease the radiation load to the leaf surface. Benefits include a lower evapotranspiration rate and reduced risk of irreversible photo-inhibition. However, they may also be associated with reduce RUE, which would reduce yield under more favorable conditions.

The effects of photo-inhibition can be alleviated by antioxidants such as superoxide dismutase (SOD) and ascorbate peroxidise, which have been shown to increase in quantity in response to drought stress (Mittler and Zilinskas, 1994). Thermal dissipation through the xanthophyll cycle is another protective mechanism that can dissipate as much as 75 percent of absorbed light energy (Niyogi, 1999). In a study comparing a drought-adapted barley landrace with a modern cultivar, the former displayed two mechanisms of photoprotection: (1) rapid xanthophyll cycling; and (2) up to 50 percent less leaf chlorophyll, resulting in a passive reduction of light absorbance (Havaux and Tardy, 1999).

Application in breeding

While many traits have been studied for their use in breeding for drought resistance, there is a general consensus among breeders that only a few of them can be recommended for use in practical breeding programmes at this time (**Table 3**). For example, CIMMYT (Reynolds et al., 2001a) recommend the use of flowering and maturity dates, spike fertility, changes in green biomass (e.g., leaf death score), and canopy temperature. In practical terms, these traits seem valuable when breeding for higher yield potential and adaptation to some degree of stress. Development of new equipment like spectroradiometers will facilitate future measurement of new physiological traits in the field. Most other traits cannot yet be recommended as part of an ongoing breeding programme, particularly those that are expensive or difficult to measure. However, some such as $\Delta^{13}C$ can be used for the selection of parents (Misra et al., 2006; Xu et al., 2007). Thermal imaging and color imaging techniques are expected to greatly facilitate large scale evaluations in the next future (Cabrera-Bosquet et al., 2012).

CONCLUSIONS

Many drought-adaptive traits have been investigated in wheat. However, association of these traits with genetic gains for yield under drought has been poorly tested and documented. Most difficulties encountered in the identification of accurate drought tolerance traits are due to the fact that wheat is cultivated under very different climatic conditions and faces very different drought scenarios worldwide.

While some single traits have benefited from tremendous research efforts and have generated considerable debate in the literature (e.g., OA, ABA), relatively little emphasis has been placed on research that can be extrapolated and used directly to crop genetic improvement in target environments.

Most drought physiology research in wheat has been conducted in controlled environments and has been poorly

[2]http://www.specmeters.com/Chlorophyll_Meters/Minolta_SPAD_502_Meter.html

integrated into breeding programmes. Multidisciplinary approaches involving physiologist, breeders, genebank managers, and biotechnologists are still scarce, holding back the exploitation of genetic diversity and the use of MAS for drought tolerance improvement.

Despite the tremendous potential offered by access to genetic resources from related species, and well-documented success in using them (e.g., 1B/1R translocation, synthetic wheats), wild *Triticeae* have been poorly exploited until now to improve drought tolerance in wheat.

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Phenotyping maize for adaptation to drought

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The need of a better adaptation of crops to drought is an issue of increasing urgency. However, enhancing the tolerance of maize has, therefore, proved to be somewhat elusive in terms of plant breeding. In that context, proper phenotyping remains as one of the main factors limiting breeding advance. Topics covered by this review include the conceptual framework for identifying secondary traits associated with yield response to drought and how to measure these secondary traits in practice.

Keywords: breeding, drought, maize, phenotyping, yield

GENERAL INFORMATION

CULTIVATED AREA AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

Maize is grown in virtually every country in the world, with a total production in 2002–2003 of 637,444,480 tons on 142,331,335 ha [Food and Agriculture Organization of the United Nations (FAO, 2007)]. This represents an average yield of 3.41 t ha^{-1}, albeit very variable across countries. The United States of America and the People's Republic of China each produced over 100 million tons in 2002–2003, with US production being 2.25 times that of China. During the last decade, these two countries accounted for near 60% of total corn production. Six other countries produced at least 10 million tons during 2002–2003. These were, in order of production: Brazil, Mexico, Argentina, India, France and Indonesia. In 2020, demand for maize in developing countries is expected to exceed 500 million tons, and will surpass the demand for both rice and wheat (Pingali and Heisey, 2001). This projected rapid increase in demand is mainly explained by growth in the demand for maize as livestock feed (for poultry and pigs, particularly in East and Southeast Asia).

Genetic contributions to grain yield improvement in maize, attributable to plant breeding, have been estimated from studies which compare side-by-side the performance of hybrids and open-pollinated cultivars from various eras (Tollenaar and Lee, 2006). Most of the available literature concerns temperate maize and, with some reservations, may be applicable to tropical maize. Maize grain yield in the USA has increased by about 100 kg ha^{-1} $year^{-1}$ or 2% $year^{-1}$ from the start of large-scale adoption of hybrids by maize growers in the late 1930s until the first decade of the twenty-first century. About 75% of the yield improvement has been attributed to genetic gain and the rest to improved agronomical practices. The genetic gain was not associated with an increase in heterosis but rather with more stress tolerance (Duvick, 1999; Tollenaar et al., 2000) related to a higher leaf area per plant and higher harvest index (HI; Tollenaar and Lee, 2006). Two important physiological processes appear to be involved: (1) sustained leaf photosynthesis during grain-filling, which contributes to increases in dry matter accumulation; and (2) an increase in kernel number due to higher partitioning to the kernels during the sensitive period of kernel number determination. As a consequence, genetic gain is not associated with a change in HI because the increase in kernel number and the increase in dry matter accumulation during the grain filling period have been proportional.

The stability in HI rejects an increase in heterosis as being responsible for the genetic gain (Tollenaar and Lee, 2006). The higher dry matter accumulation in newer than in older hybrids during grain filling can be attributed, in part, to a longer duration of the grain-filling period in the former (Tollenaar and Lee, 2006). However, the silking date as well as the relative maturity do not differ between modern and old hybrids (Cavalieri and Smith, 1985), which further refutes changes in heterosis as responsible for genetic gain (Tollenaar et al., 2004). There is evidence that supports higher tolerance to low resource availability in newer maize hybrids; they performed better than older ones under stress, due to parental line involvement (Duvick, 1997) associated with better tolerance to high plant density (Tollenaar and Lee, 2006). In fact, plant water deficit will occur more readily at high rather than at low density, and resistance to high plant density involves resistance to drought stress when moisture becomes limiting (Tollenaar and Wu, 1999).

Anthesis-silking interval (ASI) under drought has become shorter in modern hybrids, and selection has possibly led to an increase in the growth of spikelets and ears and a reduction in final spikelet number (Bänziger et al., 2000). Moreover, "stay-green" or a reduction in the rate of leaf senescence during grain filling has been one of the traits that were the most visually distinctive between older and newer hybrids (Duvick et al., 2004a). Changes in constitutive traits such as plant phenology also seem to be involved in the different response to limiting resources.

Older hybrids suffered a greater yield loss, in part, because they had extracted most of the plant-available water before entering the critical flowering period (Nissanka et al., 1997; Campos et al., 2004). In temperate maize hybrids there has also been a significant reduction in tassel size. From 1967 to 1991, tassel dry weight decreased by 36% (Duvick and Cassman, 1999). However, in tropical maize, the indirect pressure of selection to reduce tassel size by selecting for increased grain production has had relatively modest effects on tassel size. Therefore, tropical inbreds usually still possess a relatively large tassel, which may eventually have a negative effect on the development of ear and silk when the supply of photoassimilates is limited by drought stress (Ribaut et al., 2004; Sawkins et al., 2006).

Retrospective studies also show a large hybrid-by-environment interaction in terms of grain density. The genotype-by-environment interaction (GEI) could be a result of: (1) a greater genetic yield potential of newer hybrids; (2) a greater ability of newer hybrids to tolerate low resource availability; and (3) a greater general stress tolerance in newer hybrids (Tollenaar and Lee, 2006). Increased yield of newer hybrids could be a result of the synergistic effect between increased yield potential and increased resource availability (Duvick and Cassman, 1999). In general, increased yield potential will place a greater demand on all resources, resulting in increased stress frequency unless the greater yield potential is associated with an increase in general stress tolerance. In fact, yield stability and general stress tolerance are highly associated and yield stability does not appear to have declined with increasing yield potential (Tollenaar and Lee, 2002; Duvick et al., 2004b).

GENETIC AND GENOMIC RESOURCES

Hybrids tend to concentrate on a few inbred lines and their derivatives; less than 5% of the world's maize germplasm has been used by US breeders (Taba et al., 2004). In years to come, the ancestral base of US maize hybrids will increase as exotic germplasm is introgressed. Genetic diversity really is available to minimize the risk of a widespread catastrophe. Goodman (1998) has already shown a twofold increase in the use of exotic germplasm in a 12 year period from 1984 to 1996. In addition to having the right technologies, the other pillar of future breeding is to use more of the useful genetic variation that is available. This fact is of concern to all involved with maize germplasm, breeding and production (see Taba et al., 2004 for a comprehensive review). In that context, maize germplasm collections such as that hosted by CIMMYT (Centro Internacional de Mejoramiento de Maiz y Trigo; the International Maize and Wheat Improvement Center), which preserves genetic diversity and makes it fully available to all researchers with no restrictions on use, are the obvious source of genes for breeding efforts to develop tropical and subtropical maize better adapted to drought.

RELEVANT RESULTS PUBLISHED IN THE AREA OF DROUGHT ADAPTATION

In general, average yields in tropical and subtropical regions are far lower than in temperate ones, with sub-Saharan Africa way below other regions with average values across countries of around 1 t ha^{-1}. This is in spite the fact that maize is one of the main crops in these regions, where the effects of climate change including rising temperatures, evapotranspiration losses and, eventually, decreasing rainfall are expected to be particularly negative (World Bank, 2007). The possibilities for alleviation of water stress are limited. The majority of tropical maize is grown under rainfed conditions and poor farmers from these regions are unable to implement crop management strategies that might at least mitigate such constraints. In such a scenario, breeding for drought adapted maize remains the best alternative.

However, advances in breeding are frequently hindered by methodological bottlenecks. Among these, proper phenotyping is perhaps one of the most obvious today. This was not so evident few years ago, when phenotyping was considered as something already achieved, whereas emphasis was placed on other more fashionable breeding approaches such the adoption of molecular marker-assisted selection (MAS), genetic modification and the different "omics." Fortunately, the situation seems to have changed and awareness is now increasing that new genetic and genomic tools will enhance but not substitute for the conventional breeding evaluation process (Varshney et al., 2005), and that only through an integrate use of different disciplines (including proper phenotyping) will breeding be speeded up. In that context, identification of key physiological processes associated with yield improvement and the determination of gene-to-phenotype associations can potentially increase the efficiency of breeding, whether through traditional or molecular methods (Araus et al., 2003, 2008; Tollenaar and Lee, 2006) including genomic selection propitiated by the availability of dense molecular markers (Crossa et al., 2010; Cabrera-Bosquet et al., 2012).

PRIMARY DETERMINANTS OF GRAIN YIELD AND DROUGHT ADAPTATION

Grain yield may be expressed as the integrated response of different plant processes to a limiting resource such as radiation or water. Two main steps are involved: production of photoassimilates, and its further transformation onto an economic (usually harvestable) component. An additional factor to consider is the phenological stage of the plant when the limiting resource acts.

Radiation limited yield

Grain yield (GY) can be considered the product of the following:

$$GY = RAD \cdot \%RI \cdot GLD \cdot RUE \cdot HI$$

where: RAD = incident radiation received per day (e.g., 20 MJ m^{-3}); %RI = % intercepted radiation over crop life cycle (e.g., 50%); GLD = green leaf duration (e.g., 100 days); RUE = radiation-use efficiency, taken as 1.5 g MJ^{-1}; HI = harvest index (0.45; range 0.4–0.55 under well-watered conditions). Thus:

$$GY = [20 \cdot 0.5 \cdot 120 \cdot 1.5] \cdot 0.45 = 810\,g\,m^{-2},\ \text{or } 8.1\,t\,ha^{-1}$$

Grain yield can be reduced by the effects of drought on most of these factors (Andrade et al., 1996). Drought during establishment can reduce plant germination, while water stress during leaf area expansion reduces leaf area and radiation interception. Later in growth, it will reduce green leaf duration from accelerated

senescence, and reduce RUE by direct effects on photosynthesis (Dwyer et al., 1992). It can also have direct effects on yield components through induced barrenness, kernel abortion or shriveled grain, which can in turn reduce HI. The rate of seasonal dry matter accumulation is a function of interception and utilization of incident solar radiation. Differences in the rate of dry matter accumulation can be attributable to increased light interception due to: (1) greater maximum leaf area index (LAI); and (2) reduced leaf senescence (greater "stay-green") during grain filling and a greater canopy-level efficiency of utilization of intercepted radiation, due to higher leaf angle and a reduced functional leaf senescence sustaining leaf photosynthesis during grain filling (Tollenaar and Lee, 2006). Reduction in leaf growth with water deficit may be coregulated with several mechanisms, each controlled by a large number of genes. Therefore, it may well be naïve to seek a single mechanism that accounts for the effect of water deficit on leaf growth and for the genetic variability of this process (Tardieu, 2006).

Water limited yield

Passioura (1977) proposed a parallel way of considering grain yield in a water limited situation:

$$GY = W \cdot WUE \cdot HI$$

where: W = water transpired by the crop (e.g., 400 mm); WUE = water-use efficiency, biomass/unit water transpired (e.g., 4.5 g m^{-2} mm^{-1}). Thus:

$$GY = [400 \cdot 4.5] \cdot 0.45 = 810\,g\,m^{-2},\ \text{or}\ 8.1\,t\,ha^{-1}$$

In the same sense, Blum (2006) summarized the primary factors responsible for superior performance of drought-adapted cereal cultivars, grouping them into four categories:

- capturing more soil water—thus, where deep soil moisture is available, deep-rooted cultivars demonstrate a clear yield advantage under drought (Lorens et al., 1987)
- economizing water use
- maintaining cellular hydration
- utilizing stem reserves for grain filling under stress—perhaps less applicable to maize than to small grain cereals.

Seedling establishment and pre-flowering growth

A requirement for high yield is an adequate plant stand. If drought severely reduces the stand at the onset of the season, farmers can replant fields with a shorter duration cultivar or a different species, although this entails additional cost. A limited research effort directed toward improving seedling establishment suggests that natural selection may have exploited most of the genetic variation for this trait. Recurrent selection based on stressed seedlings in the field showed only modest increases in survival under water deficit (Bänziger et al., 1997). Selection for improved survival and biomass production under post-emergence drought stress is also difficult because environmental variation is high in field screens. A recent study of the effects of pre-flowering growth on maize has demonstrated that this type of stress leads to significant reductions in plant height, in leaf area per plant and in grain yield, but to an increase in HI of several percentage points (Moser et al., 2006). However, the number of kernel rows was also reduced by stress prior to flowering, leading to a reduced kernel number per plant. Genotypes showing tolerance at flowering were not necessarily the most drought-tolerant in the pre-flowering phase. Early seedling vigor is a general expression of heterosis in cereals and is beneficial for reasons that may be related to reduced evaporation, thereby economising on water use.

Flowering

A failure of the rains later in the season when replanting is not possible may lead to a total crop loss, since maize yield in conventionally selected cultivars is often reduced two to three times more when water deficits coincide with flowering, compared with other growth stages (Shaw, 1977; Grant et al., 1989). Maize is thought to be more susceptible than other rainfed crops because of its near-synchronous development of florets, usually on a single ear, and because of the exposure of silks and pollen caused by the physical separation of male and female flowers on the same plant. Spikelets that are growing rapidly are more likely to set seed; one indicator of this is rapid silk extrusion. Since the date on which anthesis occurs is affected little by drought, slow silk growth results in a long ASI, a trait that is easily observed by breeders. A long ASI is an external indicator of a reduced partitioning to the ear, resulting in a slow spikelet growth rate (Edmeades et al., 2000b; Monneveux et al., 2006). Plants with a large ASI under drought are often barren, or have few grains per ear. Grain yield of maize grown under severe water stress at flowering is highly correlated with kernel number per plant ($r = 0.90^{**}$) and quite strongly with ASI ($r = -0.53^{**}$; Bolaños and Edmeades, 1996).

Factors affecting grain set under drought have been extensively reviewed by Westgate (2000). Grain number per plant in water-deficient maize appears to depend directly on the flux of current photosynthates during the 2 weeks bracketing flowering (Schussler and Westgate, 1995). It appears that reserves of pre-flowering assimilate are simply not attracted to the ear; perhaps the carbohydrate metabolism of the ovaries of water-stressed plants is disrupted, thereby impairing sink strength (Zinselmeier et al., 1995c; Westgate, 1997; Saini and Lalonde, 1998). However, once kernels enter the linear phase of biomass accumulation, they develop the sink strength needed to remobilize carbon reserves. This, along with continued photoassimilation, determines final kernel weight. The critical step in determining HI appears to take place 10–15 days either side of flowering. When assimilate flux per plant is reduced by competition, it has been shown that tassel growth is favored over ear growth (Edmeades et al., 2000a), and a similar tendency has been observed by Bolaños and Edmeades (1993a,b) under drought. Reductions in plant height and tassel size have also been associated with a reduction in ASI (Fischer et al., 1983, 1987). Although little is known about competing effects of root growth on ear growth, Bolaños et al. (1993) reported that, in one tropical maize population, reduced root biomass was associated with increased ear growth under drought.

Leaf growth and anthesis-silking interval

Leaf growth and ASI are the main determinants of source and sink strengths of maize, via their relations with light interception and

HI, respectively. They depend on the ability of leaves and silks to expand under fluctuating environmental conditions, so the possibility is raised that they may have a partly common genetic determinism. This was tested in a mapping population segregating for ASI. For well-watered plants, the alleles conferring high leaf elongation rate conferred a low ASI (high silk elongation rate). Under water deficit, the allele for leaf growth maintenance was, in all cases, that for shorter ASI (maintained silk elongation rate). By contrast, other regions influencing ASI had no influence on leaf growth. These results may have important consequences for modeling the GEI and for designing drought-tolerant ideotypes (Welcker et al., 2007).

The relationship between anthesis-silking interval and grain yield

Stress susceptibility varies considerably throughout the life cycle of the maize plant, and is greatest at flowering. Much of our conventional thinking on the degree of susceptibility to stress has been based on research published by Shaw (1977), in which stress-induced loss of yield per day was related to developmental stage in a hybrid that is now almost 40 years old (**Figure 1**).

There is good evidence that this marked susceptibility to drought stress at flowering has diminished with selection. However, there remains considerable genetic variation for tolerance to drought at flowering in modern commercial Corn Belt germplasm (Campos et al., 2004; Barker et al., 2005). One clear indicator of stress at flowering is a delay in silk exsertion in conjunction with very little or no delay in anthesis, giving rise to an easily observed ASI. Correlation analyses relating secondary traits to grain yield under drought stress at flowering in tropical germplasm show a close dependence of yield on kernel number per ear (KPE; up to $r = 0.9$), and moderate to strong associations of grain yield and KPE with ASI ($r = -0.4$ to -0.7; Bolaños and Edmeades, 1996). Others have reported similar correlations between ASI and grain yield in a wide array of germplasm (DuPlessis and Dijkhuis, 1967; Jensen, 1971; Bolaños and Edmeades, 1993b; Chapman and Edmeades, 1999; Monneveux et al., 2006). These are among the largest correlations of any secondary trait with grain yield under drought (e.g., correlation of grain yield under stress with stay-green: $r = 0.3$ to 0.5; with weight per kernel, $r = 0.2$ to 0.4; Bolaños and Edmeades, 1996), and emphasize the critical importance of the flowering process in establishing KPE and in stabilizing yield under stress.

Where stress is severe enough to induce barrenness, ASI is also highly correlated with the number of ears per plant ($r = -0.5$ to -0.7). Thus, ASI measured at flowering can predict a significant proportion of variation observed in grain yield that is only revealed 2 to 3 months later. These results are not confined to older hybrids or tropical germplasm. Evaluation of a representative sample of 54 modern precommercial Corn Belt hybrids has shown a correlation between grain yield and ASI across water stress levels of -0.72^{**}, and between kernel number per plant and ASI of -0.71^{**} (Edmeades, 2002, unpublished data). Andrade et al. (2002) reported a common relationship between kernel number per plant and plant growth rate when both water and nitrogen supplies varied. Evidence of this nature led Edmeades et al. (2000b) to conclude that variation for stress tolerance at flowering exists, and that ASI is a convenient external indicator of this and may be a reasonable indicator of tolerance to reduced photosynthesis per plant at flowering arising from many causes.

The heritability of ASI is often slightly higher than that for grain yield, and several QTLs associated with this trait under drought stress have been identified (Ribaut et al., 1996). Other studies have subsequently identified similar regions and confirmed those originally identified in several other crosses (Welcker et al., 2007). These authors have also reported a QTL that colocalises for leaf elongation as well as for short ASI, suggesting a common genetic control or that turgor maintenance affects both. Marker-assisted backcrossing of some of these QTLs has

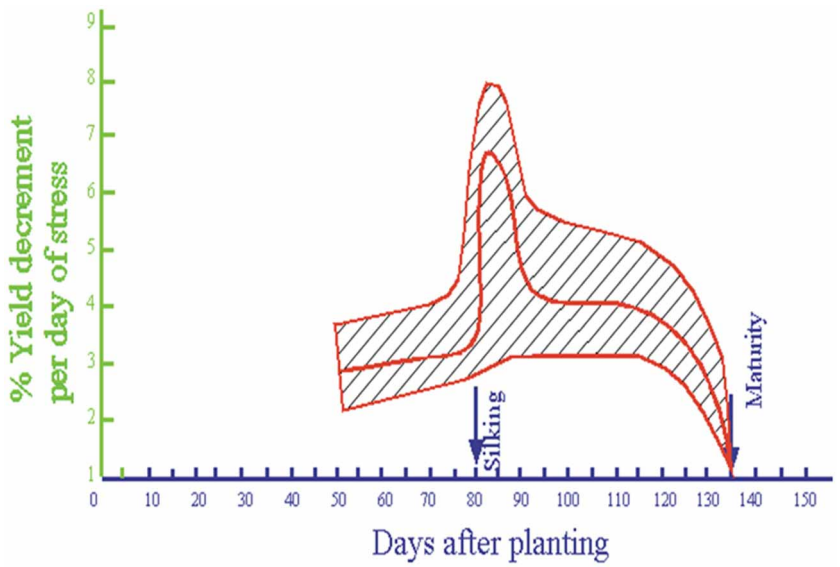

FIGURE 1 | Relationship between yield loss per day of stress and growth stage in a maize hybrid bred in the 1960s (Redrawn from Shaw, 1977).

demonstrated significant improvement in grain yield under flowering stress (Ribaut and Ragot, 2007). Managed drought stress environments, where stress is imposed by withdrawing irrigation during an otherwise dry growing season, are a highly effective means of exposing genetic variation for ASI in a repeatable, reliable manner (see Bänziger et al., 2000 for a useful practical guide to their use).

Selection for traits that govern kernel set

Given this strong relationship between ASI and grain yield and/or kernel number, can selection for these traits lead to greater yield stability when drought stress coincides with the flowering period? If so, what are the limits to progress, and are there concomitant penalties in non-stressed performance? The best current examples of selection are found in tropical germplasm. Here, selection for improved grain yield under drought stress at flowering, achieved mainly by emphasizing increased grain yield and reduced ASI and barrenness, resulted in gains per selection cycle in yield, ASI, ears per plant (EPP) and HI under severe stress. These gains averaged, respectively 100 kg ha^{-1}, −1.1 day (or around 15°C day), 0.03 and 0.013 respectively (Edmeades et al., 2000a). There were also modest increases in the KPE. The increase in HI occurred under both stressed and unstressed conditions (Edmeades et al., 2000b). Similar results have recently been reported in another tropical population (Monneveux et al., 2006). Selection for more rapid silk emergence also improved tolerance to low nitrogen (Bänziger et al., 1999; Zaidi et al., 2004). Subsequently, Bänziger et al. (2005) reported that hybrids selected under managed stress using similar protocols significantly outyielded commercial hybrids in Southern and Eastern Africa by an average of 17% at yield levels in the 0–3 t ha^{-1} range, 11% in the 3–6 t ha^{-1} range and 4% in the 6–9 t ha^{-1} range.

Are the changes brought about by this type of selection subject to GEI? Tropical germplasm was largely selected in dry winter seasons where stress intensity and timing could be managed. Byrne et al. (1995) tested several selection cycles of tropical maize in the target environment, i.e., a normal summer crop season in a number of tropical sites away from the selection location. They found that 83% of the gains reported at the selection site carried over into the target environment. Pioneer Hi-Bred International Inc. tested initial and advanced selection cycles from three tropical populations, along with an older temperate drought tolerant population, at sites where the tropical germplasm was not adapted because of its photoperiod sensitivity. Although mean yields of tropical selections were not competitive with adapted temperate germplasm, gains due to selection for increased grain yields and due to reduced ASI and barrenness under stress were very similar to those observed at the selection site (**Figure 2**). These data suggest that changes due to selection targeted at the flowering period provide stability of performance across locations, even in locations where overall adaptation is poor. Zaidi et al. (2004) reported correlations between hybrids selected under drought versus conventional selection for yields under drought and under low nitrogen of

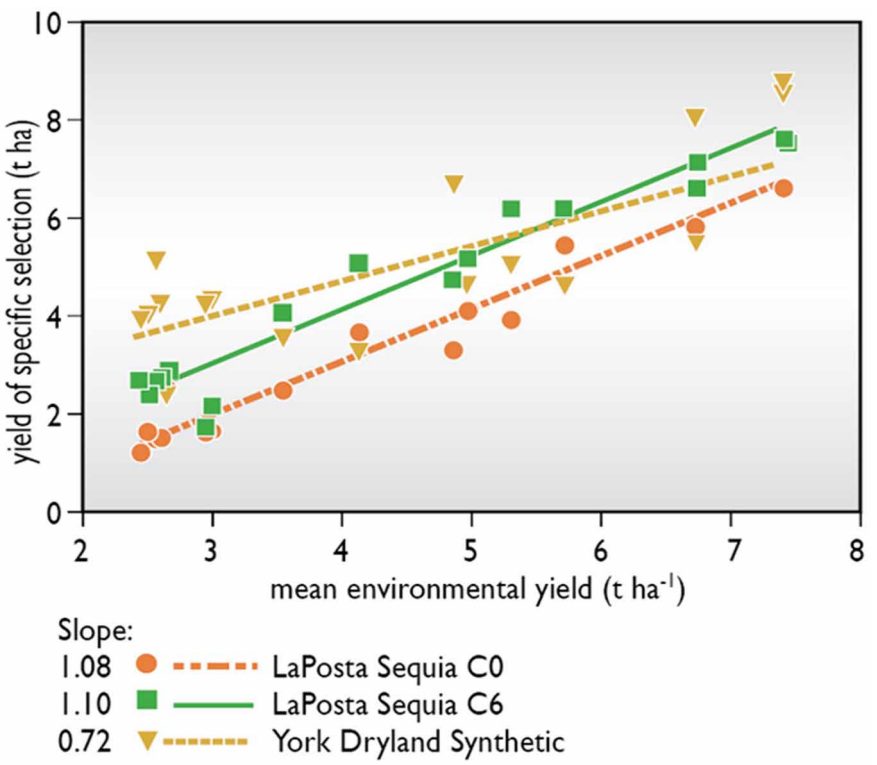

FIGURE 2 | Yield of unselected and selected versions of a tropical population, "La Posta Sequia," when grown in environments to which it was not adapted. Yields of an adapted Corn Belt population, "York Dryland Synthetic," are given as reference.

0.65*–0.67* versus 0.44–0.46 ns, suggesting selection for tolerance at flowering reduced GEI. Reviews of progress in the ERA Corn Belt hybrid set, spanning improvement through pedigree breeding over the past 70 years, suggest that multilocation testing and screening at high plant densities have provided gains in yield in stressed and unstressed environments, although rates of gain in stressed environments were less than half of those in unstressed fields (Duvick, 2005; Campos et al., 2006).

Underlying causes for the relationship between anthesis-silking interval and grain yield

The dependence of kernels per ear on ASI suggests that germplasm that has not been previously exposed to strong selection under stress at silking may respond to stress by giving a large spread in silk emergence. Is an extended ASI a symptom of some deeper problem associated with spikelet fertility? There are three main reasons for the association between ASI, kernel set and grain yield (Hall et al., 1981; Otegui et al., 1995), as follows:

- *Lack of pollen because of heat, asynchrony, or because anthers do not exsert:* Hot dry weather during pollination may cause tassels to blast and kill the pollen before it is shed (Lonnquist and Jugenheimer, 1943; Schoper et al., 1987). Pollen quantity and viability are reduced in some genotypes when tassel temperatures reach 38°C (Lonnquist and Jugenheimer, 1943; Schoper et al., 1987), although drought *per se* does not appear to affect pollen viability (Hall et al., 1982; Schoper et al., 1986; Westgate and Boyer, 1986). Lizaso et al. (2003) have created algorithms that predict the effect of pollen viability on pollen concentrations considered critical for full kernel set, but have not provided any in situ measurements of pollen viability in the field. Asynchrony, caused by delayed silking, may simply result in a shortage of pollen for late emerging silks. Bassetti and Westgate (1994) have shown in one hybrid, P3790, that a reduction in kernel set occurred when pollen shed fell below 100 grains cm^{-2} d^{-1}. This value agrees fairly well with that provided by Sadras et al. (1985), who reported that a mean pollen density of five grains per silk was necessary for 90% kernel set. Bassetti and Westgate (1994) also observed that this threshold pollen concentration increased if silks emerged more than 3 days after the start of anthesis. This suggested that the competence of silks and ovaries in late emerging silks, typically originating from the tip of the ear, had declined. In tropical genotypes that are usually characterized by large tassels, the period of shed is lengthened.
- Marked reductions in tassel size have occurred in temperate maize over the past 50 years of selection (Campos et al., 2006). However, in single cross hybrids that have been selected for high yield, tassels are typically half the biomass per plant of landraces. This means that the window of pollen availability is narrower, and the numbers of grains shed per day and per tassel are less. For example, Hall et al. (1982) cite pollen production per tassel of large open-pollinated varieties as 42.2 million versus 14.8 million for a Corn Belt synthetic. This can be compared with only 4.5 million pollen grains per tassel in modern hybrids in mid-Western environments (Westgate et al., 2003) and as little as 1.4 million per tassel in inbred lines (Fonseca et al., 2004), amounts that are undoubtedly affected by the environment (Uribelarrea et al., 2002). Male sterility can also be a cause of pollen shortage. Interplanting male sterile inbreds in varying proportions has been used as a means of altering pollen supply in quantitative studies of kernel set response to pollen supply (Lizaso et al., 2003; Westgate et al., 2003), and recurrent selection in populations for short ASI has sometimes resulted in a sharp increase in male sterile plants (Edmeades et al., 2000a).
- *Damage to the embryo sac during megasporagenesis:* This will normally prevent pollination, although silking may occur (Moss and Downey, 1971). Damage of this nature only occurs when severe water stress is encountered 1 to 2 weeks before silking, and is not reversible.
- *A slow rate of spikelet growth:* This results in a large ASI, silk senescence and abortion following pollination; drought reduces plant growth rate generally, and slows ear and spikelet growth.

Bolaños and Edmeades (1993b) found that selection for short ASI and increased grain weight under drought in a tropical population resulted in a significant increase in ear relative growth rate and a decrease in tassel relative growth rates. These changes are usually considered to reflect alterations in carbon partitioning. In this study, biomass of the upper ear at anthesis more than doubled over eight cycles of selection, and ear biomass per spikelet at anthesis increased by 12% per selection cycle. Rapid silk growth could be related to increased spikelet size, perhaps because there were fewer spikelets growing (Edmeades et al., 2000b; Monneveux et al., 2006). It is also possible that the earlier cessation of spikelet initiation in advanced selection cycles released already-initiated spikelets from a type of apical dominance, and permitted their more rapid growth.

The reduction in growth of tassels, stems, and roots that also accompanied selection probably released current assimilates to support accelerated ear growth. Reduced stem growth near flowering appears to accelerate ear growth, and results in reduced ASI (Sowell et al., 1961; Johnson et al., 1986; Edmeades et al., 2000b). Several recent studies have related kernel set to plant growth rate in the period of 10–15 days either side of flowering (Vega et al., 2001a), a technique that sharply reduces sampling errors. Lower plant and ear growth rates indicate lower assimilate flux to the growing plant and to the ear, a scenario that often results in kernel abortion within a few days after pollination (Schussler and Westgate, 1995). Increased rates of ear growth result in a rapid exsertion of silks, a higher rate of reproductive success, increased grain yield under all conditions, but especially under stress, and a general increase in HI (Bolaños and Edmeades, 1993a; Edmeades et al., 1999) in stressed and unstressed conditions. When slow growing silks of water-stressed plants were pollinated with fresh pollen, the majority of egg sacs were fertilized, but many ceased development 2 to 3 days after pollination (Westgate and Boyer, 1986; Bassetti and Westgate, 1993c). Others have also noted that when silks on plants exhibiting a long ASI are pollinated with fresh pollen, they will often not form grain (Lonnquist and Jugenheimer, 1943; Moss and Downey, 1971; Hall

et al., 1981; Otegui et al., 1995). This failure probably reflects the state of health of the silks and the ovaries.

More recent research suggests that sucrose serves as a substrate for ovary growth, and that its concentration is a signal for gene expression (Boyer and McLaughlin, 2007). When the sucrose concentration is low, invertase genes are downregulated and genes associated with senescence are upregulated. Quantification of the extent of this type of abortion is difficult, since no trace of the aborted floret remains at maturity. It is possible that pollination with transgenic pollen, followed by testing for the presence of the transgene in specific kernel rings of immature ears with a quantitative polymerase chain reaction (PCR) assay, could detect abortion by comparing the position of the signal with that of filled kernels on mature unstressed ears. Infusion of sucrose into the internode near the point of ear insertion has been successful in reversing a large proportion of the grain loss associated with severe drought stress near flowering (Boyle et al., 1991). However, studies by Schussler and Westgate (1991b) and Zinselmeier et al. (1995a) both noted that there were direct effects of water stress on carbohydrate metabolism in the ovaries at silking.

In an elegant set of sucrose feeding studies, Zinselmeier et al. (1999) showed that ovary abortion under stress was related to the disappearance of starch reserves around the ovary walls. Both this and previous work showed that sucrose fed to stressed plants at flowering accumulated in the ovary tissues, and was apparently not broken down to hexose sugars in the first steps needed to form starch. It was hypothesized that water stress sharply inhibited the activity of acid invertase that catalyzes this step (Zinselmeier et al., 1995c, 1999).

It is apparent that the developing maize ear is a weak sink at a time when stem reserves of assimilate formed from previous photosynthesis are at a relatively low concentration (Westgate and Boyer, 1985). At silking, the ear appears unable to mobilize and attract these reserves and, instead, relies heavily on current photosynthesis (Schussler and Westgate, 1991a,b, 1994). This source of assimilate also supports concurrent stem, husk, tassel, and root growth (Zinselmeier et al., 1995b; Edmeades et al., 2000a). If this flux is reduced, or stems and tassels are growing aggressively, then the flux to the ear also falls, and kernel set can be reduced substantially. Therefore, accelerated silk emergence and a short ASI appear to be manifestations of increased partitioning of biomass to the developing ear and of a larger ear growth rate. Thresholds may be important. If assimilate flux to the ear falls below a certain threshold (**Figure 3A**), the normal pattern of silking is disturbed and the ear will abort completely or produce 30–50 kernels unevenly scattered over the rachis (Edmeades et al., 2000b).

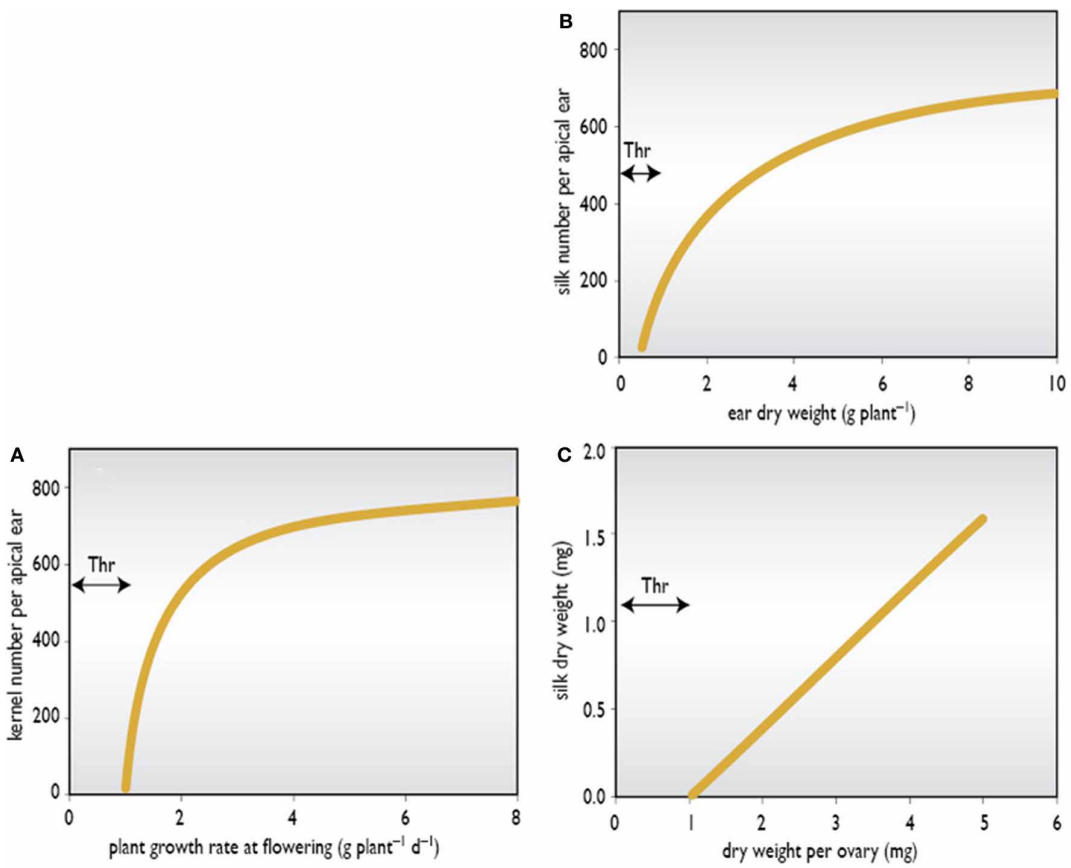

FIGURE 3 | Theoretical thresholds (Thr) in ear growth. (A) Kernel number versus plant growth rate at flowering; **(B)** Silk number versus ear dry weight; and **(C)** Silk dry weight versus ovary dry weight.

Another threshold could be the ear dry weight needed to generate silk growth (**Figure 3B**). We also hypothesize that there is a threshold weight or growth rate for each ovary before silk growth will commence (**Figure 3C**). Non-destructive morphometric methods for estimating the thresholds of reproductive growth versus plant growth rate and kernel set versus ear growth rate have been described for maize by Vega et al. (2001a,b). They related kernel number per plant to plant growth rate around flowering to estimate threshold growth rates of the type illustrated in **Figure 3A**. Using similar methods in a series of hybrids released in Argentina over a 30 year period, Echarte et al. (2004) showed that the threshold plant growth rate for kernel set has fallen with selection, implying that modern hybrids can set kernels at lower plant growth rates than older hybrids.

Assuming that the methodology exists to estimate these thresholds precisely, it is very likely that genetic variation will be detected for all the thresholds described. Silks age, and if the silk has been emerged for 7 to 8 days, it will begin to senesce at its base. This will prevent the growth of pollen tubes to the ovary (Bassetti and Westgate, 1993a,b). When evaluating the effects of time of pollination on kernel set, Anderson et al. (2004) reported a rise in kernel set until 6–8 days after first silk, at a time when the maximum number of silks were exposed (Fonseca et al., 2004) and then a general decline in kernel set that, presumably, reflected senescing silks. The timing of the decline varied with year, suggesting that environmental conditions may affect the speed at which senescence occurs. When growth of silks is slowed by water stress early in their lives (e.g., 3 days after first silk), silk senescence is delayed. However, when the stress occurs a few days later, it serves to accelerate the senescence process (Bassetti and Westgate, 1993c).

Grain filling and stay-green

Provided that an ear has been established, the maintenance of a green functional canopy and a capacity to remobilize carbohydrates stored in the stem and husk should contribute to high yield under terminal drought stress. Associations between foliar stay-green and yield are often weak (Bolaños and Edmeades, 1996), and reasons for this must be sought in the nitrogen balance of the crop at that growth stage. Selection for more grains per plant will likely increase the internal demand for nitrogen and, since nitrogen uptake from a dry soil is low, this may result in "mining" of nitrogen from leaves, thus offsetting improvements in stay-green resulting from directed selection (Chapman and Edmeades, 1999). Duvick (2005) reported that stay-green had improved significantly over 50 years of breeding in Corn Belt maize, although the improvement was much greater under unstressed conditions than under terminal drought. However, QTLs have been identified in sorghum that significantly extend stay-green under drought (Harris et al., 2007), and it seems likely that they will also be identified under moderate terminal drought in maize.

Under drought stress, delayed senescence (commonly termed "stay-green") during post-anthesis can sometimes be accompanied by maintenance of leaf water status, as in the case of stay-green sorghum (Xu et al., 2000). However in maize, stay-green was associated with higher yield (Ma and Dwyer, 1998), probably because of nitrogen use factors rather than plant water status effects (Blum, 2006). Thus, stay-green and kernel numbers are affected by nitrogen uptake and use efficiency, and by nitrogen remobilization (Gallais and Hirel, 2004). The most important single factor influencing nitrogen use efficiency is glutamine synthetase (Hirel et al., 2007). Other factors affecting stay-green are growth regulators. Thus, increasing the amount of endogenous cytokinin (Ori et al., 1999) or reducing the production of ethylene led to a delay in senescence (John et al., 1995). In fact, 1-methylcyclopropene has recently been commercialized for application in maize and other crops; it apparently binds with ethylene receptor sites in plants, reducing the negative effects of ethylene. In other cereals, ethylene has been related to decreased kernel number (Hays et al., 2007).

Irrespective of the underlying cause, stay-green may be a consequence of a plant's being able to keep a better water or nitrogen status rather than a primary factor in itself. Whatever the physiological mechanism involved in the adaptive trait, stay-green is a major factor that may contribute to improving grain yield when water shortage occurs during flowering and at the beginning of grain filling, provided that water is available further during grain filling (Ribaut et al., 2004). Regarding the use of stem reserves stored before and during heading for grain filling under stress, it seems that this characteristic is not evident in maize.

METHODOLOGY

BREEDING STRATEGY

Multilocation testing

Conventional breeding for drought tolerance based on extensive multilocation testing of progenies and GEI analysis has successfully increased grain yield under well-watered and moderately stressed environments. However, the use of nurseries where timing and intensity of water deficits are carefully managed, combined with the use of secondary traits, is more efficient and generally cheaper than multilocation testing (Monneveux and Ribaut, 2006). Proper control of the spatial variability inherent to field testing may also help to improve the efficiency of maize breeding for abiotic stresses (Cairns et al., 2012; Prasanna et al., 2012). Moreover to facilitate the full potential of molecular tools greater emphasis needs to be given to reducing the within-experimental site variability, application of stress and characterization of the environment and appropriate phenotyping tools (Masuka et al., 2012).

Empirical versus analytical breeding

Grain yield and its response to stress are highly complex traits involving a long-term (the full crop cycle) interaction between the environment and plant characteristics and regulatory pathways at different scales of organization (from molecular to the whole canopy). Empirical breeding, which is based on selecting directly by yield, has limited success under drought, due to large genotype-by-season and genotype-by-location interactions, which cause a low heritability of yield (Araus et al., 2002, 2008; Monneveux and Ribaut, 2006; Lopes et al., 2011; Prasanna et al., 2012). Alternatively, analytical breeding consists of the use of secondary traits to either complement phenotypic selection or eventually replace selection based on yield as the only phenotypic

trait. This approach may improve the selection response because heritability of some secondary traits remains higher than that of yield, where those traits exhibit enough genetic variability, and are genetically correlated with yield.

Even if analytical breeding is not widely recognized, in practice, many breeders use secondary traits in addition to yield to improve the selection response. This kind of evolved empirical breeding has the concept of ideotype as a cornerstone and, de facto, integrates the concept of secondary traits. In a purely analytical breeding scheme, ideotype would be replaced by a selection index formulated based on the adjusted weight of the different traits considered during phenotyping (see below).

Farmer participatory approach

In addition to the above considerations, the optimal managed growing conditions (for the available water) of an experimental station are far different from the conditions prevailing in the fields of resource-poor farmers. In recent years, due to the lack of impact of traditional plant breeding approaches in low-income countries, there has been a movement toward the greater involvement of farmers in variety selection, the so-called "farmer participatory approach." This may be considered as an adaptation of phenotyping protocols that have been discussed elsewhere (Bänziger et al., 2000; Sawkins et al., 2006).

TRIAL PLANNING

Choice and characterization of the testing environment

The difficulty in choosing appropriate selection environments has restricted breeding progress for drought tolerance in highly variable target environments. GEI are common under drought and make breeding progress difficult. GEI may originate from environmental variation in the timing and severity of water deficits, from genetic variation in flowering time, and from nutrient deficiencies and toxicities whose occurrence and severity interact with water deficits (Bänziger and Cooper, 2001). Also, high error variances such as induced by variable plant stand or variable soil water holding capacity are intrinsic to many field trials grown under drought, and impede selection decisions. Even though there is extensive evidence that selection under target stresses may accelerate breeding gains for stress environments (Bänziger et al., 1997), the difficulty of choosing appropriate selection environments, given a highly variable target environment, may limit the identification of superior genotypes (Cairns et al., 2012; Masuka et al., 2012; Prasanna et al., 2012). While breeding programmes in high-income countries may resort to real-time geographic information system (GIS) information for adequately weighting information from multienvironments trial (Podlich et al., 1999), those opportunities rarely exist in low-income countries because there is a lack of both real-time GIS information and resources for conducting a large number of multienvironment trials.

WATER STRESS MANAGEMENT AND CHARACTERISATION

Phenotyping for drought performance is not just a matter of choosing the right combination of traits and measuring them at the right time. It is also necessary to cope with other sources of uncertainty relating to the need for suitable test sites with a drought cycle, irrigation system, and trained staff. Efficient phenotyping (frequently termed "precision phenotyping") implies meeting two requirements. The first requirement is proper stress management of the agronomic conditions (including irrigation management and agroclimatic record) in order to impose as closely as possible the desired stress in terms of severity and occurrence during the crop cycle. For example CIMMYT has traditionally put emphasis on inducing drought stress around the time of flowering rather than at earlier stages of the crop cycle. The second requirement is to phenotype the critical traits using the right procedure and/or tools. The main principles of drought environment management have been described by Bänziger et al. (2000), and its successful translation to into practical breeding has been well illustrated in a recent study (Bänziger et al., 2005).

PLANT WATER STRATEGY

When consider the response of any crop to drought stress, it is convenient to distinguish between moderate and severe stress. Yield under moderate stress conditions is highly dependent on the yield potential of the cultivar. For most cereals, moderate stress means a yield reduction of no more than about 50% compared with non-stress conditions, where drought resistance is less of an issue than is the yield potential of the cultivar (Araus et al., 2002, 2008; Blum, 2006). When yield is further reduced by stress to a level far below 50% of yield potential, then yield potential becomes irrelevant or even a liability, and a plant cannot yield well without some protection against this dysfunction. However, there is a range of growing environments where the combined effect of both factors eventually makes selection more complex (Sawkins et al., 2006). Therefore, germplasm screening in the absence of water stress as well as under stress environments is usually required. This approach, with the simultaneous use of selection under different contrasted environments, has been successfully implemented in sub-Saharan Africa, where hybrids developed by CIMMYT have outyielded hybrids from commercial companies (Bänziger et al., 2005).

Experience from drought-resistant cereal cultivation during a century of scientific breeding (Araus et al., 2002, 2004, 2008; Blum, 2005; Tollenaar and Lee, 2006) clearly indicates that drought resistance in crop plants at this level of stress is mainly derived from their ability to sustain tissue hydration under drought (i.e., dehydration avoidance), rather than an ability to sustain biological function when tissues are dehydrated (i.e., dehydration tolerance). However, until recently, most molecular biology approaches involving plant transformation, for example, have traditionally dealt with dehydration tolerance rather than avoidance (Araus et al., 2003). Dehydration avoidance in drought-resistant cereals cultivars is largely derived from constitutive traits (i.e., traits expressed in the absence of stress) rather than from drought-responsive traits (Blum, 2005). Constitutive traits may include seedling vigor, early, or synchronized flowering, leaf area, potential root length and plant size. In a historical perspective, the role of drought-responsive genes in comparison to genes that control constitutive traits seems to have had a relatively moderate role in the development of drought-resistant cereal cultivars, perhaps with the exception of osmotic adjustment (Blum, 2006), which does not seem to play an important role in maize (Tardieu, 2006).

PHENOTYPING TRAITS

For a secondary trait to be useful in a breeding programme, it has to comply with several requirements (Araus et al., 2002, 2008; Lafitte et al., 2003):

- It should be genetically correlated with grain yield in the environmental conditions of the target environment, i.e., the relationship with yield has to be causal not casual.
- It should be less affected by environment than grain yield is; i.e., it should have higher heritability than the yield itself, and so less GEI.
- Genetic variability for the trait must exist within the species.
- In the case of traits addressed in breeding for stress-prone environments, the trait should not be associated with poor yields in unstressed environments. Unfortunately the latter is the case for many traits selected because they confer tolerance instead of avoidance of a given stress (Araus et al., 2002, 2003).
- It should be possible to measure the trait rapidly, more economically than yield itself, and in a reliable way.
- The trait must be able to be assessed in individual plants or in very small plots, preferably by non-destructive means.

Most successful traits are "integrative," either in time (reflecting physiological activities throughout the growing cycle), or in level of organization (i.e., at the whole plant level or, even better, at the level of the canopy), or both (Araus et al., 2002, 2008). In such a category we may include phenological traits (either constitutive or affected by stress) having an effect on HI (such as time to anthesis and ASI) or on energy uptake (stay-green), as well as other traits related with water status (such as transpiration and stomatal conductance).

Anthesis-silking interval

By determining genotypic correlations between a range of secondary traits and grain yield under drought, Bolaños and Edmeades (1996) found that reproductive traits related with HI, such as ASI, explained much more of the variation in yield than did traits related to plant water status, water use and WUE (e.g., leaf extension rate, canopy temperature, leaf erectness, leaf rolling, and leaf senescence). Indeed, ASI is one of the few examples of secondary traits widely used for maize selection under drought. The trait was developed by CIMMYT (Bolaños et al., 1993; Bolaños and Edmeades, 1996). ASI is an excellent secondary trait since it exhibits a significant negative correlation with grain yield and relatively high heritability, plus the other requirements indicated above. However, because continued selection for secondary traits results in changes in the underlying genetic correlation between traits, these relationships require reevaluation over time (Edmeades et al., 1997). Moreover, a short ASI has already been incorporated into the genetic background, which means that phenotyping for other traits is increasingly important. As noted above, there is a consistent correlation between ASI and kernels per ear under drought stress at flowering, normally ranging from -0.3 to -0.7. Gains have been made in yield and through reduced barrenness under stress when ASI has been used directly in selection.

Why not simply continue to use this trait as an integrated indicator of reproductive competence under stress? The following are some of the limitations of ASI as a selection trait:

- It does not capture variation in flowering behavior within and among plants. It is not clear what a plot value for ASI means at the individual plant level. Fifty percent anthesis and silking dates do not reflect the trajectory of anthesis or silking over time, but merely capture the median behavior of the population of plants. Thus, ASI does not describe attributes of a population of silks or pollen grains, nor can it quantify the asynchronous exsertion of silks within ears. It does not describe the fate of later emerging silks, nor the probability of these silks encountering pollen. ASI *per se* provides no information on changing spikelet numbers. Fewer spikelets appear to result in a greater reproductive efficiency per spikelet, but under unstressed conditions this reduction in spikelets may ultimately restrict yield potential, unless additional spikelets are added through a second ear per plant (Tollenaar et al., 1992).
- ASI is subject to error when silk delays are small. Since ASI is the difference of two measurements, both of which are subject to error, it can only be estimated precisely when the difference between anthesis and silking is reasonably large. Typically, errors in high-throughput visual estimation of flowering are ± 1 day, so errors in ASI from rapid estimates are likely to be ± 2 days. Other types of error can also occur when stress is severe. Some Corn Belt hybrids with small tassels enclose the tassel in the flag leaf during anther exsertion, and pollen shed cannot be observed. Similarly some hybrids will exsert silks in the gap between the stem and the ears leaf sheath, and are easily overlooked. ASI attains its greatest value for selection when it is >3 days, and when the exsertion of tassels and silks is clearly visible. Large ASI values of 5–8 days have no better heritabilities than shorter ASI of 3 days (Bolaños and Edmeades, 1996).
- ASI is time consuming to observe in the field. It is probably more economical to record the level of barrenness at harvest, provided the stress at flowering has been severe enough to induce barrenness in about 20% of the plants. The strong genetic correlation between ASI and ears per plant under severe drought stress (-0.7 to -0.9) can be used to advantage here (Bolaños and Edmeades, 1996).

We conclude that, while ASI continues to be a very useful trait that provides a snapshot of female versus male reproductive development, it does not provide useful information on the rate of silk appearance or the quantity of pollen shed per exposed silk.

Flowering parameters and kernel numbers

Details on applying these procedures are given in Bänziger et al. (2000). In brief, in the field they should be observed on a well-bordered area of known size in each plot, where there are no or very few missing plants.

- *Fifty percent silking and 50% anthesis and ASI*: Observe a known number of plants (guideline: $N = 20$ for inbred lines

or hybrids; $N = 35$ for open-pollinated varieties) per plot at the same time each day until 50% of the plants in the plot have produced at least one visible anther or have at least one silk emerged, and record the date when this occurs. The ASI is: (days to 50% silk – days to 50% anthesis). Each of these parameters can also be expressed as heat units (°C day) if temperature data are being recorded near or in the plot.

- *Pollen density*: There are no known easy ways of measuring pollen production per genotype in plot sizes of less than 6 rows × 5 m that do not involve the slow process of bagging tassels to avoid cross contamination, followed by weighing the pollen shed into the bag each day. Pollen subsamples are then counted and weighed to provide an estimate of the number of pollen grains shed per plant (Hall et al., 1982). Aylor (2005) suggests that this method overestimates pollen at the silk level fourfold, because a proportion of grains lodge on leaves above the ear. The internal bag environment may also hasten anther dehiscence and increase shed. Where the goal is simply to measure the pollen density present in the plot from all shedding tassels (i.e., to assess if pollen is limiting seed set in a specific genotype growing in a trial) a sticky or liquid trap placed at silk level is usually used and is changed daily to avoid contamination with anthers, insects, etc. Counting is usually done either by suspending in an isotonic solution and counting with a Coulter counter (Fonseca et al., 2004), or by direct counting of the sticky surface using computerized imaging methods (Bassetti and Westgate, 1994; Fonseca et al., 2002; Uribelarrea et al., 2002).
- *Silk number*: Traditionally this has been counted by hand from approximately 10 ears per plot (hybrids; Bassetti and Westgate, 1993a). Usually, a cross section of the silk brush (1–2 cm long) is cut in the field and stored in water (for a few hours) or in 95% ethyl alcohol (several months). Where newly exposed silks need to be identified, the brush must be cut daily and the newly emerged silks visually identified by their bisected apical end (Cárcova et al., 2000). Similar methods were used by Uribelarrea et al. (2002), Cárcova and Otegui (2001), and Fonseca et al. (2004). Hand counting silk samples takes 10–15 min per sample and is, understandably subject to operator error. Computer imaging of pieces cut to a standard length seems increasingly feasible (Bassetti and Westgate, 1994).
- *Ears per plant*: The number of plants in a known area of plot are counted ($N = 20$ for inbreds or hybrids, $N = 35$ for open-pollinated varieties). At harvest, when ears are removed by hand, the number of ears with one or more kernels is counted. If there are no normal kernels on the ear, the plant is barren. When plots are mechanically harvested, ears are normally not visible, so counts must be made of ears that can be felt through the husk. Usually, this means that the ear needs to have ca 5 cm of grain formed along each of several ear rows, so that it can be felt through the husk as a solid mass. Ear numbers are recorded and divided by the number of plants for ears plant^{-1} and by the plot area for ears m^{-2}.
- *Plant and ear growth rates at flowering*: The morphometric methods developed and described by Vega et al. (2001a) are recommended for this measurement, if thresholds of ear and plant growth for kernel set are required.

Measurement of source traits affecting individual kernel weight

These are largely related to the trait itself, or are measures of source (i.e., assimilate storage) activities:

- *Individual kernel weight*: When ears are being shelled, a representative sample of kernels is selected, either from the stationary sheller of from the grain stream of the plot combine harvester. Broken grains and non-grain matter are removed, and two aliquots of 100 representative kernels are each hand counted, dried to constant moisture at 80°C, and weighed. Alternatively, samples of about this number of clean representative kernels can be counted using an electronic seed counter, and weights of the samples taken as before. When using the average kernel weight obtained in this way to estimate kernels per ear, care must be taken to ensure that the moisture contents of all weights are compatible.
- *Stay-green*: This is usually assessed on a 0–9 scale, where each unit refers to 10% of the visually assessed foliage area that is green (or brown) at the time. This score is usually assessed on a plot basis once differences in foliar senescence of 2–3 units become clear among plots, and is usually repeated every 7–10 days until the leaves of about 10% of genotypes have fully senesced.
- *Remobilization of stem reserves*: Grain filling could continue in the absence of green leaf if assimilate stored in the stem and husk could be remobilized to the ear. Maize loses a significant amount of dry weight from both of these organs during grain filling, although taller maize plants with larger stem volumes are no more effective in maintaining kernel weight than their shorter counterparts when defoliated during grain filling (Edmeades and Lafitte, 1993). To measure remobilization *per se* requires an estimate of the loss of stem dry weight, either directly by destructive sampling, or from a reduction in stem diameter. Neither has been used on a large scale to assess differences among maize genotypes in remobilization capacity under drought; stable weight per kernel is the most economical way of estimating buffering capacity through remobilization.

The following measurements, carried out rapidly and precisely, are keys to successful genetic manipulation of kernel set and grain filling, and hence yield stability under drought stress:

- grain yield
- ASI
- number of silks emerging from stressed versus unstressed ears over time
- threshold plant growth rate for ear formation
- threshold ear size (or ear growth rate) for silk growth and for kernel set
- adequacy of pollen supply (its timing, intensity, and viability)
- ears per plant (or, conversely, barrenness)
- kernels per ear
- weight per kernel
- degree of kernel abortion in the first 5 days after pollination
- canopy stay-green estimates.

Water status parameters

The water status of the crop may be assessed through transpiration, i.e., the water used by the leaf or the plant. The rationale is quite straightforward: the better the status the more the plant will transpire. There are different potentials tools (or surrogates) that allow transpiration to be measured indirectly:

- *Porometry*: transpiration may be broken down into two components. One is the leaf conductance (mostly determined by how open the stomata are, i.e., the stomatal conductance, g_s) which really depends on the water status of the plant. The other is the evapotranspirative demand, which depends on environmental variables such as temperature, relative humidity and wind. Thus, g_s may be used to screen for water status in maize (Sanguineti et al., 1999), and the current generation of relatively low-cost (a few thousand US$) and easy-to-handle porometers such as the Decagon Leaf Porometer SC-1 or the Delta-T AP4 allow rapid (20–30 s) measurement of leaf conductance (**Figure 4**). However, unless several porometers are used simultaneously, it may still be impractical for a large scale evaluation.
- *Canopy temperature*: Depression of the canopy temperature reflects evaporative cooling of the leaf surface due to transpiration. Measurements are performed from a distance using infrared thermometers (**Figure 4**), which are inexpensive devices (a few 100 US$). They are frequently used on crops with homogenous canopies (e.g., cotton or small grain cereals such as wheat or barley) provided that they fully cover the soil (Reynolds et al., 2001), the atmospheric conditions are adequate (sunny days, lack of wind, high evapotranspirative demand), and there are not strong differences in phenology (e.g., heading time for cereals) between genotypes. The canopy temperature has also been measured in maize (Sadler et al., 2000; Wanjura and Upchurch, 2000). However, the characteristics of the plant make it less practical to measure temperature at the canopy level, although it is possible to do it for individual leaves (Sanguineti et al., 1999; O'Neill et al., 2006), provided that they are fully exposed to the sun and at a similar angle.

New remote-sensing tools based on the use of thermal imaging to estimate plant water status at field level are achieving increased importance (Chaerle et al., 2007; Grant et al., 2007; Möller et al., 2007). Recently the use of thermography (**Figure 5**) has been proposed for high throughput phenotyping of tropical maize adaptation in water stress (Romano et al., 2011; Zia et al., 2012).

FIGURE 4 | Different devices to evaluate plant growth, phenology and water status. (A) spectroradiometer with active sensor to measure the normalized difference vegetation index (NDVI); **(B)** porometer to measure stomatal conductance; **(C)** leaf chlorophyll meter; **(D)** infrared thermometer to measure leaf temperature.

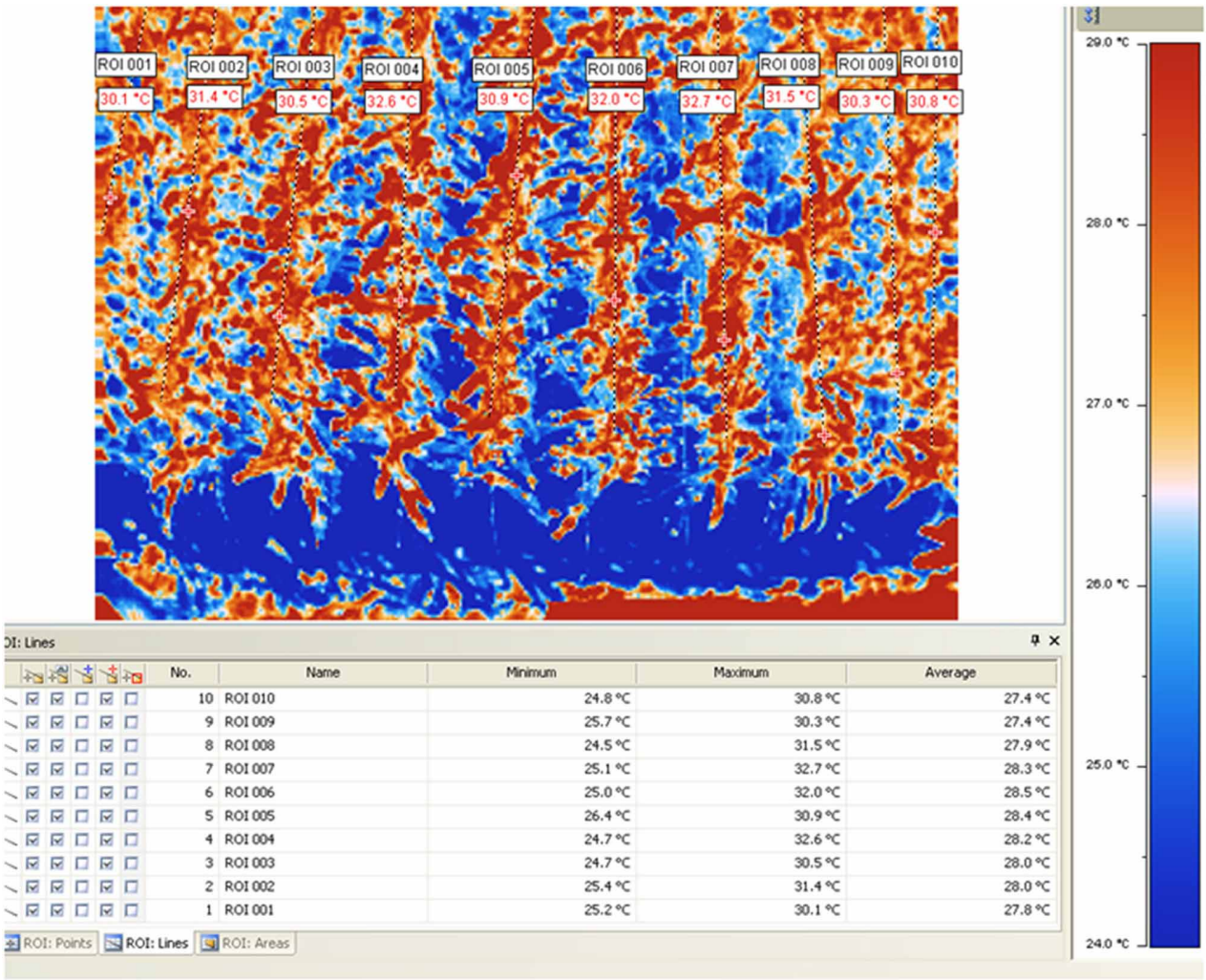

FIGURE 5 | Thermal image and corresponding temperatures of different maize testcrosses measured at CIMMYT's experimental station of Tlaltizapan (Ed. Morelos, Mexico). For more information about the procedure used see Romano et al., 2011 and Zia et al., 2012.

Oxygen isotope composition

The stable $^{13}C/^{12}C$ isotope composition ($\delta^{13}C$) measured in plant matter has been used to help breeding for drought adaptation in wheat and other small grain cereals. However, in maize, its C_4 metabolism prevents the use of $\delta^{13}C$ as a tool for screening (Monneveux et al., 2007; Cabrera-Bosquet et al., 2009b). The stable $^{18}O/^{16}O$ isotope composition of plant organic material ($\delta^{18}O$) has been shown to reflect the isotope composition of soil water, evaporative ^{18}O enrichment in transpiring leaves, and isotopic exchange between oxygen atoms in organic molecules and local water in the cells in which the organic molecules are formed (Barbour, 2007). As plant material has been shown to record leaf evaporative conditions, measurement of ^{18}O enrichment of the plant matter compared with the source water may provide a powerful tool for plant breeders (Barbour, 2007; Cabrera-Bosquet et al., 2009a). Although an integrative record of g_s may, in its own right, be of interest to breeders, the link between $\delta^{18}O$ and crop yield is likely to stimulate greater interest.

Cotton and wheat display strong correlations between g_s and yield when grown in non-limiting environments (Lu et al., 1994; Sayre et al., 1997). Barbour et al. (2000) have shown that the $\delta^{18}O$ of both whole leaf tissue and cellulose is strongly negatively related to the seasonal mean g_s and to grain yield for field-grown wheat. Therefore, the $\delta^{18}O$ composition of plant tissue is of interest to breeding for improved water use and yield in crop species. Its theoretical foundations already seem reasonably well established (Farquhar et al., 2007), which may help its further adoption as a breeding tool. Some contradictory results (Sheshshayee et al., 2005) still need to be resolved, however, and practical aspects rather than theoretical ones prevent a more widespread adoption of $\delta^{18}O$ as a breeding tool. First is its cost, which is still far higher than for $\delta^{13}C$, and second is the fact that, except for kernels, it is better to analyze chemical fractions such as cellulose rather than dry matter as a whole. In such a context, other surrogates for transpiration may be used such as the total mineral content accumulated in transpiring organs. For crops such as wheat and barley,

these have shown a good positive relationship with grain yield (Araus et al., 1998; Voltas et al., 1998). Recently the potential utility of $\delta^{18}O$ analyzed in kernels (Cabrera-Bosquet et al., 2009c) as well as total mineral (i.e., ash) content in mature but not senescent leaves (Cabrera-Bosquet et al., 2009b) has been demonstrated in maize. These approaches have also shown that phenotypic expression of heterosis in maize in linked to a better water status of hybrids compared with lines regardless of the growing conditions (Araus et al., 2010; **Figure 6**). While a potential limitation of a wider use of $\delta^{18}O$ arise in its cost and technical facilities required, a recent study concluded that near infrared reflectance spectroscopy (NIRS) can be used as a rapid, cost-effective, non-destructive method for screening $\delta^{18}O$, moreover to represent an accurate method for predicting ash and N contents in the same samples (Cabrera-Bosquet et al., 2011). Therefore, these NIRS-based analytical methodologies represent a promising application in crop management and maize breeding programs for improved water and nitrogen use efficiency and grain quality.

Plant growth, senescence, and other traits: spectroradiometrical techniques

Extensive phenotyping of large field trials for several traits is extremely expensive. Spectroradiometrical techniques allow fast and non-destructive evaluation of different characteristics of plants. They, therefore, present opportunities to develop novel phenotyping platforms that allow large screenings of genotypes for several traits in multilocation field trials (Aparicio et al., 2000; Araus et al., 2001; Babar et al., 2006). These techniques allow monitoring of several dynamic complex traits with high temporal resolution (Araus et al., 2001).

The most common use of spectroradiometrical techniques is for evaluation of chlorophyll content and related traits (such as nitrogen content, green area), based in a shift of light absorbed in the visible (400–700 nm wavelength, where the photosynthetic pigments absorb) versus the near infrared bands (700–1000 nm) of the spectrum. The same principle is used to evaluate plant status at different organization levels (**Figure 4**), from the leaf (e.g., the portable leaf chlorophyll meter like SPAD, which works using the light transmitted) to the canopy (with land-based portable spectroradiometers), where the light reflected is usually measured and vegetation indices subsequently calculated, or even to the entire crop or ecosystem (with aerial or satellite placed sensors). One of the most common vegetation indices is the normalized difference vegetation index (NDVI), which may be used to evaluate crop characteristics such as early vigor and stay-green that may be important in maize, and even grain yield (Lu et al., 2011). The use of NDVI has also been proposed as a covariate trait to remove the effect of confounding management problems (e.g., differences in plant emergence across plots) on genotype grain yield performance (Bänziger, personal communication). Besides vegetation indices, other spectral indices allow the evaluation of different traits related to photosynthetic efficiency and water status. A list of the main spectral reflectance indices potentially useful in breeding programmes is summarized by Araus et al. (2001). In addition, recent development of new formulations of the water index (WI) may open up promising perspectives for its use in drought phenotyping (Babar et al., 2006).

Canopy spectral reflectance sensors have been grouped into two categories, active and passive. Active sensors (equipped with their own source of radiation) are less influenced by environmental conditions but measure few wavelengths (Teal et al., 2006; Marti et al., 2007). The most widely known example of a land-based portable spectroradiometer with these characteristics is the

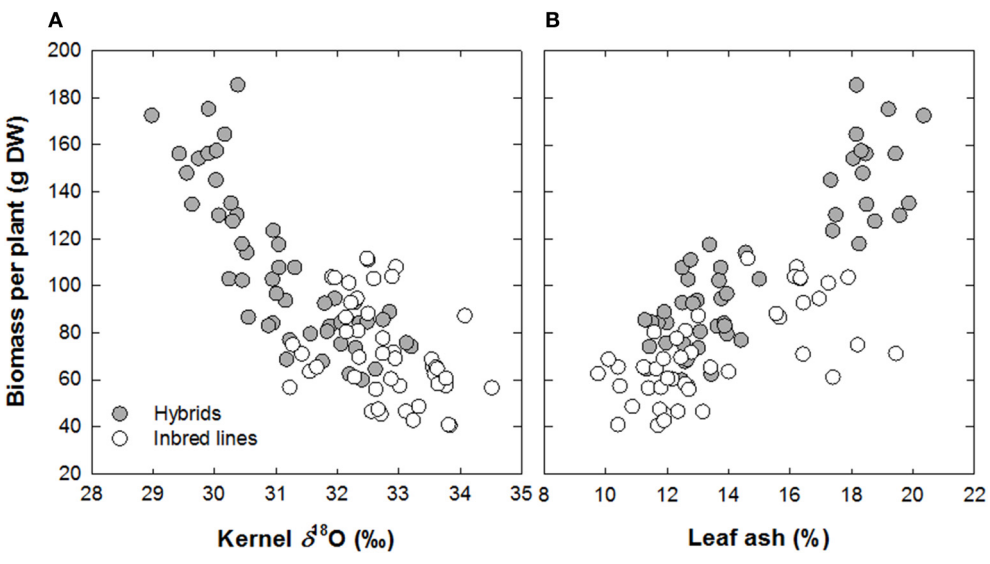

FIGURE 6 | Relationship between biomass per plant about 2 weeks after anthesis with (A) oxygen isotope composition in mature kernels ($\delta^{18}O$) and (B) ash concentration in leaves about 2 weeks after anthesis. Data from a set of maize inbred lines and derived hybrids grown under three different water regimes were plotted together ($n = 96$). Each point represents a mean value for three plots of a single genotype grown under a particular water regime (Redrawn from Araus et al., 2010).

"GreenSeeker"[1]. Passive sensors (using solar radiation) are largely influenced by environmental conditions, but measure a wide spectral range with high spectral resolution (with a bandwidth of ca 2 nm; Araus et al., 2001; Osborne et al., 2002; Babar et al., 2006). The cost of active sensors is far less than (about quarter of) that of commercialized passive sensors and they are more suitable for phenotyping multilocation field trials because data collection can be performed during a more extended time than with passive sensors. Moreover, they are configured to faster (more automatic) collection of data. However, the few measured wavelengths and low spectral resolution of active sensors might limit the prediction of complex traits; they usually measure NDVI. Therefore, the development of active sensors with increased spectral range and resolution will certainly bring forward the application of canopy spectral reflectance as a component of high-throughput phenotyping platforms.

At the leaf level, in addition to the portable chlorophyll meters developed about 20 years ago and widely used currently to evaluate differences in leaf senescence or nitrogen status, there is a new generation of sensors specifically designed to evaluate other pigments like anthocyanins (e.g., Dualex 3.3 ANTH) and flavonoids (e.g., Dualex 3.3 FLAV)[2]. Their cost, even though about one third of that of multispectral passive sensors, still prevents their wide adoption in breeding programmes.

Other more novel phenotyping techniques based on chlorophyll fluorescence (Chaerle et al., 2007), digital imaging (Casadesús et al., 2007), or even the use of spectroradiometers covering the region of 2–3 μm wavelength are promising, although still very expensive and at an early phase of their development.

Many new phenotyping tools based on remote sensing are now available including non-destructive measurements of growth-related parameters and even grain yield predictions based on spectral reflectance (Weber et al., 2012). The ability to accurately estimate grain yield using spectral reflectance measurements prior harvest could be used to reduce phenotyping time and costs. Thus in a recent study with tropical and subtropical maize grain yield of 300 maize testcrosses grown under different water and temperature regimes was predicted using spectral reflectance (495–1853 nm) of both leaves and canopy measured between tassel emergence until milkgrain stage and using partial least square regression (PLSR) was used for data analysis (Weber et al., 2012).

Is it worth measuring metabolic levels?

The role of abscisic acid (ABA) in relation to drought has been intensively studied in maize over many years (Settler, 2006). ABA is widely believed to be a major contributor to the control of plant transpiration and leaf growth (Tardieu, 2006). Moreover, ABA is thought to inhibit cell division in the endosperm; if this occurs at an early stage, the kernels will abort. A lot of research has been undertaken on the control of biosynthesis and catabolism of ABA, and the action and role of ABA under water stress (Sawkins et al., 2006; Settler, 2006). The signaling pathways of ABA and ethylene overlap because mutants affected in their sensitivity to ABA are allelic with mutants of ethylene sensitivity (Beaudoin et al., 2000). Furthermore a similar overlap is observed between the signaling pathways of ABA and of sucrose (Leon and Sheen, 2003). However, this avenue, like others dealing with transient levels of metabolites and other growth regulators, has an inherent potential limitation. In the case of ABA, it provides just a measure of drought stress at the time of sampling and in the organ sampled. Moreover, the adaptive (i.e., positive) role of ABA is under challenge. In maize, near isogenic lines (NILs) have been produced for *root-ABA1*, a major QTL that affects root architecture, ABA concentration, and grain yield across different water regimes (Giuliani et al., 2005; Landi et al., 2005). The lines producing more ABA were those showing less yield performance not only under well irrigated conditions, but also under moderate water stress.

Carbohydrates are also claimed to be another critical control factor. The supply of photoassimilates to the developing maize grain is of critical importance during conditions of water stress (Settler, 2006; Tiessen et al., 2006). Carbohydrates, along with other compatible solutes may play a role in osmotic adjustment (OA), maintaining turgor pressure in cells (in leaves as well as in reproductive organs) during water stress. Tang and Boyer (2002) observed a decrease in osmotic potential of about 1.5 MPa in growing maize tissues subjected to water deficit, whereas Bolaños et al. (1993) observed a small OA in the same species. However, several studies in maize found no correlation between accumulation of osmolytes and yield (Bolaños and Edmeades, 1991; Guei and Wassom, 1993). In fact, OA may be incomplete in maize leaves subjected to mild air or soil water deficits (Bouchabke et al., 2006). Nevertheless, osmolytes may still have a role in plant survival, helping to maintain the reversibility of cell dehydration. In fact, osmolytes can also serve as antioxidants and chaperons.

A recent study in tropical maize has shown that different organs possessed distinct metabolite compositions, with the leaf blade displaying the most considerable metabolome changes following water deficiency. However whilst a general increase in metabolite levels under drought stress was shown, including changes in amino acids, sugars, sugar alcohols, and intermediates of the TCA cycle, these changes were not differential between maize hybrids that had previously been designated based on field performance as either drought-tolerant or susceptible. Nevertheless several metabolites displayed conserved responses to drought (Witt et al., 2011).

How to use phenotypic traits

Once diverse phenotypic data have been collected, the question arises as to how to use them. Valuable traits may be combined in a selection index which is, in a way, a quantitative translation of the ideotype concept. Fischer et al. (1989) have already obtained higher yield gains under severe moisture stress conditions in maize by using a selection index combining ASI, relative leaf extension and leaf death score, rather than selecting by yield *per se*. More recently, Bänziger et al. (2000) have proposed to combine data on stressed and unstressed yield, ASI, barrenness, and stay-green under stress in a selection index used by CIMMYT to identify superior genotypes with increases in yield averaging

[1]http://www.ntechindustries.com/greenseeker-home.html
[2]ftp://ftp.dynamax.com/DynamaxPDF/Dualex.pdf

100 kg ha^{-1} per selection cycle. When defining a selection index, weights of different traits are chosen based on variance and heritability and on genetic correlation with yield. In maize, weights typically allocated to secondary traits are +3, −2, −2, −2, and −1 for ears per plant, ASI, leaf senescence, tassel size, and leaf rolling, respectively (Bänziger et al., 2000). Selection indices are continuously redefined, giving attention not only to the target environment for selection, but also with a view to incorporating new secondary traits and innovative tools for their evaluation. Selection indices still have an important empirical bias related to the assigned weights of each of the phenotypic traits considered. A step forward would consist of integrating phenotypic data into a crop model. Models may help to manage phenotyping more efficiently. However, available models are not yet developed well enough to predict differences in performance across genotypes in a reliable manner. Nevertheless more recently a selection index method based on Eigenanalysis and developed by CIMMYT (Cerón-Rojas et al., 2006) has been proposed to calculate the best selection indices for each target environment.

CONCLUSIONS

When drought occurs around flowering, grain number and, consequently, grain yield are affected markedly, particularly in maize. By contrast, losses due to drought during plant establishment are relatively low and can, to some extent, be offset by replanting. Therefore, research on traits affecting inflorescence and grain formation is and will continue to be a main priority in the tropical maize breeding research agenda at CIMMYT (Edmeades et al., 2000b; Bänziger et al., 2006). In such a context, productivity-enhancing traits become more important during flowering and grain filling. If terminal drought is the major constraint, then traits affecting grain filling (e.g., current photosynthesis, stay-green) will be more important (Monneveux and Ribaut, 2006; Monneveux et al., 2008).

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II.1.5 Phenotyping pearl millet for adaptation to drought

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Pearl millet is highly resilient to some of the driest areas of the world, like the Sahel area or fringes of the Thar desert in India. Despite this, there is a wealth of variation in pearl millet genotypes for their adaptation to drought and the object of this paper was to review some related work in the past 25 years to harness these capacities toward the breeding of better adapted cultivars. Work on short duration cultivars has been a major effort. Pearl millet has also some development plasticity thanks to a high tillering ability, which allows compensating for possible drought-related failure of the main culm under intermittent drought. The development of molecular tools for breeding has made great progress in the last 10–15 years and markers, maps, EST libraries, BACs are now available and a number of quantitative trait loci (QTLs) for different traits, including drought, have been identified. Most of the work on drought has focused on the drought tolerance index (DTI), an index that reflect the genetic differences in drought adaptation that are independent of flowering time and yield potential. The DTI is closely associated to the panicle harvest index (PNHI), a trait that relates to a better grain setting and grain filling capacity. Initial work on the DTI involved empirical breeding and selection based on PNHI. A QTL for PNHI has then been identified and introgressed by marker-assisted backcrossing. More recently, a thorough dissection of that QTL has been carried out and shows that high PNHI is related to the constitutive ability of tolerant lines to save water (lower leaf conductance and sensitivity of transpiration to high vapor pressure deficit) at a vegetative stage and use it for the grain filling period. However, there is no contribution of root traits in this QTL. Current work is taking place to map these water saving traits, understand their genetic interactions, and design ideotypes having specific genetic make-up toward adaptation to specific rainfall environments.

Keywords: drought, pearl millet

GENERAL INFORMATION

IMPORTANCE OF PEARL MILLET IN THE HUMAN DIET

Pearl millet (*Pennisetum glaucum* (L) R Br) is a hardy cereal crop, grown mostly in marginal environments in the arid and semi-arid tropical regions of Asia and Africa. It is grown primarily for grain production but is also valued for its fodder, the importance of which has been rising in recent years. Pearl millet is grown in areas with very limited rainfall (300–500 mm in the majority of cases), where crops such as maize or sorghum are very likely to fail in most years. Therefore, pearl millet is a central component of the food security of the rural poor in dry areas.

With regard to nutritional quality, pearl millet is at least equivalent to maize and generally superior to sorghum in protein content and quality, protein efficiency ratio (PER) values, and metabolizable energy levels. Pearl millet does not contain any condensed polyphenols such as the tannins in sorghum that can decrease digestibility. It is deficient in essential amino acids, although it contains 35% more lysine than sorghum (Rooney and McDonough, 1987). Pearl millet grain contains 5–6% oil (Jambunathan and Subramanian, 1988) and is also rich in important micronutrients such as iron and zinc. Moreover, among all cereals, it is the cheapest source of energy, protein, iron, and zinc. These qualities make pearl millet the major contributor to protein, iron, and zinc intake in the regions where it is grown, accounting, for example, for 20–30% of the zinc intake, and 35–50% of the total iron intake of low-income consumers.

Yet, pearl millet remains a food for the poor and is stigmatized by its frequent association with poverty. As a result, the consumer choice is to move away from pearl millet consumption whenever possible. In India, for example, the food use of coarse cereals has been declining during the last two to three decades, owing to a shift in consumption to fine cereals such as rice and wheat. Pearl millet is no exception to this; its consumption per capita and per year in India in 1999–2000 was only 3.7 kg out of 147 kg for all cereals. Despite the decline in overall per capita consumption of pearl millet, it remains an important staple in producing regions, with 66.7 kg per capita consumed in Western Rajasthan, and 62.6 kg in Gujarat. However, even though pearl millet remains at the heart of food security in large areas of the semi-arid tropics, there is a need to diversify its uses, in particular commercially, to make it more attractive and fully use its potential for these regions.

Alternative uses of pearl millet such as for poultry feed are on the increase. Indeed, Smith et al. (1989) report that pearl millet can replace maize in chick diets without affecting weight gain or feed efficiency. The crop residue/straw of dual-purpose pearl

millet is an important source of fodder, accounting for 40–50% of dry matter intake year round, and the only source of feed in the dry months. The use of pearl millet for fodder predominates in low input crop-livestock systems and is likely to become a very important component of the sustainability of such systems. In fact, the growing demand for milk and meat is reflected in the rising price of straw of cereals like pearl millet (Hash et al., 2003).

CULTIVATED AREA AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

The total area cultivated with pearl millet worldwide is 26 million ha, comprising ca 11 million ha in each of West Africa and South Asia, and ca 2 million ha in each of East Africa, Southern Africa, and Brazil [International Crops Research Institute for the Semi-arid Tropics (ICRISAT)] and Food and Agriculture Organization of the United Nations, (FAO, 1996). India is the largest producer, with 9–10 million ha in area and 7–8 million tons of grain production. Pearl millet is cultivated in the hot dry parts of India in regions receiving low annual rainfall ranging from 300 to 800 mm. Between 1970 and 2001, the area under the crop in India declined from 12.1 to 9.4 million ha but production increased from 5.7 to 6.9 million tons due to an increase in yield from 473 to 740 kg ha^{-1}. Pakistan has ca 500,000 ha cultivated to pearl millet. In Africa, the largest pearl millet growing countries are Senegal, Mali, Burkina Faso, Niger, Nigeria, Chad, and Sudan. In West and Central Africa, open-pollinated varieties are cultivated on 16 million ha, with a production of 11.5 million tons and productivity of 800 kg ha^{-1}.

The growth potential of any crop species is a function of its growth rate and the length of the growth cycle. This is obviously conditioned by the agronomic potential where it is grown (relating to water, light, and nutrients). In general, pearl millet is rarely grown in areas enjoying high agronomic potential. It is almost invariably grown in low rainfall areas (van Oosterom et al., 1996a,b) and under marginal fertility which, in fact, results in an incomplete use of the available water (Payne et al., 1990). Thus, environmental factors are usually the main limitations to its growth potential. Even under favorable conditions, pearl millet tends to have a shorter crop cycle than other cereals because it has a "built in" drought escape mechanism (early flowering) inherited from its wild progenitors, having evolved in semi-desert environments with adapted short life cycles. Therefore, pearl millet is short cycled, has a short grain-filling period and has small seed sizes. Its growth potential is no match for other longer-duration cereals growing in favorable environments. Yet, it enjoys a high crop growth rate that confers a fairly high growth potential under optimal conditions (Begg, 1965), relating in particular to its being a C_4 plant, with a large leaf area index (LAI) due to its erect type (Craufurd and Bidinger, 1989), and high radiation-use efficiency (RUE; Squire et al., 1986). The maximum RUE recorded ranges between 2.5 g MJ^{-1} (Squire et al., 1986) and 4.0 g MJ^{-1} (Ram et al., 1999), although most data range between 1.0 and 2.0 g MJ^{-1}. One limitation to RUE is early in the crop cycle, when the LAI is low. There seems to be genetic variation in the rate of leaf appearance, probably because of differences in the base temperature, although this has not been exploited in breeding (Bidinger and Hash, 2003).

Landrace open-pollinated cultivars of pearl millet usually exhibit high levels of vegetative vigor and very high biomass production. However, the harvest index (HI) of these traditionally tall cultivars is only 15–20%. This is largely due to the fact that the photoperiod-mediated change in the total growth duration mostly affects the length of the vegetative period (Carberry and Campbell, 1985). It has been reported that a crop of a local variety of pearl millet, cv Ex-Bornu, grown in Northern Nigeria under high fertility conditions without irrigation, could produce 22 tons ha^{-1} of above ground dry matter 90 days after sowing, although only 3.2 tons of this (14.5%) was grain (Kassam and Kowal, 1975). In contrast, grain yield on a field basis of over 5 tons ha^{-1} was produced by semi-dwarf hybrids maturing in 85 days in India (Rachie and Majmudar, 1980). Experimental yields of up to 8 tons ha^{-1} have even been reported (Burton et al., 1972).

GENETIC AND GENOMIC RESOURCES

Over the past decade, ICRISAT and its partners have made substantial investments in developing mapping populations (Hash and Witcombe, 1994) and in DNA-based molecular marker systems including restriction fragment length polymorphism (RFLP; Liu et al., 1994), sequence-tagged sites (STS; Devos et al., 1995), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) markers (Qi et al., 2000; Allouis et al., 2001), and a bacterial artificial chromosome (BAC) library (Allouis et al., 2001) for pearl millet. These genetic tools have been used to develop a DNA marker-based linkage map for pearl millet (Liu et al., 1994), and to map quantitative trait loci (QTLs) conferring resistance to biotic stresses (Jones et al., 1995, 2002; Morgan et al., 1998) and tolerance to terminal drought stress (Yadav et al., 2002b). They have also been used for: (1) identification of QTLs for flowering time, that appear to be largely responsible for genotype-by-environment interaction (GEI) for grain and stover yield under favorable growing conditions (Yadav et al., 2002a); (2) diversity assessment (Liu et al., 1992; Bhattacharjee et al., 2002); (3) studies of recombination rates (Busso et al., 1995; Liu et al., 1996); (4) analysis of the domestication syndrome (Poncet et al., 2000, 2002); and (5) comparative genomics (Devos et al., 1998; Devos and Gale, 2000).

Levels of DNA marker polymorphism in pearl millet are very high, even between elite inbred parental lines of hybrids adapted to growth in India. The current pearl millet DNA marker-based genetic linkage map covers about 700 cM (Haldane function) distributed across the expected seven linkage groups for this diploid ($2n = 2x = 14$) species, and at least one free-floating pair of linked RFLP markers. However, telomeric regions capping the chromosomes have not yet been mapped (Devos, pers. communication). These DNA marker-based linkage groups have not been definitively linked with the chromosome map of this species (Minocha and Sidhu, 1981; Kaul and Sidhu, 1997), which has been developed over the past 35 years using morphological markers (Anand Kumar and Andrews, 1993) and conventional cytogenetic methods (Jauhar and Hanna, 1998).

Compared to most other grasses, the pearl millet genome appears to have undergone a large number of structural re-arrangements (Devos and Gale, 2000). It seems likely that these re-arrangements could have been associated with the evolution

and maintenance of adaptive gene complexes that permit this highly cross-pollinated crop and its wild progenitors to thrive in environments where they are routinely subject to severe abiotic stresses (e.g., seedling and reproductive heat stress, sand blasting of seedlings, soil nutrient deficiencies and soil toxicities, drought stress). These structural re-arrangements continue to be common in pearl millet, although marker relationships are nearly all colinear across the 10 pearl millet mapping population skeletons mapped to date (Liu et al., 1994, 1996; Devos and Gale, 2000; Azhaguvel, 2001; Kolesnikova, 2001).

Several pearl millet mapping populations of moderate size (120–275 progenies) have been developed at ICRISAT Headquarters at Patancheru, India as sets of F_4 progeny bulks and their F_3 test-crosses, derived from individual skeleton-mapped F_2 plants (Hash and Witcombe, 1994; Hash and Bramel-Cox, 2000). These now involve some 10 pairs of genetically diverse inbred lines of Asian, African, and American origin, selected for QTL mapping of disease resistances (Jones et al., 1995, 2002), abiotic stress tolerances (Howarth et al., 1997; Yadav and Weltzien, 1999; Yadav et al., 1999, 2000, 2002b), grain and stover yield and quality components (Yadav et al., 2002a), and morphological markers (Azhaguvel, 2001). Several of these populations have parents of contrasting Indian and West African origin (e.g., PT 732B × P 1449-2; H 77/833-2 × PRLT 2/89-33; ICMB 841 × 863B; and W 504 × P 310-17) that are expected to differ for many traits.

Being domesticated from wild relatives, i.e., *Pennisetum fallax* and *Pennisetum violaceum* (Stapf and Hubbard, 1934), later reclassified as *P glaucum* (de Wet et al., 1992), living in the southern fringes of the Sahara, pearl millet has a number of characteristics that confer upon it adaptation to drought conditions. The different characteristics and whether these have been exploited for breeding purpose are discussed below.

Tillering and developmental plasticity

This is an attribute that derives from wild progenitors. Pearl millet develops primary tillers, and then secondary tillers from the primary ones, about every 45–50°C days (base temperature of 10°C). Because of this high tillering ability and because the length of the period between floral initiation and flowering is similar, plants have tillers at all stages of apical development at all times (Craufurd and Bidinger, 1988a,b). This developmental plasticity allows pearl millet to compensate for potential failure of the main and primary tillers in the case of a mid-season drought. The secondary tillers would, to a large extent, compensate for the yield loss on the main tillers by a larger number of them developing a panicle, as long as the relief from mid-season drought makes sufficient water available for the secondary tillers to reach maturity (Mahalakshmi and Bidinger, 1986). Because of this plasticity, it is often considered that pearl millet is not affected very much by mid-season drought, provided that moisture is available for the end of the season (Mahalakshmi and Bidinger, 1985a,b).

Flowering time

In most crops, matching plant phenology with the stress environment is a key factor in adaptation to drought. Flowering time, a so-called "drought escape mechanism," is the major component of pearl millet's adaptation to water-scarce environments (e.g., Bidinger et al., 1987a,b; see "Drought Resistance Index" below). The floral morphogenesis stage, GC2, which is the period between floral initiation and flowering, appears to be fairly constant across genotypes of pearl millet. The relative shortness of that period (about 350°C-days (degree-days, which represent a thermal unit of temperature accumulation above a baseline temperature of 10°C for pearl millet—for instance 1 day with a mean temperature of 25° would accumulate 25 – 10 = 15°C-days) allows pearl millet to complete it with relatively limited water (Dancette, 1983). Therefore, earliness is an important drought escape attribute of pearl millet and is, indeed, a major component of GEI. For instance, in the case where the rains stop early, a 1-week difference in the time to flowering between two genotypes brings about a 30% reduction in the grain-filling period and gives the early cultivar more chance to escape drought stress, whereas the late cultivar is likely to suffer the stress before or during reproduction. However, it appears that the prospect of breeding for earliness is limited because of the often poor predictability of rainfall events in the semi-arid tropics. Therefore, there seems to be an optimal time for flowering, suited to the average season length. It is within that particular range of flowering times for any particular environment that other traits likely to improve performance under water-limited conditions must be found.

In West Africa, the sensitivity of pearl millet to the photoperiod (Clerget et al., 2004) is a way that it has evolved to "trigger" an escape mechanism, since it appears that the timing of flowering is closely related to the end of the rainy season. In other words, pearl millet flowers "on time" to ensure that it can complete its maturation cycle with the remaining soil moisture (Kouressy et al., 1998). Any genotype with delayed flowering may be exposed to serious stress conditions during its reproduction phase.

Drought resistance index

It has been found that about 50% of yield variation under drought stress conditions could be explained by differences in the yield potential of genotypes and their flowering time (Bidinger et al., 1982, 1987a). Therefore, data on yield under stress conditions would have little relation to drought tolerance per se without removing the components that are explained by yield potential and phenology. This led Bidinger et al. (1987b) to develop an index, the "drought resistance index" (DRI), in which the effect of yield potential and drought escape (flowering time) are removed by assuming that yield under stress is a function of yield potential (control yield in the test environment), drought escape (proxied by time to flowering), and a residual that accounts for drought tolerance/susceptibility. So that:

$$\hat{Y}s = aYc + bFl + Residual$$

where $\hat{Y}s$ is the predicted yield under stress based on the yield under control conditions, respectively, the flowering time (Fl) and a residual. This residual variation in grain yield under stress that is not explained by either the potential yield (Yc) or by the flowering time (Fl) represents the DRI. The value of the residual (= DRI) is obtained as follows: DRI = Ys – $\hat{Y}s$, where Ys is the actual

grain yield under stress conditions. Therefore, the DRI represent the deviation in grain yield under stress from a baseline yield that depends on the yield potential and flowering time and it therefore allows to compare genotype's performance regardless of their yield potential and flowering time.

A similar approach has been used in other stresses, for example, to separate salinity tolerance per se from yield potential in a set of chickpea germplasm lines (Vadez et al., 2007). The DRI approach has been used in a selection programme for improved drought tolerance (see below), using the panicle harvest index (PNHI), i.e., the ratio of grain yield to panicle yield on a plot basis, as a proxy to assess the DRI.

Rooting ability

Pearl millet is known to be deep and profusely rooted, with the ability to match its rooting to water availability in a very plastic manner, leading to a highly varying root growth to shoot growth ratio, depending on the intensity of water limitation (Squire et al., 1987). During the vegetative period, root growth is very profuse, but little is known about root growth during the post-anthesis period, although it has been reported that it continues well into grain-filling in long-duration West African cultivars (Do et al., 1989). Root penetration rates between 3.5 and 4.5 cm day^{-1} have been reported in sandy soils (Chopart, 1983; Azam Ali et al., 1984). Root depth is dependent on the season length of the cultivar, and can be as deep as 3 m in long-duration varieties, in contrast to only 140 cm in short-duration cultivars (Chopart, 1983). Lateral root spreading is also a major feature of pearl millet, with the soil volume exploration at low planting density being as much as 6 m^3 (Chopart, 1983).

It is often assumed that water uptake and, consequently, water limitation is what limits pearl millet production in a low rainfall environment. However, it has been shown that water may not be the most limiting factor, at least in the sandy soils of Niger, where substantial water storage and drainage have been found below the deep root zone (Payne et al., 1990). This may not be the case in all soils where pearl millet is grown. In fact, roots appear to play an important role in pearl millet genotypes that differ in the presence or absence of a major terminal drought tolerance QTL (Vadez et al., 2005). Further efforts are needed to clarify the extent of the role of the root in the drought tolerance of pearl millet.

Water-use efficiency

Being a C_4 plant, pearl millet already has high transpiration efficiency (TE). However, it seems that the major strategy of pearl millet is to maximize carbon fixation as long as water is available. Therefore, stomatal movements adapt in such a way that the transpiration rate is kept as high as possible (Squire, 1979; Henson and Mahaklakshmi, 1985). It also appears that stomata are sensitive to the vapor pressure deficit (VPD), particularly during the pre-flowering stage, this being related to differences in the abscicic acid (ABA) content of the leaves (Henson and Mahaklakshmi, 1985). In any case, there have been no studies to assess the range of variation in TE across a diverse range of pearl millet cultivars and lines, nor on the sensitivity of stomata to VPD.

At the plot level, water-use efficiency (WUE) values of 300–400 kg biomass ha^{-1} cm^{-1} water have been reported, assuming a full ground cover (LAI > 3–4) (Singh and Singh, 1995). Under low planting density, the WUE usually drops to the range 50–150 kg ha^{-1} cm^{-1}, mostly because of an increased evaporation component (Payne, 1997), itself high because of the fertility-related low sowing density. Therefore, it seems that fertility may be the number one factor to improve the WUE at the plot level.

QTL for terminal drought tolerance

In most of the environments where pearl millet is grown, the crop is facing stress during the grain-filling period, in particular in Northern India (van Oosterom et al., 1996a,b). Therefore, work has focused on identifying QTLs for terminal drought tolerance using the PNHI as a selection criterion.

METHODOLOGY

BREEDING STRATEGY

Possible definitions

The overall goal of a breeding programme for drought stress is, ultimately, an improved genetic yield, or a more stable yield, under drought conditions. These two objectives are not necessarily related. The latter, the stabilization of yield across environments in drought-prone areas, is very important because of the large differences in the coefficient of variation of pearl millet production at the all-India level (26%) compare to that in Rajasthan state (53%), which is characterized by very low and erratic rainfall. There are different ways to assess what is commonly called "drought tolerance," and this depends mostly on how close the assessed trait/parameter is from the final target—an increased genetic yield. Therefore, the approaches to drought tolerance vary. Three categories can broadly be defined, with advantages and drawbacks as highlighted below:

- Drought tolerance is seen purely as a higher and more stable yield under drought conditions, which is fully in line with the ultimate goal. However, in almost all cases, this is related to a large GEI because yield is the integration of many different processes, each of them having a close interaction with the environment.
- Drought tolerance is considered as the maintenance of different development and growth processes, such as leaf expansion, at levels that are close to control well-watered plants. Here, we assume that these would remain well-linked to yield performance. This approach is straightforward and may be easier to capture than yield itself. However, some of these traits can be cumbersome to measure, which may not allow time to assess large numbers of accessions and progenies.
- Drought tolerance can be seen as more upstream, at the organ or cell level, and can be seen as the capacity to sustain certain biological mechanisms, such as maintaining leaf turgor, close to the level of well-watered plants. Measuring such traits requires screening under controlled environment conditions where better management and reproducibility of environmental variation can help reach low levels of GEI. However, the main drawback of this approach is that the traits may be loosely related to the final yield under stress.

Drought resistance index and its relationship to the panicle harvest index

Pearl millet is very resilient to intermittent drought because of its developmental plasticity and its capacity to compensate yield losses on the main tillers with grain production on secondary tillers. For these reasons, it is often considered that mid-season drought is a less important problem for pearl millet, and that tolerance to terminal drought affecting the plant during grain filling is the major target for drought improvement. It has been found that yield under stress is, in part, determined by the yield potential of the material tested plus some escape mechanisms related to its phenology. Bidinger et al. (1987a,b) have encapsulated drought tolerance per se from these non-stress related parameters into the DRI, through correlation analyses of yield data under stress with flowering time and yield under non-stressed conditions. The approach has been to work backwards from measured differences in grain yield in managed drought environments, to readily-measurable aspects of field performance that explain those differences (Fussell et al., 1987). From that point, various yield component parameters were measured under different watering regimes, using some pearl millet varieties differing in their tolerance to terminal drought (**Table 1**). This analysis revealed that the number of grains per panicle and the 100-grain weight were the yield components most affected under terminal drought conditions, leading to a decrease in the PNHI. The PNHI can also be called the threshing index and it represents the proportion of grain weight that a whole panicle contains. A high panicle index reflect that most florets of the panicle have successfully developed in a grain, and that this grain has filled up to its potential.

DRI represents the share of the variation in yield across a set of genotypes that cannot be explained either by differences in yield potential or time to flowering, and is closely related to yield under stress conditions (**Table 2**). Bidinger et al. (1987a,b) have also shown DRI was closely related to the PNHI and therefore, a high DRI was closely related to a higher percentage grain set and better grain filling (**Table 3**), which are the major components of the PNHI. In subsequent works, the PNHI hasthen been used as an indirect proxy for DRI, and is readily and cost-effectively measured. PNHI is a particularly effective variable for post-flowering stress, because the mass of the structural parts of the panicle (which complete their growth prior to flowering) is largely unaffected by stress, whereas the mass of grain is significantly affected by both floret abortion and reduced grain filling (Bidinger and Mukuru, 1995; **Table 1**).

Trait-based conventional approach

This approach was initially used to select genotypes achieving a high PNHI under terminal drought conditions. PNHI was initially tested in hybrid parent breeding, where it was used as a selection criterion by the following procedure:

(1) Conduct bidirectional selection for combining ability for high and low PNHI in replicated potential maintainer (B) and restorer (R) line test cross nurseries (three testers each) grown in managed terminal drought stress environments.
(2) Cross parents selected for high and low PNHI under stress conditions on three different A or R line testers from those used in the original test cross nurseries in which selection was carried out.
(3) Evaluate these test crosses for general combining ability (GCA) for PNHI, grain yield and yield components, in both fully irrigated control environments and in managed stress environments.

In both experiments, the differences between the high and low PNHI selections in the irrigated control environments were small and generally not statistically significant (1% for PNHI itself, 2% for grain yield, and 3% for seed mass). Differences in the terminal stress environment between the high and low selections were

Table 2 | Relations between the drought resistance index (DRI) and various agronomic factors measured either under fully irrigated conditions (control) or under terminal drought stress (stress) [Adapted from Bidinger et al. (1987b)].

DRI versus:	1981	1982
MID-SEASON STRESS		
Control flowering	0.06	0.08
Control yield	0.06	0.06
Stress yield	0.67***	0.58***
Stress/control yield	0.47***	0.46***
TERMINAL STRESS		
Control flowering	0.00	−0.05
Control yield	0.05	0.05
Stress yield	0.55***	0.72***
Stress/control yield	0.55***	0.61***

Stress/control represents the ratio of the yields under each respective treatment.
$^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.0001$.

Table 1 | Consequences of different levels of terminal stress tolerance on pearl millet panicle components and panicle harvest index (PNHI; Source: hypothetical data extracted from Bidinger, 2002).

Genotype level of tolerance	Panicle structural part (g)	Grains per panicle (no.)	Single grain mass (g)	Total grain mass (g)	Total panicle mass (g)	PNHI (%)
Non-stress conditions	5.0	1500	0.0100	15.0	20.0	75
Escape: early flowering	5.0	1500	0.0085	12.8	17.8	72
Tolerant	5.0	1350 (−10%)	0.0085 (−15%)	11.5	16.5	70
Intermediate	5.0	1200 (−20%)	0.0070 (−30%)	8.4	13.4	63
Susceptible	5.0	1200 (−20%)	0.0050 (−50%)	6.0	11.0	55

Table 3 | Relationships between the drought resistance index (DRI) and yield and various yield components under a range of water stress regimes, i.e., a mid-season stress or a terminal water stress [Adapted from Bidinger et al. (1987b)].

DRI versus:	1981	1982
MID-SEASON STRESS		
Grain m^{-2}	0.39^{***}	0.49^{***}
Plant m^{-2}	0.03	0.28^{*}
Panicle $plant^{-1}$	0.08	−0.19
Grain $panicle^{-1}$	0.26^{*}	0.31^{**}
Individual grain mass	0.10	0.32^{**}
Panicle m^{-2}	0.07	0.18
Grain yield $panicle^{-1}$	0.24^{*}	0.34^{**}
TERMINAL STRESS		
Grain m^{-2}	0.46^{***}	0.45^{***}
Plant m^{-2}	−0.12	−0.0
Panicle $plant^{-1}$	0.10	0.07
Grain $panicle^{-1}$	0.53^{***}	0.37^{**}
Individual grain mass	0.25^{*}	0.40^{***}
Panicle m^{-2}	0.10	0.06
Grain yield $panicle^{-1}$	0.69^{***}	0.58^{***}

$^{*}P < 0.05$; $^{**}P < 0.01$; *and* $^{***}P < 0.0001$.

Table 4 | Combining ability for PNHI, yield and yield components of restorer and maintainer lines selected for high (nine lines) and low (nine lines) combining ability for PNHI, in test cross nurseries grown under terminal drought stress at ICRISAT-Patancheru.

	PNHI (%)	Grain yield ($g\ m^{-2}$)	Grain no ($10^3\ m^{-2}$)	Seed mass ($mg\ seed^{-1}$)
RESTORER LINES				
High PNHI selections	64.8	218	31.1	6.86
Low PNHI selections	59.8	192	29.5	6.38
SED	0.4	2.7	3.7	0.69
MAINTAINER LINES				
High PNHI selections	63.6	189	29.7	6.31
Low PNHI selections	60.4	173	28.9	5.93
SED	0.4	2.8	3.9	0.57

Data are means of three test crosses per line and 3 years of replicated evaluations in managed terminal stress environments in the dry season at ICRISAT-Patancheru. [SED, standard error of the difference; Adapted from Bidinger et al. (2000)].

generally statistically significant and of a greater magnitude under stress conditions (**Table 4**). For example, the combining ability of high PNHI selections exceeded that of the low PNHI selections by approximately 5–8% for PNHI itself, by 9–13% for grain yield, and by 6–7% for seed mass. Thus, selection for or against GCA for PNHI under terminal stress had little effect on the combining ability of elite parental lines in non-stress conditions, but resulted in a significant difference in their combining ability for both PNHI itself and for grain yield under terminal stress.

PNHI was also used as a selection criterion in open-pollinated variety breeding for improved tolerance to terminal stress, using S_1 progeny selection in a random mating population (data not shown). The selection was based on PNHI under terminal stress (PNHI/stress) compared to two controls: selection on the basis of grain yield in a paired irrigated control environment (yield/control), and selection of random S_1 progenies (random check). Two cycles of selection were conducted, using 810 S_1 progenies from the parent population in cycle 1, and 400 S1 progenies from each of two subpopulations (formed from 50 progenies from the first cycle) representing the PNHI/stress and yield/control selection alternatives, in cycle 2. Overall, after two cycles of selection, selecting experimental varieties on the basis of composite progeny PNHI in terminal stress environments improved PNHI by 1–3% and grain yield by 2–8% under terminal stress (in comparison to control experimental varieties, based on randomly selected progenies).

Trait-based molecular breeding approach in current use

This is the current approach to pearl millet breeding for drought tolerance. It is based on the fact that PNHI remains a highly complex trait for which a molecular approach can increase precision during the selection process. For molecular breeding, the development of recombinant inbred lines (RILs) is needed to link phenotypic data and marker data, and potentially identify QTLs, i.e., genome portions that are related to phenotypic data. Prior to that, the parents used for crossing should comply with a number of characteristics to maximize the chances of discovering RILs. They should: (1) be chosen from large number of accessions; (2) have maximum phenotypic contrast; (3) have large genotypic contrast; and (4) be similar for certain phenotypic traits that can interact with the trait of interest (yield), such as time to flowering or photoperiod sensitivity.

Although parents chosen for crossing and development of RILs, may display large phenotypic contrast, they may have little DNA-level polymorphism. Such a situation limits the marker coverage that can be used to map the genomic portion responsible for the observed phenotypic differences. Having a limited number of polymorphic markers will, in most cases, increase the cost and time to get QTLs, and lower the resolution of the QTLs. An alternative in such cases is to develop different types of marker with a higher resolution, such as single nucleotide polymorphisms (SNPs). Finally, the crossing of parents may involve certain criteria that can have a strong influence on the response to drought or salinity. Indeed, we have shown earlier that the yield under terminal drought was a function of the yield potential under no stress, a drought escape mechanism, and DRI per se. Therefore, it not is advisable to cross parents with large variations in yield potential or flowering time if the intention is to develop a RIL population to map terminal drought tolerance.

This approach has been used successfully for the identification of terminal drought tolerance QTLs (Yadav et al., 2000, 2002b, 2003), and the introgression of a terminal drought tolerance QTL into the background of the popular pearl millet hybrid HHB67 to create the new hybrid HHB67-improved. This terminal drought tolerance QTL has a major effect, explaining over 30% of the yield variation under terminal drought. It is located on linkage group 2 (LG2) (**Figure 1**). Further efforts are still needed to reduce the size of that QTL to improve the precision of its introgression. Better

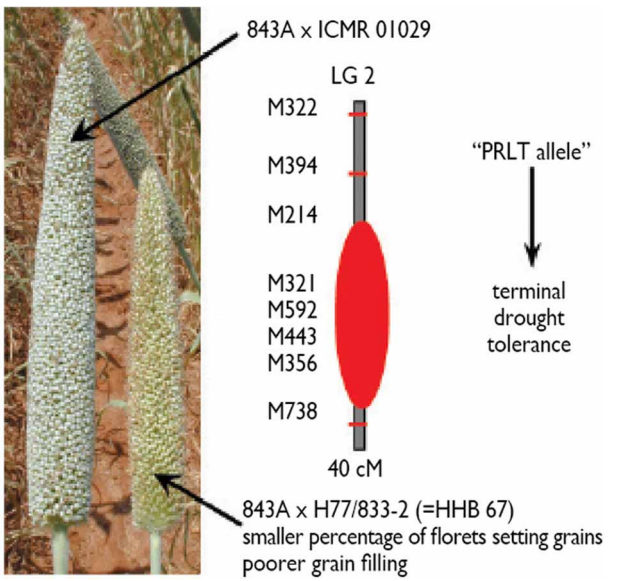

FIGURE 1 | Phenotypic expression of stress effects on the panicle of sensitive HHB67 (= 843A × H77/833-2) and an improved version of this hybrid (843A × ICMR 01029), and showing a better seed set and a better grain filling in the tolerant hybrid. ICMR01029 is an introgression line with a terminal drought tolerance on linkage group 2 from donor parent PRLT/89-33, after four backcrosses using H77/833-2 (Source: Hash, unpublished).

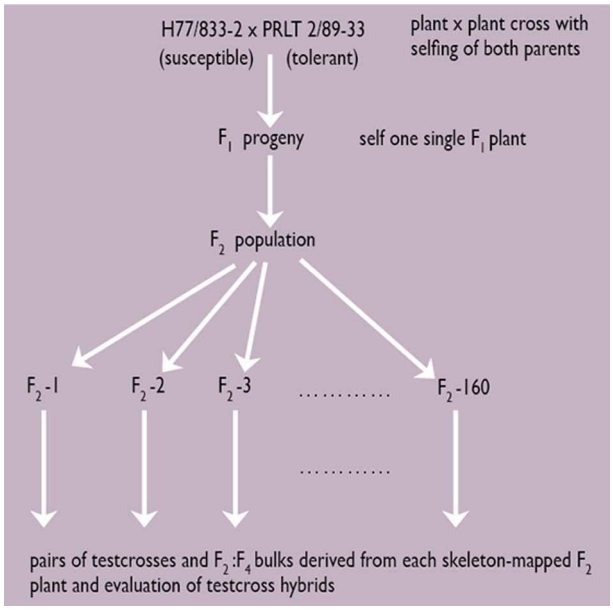

FIGURE 2 | Strategy for the development of a skeleton map and identification of drought tolerance QTLs (Source: Hash, unpublished).

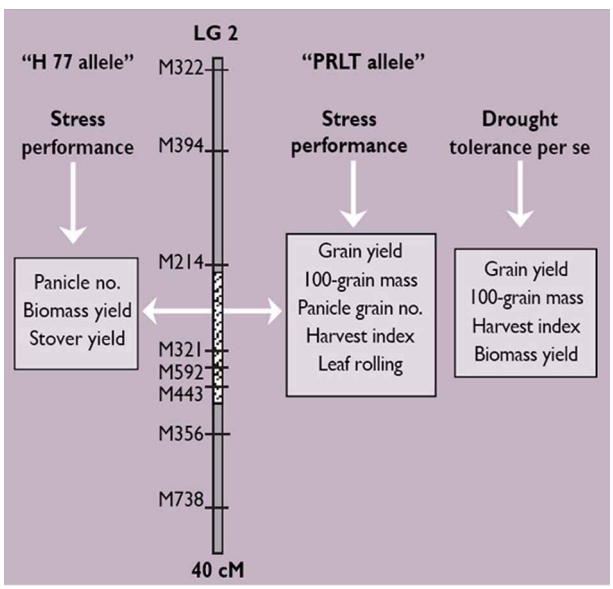

FIGURE 3 | The outcome of QTL mapping: identification of a set of QTLs for different traits (leaf rolling, biomass yield, dry straw yield, panicle number, panicle grain number, 100-grain mass, grain yield, and harvest index) in pearl millet in three drought nursery experiments.

marker coverage of the QTL region would be needed for that, and work toward that aim is in progress.

Scheme for RIL development and testcross hybrid testing

For terminal drought tolerance, contrasting parents PRLT 2/89-33 (tolerant) and H77/833-2 (sensitive) were identified and crossed. Then, selfing was done for two generations. Test crossing was done on F_4:F_2 derived progenies, using several pollinators, and measuring the GCA for PNHI (**Figure 2**). In doing this, two parents and 19 product lines all combined to five different testers were used, giving 105 Drought Tolerance QTL-near isogenic line (NIL) testcross hybrids. These materials were evaluated during the summers of 2003 and 2004 in the drought nursery at ICRISAT-Patancheru under three moisture regimes (fully irrigated conditions; early stress imposed by stopping irrigation at booting; late stress imposed by stopping irrigation at flowering). The experimental design is an alpha design with two-row plots and 4 m rows, into three replications. Usually, many QTLs are identified, each differing in the percentage of the variation in phenotypic data that they explain. QTLs can be identified for many different traits, some of these collocating at the same portion of the chromosome (**Figure 3**).

The likelihood of odds (LOD) score assesses, in part, the importance of a QTL. The higher the LOD score, the more significant is the QTL. Among the many usually identified, one or two major QTLs are chosen to be introgressed into a genetic background of either elite germplasm, or locally adapted germplasm. A few rounds of backcrosses are usually needed to end up with introgression lines having maintained most, if not all, of the recurrent parent genome, except for the portion flanked by the marker pair (**Figure 4**). Results of the whole effort are represented in **Figure 1**, where the introgression of a major QTL for terminal drought tolerance from donor parent PRLT/89-33 in the background of sensitive parent, high tillering H77/833-2, led to

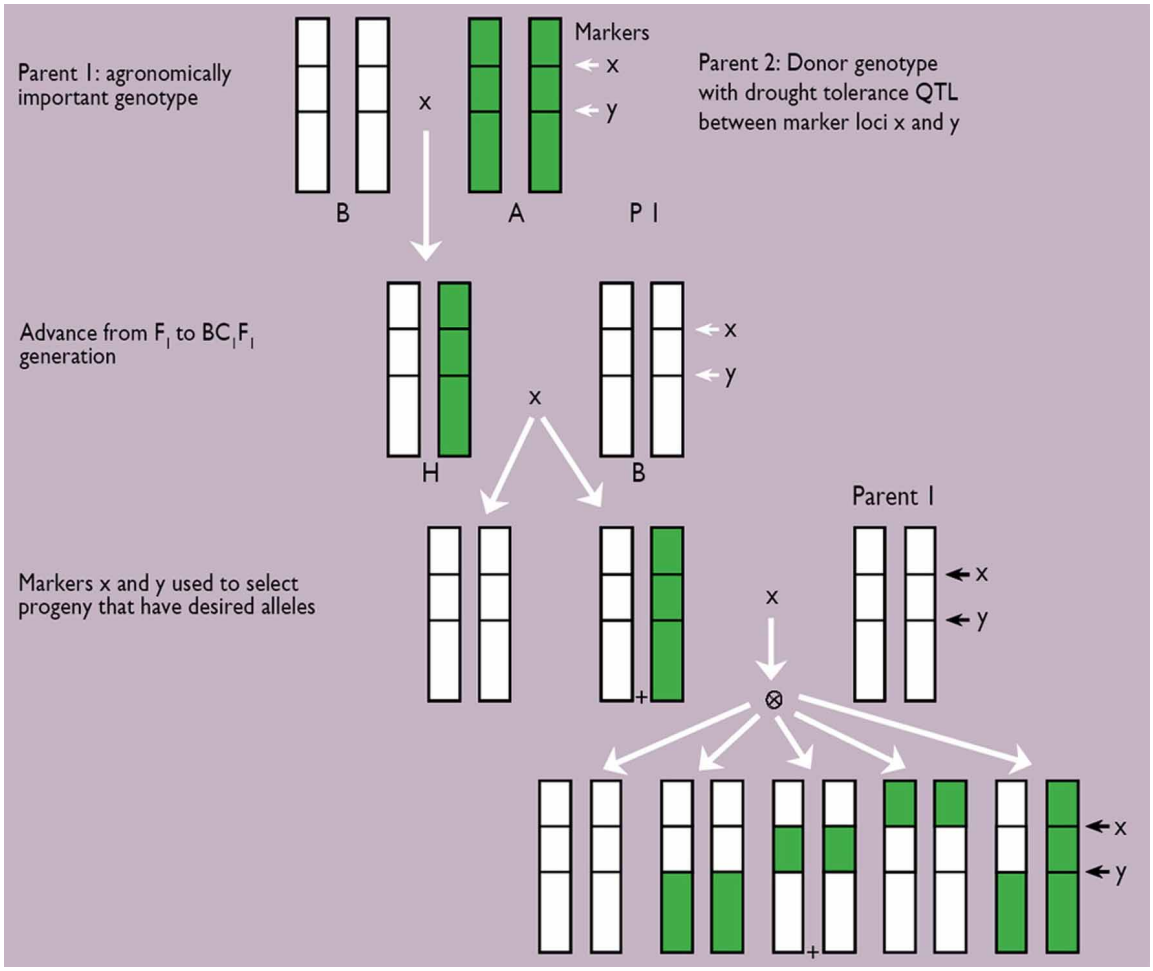

FIGURE 4 | Scheme for marker-assisted backcrossing of desired (+) segregants. A QTL was introgressed after four rounds of marker-assisted backcrossing (Source: Hash, unpublished).

panicles with a higher percentage of seed setting, and a high 100-grain weight. The output is a genotype that looks essentially like the recurrent parent but with a higher threshing index of the panicle.

TRIAL PLANNING

The creation and genotyping of mapping populations is often the most expensive part of the overall effort, but its ultimate success depends on the effectiveness of the phenotyping procedure in detecting repeatable, highly heritable differences among recombinant lines, that permit the identification of robust QTLs. Drought is a particularly difficult topic for molecular mapping, because it is not possible to define or measure tolerance with the same clarity or precision as disease resistance or morphological or physiological traits. Nor is it easy to manage experimental drought or saline environments with a high level of control and repeatability. One key aspect in the implementation of a phenotyping experiment is to carefully exclude any possibility of non-genetic variation in the measured traits. Therefore, extra effort is needed in the conceptualization, design and management of phenotyping programmes for drought, to maximize the chances of identifying highly contrasting materials and, further, QTLs that will be useful in the future improvement of tolerance in the target crop and in the target environment.

WATER STRESS MANAGEMENT AND CHARACTERISATION

Pearl millet is usually grown in areas receiving less than 500 mm of rainfall annually. It is usually planted at the start of the rains, either in the Sahelian areas or the arid semi-desert areas of north-east India (Rajasthan) and southwest Pakistan (Bidinger et al., 1987a,b). Because the duration of the rains is normally shorter than the duration of the crop, the stress that millet commonly experience is a terminal drought, whereby seed filling occurs with plants depending on the moisture available in the soil. We have previously seen that the phenotyping of terminal drought tolerance uses three water regimes: full irrigation; early stress imposed by stopping irrigation at booting; and late stress imposed by stopping irrigation at flowering (Serraj et al., 2005). However, the intensity of stress imposed is also very important, and certain genotypes can react differently to different intensities. Therefore,

line source treatments have also been set up. These are based on the fact that sprinkler irrigation provides a decreasing supply of water when moving away from the sprinkler head, and this decline is roughly linear (unpublished). This allows imposition of a gradient of irrigation and, therefore, a gradient of stress intensities. This approach (**Figure 5**) has been used to assess a limited number of promising drought tolerant QTL–NIL test-cross hybrids.

Protocol and measures

The DRI is a measure of tolerance to terminal drought conditions, as explained previously. Experiments have been conducted to expose pearl millet to a range of environments and different intensities of stress from flowering onwards. In each of the treatments, the yield components (grain number per panicle and surface area, grain size) are measured, as well as time to flowering and to maturity. To separate out the effects of yield potential and phenology from the yield under stress to obtain the DRI, the following equation is used:

$$Ys = aYc + bFl + cDRI + E$$

DRI is usually well correlated to PNHI and, therefore, PNHI is routinely calculated from the yield components.

PLANT WATER STRATEGY

Accurate field or controlled environment phenotyping of germplasm accessions or mapping populations for traits as complex as drought tolerance is almost certainly the limiting factor in our ability to detect contrasting materials and to discover molecular markers for such traits. The PNHI trait remains complex, and its measurement under field conditions remains subject to field variability and the usual experimental errors associated with field evaluations. For that reason, secondary traits that correlate well with field performance and that can be measured under more controlled conditions are very useful. Several hypotheses can explain differences in the PNHI. Better grain filling during the post-anthesis period could be due to water saving in the soil profile from the time it is wet. The water saved would then be available later on for grain filling. Another possibility is that deeper or more profuse rooting would allow the crop to sustain water uptake and continue grain filling in the latest part of the grain-filling period. Other hypotheses to explain differences in PNHI and, eventually, differences in grain yield under terminal drought stress can be formulated. Such hypotheses lead to the identification of putative secondary traits.

FIGURE 5 | Typical line source experiment, which allows the imposition of a gradient of watering regimes, from fully irrigated conditions close to the irrigation line, to severely stressed at the point most distant from the line (Source: SMH Rizvi, unpublished).

PHENOTYPING TRAITS

The hypotheses above are currently being tested. For instance, we have found that the rate of water loss per unit of leaf area and time was lower in PRLT/89-33, our terminal drought tolerant parent and donor for the major drought tolerance QTL on LG2, compared to H77/833-2, a terminal drought sensitive genotype (Vadez et al., 2007). These differences were found under well-watered conditions and were consistently found across experimental seasons, at both the pre-flowering and post-flowering stages. This trait, which appears to be constitutive and also relatively easy to measure, is very suitable for phenotyping the RIL progenies of the cross between PRLT/89-33 and H77/833-2.

We also measured the root depth and root length density in a set of pearl millet genotypes contrasting for terminal drought tolerance, and including PRLT/89-33 and H77/833-2, as well as terminal drought sensitive 841B and tolerant 863B, along with some introgression lines with the DT QTL from PRLT/89-33 in the background of H77/833-2. Root traits were measured under water stress conditions, and all terminal drought tolerant materials appeared to have more profuse rooting in the deep soil layers than did sensitive materials (Vadez et al., 2007). In contrast, there seemed to be little difference under well-watered conditions. Therefore, rooting appears to be an adaptive trait that tolerant pearl millet genotypes "develop" under stress conditions. However, the measurement of rooting was time-consuming and showed fairly large experimental errors. Since the putative role of deeper rooting would be to sustain water uptake during the latest part of the grain-filling period, the phenotyping of root trait differences would better be based on the volume of water uptake during the grain-filling period.

Phenotyping work on pearl millet has, so far, focused on terminal drought tolerance. QTLs have been identified under the screening conditions of the drought nursery at ICRISAT-Patancheru. Soils are heavy and deep Alfisols, with a significant water-holding capacity (well above 200 mm), thereby allowing the secondary traits described above to be relevant under such conditions. A similar situation may also prevail in pearl millet cultivation in certain areas of West and Central Africa endowed with heavy soils. However, the terminal drought tolerance QTL identified under these particular drought conditions may not be suited to other types of drought environment, for example, those prevalent in semi-desert areas such as northwestern India, or in areas of West and Central Africa, where sandy soils with limited moisture availability dominate.

Therefore, it is crucial in a phenotyping exercise to ensure that the traits that would be measured are the traits that are relevant for the target area. In that respect, the past 30 years have taught us a lot with respect to traits for adaptation to terminal drought tolerance. We feel that more needs to be learnt about traits that

would contribute to better drought adaptation to harsher environment. Only the improvement of phenotyping capacities in all representative types of stress environment would allow us to understand the specificity of each trait and would improve the accuracy of trait-based marker-assisted breeding for drought.

CONCLUSIONS

Phenotyping remains the foundation for success in every marker-assisted selection approach, particularly for such complex trait as drought. Precise and accurate phenotyping methods have been set up to phenotype the response to terminal drought in pearl millet, using PNHI as a proxy for an increased yield under terminal drought, independently of yield potential and time to flowering. Such precise phenotyping was possible because of the large human and physical investment made in that activity at ICRISAT's Headquarters at Patancheru. This has led to the identification of a major terminal drought tolerance QTL on LG2 of the pearl millet genome. The subsequent introgression of that QTL into the background of a sensitive hybrid, HHB67, has led to an improved version of that hybrid, HHB67-improved. This QTL remains large in size and, therefore, relatively difficult to introgress. Secondary traits for the high PNHI of the terminal drought tolerant lines would be needed to refine the QTL interval and facilitate its use in modern breeding. More work would also be needed to identify the traits involved in better performance and resilience of pearl millet under other types of drought environment. This would require significant investment in human and physical capacity to phenotype in these other environments for modern breeding to be used.

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Dr Fran Bidinger, a key contributor to this chapter, passed away in April 2008. Fran dedicated his life to investigations on pearl millet, where he paved the way to current advances in drought research. Fran was an enthusiastic and dedicated scientist, always curious and keen to discuss science, and who did science until the end, insisting on delivering a course on drought phenotyping while unwell, on his last day at work. He will be remembered for his kindness, humility and scientific rigor. I feel privileged to have had him as a "father" in my initial years at ICRISAT, always patient to explain the unexplained.

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Phenotyping common beans for adaptation to drought

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Common beans (*Phaseolus vulgaris* L.) originated in the New World and are the grain legume of greatest production for direct human consumption. Common bean production is subject to frequent droughts in highland Mexico, in the Pacific coast of Central America, in northeast Brazil, and in eastern and southern Africa from Ethiopia to South Africa. This article reviews efforts to improve common bean for drought tolerance, referring to genetic diversity for drought response, the physiology of drought tolerance mechanisms, and breeding strategies. Different races of common bean respond differently to drought, with race Durango of highland Mexico being a major source of genes. Sister species of *P. vulgaris* likewise have unique traits, especially *P. acutifolius* which is well adapted to dryland conditions. Diverse sources of tolerance may have different mechanisms of plant response, implying the need for different methods of phenotyping to recognize the relevant traits. Practical considerations of field management are discussed including: trial planning; water management; and field preparation.

Keywords: Phaseolus, field technique, abiotic stress, breeding, stress physiology

GENERAL INFORMATION

IMPORTANCE OF BEANS IN THE HUMAN DIET

Common beans (*Phaseolus vulgaris* L) are the most important grain legume for human consumption (Broughton et al., 2003; Beebe, 2012). Given that most protein consumed by the poor is from plant sources, being protein-rich, beans play an especially significant role in the human diet. Although far less important than cereals as a source of calories, beans often supply a significant proportion of carbohydrates [Food and Agriculture Organization of the United Nations (FAO), 2001]. Like other legumes, they are also a key source of minerals, especially iron (Graham et al., 2007).

In Latin American countries, national per capita consumption of beans is typically between 12 and 18 kg per year, but this does not reflect differences in urban versus rural consumption, nor income differences (Broughton et al., 2003). In rural Nicaragua, for example, per capita consumption can be as high as 36 kg per year among the more affluent, whereas the rural poor cannot satisfy their needs and consume about half that amount (FAO, 2001). In Africa, bean consumption can be as high as 60 kg per capita per year in countries like Rwanda or in western Kenya. However, here as well, availability and cost often limit bean consumption and real consumption levels may be lower. In Mexico, an estimated per capita consumption of 12 kg per year of dry beans has been registered, but almost 100,000 tons are transformed into canned beans with a yield of 3.5 kg of canned bean product per kg of dry beans. The need for ready cooked bean products is increasing, due to the inclusion of an increasing number of women in the work force.

CULTIVATED AREA AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

Total world production cannot be calculated with certainty due to confusion with other legumes in some of the data, but is between 11 and 12 million tons (FAO, 2006). Latin America is the region of greatest production of common beans, representing about 50% of world volume, followed by Africa with 25%. Brazil, Mexico and the United States of America are the three largest producers in the western hemisphere. In Mexico, runner beans (*Phaseolus coccineus* L) are a relatively important crop in the highlands, but data on this species are included with those for common beans.

In Africa, most bean production is found in the eastern and southern highlands, extending from Ethiopia to South Africa, with Kenya being the largest producer in the region. In West Africa, bean production is localized in specific environments, with Cameroon being the principal producer. Beans are a minor crop in Europe and North Africa, concentrated around the Mediterranean, in Spain, Italy, Morocco, Algeria, and the Balkan states. In Asia, beans are spread in an extensive band from Turkey through Iran and the Himalayan foothills, and east through Myanmar and China. India is cited as a major producer of common beans (FAO, 2006), but these figures undoubtedly include other legumes (Singh, 1999).

Beans are traditionally a small farmer crop, often grown in complex farming systems in association or rotation with maize, sorghum, bananas, or other crops (Broughton et al., 2003). The range of growth habits (from determinate bush types to vigorous climbers), and the range of growth cycles (from 2 to 10 months in length) make beans a crop that fits many production niches.

Nevertheless, beans are becoming increasingly commercial with the trends of urbanization and market globalization. These trends impact on both small farmers who market excess production to local urban centers, and large commercial farmers in Argentina, China, Mexico, the USA, and Canada with an eye to export markets. Small farmers are also organizing themselves to tap into opportunities to export in countries like Bolivia, Ethiopia, Nicaragua, and Peru, each of which report from US$20–100 million in bean exports annually.

Experimental yields of bush beans can be 4 t ha^{-1} or more, while climbing beans can reach 6 t ha^{-1} under a trellis system. On-farm yields, as expected, are far below experimental yields. National averages in Latin America range from 600 to 950 kg ha^{-1}, with a long-term tendency to increase, while national averages in Africa are similar but tending to decline (FAO, 2006). Total production in both regions is increasing. This trend in Latin America is driven by increasing yields on a stable total area, whereas in Africa, increased production reflects an increased area planted despite declining yields. Anecdotal reports suggest that declining yields in Africa are due to the extension of the crop into marginal production areas with poorer soil fertility and/or drought (Buruchara, pers. communication). This highlights the importance of attention to abiotic stress resistance, including water deficits, which will be emphasized in this review.

As expected, countries with technified agricultural systems present much higher yields than tropical and developing countries. In the USA, average yields in the past decade range from 1.64 to 1.96 t ha^{-1} (USDA, 2007), albeit with significant regional differences. Similarly, average yields in Argentina and Colombia are about 1.2 t ha^{-1} due to varietal selection, and in Brazil under intensive management and irrigation, yields average 1.8 t ha^{-1} (Broughton et al., 2003). Although well above yields in most developing countries, these are still as much as 3 t ha^{-1} below the yield potential of the crop. While it is clear that the yield gap is a generalized phenomenon, yields in drought-endemic regions are typically lower. The largest single drought susceptible production area in the world is in highland Mexico, where more than a million hectares of beans are cultivated, and where yields fall below 0.4 t ha^{-1} in dry years. In Northeastern Brazil, which accounts for another million hectares, yields are around 0.45 t ha^{-1}, which is far lower than the 1.5 t ha^{-1} obtained in the more developed southern state of São Paulo (Conab, 2007).

Low yields are undoubtedly due in part to the direct effect of droughts, and in part to the fact that dry areas are also poverty hot spots where there is less capital investment. The Pacific coast of Central America, where most of the population lives, is another drought-prone region, as are Haiti and eastern Cuba in the Caribbean. In Africa, an estimated 682,000 hectares of beans are cultivated in semi-arid environments, with annual yield losses to drought of 781,000 tons across all environments (Wortmann et al., 1998). The drought-endemic area stretches from eastern and central Ethiopia, south through eastern Kenya and the Rift Valley, and through northern Tanzania. Kenya has the largest area of beans under threat of drought, often resulting from failure of the short rains. Occasionally, severe droughts also occur in Southern Africa, affecting Malawi, Zimbabwe, and Mozambique. They are often associated with "El Niño" weather events. Although drought may be less frequent than in eastern Africa, southern Africa enjoys only one rainfed cropping season per year. Therefore, droughts in this region have an especially tragic impact. Moreover, climate models predict that this region will become drier with global climate change (Williams et al., 2007).

GENETIC AND GENOMIC RESOURCES

The genetic structure of common beans has been reviewed frequently (Gepts and Debouck, 1991; Singh et al., 1991; Broughton et al., 2003), and is only summarized here. Cultivated common beans display a well-defined genepool structure that originates in the wild bean ancestor. Wild common beans grow as a viny annual herbaceous plant in a sub-humid premontane forest ecology from northern Mexico to northern Argentina (Toro et al., 1990). Genetic analysis using amplified fragment length polymorphism (AFLP) reveals at least four wild bean genepools, centered in: (1) Middle America (Mexico and Central America); (2) Colombia; (3) western Ecuador and northern Peru; and (4) the southern Andes (Tohme et al., 1996). Cultivated bean genepools derive principally from the Middle American wild bean pool, and the southern Andean pool. Additionally some incipient domestication or introgression appears to have occurred in Colombia (Gepts and Bliss, 1986; Chacón et al., 1996; Islam et al., 2001b).

Wild common bean populations are under threat due to urbanization and intensive cattle grazing, and they occur disproportionately in regions where climate change will impact on natural ecosystems (Williams et al., 2007). The Andean and Middle American genepools of cultivated beans are distinguished clearly by DNA markers (for example, Beebe et al., 2000; Islam et al., 2004; Blair et al., 2006a), by plant and seed morphology (Singh et al., 1991), by reaction to diseases including anthracnose (Mahuku et al., 2003) and angular leaf spot (Mahuku et al., 2002), and by grain mineral content (Islam et al., 2001a), among other traits.

The major genepools in turn have been divided into races based on plant morphology, adaptation range and agronomic traits. The Middle American genepool was divided into the races Durango (prostrate bush types with medium-sized seed from dry highland Mexico), Jalisco (climbing beans from the moist highlands of central Mexico), and Mesoamerica (small seeded types, mostly bush habits, from lowland Central America and Mexico; Singh et al., 1991). Beebe et al. (2000) suggested the existence of a fourth race—Guatemala (mostly climbing beans from Guatemala and southern Mexico)—as well as some systematic variation within races. Chacón et al. (2005) found that the races Durango and Jalisco shared a common chloroplast DNA pattern, while the races Guatemala and Mesoamerica each presented a distinctive pattern, emphasizing their evolutionary uniqueness. Díaz and Blair (2006) found a dichotomous structure in the Middle American genepool, with grouping of the Durango and Jalisco races apart from the race Mesoamerica, and novel diversity in some climbing bean accessions potentially from the race Guatemala.

Three races have also been proposed within the Andean genepool (Singh et al., 1991), but their differentiation by restriction fragment length polymorphism (RFLP) or random amplified

polymorphic DNA (RAPD) markers is not as clear as in the Middle American genepool (Becerra Velásquez and Gepts, 1994; Beebe et al., 2001). All such races display a common chloroplast DNA composition, suggesting that a single population might have been domesticated (Chacón et al., 2005). Andean races can, however, be distinguished by microsatellite alleles (Blair et al., 2007), and are known to be different in terms of growth habit prevalence and adaptation ranges. They include the races Peru (predominantly highland climbing beans), Nueva Granada (mostly bush beans with mid-altitude adaptation), and Chile (prostrate bush or weak climbers, with temperate adaptation to higher latitudes). Significant introgression has occurred into the Andean genepool from Middle American types that have filtered into the northern Andes since pre-Colombian times (Islam et al., 2004; Blair et al., 2007). Cultivars of the Andean genepool are distinguished by more attractive colors and by larger seed (35–50 g 100 seed^{-1}, compared to 20 g 100 seed^{-1} for Mesoamerican beans or 30 g 100 seed^{-1} for Durango types; Singh et al., 1991). A unique white bean cultivated in Spain, the Fabada bean, has grain as large as 100 g 100 seed^{-1}! These traits add value to Andean beans and make them an attractive crop for sale. In regions of Africa in which both Andean and Middle American beans are grown, many market-oriented farmers would prefer Andean types, whereas small seeded Mesoamerican types might be used for home consumption.

Over the past decade and a half, the genepool structure and the concept of races within genepools has become the intellectual framework for the improvement of common beans. Breeders now routinely speak of commercial grain classes within the context of races. It is appreciated that crosses among genotypes of the Andean and Middle American genepools represent wide crosses with a low probability of success. There is a sense of which parental types can combine more readily to produce useful progeny. There is a structure within which to explore genetic diversity systematically for useful traits. This represents a significant systematization of knowledge about bean genetic resources accompanied by practical application. Unanswered questions regarding the genetic structure of common beans and its significance include: the extent and potential value of wild and weedy types from Colombia (Beebe et al., 1997); the existence of other possibly unique wild bean populations in Nicaragua, Venezuela, and/or parts of Peru; the origin and potential value of Brazilian landraces of the Andean genepool (Beebe et al., 2001); and the origins of the races, especially Chile and Guatemala (Díaz and Blair, 2006; Blair et al., 2007). A deeper understanding of genetic diversity and population structure within cultivated common beans is necessary both for greater utilization of useful germplasm but also for association mapping of valuable traits such as drought tolerance.

In addition to wild ancestors in the primary genepool, common beans enjoy an extensive secondary genepool that can be crossed quite readily with *P. vulgaris*. Singh (2001) has reviewed the use of these genetic resources extensively. Briefly, two other cultivated species, runner beans (*P. coccineus)* and year-long beans (*Phaseolus dumosus*; = *polyanthus*) are found in this genepool, as well as wild species such as *Phaseolus costaricensis*. All three species are vigorous vines with perennial or semi-perennial tendencies, and are found in moist environments. Wild or escaped types experience intense competition from surrounding vegetation, making aggressive vegetative growth necessary for survival. Of these species, runner beans display wider genetic variability, even at the level of the chloroplast (Tovar, 2001). Runner beans and year-long beans have both been employed as sources of resistance to a wide array of bean pathogens (Singh, 2001), although their use for other traits has been very limited. Some accessions of *P. coccineus* are very tolerant of aluminum toxicity in the field and in greenhouse hydroponic systems [Centro International de Agricultura Tropical (CIAT), 2005; Butare et al., 2011]. Field observations and subsequent greenhouse studies of root systems have revealed that runner beans have thick roots that might have a better potential to penetrate compacted soil than common beans. These are traits that could well contribute to drought resistance, and merit further investigation.

Tepary beans (*Phaseolus acutifolius*) are a fourth domesticated species of the genus pertaining to the tertiary genepool, and are native to the desert highlands of northwest Mexico and the southwest of the USA. As such, they are extremely resistant to drought, heat and cold (Martinez-Rojo et al., 2007), and have been viewed as a potential source of drought resistance for common beans. Greenhouse studies of tepary bean root systems reveal extremely fine roots that penetrate soil rapidly and branch profusely, offering quick access to limited soil water reserves (Butare et al., 2011). Crosses between common beans and tepary beans have normally been difficult, requiring the use of *P. vulgaris* cytoplasm and embryo rescue to obtain F_1 plants. In spite of difficulties, tepary beans have been used as a source of resistance for biotic constraints, especially common bacterial blight (Coyne et al., 1963; McElroy, 1985). An innovative breeding method called "congruity backcrossing" involving alternate crossing to common bean and tepary bean parents, has permitted a greater degree of cross compatibility between these two species, possibly by gradually improving chromosome pairing (Haghighi and Ascher, 1988). A modification of this system now permits crossing into *P. acutifolius* cytoplasm (CIAT, 2002b). An evaluation of introgression from the tepary bean genome shows that DNA markers in the tepary bean parent can be transferred to the interspecific progeny (Muñoz et al., 2004). Thus, the introgression of drought resistance might be feasible. Modest levels of drought resistance have already been introgressed from tepary beans into common beans, but not yet at the levels of tepary beans, nor at a levels superior to that available within *P. vulgaris* (CIAT, 2002a). As an alternative, the cloning of genes from tepary beans could lead to wider exploitation.

Lima beans (*P. lunatus*) are the fifth domesticate within the *Phaseolus* genus. Lima beans grow over an even wider range of environments than common beans, since they are very tolerant of heat and edaphic problems. It is tempting to introgress traits from lima beans into common beans. However, efforts to date to cross lima beans with common beans have resulted in no more than totally sterile F_1 plants (Mok et al., 1978). For the foreseeable future, it will be more productive to view lima beans as crop in its own right, unless genes can be extracted from it through molecular biological techniques.

With the advent of DNA markers in the 1980's, multiple mapping populations were created. A list of 14 such populations has been published, including 10 of recombinant inbred lines (RILs; Broughton et al., 2003). The first two maps based on RFLP, and therefore of wider accessibility, were published in the early 1990's (Vallejos et al., 1992; Nodari et al., 1993). Many subsequent maps were based on RAPD markers. Eventually, a total of five maps were harmonized around a core map (Freyre et al., 1998). A sixth map created at CIAT has, likewise, been cross-referenced with the core map (Blair et al., 2003). Cross-comparable RFLP, RAPD, and microsatellite markers form the basis of these maps. A sizable set of microsatellites has been evaluated for polymorphism across parents of multiple mapping populations, as a means of integrating genetic studies through known map positions (Blair et al., 2006a).

CIAT holds other sets of RILs, several created specifically for drought studies, and others for studies of root structure and function in plant nutrition (Blair et al., 2011). A series of publications on roots and phosphorus nutrition resulted from a population of DOR 364 × G 19833 (Liao et al., 2004; Yan et al., 2004; Beebe et al., 2006a). This population proved to be especially useful to reveal the relationship between the acquisition of soil phosphorus and specific root traits, by demonstrating the association of each with common genomic regions. This methodology can readily be extended to drought resistance traits. Other RIL populations include drought-resistant parents BAT 477, G 21212, ICA Quimbaya, and SEA 5.

However, most maps with ample genome coverage have been based on segregation of crosses between Mesoamerican and Andean genotypes to facilitate abundant DNA polymorphism (Blair et al., 2006a). Many polymorphic markers in these crosses are genepool specific, and do not discriminate between DNA of genotypes from the same genepool or race. Since most genetic improvement is carried out within genepools, such markers are seldom useful for gene tagging, mapping or marker-assisted selection (MAS). Thus, there is still a need for a larger set of simple sequence repeat (SSR) or other markers of similar attributes that will permit better genome coverage of crosses among genetically similar materials. CIAT and partners are currently mining additional genomic and complementary DNA (cDNA)-based SSRs for marker development others are developing SNP platforms.

Genomic and cDNA clones have been useful for marker development and gene mining, and they form the basis for some recent sequencing projects. These include an expressed sequence tag (EST) effort consisting of 22,000 sequences derived from four cDNA libraries made from the Mesoamerican genotype, Negro Jamapa, and one cDNA library made from the Andean genotype, G 19833 (Ramírez et al., 2005). Given that these sequences are from drought-susceptible genotypes and represent 3′ end sequences, one option is to develop more cDNA libraries for drought-tolerant genotypes and obtain a set of full-length and 5′ end sequences. In addition, a bacterial artificial chromosome (BAC) library made for the genotype G 19833 is being end-sequenced as part of a physical mapping project for common beans, which will be a useful source of additional markers and partial gene sequences. Finally, for functional analysis of candidate genes of interest, TILLING populations are being created between CIAT and partners at the University of Geneva and the United States Department of Agriculture (USDA)—Puerto Rico which can be screened for mutations in drought gene pathways.

The approach of using ESTs and functional analysis for gene analysis in legumes was described by VandenBosch and Stacey (2003), and includes discussion of applications to improve common beans for nutritional quality and abiotic stress tolerance. Furthermore, an international network of *Phaseolus* researchers called "Phaseomics" maintains communications among bean scientists. A description of their interests and stocks under development has been published in Broughton et al. (2003) a reference genome sequence will soon be published.

RELEVANT RESULTS PUBLISHED IN THE AREA OF DROUGHT ADAPTATION

For common beans, the working definition of drought would be the inadequacy of water availability, including precipitation and soil moisture storage capacity, in quantity and distribution during the life cycle of the crop, which restricts the expression of the full genetic potential of the cultivar. Terminal or intermittent drought stress affects over 60% of the dry bean production worldwide (White and Singh, 1991). As described earlier, the bean growing areas in Latin America most affected by drought are Northeastern Brazil and the central and northern highlands of Mexico. These are the areas where drought tolerance screening has been undertaken. In Africa, droughts are frequent and severe in bean growing areas of eastern Kenya and eastern Transvaal, while other areas such as parts of northern Tanzania, the Kasese area of Uganda, and parts of the Hararghe Highlands and the Rift Valley of Ethiopia are affected by water deficits (Wortmann et al., 1998).

Breeding for drought resistance has a long history in Mexico, Honduras, and Brazil, and at CIAT in Colombia. It has gained momentum in recent years, with field studies of advanced lines in Cuba, Ecuador, El Salvador, Guatemala, and Nicaragua in Latin America, and with the creation of international drought nurseries in Kenya, Sudan, Ethiopia, and other countries in Eastern Africa. Most research has concentrated on germplasm evaluation. For example, White et al. (1994a,b) carried out genetic studies of parental materials from CIAT and from Mexico, finding that combining ability was determined largely by local adaptation. Thus, Mexican lines were good parents in Mexico and lines selected in Colombia served better as parents in Colombia, indicating that genes that were specific for drought resistance required an adapted genetic background for expression.

In the drought prone region of northern Mexico, two different sets of germplasm were tested under the typical conditions of insufficient and erratic rainfall. The primary trait measured was seed yield. The first set included more than 7000 accessions from the INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias) germplasm bank grown in subdivided sets over 3 years at two locations. Drought-resistant genotypes were identified mainly in the Durango (type III growth habit) and Mesoamerica races (types II and III), whereas genotypes from the Jalisco race were susceptible. The second set included 800 bush genotypes of the worldwide core collection assembled at CIAT (Tohme et al., 1995). After 2 years of evaluation, a set of 20

genotypes mostly from the Durango race originating in Mexico were identified as drought-resistant, plus a few accessions from other latitudes.

Subsequently, all resistant genotypes, along with locally improved cultivars previously identified as drought-resistant, were tested in eight trials across three different regions in Mexico: lowland, mid-altitude and highlands. In the four trials at the lowland tropics and in the mid-altitude El Bajio region, "G" accessions from the CIAT core collection (mostly Pinto landraces from the Durango race) performed poorly, as expected. In the former location, this was mainly due to a short growing cycle (due to photoperiod sensitivity coupled with short winter days), whereas in the latter location it was due to an inherent susceptibility to diseases (rust and common blight) and to leafhoppers (*Empoasca kraemeri*). In the lowland and mid-altitude sites, locally adapted genotypes along with introduced bred cultivars were among the top 25% yielders, a few of them yielding well in the four trials, e.g., TLP 19 and SEA 10, improved CIAT lines from the Mesoamerican race, and 97-RS-101 from the Durango race. In contrast, at the semi-arid highland location, CIAT "G" accessions were outstanding under both rainfed and rainfed-plus-irrigated conditions. In the four semi-arid highland trials, superior cultivars included the improved cultivar Pinto Villa and landraces G 13637 (Apetito) and G 842 (PI 201331). Furthermore, cultivars 97-RS-101 and SEA 10 were among the top yielders in six out of eight trials, and Pinto Zapata and 97-RS-110 in five trials. Superior cultivars at each site included genotypes from the type II and type III growth habit, early to mid-season types and those with disease resistance (Acosta-Gallegos et al., 2004). In addition, cultivars that displayed broad adaptation across regions were of neutral reaction to photoperiod. Under both terminal and intermittent drought stress, an accelerated partitioning of photosynthates toward the reproductive structures under stress seemed to be the chief trait for seed yield (Rao, 2001; Rosales-Serna et al., 2004; Rao et al., 2006a).

Singh (1995) reported the results of breeding for drought resistance in a tropical environment at CIAT in Colombia. One line, SEA 5, was especially resistant (Singh et al., 2001; Terán and Singh, 2002b). At higher latitudes in Mexico, cultivars Pinto Villa, and Pinto Saltillo were developed in and released for drought stressed environments of the northern highlands (Acosta-Gallegos et al., 1995; Sánchez-Valdez et al., 2004). They are extensively used in commercial production and as parents in the bean breeding programme. Although successful cultivars have been released (Beaver et al., 2003), sources of resistance outside the Durango race are being sought, based on the hypothesis that different mechanisms to cope with drought stress might exist in other genepools, plus the need to widen the genetic base in the Mexican breeding programme. A few promising cultivars from the Middle American (SEQ 12, SER 16, Negro Cotaxtla 91, Negro Veracruz, Black Jack) and Nueva Granada (ICA Palmar, A195) races have been identified. Muñoz-Perea et al. (2006), working in Idaho, found that Durango landraces of the red Mexican grain class presented a relatively better yield under drought than bred cultivars released over the past 30 years, whereas improved varieties released in other grain classes were superior to landraces. In other recent efforts, Beebe et al. (2008) have reported improvement of lines of small-seeded race Mesoamerica commercial classes (small red, small black and cream striped or carioca types). Besides improved yield under drought, the drought-selected lines presented shorter days to maturity, improved yield per day and, in some cases, better yield potential under favorable conditions.

The perspective of genepool structure, and especially of races, offers useful insights into past successes in breeding for drought resistance. Singh et al. (1991) noted that the race Durango often presents drought resistance and a good harvest index (HI). Much of the subsequent work on drought resistance has been built around the race Durango or genes extracted from it. The combination of Durango and Mesoamerica races has resulted in lines with higher yield in drought environments (Singh et al., 2001; Terán and Singh, 2002a) as well as in non-stressed environments (Nienhuis and Singh, 1986). This combination of races has also resulted in improved materials in other reports (Schneider et al., 1997a; Frahm et al., 2004; Beebe et al., 2008). Thus, these two bean races have complementary genes and/or mechanisms that permit the expression of transgressive segregation for drought resistance. It is notable that more progress has been made in small-seeded Mesoamerican types for Central America and Brazil, than in the large seeded Andean types that are more popular in Africa and parts of South America. This is possibly because crosses have rarely been made between Durango drought tolerance sources and Andean genotypes.

Schneider et al. (1997b) studied the genetics of drought resistance in Mexico and in Michigan, USA using quantitative trait loci (QTLs) detected with RAPD markers and multiple regression analysis. Four markers in one population and five in a second population of RILs were reported as important for drought resistance, although all of the non-anchored linkage groups were associated with some yield trait in some site and year. In a simulation of the application of MAS for a drought resistance QTL, the authors found that MAS would have been effective in one population in Mexico, and in the other population in Michigan. A second experience in the identification of markers for QTLs was reported by Beebe et al. (2006b). A population of RILs of the cross of SEA 5 × MD 23-24 was evaluated under drought and irrigated conditions in two seasons with contrasting patterns of drought. One QTL was common to two drought seasons, one QTL was specific to each of two seasons, and some were common to unstressed environments. What was perhaps most significant was that in no case were the two alleles at an important locus specifically adapted to the contrary environments (i.e., one allele to drought conditions and the other allele to favorable conditions). Rather, a drought allele (or an allele for a favorable environment) was accompanied by a neutral allele for the other environment. This implies that yield under drought and yield under well-watered conditions are not mutually exclusive and can be combined. In fact, cultivars that are high-yielding under irrigated conditions have shown, despite a large reduction, higher than average yields under terminal drought-stressed conditions (Acosta-Díaz et al., 2004).

PHYSIOLOGICAL MECHANISMS OF ADAPTATION TO DROUGHT

Adaptation to drought encompasses a diversity of mechanisms that enable plants to survive and produce in periods of dry

weather. The mechanisms of drought resistance are grouped into three categories: drought escape; drought avoidance; and drought tolerance (Levitt, 1972).

Drought escape is defined as the ability of the crop to complete its life cycle before serious soil and crop water deficits develop. This mechanism involves rapid phenological development (early flowering and early maturity), developmental plasticity (variation in duration of growth period depending on the extent of water deficit), and remobilization of photosynthates to the grain.

Drought avoidance is defined as the ability of the crop to maintain relatively high tissue water potential, despite a shortage of soil moisture. It is achieved through increased rooting depth, an efficient root system and increased hydraulic conductance, and by reduction of water loss through reduced leaf conductance, reduced absorption of radiation by leaf movement/rolling, and reduced evaporation surface (leaf area).

Drought tolerance is defined as the ability of the crop to withstand water deficit with low tissue water potential. It is achieved through maintenance of turgor through osmotic adjustment (a process which induces solute accumulation in the cell), increase in cell elasticity and decrease in cell size, and desiccation tolerance by protoplasmic resistance. Blum (2005) indicated that an effective drought tolerance mechanism in crop plants is stem reserve utilization for grain filling under drought stress. Research approaches that have most successfully improved drought performance of crop plants: (1) used realistic soil conditions; (2) tested with adequate water and with limited water; (3) understood the sources of crop failure in the proposed growing area; and (4) targeted a limited number of traits for genetic improvement (Boyer, 1996).

Significant research efforts have been made, particularly over the past two to three decades, to improve common bean adaptation to drought (Laing et al., 1984; White and Singh, 1991; Subbarao et al., 1995; Rao, 2001; Amede et al., 2004; Hall, 2004; Ishitani et al., 2004; Beebe et al., 2008; Beebe, 2012). These have involved:

- Studying the effects of drought stress on plant growth, development, and seed yield (Robins and Domingo, 1956; Acosta-Gallegos and Kohashi-Shibata, 1989; White and Izquierdo, 1991; Nielsen and Nelson, 1998; Nleya et al., 2001; Ontiveros-Cortes et al., 2005).
- Developing field screening methods (Bascur et al., 1985; Sponchiado et al., 1989; White and Castillo, 1989).
- Evaluating and identifying sources of drought tolerance in germplasm (Da Silveira et al., 1981; Miller and Burke, 1983; Jara-R, 1990; White and Singh, 1991; Singh, 1995; Terán and Singh, 2002a,b; Muñoz-Perea et al., 2006; Singh, 2007).
- Evaluating physiological traits related to underlying mechanisms of adaptation to drought (see **Table 1** for details and references).

Common beans are grown over a wide range of habitats where they can be exposed to seasonal droughts and wide fluctuations in soil moisture availability between years. Therefore, they have evolved several mechanisms to maintain plant water status within reasonable limits for normal metabolic functioning under drought stress (Beebe, 2012). Results from a set of the same genotypes that were evaluated in several countries in the 1980's indicate that local adaptation is an important component of drought resistance (White, 1988). A number of shoot and root traits contribute to improved drought adaptation. The root traits maximize water uptake, and the shoot traits optimize the use of absorbed water for producing grain during drought stress. Loss of leaf area is the most important morphological adaptation. It results from a reduced number of leaves, reduced size of younger leaves, inhibited expansion of developing foliage, or leaf loss accentuated by senescence, all of which result in decreased seed yield (Acosta-Gallegos, 1988). Through field screening, some relatively drought-tolerant lines of bean germplasm have been identified, such as BAT 477, A 195, and BAT 1289 (White, 1988; White and Singh, 1991). The superior adaptation of BAT 477 to water deficits was attributed to drought avoidance through greater root length density and deeper soil moisture extraction (Sponchiado et al., 1989).

White and Castillo (1992) grafted diverse shoot genotypes onto selected root genotypes of common beans and evaluated yield under drought. They found variation with shoot genotype, but the effect on growth and yield under drought was found to be small, compared with the effect of root genotype. Sanders and Markhart (1992) also used grafting to examine the importance and mechanisms of the root system's effect on leaf water status in *P. vulgaris* and *P. acutifolius*. They found that the root genotype determined leaf water potential in the most stressed plants, and that roots of tepary beans had greater hydraulic conductivity than those of common beans. Castonguay and Markhart (1991) measured saturated rates of photosynthesis in water-stressed leaves of common and tepary beans, and found that genotypic variability in drought tolerance between the two was not related to differences in mesophyll tolerance of dehydration. Tepary beans relied more on drought avoidance than on drought tolerance. Severe drought impaired nitrogen mobilization, HI and water-use efficiency (WUE) in common beans (Foster et al., 1995).

Further research work by White (1993) under field conditions indicated that WUE (based on carbon isotope discrimination, CID) was not a promising indicator of adaptation to drought. Since this work included a limited number of parental genotypes in a single year, further research work is needed on WUE using CID values in leaves and grain. Other physiological traits such as shoot dry weight and leaf nitrogen concentration appeared the most promising based on heritability, strong general combining ability effects, and correlations with seed yield across trials (White et al., 1994a,b). Phenotypic plasticity is considered to be another mechanism contributing to increased performance under drought (Acosta-Gallegos and White, 1995). This particular attribute, accentuated in photoperiod-sensitive cultivars, allows genotypes to shorten their growing cycle dramatically at later planting dates to avoid drought conditions later in the growing season.

Rao et al. (2004) evaluated 36 promising bred lines and accessions under field conditions over two seasons at CIAT in Colombia, and found that two accessions of *P. acutifolius* (G 40159 and G 40068) and two bred lines (RAB 650 and SEA 23) were outstanding in their adaptation to water stress conditions.

Table 1 | Physiological studies with a focus on shoot and/or root traits that contribute to improved adaptation to drought in common beans.

Studied	Shoot traits measured	Root traits measured	References
NUMBER OF GENOTYPES			
1	Photosynthesis, transpiration, leaf water potential, ribulose bisphosphate carboxylase (Rubisco) activity	–	O'Toole et al., 1977
3	Leaf water potential, osmotic potential, turgor potential, relative water content	–	Parsons and Howe, 1984
2	Leaf area, leaf dry weight, stem dry weight, leaf water potential, leaf osmotic potential, stomatal resistance	Rooting depth, root dry weight, root distribution	Markhart, 1985
4	Seed yield, crop dry weight, leaf area duration, number of seeds/pod, canopy temperature	Root length density	Sponchiado et al., 1989
6	Seed yield	–	White and Castillo, 1989; White et al., 1990
27	Carbon isotope discrimination, ratio of intercellular to ambient CO_2 concentration, transpiration efficiency	–	Ehleringer et al., 1991
2	Water potential, relative water content, photosynthesis, chlorophyll fluorescence, quantum yield	–	Castonguay and Markhart, 1991
16	Seed yield, shoot dry weight, harvest index (HI), leaf conductance, days to maturity	–	White and Castillo, 1992
12	Net photosynthesis, transpiration, leaf nitrogen, specific leaf area, leaf conductance, photosynthetic nitrogen use efficiency, carbon isotope discrimination	–	Comstock and Ehleringer, 1993; Kao et al., 1994
9-parent diallel	Carbon isotope discrimination, leaf optical density, leaf N and K, relative duration of podfilling, shoot dry weight, HI, seed yield, 100 seed weight	–	White et al., 1994a
20	Days to flowering, days to maturity, phonological plasticity, relative response to photoperiod	–	Acosta-Gallegos and White, 1995; Foster et al., 1995
3	–	Root length density, root efficiency in water absorption	Guimarães et al., 1996
5	Stomatal conductance, water content, relative water content, moisture retention capacity, HI, phenology, biomass and yield components	–	Pimentel et al., 1999; Ramírez-Vallejo and Kelly, 1998; Serraj and Sinclair, 1998
4	Relative growth rate, leaf water potential, stomatal conductance, net assimilation rate, osmotic adjustment	–	Costa Franca et al., 2000
4	Leaf area index (LAI), shoot biomass, number of pods per plant, grain yield	–	Dowkiw et al., 2000; Gomes et al., 2000
2	Water potential, stomatal conductance, assimilation rate, leaf abscisic acid (ABA)	–	Menuccini et al., 2000
4	Leaf area, relative water content, transpiration rate, stomatal conductance, leaf dry mass, stem dry mass, shoot dry mass, seed yield	Root dry mass, root distribution	Mohamed et al., 2002
1	Leaf water potential, leaf osmotic potential, leaf turgor potential, sugars and sugar alcohols, proline	–	Amede and Schubert, 2003a
1	Leaf water potential, plant dry weight, stomatal conductance, water-use efficiency (WUE), leaf sugars	Root dry weight	Abebe and Brick, 2003; Amede and Schubert, 2003b
2 RIL	Seed yield, yield components populations	–	Frahm et al., 2004; Pastenes et al., 2004, 2005
2	Relative water content, shoot water content, photosynthesis, transpiration, WUE, leaf area, shoot dry mass, 100 seed weight, seed set, seeds/pod, pod length, number of pods, seed yield	Root dry mass, root distribution, root depth	Mohamed et al., 2005; Wakrim et al., 2005
6	Seed yield, HI, leaf area, relative growth rate, relative water content, leaf water potential, leaf osmotic potential, leaf turgor pressure, photosynthesis, leaf ABA, stomatal conductance, dark respiration, leaf sugars, leaf starch, stem sucrose, seed starch, seed protein	–	Gebeyehu, 2006

(Continued)

Table 1 | Continued

Studied	Shoot traits measured	Root traits measured	References
24 (2 for detailed analysis)	Seed yield and yield components, abscission of reproductive organs, relative growth rate, relative water content, stomatal conductance, transpiration rate, photosynthetic capacity, leaf ABA, leaf rotation, chlorophyll fluorescence, leaf anthocyanin and malondialdehyde	–	Lizana et al., 2006
2	Photosynthetic rate, chlorophyll fluorescence, electron transport rate, leaf area, leaf thickness, carotenoid composition, stomatal density, leaf cell organization	–	Wentworth et al., 2006
16	Biomass yield, seed yield, HI, 100 seed weight, days to maturity	–	Muñoz-Perea et al., 2007
2	Relative growth rate, photosynthesis and transpiration rates, stomatal conductance, water-use efficiency, relative water content, proline accumulation, glycolate oxidase activity, peroxidation, antioxidant enzyme activities, ascorbate, phenolic and flavanoid compounds	–	Rosales et al., 2012

The superior performance of these two accessions under drought was associated with their ability to mobilize photosynthates to the developing grain and to utilize the acquired nitrogen more efficiently for grain production. More recent field evaluation of advanced lines at CIAT resulted in identification of three lines (SER 16, SEA 5, and SER 5) that were superior in their adaptation to drought stress conditions (Rao et al., 2006a). The superior performance of these lines was associated with higher values of pod harvest index (PHI), pod partitioning index and leaf area index (LAI), and a lower proportion of pod wall biomass and lower value of seed phosphorus content. The findings indicate the importance of greater mobilization of photosynthates to pods and seed per unit of seed phosphorus in common beans under rainfed conditions. The SER lines that were developed in the last few years seem to combine these desirable traits for drought adaptation (Beebe et al., 2008). The above field studies conducted at CIAT have contributed to the analysis of phenotypic differences in shoot traits that contribute to superior adaptation to drought stress conditions. From these studies, it has been learned that superior PHI, pod partitioning index and lower proportion of pod wall biomass are important phenotypic traits that reflect greater ability to mobilize photosynthates to grain under drought stress. Recently, Klaedtke et al. (2012) reported that photosynthate mobilization capacity from drought adapted common bean lines can improve yield potential of interspecific populations within the secondary gene pool.

The candidate genes underlying drought tolerance are beginning to be understood at a molecular level as well as for their physiological effects (Ishitani et al., 2004). At CIAT, candidate genes for drought tolerance are being pursued based on genes for osmotic adjustment, transpiration/WUE, and root development. Some, such as the dehydration responsive element binding protein (DREB) genes, could be converted to molecular markers for physical mapping and MAS. Subtractive libraries based on differential display of genes expressed in roots under stressed and non-stressed conditions, or by tolerant and sensitive genotypes, may lead to the identification of genes specific to the deep-rooting trait.

METHODOLOGIES FOR IMPROVING ADAPTATION TO DROUGHT

BREEDING STRATEGIES

Since the initiation of the breeding effort in the semi-arid highlands of Mexico, local landraces from the Durango race have been utilized in the development of improved cultivars, along with sources of specific traits, mostly disease resistance and earliness. Successful cultivars include in their pedigree parents from the Nueva Granada race chosen on the basis of yield, disease resistance and earliness (Acosta-Gallegos et al., 1995; Beaver et al., 2003; Sánchez-Valdez et al., 2004). The yield testing of the bush core collection in the semi-arid highlands of Mexico mostly identified accessions from the Durango race as resistant under intermittent drought stress. The resistant accessions are of indeterminate prostrate type III growth habit, root-rot resistant, photoperiod sensitive and originating in the region (Acosta-Gallegos et al., 2004). Under intermittent stress environments, mid-season genotypes of indeterminate prostrate growth habit that flower in flushes are best suited to cope with such variable conditions (Acosta-Gallegos and Kohashi-Shibata, 1989; Rosales-Serna et al., 2004).

For the improvement of small seeded cultivars of the race Mesoamerica, Durango genes continue to be valuable, but these are now introgressed into this race, so no direct use of Durango is being practiced. For Central America, Northeastern Brazil, and the Caribbean, lines in the small red, small black, and carioca grains are being developed, which present double or more yield under severe stress compared to the respective commercial control (Beebe et al., 2008). Small seeded beans are often planted in warmer climates where high temperatures exacerbate drought, or under conditions of low soil fertility or aluminum toxicity that can limit vigor and root development. Thus, tolerance of low soil fertility, especially to low soil phosphorus availability, and heat

tolerance should be combined with drought resistance. The combination of drought and low soil fertility tolerance has proven to be practical, since several drought-resistant lines already express a relative degree of tolerance to low soil phosphorus availability (Beebe et al., 2008). If progeny of interspecific crosses with *P. acutifolius* become available as sources, they will probably be small seeded, and thus of more immediate use to improve small seeded cultivars. Similarly, to date, it has been easier to introgress genes for aluminum resistance from *P. coccineus* into the small seeded genotypes than into the large seeded Andean types. These are all options to be pursued. Furthermore, in the light of results that demonstrated that differences in photosynthate mobilization during terminal drought were related to drought resistance, field selection for well-filled grain has been used to improve drought resistance in the small red and black seed classes (Beebe et al., 2008). This trait apparently integrates the effects of several elements of a physiologically complex process, and the practice of selecting for good seed filling has worked well when terminal drought was severe enough to have visible effects on seed quality.

Within the Andean genepool, drought resistance is needed most in bush beans, since climbing beans are usually planted in moister environments. Parental sources combining the race Durango with Andean types have resulted in lines with modest gain over the Colombian cultivar ICA Quimbaya—one of the best Andean genotypes available previously—and have produced resistant lines in a much wider range of grain colors (CIAT, 2006). Screening of potential parental genotypes has identified drought resistance in the SEQ and BRB series of advanced lines from CIAT, as well as in some dark red kidney (DRK) genotypes derived from ICA Quimbaya. In the case of Andean beans, drought resistance traits often need to be combined with vegetative and reproductive heat tolerances, especially for intermediate elevation production sites since, with the exception of some heat-tolerant germplasm accessions, Andean beans are notably poor at seed set in high temperatures.

The breeding strategies needed for improvement of drought tolerance in commercial classes of either genepool must take into account the quantitative nature of inheritance of this trait. This fact will circumscribe the breeding methods that can be applied to drought resistance, and calls for the application of novel approaches that are not widely practiced in common bean breeding. A particularly useful method for drought resistance breeding where sufficient drought resistance is found within a given genepool is recurrent selection (Beebe et al., 2008). Prebreeding can be used to create a sufficient number of potential parents with drought resistance component traits to initiate recurrent selection. After generating drought-resistant advanced lines, these can be used with standard common bean breeding techniques to incorporate new traits into the drought-resistant background. Among these techniques, gamete selection is a method recommended for use in common beans. It involves complex crosses and selection among F_1 plants and F_1-derived families (Singh, 1994), whereas pedigree selection is widely practiced by bean breeders to obtain fixed lines (Miklas et al., 2006).

Another method termed "advanced backcrossing" is a potentially useful method for improving drought resistance traits using crosses across genepools. Advanced backcrossing is valuable because it can be used to transfer multiple gene combinations from source germplasm to recipient genotypes. One advantage of the advanced backcross technique is the creation of improved lines that are useful simultaneously in an agronomic context and for genetic analysis. Blair et al. (2006b) showed that advanced backcrossing could be used for both QTL detection and discovery of transgressive segregation for yield traits in common beans. In summary, it can be seen that, whether the approach is advanced backcross, recurrent, gamete, or pedigree selection, good drought resistance sources must be amply represented in the genetic makeup of any of the populations developed.

While resistance to local diseases is a requisite for cultivar development in general, resistance to soil pathogens is especially important in drought-prone areas. Soil pathogens can infect when moisture is adequate and inhibit the root growth that is necessary for drought resistance later in the season. *Fusarium* spp in Mexico exacerbate drought by causing deterioration of the root system and reducing absorptive capacity for nutrients and water (Navarette-Maya et al., 2002). Charcoal rot caused by *Macrophomina phaseoli* is most severe under drought (Frahm et al., 2004). If early rains are abundant and result in serious infection and damage to hypocotyls and tap roots by fungi such as *Rhizoctonia solani* or *Sclerotium rolfsii*, this will also expose the crop to more severe late season drought.

As yet, there has been no routine use of MAS for improving drought resistance in common bean breeding programmes. However, MAS offers great potential for incorporating disease resistance into drought-resistant genetic backgrounds. The application of MAS for resistance to bean golden yellow mosaic virus (BGYMV) is the best example to date of selection of QTLs in common beans (Miklas et al., 2006). Two important QTLs have been tagged, sequence characterized amplified region (SCAR) markers created, and protocols defined. Selection has been practiced on populations ranging from F_1 plants of complex crosses to advanced families. Subsequently, it will be necessary to confirm resistance of lines in field trials because the full resistance complement cannot be assured by MAS, markers run the risk of genetic recombination and, finally, an entire suite of traits is required in a commercial cultivar. Application of MAS for drought would follow a similar scheme: the identification of relatively important QTLs and the creation of robust markers, followed by their use in various generations including in the F_1 of complex crosses, and subsequent field evaluation to confirm drought resistance and to distinguish levels of resistance.

However, compared to MAS for disease resistance, QTLs for drought resistance would require an additional step to validate the value of QTLs over sites, seasons, patterns of drought, soil types, etc. The nature of drought and its interaction with multiple environmental factors make the validation of QTLs much more complex. Ideally, the validation would be carried out within the target production zone, but this is normally difficult, since finding uniform experimental conditions for evaluation of large RIL populations close to production zones is usually not practical. A compromise might be to test a subsample of 30–40 phenotypically extreme segregant RILs in a smaller trial over multiple sites, with the sole purpose of validating the QTL. This population size could serve to confirm the effect of a relatively major QTL, assuming

that this is the target for eventual MAS. Schneider et al. (1997b) were able to validate markers using a small set of selected RILs. In this scheme, a multi-trait analysis (considering yield at different sites as independent traits) can augment the statistical power lost due to small population size (Jiang and Zeng, 1995).

TRIAL PLANNING

In planning trials for drought resistance testing it is important to consider carefully the choice of field sites and management of collateral factors. Principal among these are seasonal rainfall patterns, aspects of water control provided by irrigation systems and/or rainout shelters, soil bulk density, and prevalent abiotic and biotic stresses including soil fertility/toxicity problems and diseases or insect pests, some of which are more prevalent during dry season testing.

Crop yields in farmers' fields are as much affected by the timing of water deficits during a season as by the total seasonal water supply (Passioura, 2007). Field evaluation under realistic production conditions is the "gold standard" of drought resistance. In general, reliable and uniform field agronomy continues to be the key to genetic advance. This requires a uniform soil profile and texture, to the extent possible, since these affect the available soil moisture. Sampling of soil cores can reveal hidden soil variability that does not affect crop performance under optimal conditions but that would later affect the crop response under stress. Uniform field preparation with regard to subsoiling, plough depth, and bedding is critical, since this determines much of the effective rooting volume of the crop. Often, spatial variability in reaction to drought can be traced to variability in soil preparation. Gradients are often observed down the length of the field in the direction of field preparation. In this case, experimental designs might best be oriented in the same direction instead of across the field, and lattice designs often reduce experimental error. The testing of genotypes under stress and non-stress conditions allows for the gathering of more information in a single season and site, and for the calculation of several indices such as the geometric mean, the reduction of the yield, and the drought susceptibility index (Fischer and Maurer, 1978). As a rule of thumb, the more severe the drought-stressed environment, the more replicates are needed.

The interaction of drought with other stresses is notorious, especially with edaphic stresses (fertility, toxicities, and high soil bulk density) that affect root development. Also, drought effects are frequently exacerbated by pathogens causing root rots—*Fusarium* spp and *Rhizoctonia solani* in highland environments and *Macrophomina phaseolina* in lowland environments. If these are relevant stresses in the target production zones, it is important to understand their impact on the expression of drought resistance in order to have a realistic expectation of the benefits to be derived from drought resistance. For example, in Nicaragua, drought-resistant lines in fertile environments yield 50% more than local varieties under drought, but only 15% more in infertile environments (Llano, pers. communication). This requires a careful strategy that takes into consideration all factors and allows interactions among stresses to be examined. Inclusion of multiple stresses during the selection process is normally too complex and would obscure useful genetic variability in drought resistance. It is more practical to practiced selection for individual stresses in tandem, and to study the reaction to combined stresses with advanced lines.

Biotic stresses that are more prevalent during dry season testing include a range of insect pests such as leafhopper and whiteflies. These must be controlled for valid testing of drought resistance, since these pests can have a large effect on plant phenotype either through direct feeding or virus transmission. It is often necessary to control soil fungal pathogens, such as *Macrophomina* and *Sclerotium*, which are a major concern under water stressed conditions and which are easily spread through irrigation water used to establish drought nurseries. This can be achieved by seed dressing and directed fungicide application early in the growing season, or by planting into plots that have not had a long history of common bean testing. This is often the only solution when faced with high levels of *Fusarium* infestation.

Segregating population analysis in particular requires uniform conditions of soil and stress, and is best managed with the interplanting of parental genotypes plus frequent checks throughout the field for ready comparison. As noted above, variability in crop response often follows lengthwise field preparation. Thus, the identification of superior early generation materials for which replication is not practical must take into account not only neighboring plots to the right and left but also in the same row above and below the plot of interest. Segregating populations can also be replicated across sites to minimize the effects of selection at a single site, where the risk of out-of-season rainfall can interfere with drought selection pressure. Selection for traits of high heritability such as disease resistance in early segregating generations, and delaying the selection for yield under drought stress to intermediate generations, are common practices.

For family and line evaluation, lattice designs are a valuable tool, especially to control error that results from soil variability. At the family stage of testing that normally involves several hundred entries, unbordered two-row plots can be used for economy of space. Small lattices permit even smaller sub-plots. A six-by-six lattice is relatively easy to accommodate in the field, with compact sub-plots that are two plots by three plots. Where a larger number of families and lines are to be evaluated, common controls among trials permit comparison of the relative degree of drought resistance across materials in different trials. Lattice-design experiments are also used effectively for testing of recombinant inbred line populations in QTL studies. After the numbers of families or lines have been reduced, duplicated trials under two moisture regimes at two locations allow for the identification of highly responsive genotypes or for those that show the least genotype-by-environment interaction (GEI). In environments with a history of severe drought stress, trials need to be established with a higher number of replications than usual, due to large experimental errors.

WATER STRESS MANAGEMENT AND CHARACTERISATION

With irrigated conditions, drought may be predictable in both timing and intensity but, under rainfed conditions, unpredictability is the rule. In bean growing areas in the tropics, terminal drought stress is more common than intermittent drought stress. In Latin America, terminal drought stress affects

Central America and Northeastern Brazil, while intermittent drought stress is common in the semi-arid highlands of Mexico. In Africa, terminal drought is more common than intermittent drought under the short rains (late October to January) in the Eastern Highlands, while in the long rains (February to June) and in regions of Ethiopia, intermittent drought is common.

The tap root of the bean plant may reach a depth of 1–1.5 m. The lateral root system is extensive and is mainly concentrated in the first 0.3 m. At emergence, the rooting depth is about 0.07 m, at the start of flowering it is 0.3–0.4 m, and at maturity 1–1.5 m. Water uptake occurs mainly in the first 0.5–0.7 m of depth. In areas of intermittent drought stress, indeterminate plants with profuse branching above and below ground (i.e., roots) are better equipped to cope with drought spells of variable duration. Under these conditions, lateral and even adventitious roots are important to take up moisture during the scarce rain events. Under conditions when evapotranspiration is 5–6 mm day^{-1}, 40–50% of the total available soil water can be depleted before water uptake is affected. When water levels are reduced beyond this point and drought effects begin to occur, water stress in the plant can be detected by eye, because the leaves turn dark bluish-green in color. When the crop is grown for grain production, seed yield will be seriously affected if the soil water depletion level during the grain filling period reaches 60–70% of the total available soil water. The water utilization efficiency for harvested yield or crop water productivity for dry beans containing about 10% moisture is 0.3–0.6 kg m^{-1}.

The complexities of water deficit are apparent when one considers the effects of variation in climatic and edaphic conditions on the extent of dehydration which develops in a crop, and possible interference from biotic stresses as cited above. Management aspects that improve drought adaptation include improvement of soil water holding capacity through incorporation of organic matter, reduction of soil erosion or improvement of tillage practices, development of water catchment systems, use of tied ridges, and changes in planting dates. These practices involve many location-specific considerations and require a cropping systems approach to production under water-limited conditions.

Achieving the desired level of stress is one of the most important and yet difficult facets of managing drought trials. Extreme level of drought stress could reduce seed yields to very low levels such that genotypic differences disappear, whereas insufficient stress could result in selection of non-resistant genotypes. Since very few bean growing areas in the developing world are dependent on irrigation, most strategies to manage drought stress have to focus on alternatives under rainfed conditions, with the possibility of supplemental irrigation. Trials could be established with the minimum amount of water needed to assure vigorous seedling establishment, and then irrigation is withheld to simulate terminal drought stress. Use of rainout shelters where available, can assure good terminal stress conditions. Use of furrow irrigation or sprinkler irrigation and withholding water at different growth stages of the crop can help to quantify the effects of water stress on crop growth, development, and yield.

Line source sprinklers offer a specialized irrigation system for producing a gradient of water stress or a range of levels of stress in the field. By closely spacing sprinklers along a single line, and planting genotypes in strips perpendicular to the line, water could be applied in a gradient. The main advantage of this system is to quantify the reaction of a genotype to different levels of stress, although wind speed and direction can influence the water stress gradient in the field.

WATER STRATEGY

The status of water in soils, plants and the atmosphere is commonly described in terms of water potential (Ψ_w) i.e., the chemical potential of water in a specified part of the system compared with the chemical potential of pure water at the same temperature and atmospheric pressure. It is measured in units of pressure (MPa; megapascal). The total water potential at any point in the plant can be partitioned into: (1) the osmotic potential arising from the presence of dissolved solutes; (2) the turgor potential arising from the forces exerted on the cell walls from the water attracted to the cell by the solutes and the solids in the protoplast; (3) the matrix potential arising from capillary or electrostatic forces associated with cell walls and colloidal surfaces; and (4) the gravitational potential arising from gravitational forces on the water in the plant.

Plants require vast quantities of water. Whereas they incorporate more than 90% of the absorbed nitrogen, phosphorus, and potassium, and about 10–70% of photosynthetically fixed carbon into new tissues (depending on respiratory demands for carbon), less than 1% of the water absorbed by plants is retained in biomass. The remainder is lost by transpiration, involving the absorption of water by the plant roots, the transport of water through the conducting tissues of the plant, and the passage of evaporated water through the leaves and into the air, primarily through the stomata. The essential need for water for crop growth, development and yield arises from four features of plants (Bennett, 2003): (1) When plants open the stomata of their leaves to admit atmospheric CO_2 for photosynthesis, they lose water vapor through the same pores, a process known as "stomatal transpiration." Stomatal conductance is more strongly correlated with several photosynthetic parameters (electron transport rate, carboxylation efficiency, intrinsic WUE, and respiration rate in the light) than with leaf water status (Medrano et al., 2002). (2) Leaves and stems may lose water by transpiration through non-stomatal surfaces even when stomata are closed. (3) Transpiration serves to cool leaves that are exposed to high air temperatures, low atmospheric water vapor pressures, or the heating effect of light (Radin et al., 1994). (4) The transpiration stream also serves to transport to the leaves both inorganic nutrients from the soil and a range of chemicals synthesized in the roots, including signal molecules that contribute to the integrated response of the whole plant (Peuke et al., 2002). Thus, the growing bean crop will transpire several hundred times more water than is present in its tissues at any one time.

Bean cultivars adapted to drought would require less water for irrigation and would, therefore, contribute to the conservation of an important natural resource. The short growing season reduces water requirements in common beans to levels below those of other species generally considered as more drought-adapted (White, 1993). The water requirements of a bean crop

depend on its environment and nutrition. Water infiltrates the pores between soil particles and is held there with varying degrees of tenacity. Water tension (a negative pressure) in soil at any moment controls the movement of soil water in the soil and its use by plants. This water tension is expressed in units of MPa. When tension is low (between −0.01 and −0.03 MPa), water moves to lower soil layers because of gravitational pull. But when soil water tension is −1.5 MPa or less, the adhesive force is so strong that plant roots can hardly extract water from soil. At approximately this water tension, most crops permanently wilt and stop growing. The permanent wilting point is species specific and in the case of common bean, soil water tension values above at or lower than −0.8 MPa could impose significant drought stress and limit grain yield. Soil water at a tension between about −0.01 and −1.5 MPa is considered available for plants.

An assorted range of methods and instruments have been developed to measure and express soil water. Basically there are three ways: (1) weight percentage; (2) volume percentage; and (3) tension. The choice of whether to express soil water content on a weight or a volume basis is not a critical one if the information necessary to convert one to the other is also provided. Field capacity (FC) is defined as the water content after the soil becomes saturated, followed by complete gravitational drainage. There is a higher soil water content at FC in fine-textured soils with a high clay or organic matter content. The amount of available water is higher in clay than it is in sandy soils. If the bean crop does not receive enough water either through rainfall or through irrigation to maintain leaf expansion and high rates of net photosynthesis per unit leaf area, total canopy dry matter accumulation will decline, crop development will be affected, and grain yield will be reduced. The extent of yield loss is very much dependent on the timing, duration and intensity of water deficit.

Water requirements for maximum production of a 60–120 day bean crop vary between 300 and 500 mm depending on climate (Allen et al., 1998). Crop coefficient (K_c) values that relate reference evapotranspiration (ET_o) to water requirements (ET_m) for different development stages of dry beans are: during the initial stage 0.3–0.4 (15–20 days), during the development stage 0.7–0.8 (15–20 days), during the mid-season stage 1.05–1.2 (35–45 days), during the late-season stage 0.65–0.75 (20–25 days), and at harvest 0.25–0.3.

Guerra et al. (2000) found the highest bean seed yield with irrigation at -41 kPa soil water tension measured at a soil depth of 10 cm. Recently, Muñoz-Perea et al. (2007) examined differences among dry bean landraces and cultivars (pinto and red market classes) in terms of WUE under intermittent drought-stress and non-stress environments. Under severe drought stress, WUE in pinto beans ranged from 1.5 to 4.4 kg ha^{-1} mm^{-1} water. Under favorable milder climatic conditions, the mean WUE value was 10 kg ha^{-1} mm^{-1} water in the drought stress environment and 8.7 kg ha^{-1} mm^{-1} water in the non-stress environment. Using one of the drought adapted small seeded red lines (SER 16), Builes et al. (2011) reported WUE values up to 9.2 kg ha^{-1} mm^{-1} water under drought stress.

Under rainfed conditions, water deficit can occur more than once during a crop's growth cycle, caused by erratic patterns of rainfall distribution, and may kill the crop under a severe and prolonged period of drought (Thung and Rao, 1999). The intensity and duration of stress determine the degree of yield reduction relative to yield potential. Different problems are created by water deficits at different key developmental stages of the bean crop, i.e., at sowing, establishment, branching, flowering, and grain filling.

All other factors being equal, genotypes with high WUE will survive and grow better in water-limiting environments than genotypes with low WUE. However, in nature, all other factors are rarely equal. The physiological basis for variation in drought resistance in common beans may be due to a wide and potentially unrelated array of mechanisms including earliness, rooting depth and distribution, carbon allocation patterns, leaf morphology, gas exchange patterns, osmotic adjustment, and photosynthate mobilization to grain. In general, selection for improved WUE through analysis of carbon isotopes will be most useful in selection for maintenance of growth under drought rather than survival. Survival mechanisms may relate more to growth phenology and carbon allocation patterns than improved carbon gain per unit water loss. Thus, increased survival under imposed drought could be related more strongly to allocation to roots than to gas exchange characteristics. It is possible that the lack of a positive relationship observed in common beans between carbon isotope discrimination ($\Delta^{13}C$) and seed yield under acid soil conditions, where root growth is restricted under dry conditions (White, 1993), may be due to genotypic differences in plant survival mechanisms.

PHENOTYPING TRAITS

The phenotype is a complex expression of the genotype and its interaction with the environment. Field trials for drought breeding and associated goals are normally conducted in the dry season of the year to determine genotypic differences for resistance. The trials could include germplasm accessions, advanced generation bred lines, and recombinant inbred lines as entries. Two levels of water supply (irrigated for no stress and rainfed for drought stress) need to be applied to quantify the effects of the intensity and duration of drought on crop growth and seed yield of genetically fixed materials. When the bean crop is grown with a sufficient water supply, the timing of irrigation is important and applications of water should be directed toward meeting water requirements during the establishment period, the early part of the flowering period, and at grain filling. Non-irrigated treatments generally receive water only during the establishment period, usually through pre-seeding and/or post-emergence applications of water. Depending on the number of genotypes, a partially balanced lattice design (4 × 4 or 6 × 6 or 10 × 10) with three replications can be used. The field trials can be planted in continuous rows with each genotype per replication planted in four rows of 5 m length with a row-to-row distance of 0.6 m and a plant-to-plant spacing of 0.075 m (with 15 seeds for a 1 m-long row to have a final number of 10–15 plants per 1 m-long row). The middle 2 rows are used for seed yield determination.

Climate data (daily rainfall, minimum and maximum temperature, relative humidity, and pan evaporation) need to be recorded. Depending on the rainfall and soil texture, two to three

gravity irrigations are needed to establish the trials with control and drought treatments (one irrigation at 6 days before planting, and another irrigation at 10–12 days after emergence). The specific management practices and amount of water to be applied by either furrow or sprinkler irrigation will need to be calibrated empirically for local conditions. However, the rule of thumb should be to seek a 60–80% yield reduction in susceptible controls compared to the irrigated treatment, assuming yield potential of around 2.5 t ha^{-1} in control plots. In other words, the susceptible controls would ideally yield in the range of 0.5–1 t ha^{-1} to have maximal discrimination among genotypes and better chances of selecting true drought resistance. The control treatment will require additional irrigations (four to five) depending on the rainfall. The drought treatment will not receive any additional irrigation if furrow irrigation is used, but to induce drought stress with sprinkler irrigation, a reduction of about 50% in the amount of water applied to control plots may be considered. It is important to monitor the amount of water applied (e.g., 35–50 mm) for each furrow irrigation. Also, soil samples from each replication (that includes all genotypes) need to be collected at the time when irrigation is stopped for rainfed treatment, and followed at flowering, mid-podfilling and at physiological maturity. Soil samples need to be collected with a soil corer up to 80 cm in depth (at 0–5, 5–10, 10–20, 20–40, 40–60, 60–80, and 80–100 cm) to quantify soil moisture content gravimetrically. This includes weighing the fresh and dry weight of each soil sample for each soil depth. These measurements will allow quantification of the degree of drought stress at different growth stages.

Crop development needs to be monitored by recording days to flowering and days to maturity. For quantifying physiological differences in drought resistance, a number of plant attributes can be measured at the mid-podfilling growth stage. To measure plant attributes, a row length of 0.5 m (0.3 m^2 area) for each plot should be selected for destructive sampling. During the sampling, the plants are counted (number per 0.5 m) and cut to the soil surface, put in a plastic bag and transported to the station or field room to process. Plants are separated into leaves, stems and the remaining plant parts (pods and reproductive structures). If a leaf area meter is available, the leaf area can be determined. The plant parts need to be put in separate paper bags for oven drying (70°C for 2 days). After drying of the samples, the dry weight of each is recorded. From these dry weights, total dry matter production and dry matter distribution into different plant parts as well as the leaf to stem ratio at mid-podfilling can be quantified.

Yield components should be measured at harvest time. Again, a 0.5 m long row (0.3 m^2 area) is selected, and the number of plants counted and cut to the soil surface. The plants are put into a paper bag and transported to the station or field room. They are separated into stems and pods, and the number of pods and number of seeds per harvested area counted. The stem, pod wall and seed samples are oven dried at 70°C for 2 days and their dry weights recorded.

Target traits and how to measure them

Many drought adaptation traits, such as phenology, root size, and depth, hydraulic conductivity and storage of reserves, are associated with plant development and structure, and are constitutive rather than stress-induced (Chaves et al., 2003). Condon et al. (2004) have suggested that the consequences of various plant traits and environmental conditions have to be evaluated in the specific field environments in which the crop is to be grown. The target shoot and root traits that are pertinent for drought resistance breeding in common beans are described below.

Target shoot traits

From the phenotyping protocol described for field conditions, the following shoot traits that are related to seed yield can be quantified:

- At mid-podfilling: dry weights of leaf biomass, stem biomass, pods plus reproductive structure biomass, total shoot biomass, and leaf to stem ratio of dry weight.
- At harvest: dry weights of stem biomass, pod biomass and seed biomass, number of pods per plant, dry weight of pod wall biomass and proportion of pod wall biomass to pod biomass, seed number per pod, 100 seed dry weight, seed number per area and pod number per area.
- Seed yield: the two central rows of each plot are used to determine seed yield.
- Geometric mean (GM): this is determined for seed yield, 100 seed weight and days to maturity as $GM = (ns \times ds)^{1/2}$ where ns is no stress and ds is drought stress.
- Harvest index (HI): seed biomass dry weight at harvest/total shoot biomass dry weight at mid-podfilling × 100.
- Pod harvest index (PHI): the PHI for each genotype is determined by seed biomass dry weight at harvest/pod biomass dry weight at harvest × 100.
- Pod wall biomass proportion (%): pod wall biomass dry weight at harvest/pod biomass dry weight at harvest × 100.
- Pod partitioning index: pod biomass dry weight at harvest/total shoot biomass dry weight at mid-podfilling × 100.
- Stem biomass reduction (%): (stem biomass dry weight at mid-podfilling—stem biomass dry weight at harvest)/stem biomass dry weight at mid-podfilling × 100.
- Grain filling index (GFI): the GFI for each genotype can be estimated from 100 seed dry weight under rainfed conditions/100 seed dry weight under irrigated conditions × 100.
- Seed production efficiency (number g^{-1}): seed number per area/total shoot biomass dry weight at mid-podfilling per area (adapted from Board and Maricherla, 2008).
- Pod production efficiency (number g^{-1}): pod number per area/total shoot biomass dry weight at mid-podfilling per area (adapted from Board and Maricherla, 2008).
- Drought intensity index (DII): the DII for each growing season can be calculated as $DII = 1 - X_{ds}/X_{ns}$, where X_{ds} and X_{ns} are the mean of all genotypes under drought stress and no stress treatments, respectively.
- Drought susceptibility index (DSI) for seed yield: the DSI for each genotype is calculated as follows: $DSI = (1 - Y_{ds}/Y_{ns})/DII$, where Y_{ds} and Y_{ns} are mean yields of a given genotype in drought stress and no stress environments, respectively (Fischer and Maurer, 1978).

Additional shoot traits

These include non-destructive measurements that are related to physiological processes such as photosynthetic efficiency, total chlorophyll content (Soil–Plant Analyses Development or SPAD measurement), stomatal conductance, transpiration rate, leaf temperature (in both the morning and afternoon), and leaf water potential. The destructive measurements that are related to growth and metabolism include LAI, canopy dry weight per plant (leaf, stem and pod biomass), shoot nutrient (nitrogen and phosphorus) uptake, shoot and seed ash content, and shoot and seed total non-structural carbohydrates (TNC). Seed nitrogen, phosphorus, ash content, and TNC can be measured at the time of harvest.

Field evaluation of 121 RILs of the cross MD 23-24 × SEA 5 over 2 seasons at CIAT in Colombia using the above phenotyping protocol resulted in identification of one line (MR 81) that was superior in its adaptation to drought stress conditions (Rao et al., 2005). The superior performance of this line was associated with higher values of PHI, pod partitioning index, HI, and seed TNC, and a lower proportion of pod wall biomass and lower value of seed phosphorus content, indicating the importance of greater mobilization of photosynthates to pods and seeds per unit of seed phosphorus in common beans under rainfed conditions.

Target root traits in the field

Root traits associated with drought tolerance can be measured either in the field or in the greenhouse, and these include root depth and root architectural traits. Rooting depth and root distribution under field conditions can be quantified using soil cores taken at different soil depths followed by root washing, scanning and weighing as described below for greenhouse root phenotyping. Deep rooting has been positively correlated with seed yield, crop growth, cooler canopy temperature, and soil water extraction in common beans (Sponchiado et al., 1989). In another study by White and Castillo (1988), drought-tolerant bean genotypes were able to extend their roots to a depth of 1.2 m in drought environments, whereas sensitive genotypes could not extend their roots beyond 0.8 m. These differences in rooting depth were reflected in overall shoot growth and seed yield.

Rooting behavior and shoot development under greenhouse conditions

When grown in the greenhouse, beans are planted in a mix of a soil (4–8% soil organic matter) with river sand (2:1 w/w) and grown for ca 35–45 days in small plastic tubes (80 cm long and 7.5 cm in diameter) covered with polyvinyl chloride (PVC) tubes. The plastic tubes are filled to 75 cm of their total length with 2400 g of moistened soil–sand mix (made by mixing a ratio of 500 g of soil to 100 ml of water and packing into the tubes in aliquots to ensure uniform settling). Trials are planted as a randomized block in a split plot arrangement with three levels of water supply: 80% FC (well-watered), 40% FC (simulation of intermittent drought), and without irrigation (simulation of terminal drought conditions) as main plots, and genotypes as subplots. Watering the plastic tube and allowing it to drain and then registering the amount of soil moisture left determines FC. Soil is fertilized with an adequate level of nutrients based on soil analysis. Water stress treatments can be imposed after 10–14 days of initial growth of the plants. The initial soil moisture level for the three treatments is 80% of FC. Plants in the well-watered (80% FC) and intermittent drought (40% FC) treatments are maintained by weighing each plastic tube every 3 days and applying water to the soil at the top of the plastic tube. Plants with terminal drought receive no water application after the initial establishment. Each plastic tube is weighed to determine the soil moisture content at 3-day intervals until harvest.

Traits measured in greenhouse trials

A number of shoot physiological characteristics are measured in a soil tube screening system assay. These include photosynthetic efficiency, total chlorophyll content (SPAD), stomatal conductance and transpiration rate, leaf temperature (both in the morning and afternoon), and leaf water potential. At the time of harvest (ca 35–45 days after planting and 3 weeks of drought stress), leaf area, shoot biomass distribution (leaf, stem, pod, and root biomass), leaf TNC content, and root characteristics are determined. The soil tube is sliced into 5 layers (0–5, 5–10, 10–20, 20–40, and 40–75 cm). Roots in each soil layer are washed free of soil, and length, diameter, specific root length, and dry weight are determined. Root length and diameter are measured with an image analysis system (WinRHIZO, Regent Instruments Inc.) [1]. Root weight is determined after the roots are dried in an oven at 60°C for 48 h.

Rao et al. (2006b) used the above soil tube screening system to evaluate the impact of drought on different genotypes of common beans in terms of root growth and root distribution. Results on five genotypes grown in large soil cylinders indicated that SEA 5, BAT 477, and G 21212 were deep rooted compared with BAT 881 and MD 23-24. Terminal drought simulation studies in soil tubes indicated that BAT 477 has the ability to grow tap roots under drought conditions, whereas tap root growth was inhibited in DOR 364. Meanwhile, BAT 477 was found to have vigorous lateral root growth without drought stress. This constitutive trait may help it to cope with water deficiency, although the lateral root growth of both genotypes was inhibited under the drought conditions tested. Greenhouse evaluation of 30 RILs of the cross of DOR 364 × BAT 477 using the same method for root phenotyping resulted in identification of two RILs (BT 21138-124-1-4 and BT 21138-6-1-1) with greater ability for fine root development at deeper soil depth than the other RILs tested.

This greenhouse screening technique using soil tubes to determine phenotypic differences in rooting ability under drought stress has been found to be very complementary to field studies to evaluate shoot traits for drought resistance in both parents and advanced lines of common beans.

CHALLENGES AND OPPORTUNITIES FOR IMPROVING COMMON BEAN FOR ADAPTATION TO DROUGHT

Although physiological studies have revealed the role of some traits, especially rooting depth and photosynthate remobilization, the mechanisms behind these traits are not yet defined. Furthermore, the relative importance of other traits is still not

[1] http://www.regentinstruments.com/

understood, for example, the control of stomatal behavior. Nor has the role of metabolites in drought resistance been well studied. In a species as diverse as common beans, and with the potential that it has for introgression from sister species, useful genetic variability may yet be found for other traits and mechanisms that may have a role in drought resistance. Therefore, study of those physiological traits and mechanisms needs to continue. Furthermore, screening conditions to optimize the expression of traits need to be fine-tuned, and the relationship between physiological traits and the QTLs that control them needs to be explored.

While initial QTL studies have been promising, these have mostly been in a limited number of RIL populations, all so far created from crosses within the Middle American genepool (Schneider et al., 1997b; Beebe et al., 2006a). Further studies with populations developed from crosses between genepools or from crosses within the Andean genepool are needed to explore additional diversity for drought resistance QTL alleles, and to analyze the effect of genetic backgrounds on the QTL alleles that have already been identified. As mentioned earlier, there is a need for a larger number of high polymorphism microsatellites to analyze populations derived from intra-genepool crosses. In addition, a highly saturated marker system such as diversity arrays technology (DArT) would be valuable for fine mapping of QTLs. To do this effectively, larger populations are needed for genetic analysis, since most RIL populations in common beans have only been developed with around 100 lines. However, the creation of RIL populations of more than 300 lines presents its own difficulties, given that common beans are a low multiplication species compared to cereals. This also affects the maintenance of RILs.

Alternative population types would also be of interest for the analysis of drought resistance. In this sense, the advanced backcross strategy holds promise for the determination of QTLs that function without the confounding effect of epistasis with alleles from non-commercial sources, since advanced backcross breeding fixes valuable alleles in the genetic background of a commercial parent. If MAS for drought tolerance is to be successful, then understanding the interaction of QTL alleles with multiple genetic backgrounds is important, since breeding programmes usually deal with a range of commercial classes and seed colors representing different genetic backgrounds, genepools and races.

An additional challenge to the genetic understanding of drought resistance is to associate QTLs with their underlying genetic and mechanistic factors, whether these be regulatory genes such as those governing transcription factors, or structural genes such as those involved in hormone pathways, carbon or nitrogen metabolism under drought stress and drought-associated secondary metabolite production. Structural genes for biosynthesis of metabolites such as proline and trehalose would be of interest for common beans, since these two metabolites have had an effect on drought resistance (Farías-Rodríguez et al., 1998; Amede and Schubert, 2003a; Chen, pers. communication; Suárez et al., 2008).

Parts of the abscisic acid (ABA) hormone response pathway would also be sources of candidate genes that may underlie some of the QTLs identified to date, or that are still to be discovered. In addition, candidate genes for carbon accumulation and remobilization from leaves and stems to pods and to seeds such as those encoding sucrose synthase, sucrose-phosphate synthase, and vacuolar or cell wall invertases might also be of interest (Sturm, 1999; Pinheiro et al., 2001). In this regard, the analysis of drought related candidate genes can be an important offshoot of translational genomics that makes use of sequence information in well-studied model species to understand genes that are important to agricultural traits in crop species. Comparative genomics has been exploited to a greater extent in cereals (Bennetzen, 2000) than in legumes, but it may be possible in the future to align QTLs for drought resistance discovered in soybeans (Mian et al., 1996, 1998) or even candidate genes analyzed in this species with their putative orthologous loci in common beans.

Candidate genes important for hormone or metabolite production are relatively straightforward to clone, whereas transcription factors are often members of multigene families that are relatively more difficult to analyze on either a transcriptome or gene-by-gene basis (Udvardi et al., 2007). Furthermore, subtle difference in the expression of transcription factors can have major effects. Therefore, they are difficult to detect with differential display, subtractive library production or array-based analyses (Torres et al., 2006). One example of a transcription factor that is specifically expressed in roots of both tepary beans and common beans was discovered by Rodriguez-Uribe and O'Connell (2006) and is a member of the basic-leucine zipper (bZIP) transcription factor family. Some members of the DREB gene family also appear to be root specific and induced by drought (CIAT, unpublished data). It is important when analyzing candidate genes to base the analysis on the evaluation of gene sequences or expression levels in the best drought resistance source genotypes or species available to the bean researcher.

In terms of breeding, interspecific crosses with *P. acutifolius* in particular continue to be attractive from the standpoint of the very high levels of drought resistance in this species. However, so far, such crosses have been disappointing in terms of what has actually been transferred to common beans. Progress may be facilitated by a better understanding of the mechanisms and traits involved, such that selection in populations may be focused on them. Accessions of *P. acutifolius* are included in physiological studies and, to date, it is evident that deep fine roots are typical of this species and probably contribute to drought resistance. Characterization of *P. acutifolius* accessions for drought resistance should continue to elucidate how its traits and mechanisms can complement those existing in common bean, and to focus selection on the most important and unique traits. At the very least, *P. acutifolius* can serve as a model of how multiple drought resistance traits and mechanisms can combine for high levels of resistance. Interspecific crosses with *P. coccineus* are another opportunity to modify the root structure in potentially useful ways. The root system of *P. coccineus* is much thicker than that of *P. vulgaris* and may be better able to penetrate soil of high bulk density than roots of common beans—a useful trait for compacted soils where root development is limited. This must be confirmed, and the potential value of this trait be evaluated.

In the medium to long term, the challenge will be to match traits and mechanisms to specific environments with regard to patterns of drought (terminal versus intermittent), and

associated limitations (e.g., low soil fertility, high temperatures, and local pathogens). This will need to be an iterative process of identifying genetic diversity, defining general classes of traits, and testing these across broad classes of environments. The complexity of this task will defy a strictly rational approach for the foreseeable future, and much will depend on an empirical approach, followed by more cycles of physiological analysis and testing.

Great potential exists for improving drought resistance in common beans. Exploiting this potential will be enhanced by more systematic application of physiological and genomic tools and continued genetic and mechanistic analysis of a range of diverse germplasm both from within the species and from close relatives. At present, the most important traits appear to be those associated with rooting depth and photosynthate remobilization, but other traits may emerge in the future. Effective use of genomic tools will be aided by a better understanding of the physiology of drought response and drought resistance mechanisms. Beans in particular are sensitive to other soil factors, such as compaction or low soil fertility, that will influence the expression of favorable rooting traits. This fact makes the study of drought resistance in beans especially complex, and has important implications for the ultimate expression of drought resistance in farmers' fields. Beans may also be sensitive to environmental factors that influence mobilization of photosynthates to grain. Efficient breeding schemes, managed stress conditions, scaling-up of the use of phenotyping tools, together with genomics and MAS, are expected to improve the efficiency of genetic enhancement for drought resistance in common beans.

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Phenotyping chickpeas and pigeonpeas for adaptation to drought

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The chickpea and pigeonpea are protein-rich grain legumes used for human consumption in many countries. Grain yield of these crops is low to moderate in the semi-arid tropics with large variation due to high GxE interaction. In the Indian subcontinent chickpea is grown in the post-rainy winter season on receding soil moisture, and in other countries during the cool and dry post winter or spring seasons. The pigeonpea is sown during rainy season which flowers and matures in post-rainy season. The rainy months are hot and humid with diurnal temperature varying between 25 and 35˚C (maximum) and 20 and 25˚C (minimum) with an erratic rainfall. The available soil water during post-rainy season is about 200–250 mm which is bare minimum to meet the normal evapotranspiration. Thus occurrence of drought is frequent and at varying degrees. To enhance productivity of these crops cultivars tolerant to drought need to be developed. ICRISAT conserves a large number of accessions of chickpea (>20,000) and pigeonpea (>15,000). However only a small proportion (<1%) has been used in crop improvement programs mainly due to non-availability of reliable information on traits of economic importance. To overcome this, core and mini core collections (10% of core, 1% of entire collection) have been developed. Using the mini core approach, trait-specific donor lines were identified for agronomic, quality, and stress related traits in both crops. Composite collections were developed both in chickpea (3000 accessions) and pigeonpea (1000 accessions), genotyped using SSR markers and genotype based reference sets of 300 accessions selected for each crop. Screening methods for different drought-tolerant traits such as early maturity (drought escape), large and deep root system, high water-use efficiency, smaller leaflets, reduced canopy temperature, carbon isotope discrimination, high leaf chlorophyll content (drought avoidance), and breeding strategies for improving drought tolerance have been discussed.

Keywords: carbon isotope, composite collection, core collection, genetic diversity, mini core collection, reference set, root traits, SSR markers

GENERAL INFORMATION

IMPORTANCE OF CHICKPEAS AND PIGEONPEAS IN THE HUMAN DIET

Chickpeas (*Cicer arietinum* L) are the fourth largest grain legume crop in the world, with a total production of 10.9 million tons from an area of 12.0 million ha and a productivity of 0. 91t ha^{-1} (Food and Agriculture Organization of the United Nations (FAO, 2010b)). Large variations in chickpea yield are reported, ranging from 0. 45t ha^{-1} in Tanzania to 1.67t ha^{-1} in Canada. Chickpea productivity records in the last four decades reveal an interesting trend: productivity consistently increased in India and Mexico while it declined in Turkey, Pakistan, and Iran.

The global production of pigeonpeas (*Cajanus cajan* L) is 3.7 million tons from an area of 4.8 million ha with a productivity of 0.77t ha^{-1}. Large variations in pigeonpea yields from 0. 3t ha^{-1} in Haiti to 1.2t ha^{-1} in The Philippines are reported. Pigeonpeas are grown as a field and as a backyard crop in several countries, but as a field crop only in 21 countries (FAO, 2010b).

Both chickpeas and pigeonpeas are important grain legumes grown for their protein-rich seeds used in human consumption, for their ability to restore and maintain soil fertility by nitrogen fixation, and for their suitability to fit very well into various cropping patterns. Globally, over 90% of chickpeas and pigeonpeas are produced and consumed in Asia. Chickpea seeds contain 23% protein, 64% carbohydrates, 5% fat, 6% crude fiber, 6% soluble sugar, and 3 percent ash (William and Singh, 1987), whereas pigeonpea seeds contain 20.5% protein, 64.2% carbohydrates, 6.8% lysine, 3.8% fat, 5% fiber, and 4.2% ash (Faris and Singh, 1990).

CHARACTERIZATION OF GROWING ENVIRONMENTS

Chickpeas are largely grown in arid and semi-arid environments in Asia and Africa, with more than 80% of the annual rainfall occurring during the rainy season (June–September). The rainfall variability within the region is usually high, leading to varying intensities of drought. In the Indian subcontinent, chickpeas

are grown during the post-rainy season. In northern Pakistan, Afghanistan, Iran, the Middle East, and Mediterranean Europe, they are cultivated during the wetter winter months or, where snow occurs, during the cool dry springtime period, wherein more than 70% of the annual precipitation (i.e., snow plus rain) falls during the 5–6 months from November/December to April, with summers typically dry and warm (Khan, 1980). Although the mean total annual precipitation throughout the region rarely exceeds 500 mm, it is conserved and used rather effectively during the cool winter season by a crop that has a relatively small evapotranspiration requirement (200–250 mm). Mean annual air temperatures are often cooler than 20˚C, except in some areas where the rainfall distribution is bimodal.

The alluvial soils (Entisols) in northwest India and Nepal may retain up to 200 mm of available water in a 120 cm deep soil profile. Over similar depths, the black cotton soils (Vertisols) of the Indian subcontinent have the potential to store 250 mm of available water. Potential evapotranspiration demand during the 5–6 month period from October/November to March is typically within the range 200–300 mm for most chickpea-growing areas in the region. Thus, chickpeas are usually grown under stored residual soil moisture with the moisture receding to deeper soil layers with the age of the plants, leading to terminal drought stress. The intensity and the timing of the stress can, of course, vary depending on the previous rainfall, soil type, crop duration, and crop growth.

Pigeonpeas are commonly sown during the rainy season and flower and mature in the post-rainy season. The rainy months are hot, average diurnal air temperatures varying between 25 and 35˚C, with daily maximum values typically close to 35˚C and warm nights (20–25˚C). There can be large gaps between the two rainfall events leading to spells of intermittent drought stress. Pigeonpeas are grown on a wide range of soils in the tropics and subtropics including Entisols, Vertisols, Alfisols, Inceptisols, Ultisols, and Oxisols, with a wide variation in water-holding capacity. Both Entisols and Vertisols are generally deep and hold more than 200 mm of plant-available water to a depth of 1.5 m at the end of the rainy season, whereas Alfisols are usually less than 1 m deep and hold less than 90 mm plant-available water to a depth of 1 m (Reddy and Virmani, 1981). The crop grows well on Entisols, but suffers moisture deficits of different intensities as intermittent and/or terminal drought on Alfisols and Inceptisols.

Clearly, there is a need to match the duration of the soil moisture availability to that of the genotype duration for maximizing productivity in any given environment.

GENETIC AND GENOMIC RESOURCES

Germplasm in CGIAR and NARS genebanks

Plant genetic resources are the most valuable among all of the natural resources. The widespread cultivation of modern and high-yielding cultivars has posed a great threat to the reservoir of local plant biodiversity that has evolved over millennia. To safeguard this diversity, large-scale collecting and conservation efforts have been made in recent years, resulting in the assembly of more than 7 million accessions held worldwide in over 1750 genebanks of the Consultative Group on International Agricultural Research (CGIAR) or of national agricultural research systems (NARS; FAO, 2010a). There are over 98,000 chickpea accessions in genebanks, predominantly preserved at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and the International Center for Agricultural Research in the Dry Areas (ICARDA). There are 28,000 pigeonpea accessions in genebanks, with ICRISAT holding 13, 632 accessions. In addition, substantial numbers of chickpea and pigeonpea accessions are stored at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. The other two genebanks holding large collection of chickpea are the United States Department of Agriculture (USDA) Regional Plant Introduction Station, Pullman, Washington State and the Australian Temperate and Field Crops Collection (ATFCC), Victoria, Australia. These genebanks also maintain ca 900 wild relatives of chickpeas and ca 670 of pigeonpeas. In addition, 269 chickpea and 1619 pigeonpea elite germplasm lines have also been registered in the genebanks.

Assessing genetic diversity for phenotypic traits

The assessment of diversity in germplasm is important to plant breeders for crop improvement and to genebank curators for efficient and effective management of their collections. A large collection of chickpea germplasm has been characterized for a number of morphophysiological and reproductive traits at ICRISAT, Patancheru, India. Diversity assessment, based on 16,820 accessions and 13 traits, revealed an interesting trend, namely significant differences in means and heterogeneous variances for agronomic traits among regions. Accessions from Africa were earliest to flower, and those from Southeast Asia shortest in stature. Cluster analysis delineated two regional clusters consisting of Africa and South and Southeast Asia in the first, and the Americas, Europe, West Asia, the Mediterranean, and East Asia in the second (Upadhyaya, 2003).

Diversity assessment in pigeonpeas (based on 26 traits in 11,402 accessions) also revealed significant differences in means and heterogeneous variances among regions. Accessions from Oceania were conspicuous by their short growth duration, reduced plant height, fewer branches, pods with fewer seeds, smaller seed size, and lower seed yields. In contrast, accessions from Africa were of longer duration, taller, with multi-seeded pods, and larger seeds. Cluster analysis delineated three clusters: cluster 1 including accessions from Oceania; cluster 2 from India and adjacent countries, and cluster 3 from Indonesia, Thailand, Philippines, Europe, Africa, America, and Caribbean countries (Upadhyaya et al., 2005).

Core and mini core collections to sample representative diversity in the entire collection

The main reason for the low use of germplasm in crop improvement programs is the lack of information on a large number of the accessions, particularly for traits of economic importance which display a great deal of genotype-by-environment interaction (GEI). Frankel (1984) introduced the concept of developing a core collection, which consist of about 10% of the entire collection and represent at least 70% of the genetic variability of the entire collection (Brown, 1989) as a gateway to the enhanced utilization of germplasm in breeding. Core collections of chickpeas and pigeonpeas have been reported (Upadhyaya et al., 2001; Reddy et al.,

2005). However, it soon became evident that developing core collections would not solve the problem of low use of germplasm, because even the core collection could still be large. To overcome this, Upadhyaya and Ortiz (2001) proposed the "mini core collection" concept (consisting of 10% of the core or 1% of the entire germplasm), and developed mini core subsets in chickpeas and pigeonpeas (Upadhyaya and Ortiz, 2001; Upadhyaya et al., 2006b).

Core and mini core collections to identify trait-specific germplasm

Core and mini core subsets provide easy access to the wider spectrum of germplasm collections for discovering useful variation for breeding and genomics applications. When evaluated, new sources of variation have been reported in chickpeas for, for example, high yield (Upadhyaya et al., 2007a), early maturity (Upadhyaya et al., 2007b), large seed size, drought and salinity tolerance (Serraj et al., 2004a,b; Kashiwagi et al., 2005, 2006a), and disease resistance (Pande et al., 2006). In pigeonpeas, new sources of early maturity with high yield (Upadhyaya, unpublished) and salinity tolerance have been discovered (Srivastava et al., 2006).

Polymerase chain reaction-based markers, genotypic diversity, and genetic maps

Development and use of polymerase chain reaction (PCR)-based molecular markers and genetic maps in chickpeas started as early as 1990 (Gaur and Slinkard, 1990). Subsequently, several hundred simple sequence repeat (SSR) markers have been developed in chickpeas (Varshney et al., 2007). The majority of these markers have been mapped in two inter-specific mapping populations: *C. arietinum* ICC 4958 × *C. reticulatum* PI 489777 (Winter et al., 1999, 2000; Pfaff and Kahl, 2003) and *C. arietinum* FLIP 84–92C × *C. reticulatum* PI 599072 (Tekeoglu et al., 2002), and genetic linkage maps of varying genome coverage have been reported.

Molecular markers in pigeonpeas were used to study genetic diversity (Nadimpalli et al., 1994; Ratnaparkhe et al., 1995). The level of polymorphism among wild species was high, while little polymorphism was detected within *C cajan* accessions. Recently, amplified fragment length polymorphism (AFLP) and diversity arrays technology (DArT) analysis have been conducted on a few cultivars and wild species, with similar results of low polymorphism being observed among pigeonpea cultivars (Panguluri et al., 2006; Yang et al., 2006).

Upadhyaya et al. (2006a) developed a composite collection of chickpea (3,000 accessions), representing the entire spectrum of genetic diversity present in ICRISAT and ICARDA genebanks. They genotyped the 3,000 accessions using high-throughput assay and 50 SSR markers. Data on two markers (TA28 and TR2) were not used in the analysis and only a dataset of 48 SSR loci on 2,915 accessions (with less than 3.25% missing data) of the composite collection was used to study structure and diversity and thereby identify a reference set of the 300 most diverse accessions (Upadhyaya et al., 2008). This composite collection showed rich allelic diversity (1,683 alleles, and 35 alleles per locus, with 935 rare alleles, 748 common alleles, and gene diversity from 0.534 to 0.975), and a number of group-specific unique alleles (114 in Kabuli, 297 in Desi, 69 in wild *Cicer*, 114 in Mediterranean, 114 in West Asia, and 117 in South and Southeast Asia groups). The Kabuli group was more genetically diverse than other types. Only four alleles in pea-shaped chickpeas differentiated them from other biological groupings. South and Southeast Asia and West Asia groups shared 74 common alleles, Mediterranean and South and Southeast Asia groups shared 33, and Mediterranean and West Asia groups shared 38. Desi and Kabuli types shared 436 alleles. DARwin structure analysis revealed that Desi and Kabuli chickpeas formed two distinct clusters. A reference set consisting of 300 accessions captured 78% (1,315 alleles) of allelic richness from the composite collection (1,683 alleles).

A pigeonpea composite collection of 1,000 accessions was developed that has been profiled using 20 SSRs and high-throughput assays at ICRISAT. After quality control, a complete dataset of 20 SSRs on 952 accessions (<3% missing data point) was used to dissect the structure and diversity in the composite collection and for the formation of a reference set. A total of 197 alleles were detected in the composite collection, of which 115 were rare and 82 common alleles. Gene diversity varied from 0.002 to 0.726. Biologically, group-specific unique alleles were 60 in wild types and 64 in cultivated types. Simple matching allele frequency-based distance matrix was used to identify a reference set of the 300 most diverse accessions, capturing 95% (187 alleles) of the 197 alleles of the composite collection (952 accessions). The reference set will be profiled with additional markers and extensively phenotyped for traits of economic importance to identify accessions for beneficial traits for utilization in pigeonpea breeding and genomics.

RELEVANT RESULTS PUBLISHED IN THE AREA OF DROUGHT ADAPTATION

Improving the drought tolerance of crop plants has been a difficult challenge under rain-fed environments because: (i) the rainfall received and the frequency of rainfall events vary among the seasons/years and locations; and (ii) large genotype-by-season or genotype-by-location interactions mask the genetic variation of yield. It is difficult to develop phenotypic screens for intermittent drought tolerance since the timing and intensity of this type of drought are fairly unpredictable, whereas screening for terminal drought has been successful in many crop plants (Turner, 1986; Subbarao et al., 1995). The strategies through which crops cope with soil water deficit can be categorized into three groups (Loomis and Connor, 1992): (i) drought escape in which the crops try to complete their reproductive growth before the soil water deficit becomes too severe; (ii) drought avoidance where the crops either minimize the water loss from their tissues or enhance water absorption even under drought conditions; and (iii) drought tolerance where the crops enhance the physical and/or physiological capability of their cells to continue metabolism at low leaf water status.

METHODOLOGY

BREEDING STRATEGY

Chickpeas

Terminal drought escape through early phenology (short-duration) has been the most successful breeding strategy in chickpeas (Gaur et al., 2008). The number of days taken from sowing

to flowering initiation can be recorded easily, providing a good indication of the succeeding phenological traits (days to podding and to maturity), since these traits are intercorrelated. Pundir et al. (1988) reported a range from 33 to 107 days for time to 50% flowering in a collection of 12,018 accessions. This is a wide range and provides good scope for developing cultivars with the desired earliness. In segregating generations, plants that flower early, for instance in 25–30 days at ICRISAT–Patancheru, are tagged and their progenies are evaluated further. Selection for time to flowering is effective even in early segregating generations, since the trait is recessive and controlled by a few major genes (Or et al., 1999; Kumar and van Rheenen, 2000). Several early maturing high-yielding cultivars have been developed, for example, ICCV 2 (released in India, Sudan, and Myanmar), ICCV 92311, JGK 1, and KAK 2 (released in India) and ICCV 92318 (released in Ethiopia) in Kabuli types, and ICCC 37, JG 11, and ICCV 93954 (released in India) and ICCV 88202 (released in Australia, Myanmar and India) in Desi types. Adoption of early maturing varieties such as KAK 2, JG 11, Vihar etc., has shown high impact on enhancement of the chickpea area under cultivation and productivity in short-season environments such as Myanmar (Than et al., 2007) and southern India (Gaur et al., 2008).

It has been possible to develop breeding lines that mature earlier than both the parents by accumulating earliness genes from the two parents. For example, the super-early line ICCV 96029, which flowers in about 24 days at Patancheru, was developed from a cross between two early lines, ICCV 2 and ICCV 93929, which flower in 30 and 32 days (Kumar and Rao, 1996). Super-early lines have further expanded opportunities for cultivation of chickpeas in areas and cropping systems where the cropping window available for chickpeas is narrow and in specific situations where early podding is highly desired, for example when immature grains are used as vegetables (Sandhu et al., 2007).

The prolific root system in chickpeas contributes to grain yield under terminal drought conditions (Kashiwagi et al., 2006a). Reports on the relationship of other morphophysiological traits to grain yield under drought conditions are variable. Thus, breeding efforts using any of these traits as criteria for drought tolerance are few. Although the importance of a prolific root system in terminal drought tolerance is well recognized, only limited efforts have been made to breed for improved root traits. This is because screening for root traits is a destructive and labor intensive process, and difficult to use in large segregating populations.

Combining different drought resistance mechanisms is a potential strategy for enhancing levels of drought resistance. Efforts have been made to combine the large root trait with few leaflets, and breeding lines have been developed combining these traits (Saxena, 2003). However, no information is available on their drought tolerance.

It is well recognized that molecular markers linked to major genes controlling root traits can facilitate marker-assisted breeding (MAB) for those traits. A major quantitative trait locus (QTL) contributing one third of the variation for root length and root biomass has been identified (Chandra et al., 2004) and efforts are being made to identify additional QTLs for root traits. MAB for root traits in chickpeas is in progress.

Pigeonpeas

Traditional long- and medium-duration pigeonpea landraces have evolved under, and have apparently adapted to, terminal drought stress conditions. However, studies show that prevalence of drought during the reproductive phase usually reduces grain yield in pigeonpeas (Chauhan et al., 1992). This is more apparent in environments closer to the equator where evapotranspiration is high. Since a large spectrum of maturity is now available in pigeonpeas, the development of genotypes with the duration that matches well with the duration of soil moisture availability is the first line of defense against terminal drought stress. Another strategy may be to select the single plants from segregating populations that show good yield in hotspots for terminal drought conditions. Furthermore, opting for a shorter duration cultivar than those traditionally used in a region does not necessarily mean sacrificing yield potential, since even extra-short-duration cultivars can produce yields above 2.5t ha^{-1} (Nam et al., 1993).

Hybrids in most crops have been found to perform well under moisture stress conditions. Two pigeonpea hybrids, ICPH 8 and ICPH 9, exhibited higher yield levels than controls irrespective of soil moisture regimes. This suggests that pigeonpea hybrids have the potential to perform well in both dry as well as optimum soil moisture environments (Saxena et al., 1997). This may be related to their superior ability to maintain relative water content (Lopez et al., 1994).

TRIAL PLANNING

Segregating populations originating from hybridization between drought-tolerant and susceptible lines should be grown under drought stress situations, and may be advanced following the single seed descent method until the lines attain a good level of phenotypic uniformity. The advanced lines should be evaluated for at least 3 years to assess their yield potential under terminal drought stress conditions. In the first year, all the lines along with controls should be grown in a preliminary trial with two to three replications on a small plot size, using appropriate experimental designs. In the second and third years, the selected lines along with controls should be promoted to advanced and elite trials, respectively, and should be evaluated multilocationally, preferably with a higher number of replications and a bigger plot size. These trials should be grown under rain-fed conditions (hotspot locations) prone to terminal drought. The entries that outperform (at least by 10%) under drought stress situation may be selected for further evaluation. The best performing drought-tolerant lines should be involved in a more detailed study to dissect the genetic, physiological, and molecular basis of drought tolerance. In all of the trials, soil and climate data must be recorded to document the contribution of these variables to the performance of test entries and also to explain GEI.

DROUGHT STRESS MANAGEMENT AND CHARACTERIZATION

Rainout shelters are designed to protect a certain area of the land against receiving precipitation so that a controlled drought stress can be imposed on that area. Static and moveable rainout shelters have been constructed, with the latter having either

automatic/motorized or manual versions. The automatic version is activated to move over the protected plot by a rain sensor and an electronic drive system. The manual version is moved either by manually switching the drive on or by manually pushing it over the protected plot. The manually handled rainout shelters are lightweight and therefore cheaper to construct. ICRISAT has designed manually driven rainout shelters for use in drought research (Chauhan et al., 1997). One unit made from gabled metal frames covered by polythene sheets is 7.5 m wide, 15 m long, and 2 m high (at the mid-point).

Line-source sprinkler irrigation (Hanks et al., 1976) is the most common method to create a moisture gradient to screen for mid- and terminal drought stress. The plot nearest to the sprinkler head serves as a control (fully irrigated). The amount of water then decreases as the distance of the plot from the sprinkler head increases, allowing increasing intensities of drought stress. Catch cans (plastic buckets) are kept on each plot to measure the amount of water applied by sprinklers. A neutron probe (Model 2651 Troxler Electronic Laboratories Inc, USA), is used to assess the soil moisture at various depths at regular interval through access tubes buried up to the desired depth. However, neutron probe readings need to be calibrated, at least once, against the gravimetrically estimated soil moisture content. The readings derived from calibration of the count ratio of the neutron moisture meter are further converted into volumetric moisture content. A summation of volumetric water present at each soil depth, up to the maximum known depth of root penetration, would provide the amount of available soil water (in cm) in the whole soil profile.

When to impose drought stress – as mid-season or terminal drought – depends upon the crop phenology, guided mainly by the crop duration. In general, test materials are grouped according to similar maturity and then subjected to drought stress. Mid-season drought is imposed at flowering, while terminal drought is imposed during the post anthesis period (preferably 30–40 days prior to maturity). Water is withheld during this period and the drought response is measured against the fully irrigated control.

PLANT WATER STRATEGY

Drought escape

Crops that mature early have a better chance to escape terminal drought. Even in segregating populations, it is easy to score for early maturity, since the number of days taken to flowering correlates fairly well with crop phenology (Murfet and Reid, 1985; Kumar and Abbo, 2001). A faster rate of partitioning has been shown to be associated with drought tolerance, permitting a relatively higher biomass at flowering and escaping part of the terminal drought periods (Krishnamurthy et al., 1999). This can be assessed in any conventional field studies. The traits to measure under drought stressed environments are vegetative and reproductive growth periods, shoot biomass at 50% flowering, and shoot biomass and grain yield at maturity. Similar sets of data under optimally irrigated conditions as well as under drought would permit comparison of the rate of partitioning between the different environments (Krishnamurthy et al., 1999).

Drought avoidance

Stomatal conductance, root traits, water-use efficiency (WUE), and osmotic adjustment (OA) are some important mechanisms allowing selection for drought avoidance. Stomatal conductance regulates transpiration activity through which the plant can minimize water loss under drought stress conditions. It can be estimated by using a gas exchange system such as LI-COR Biosciences' LI-6400 portable photosynthesis system. However, it is time-consuming and, hence, not suitable for large-scale phenotyping of populations – a requisite in molecular breeding approaches. As canopy temperature is a consequence of stomatal activity, it can serve as a proxy to estimate stomatal activity. Plant canopy temperature differences can be quantified using an infra-thermo camera (**Figures 1A,B**) and such differences have been shown to correlate reasonably well with the transpiration status in rice, potatoes, wheat, and sugar beet (Fukuoka, 2005). This sophisticated device can record the thermal digitized image of the plant canopy within a short time (1 min), thus allowing phenotyping for transpiration (stomatal conductance) in large populations. Now with the availability of a macro program, it is also possible to remove the image background (**Figures 2A,B**) of the soil surface (or the soil reflection) before estimating the canopy area alone. These images are also readable through the Macro for the estimation of the range and the extent of canopy fraction with a specific temperature as well the average temperature of the whole canopy (**Figures 3A,B**) and phenotype chickpeas and pigeonpeas for canopy temperature or transpiration status.

Variations in root traits have been associated with enhanced drought tolerance in some crops (Subbarao et al., 1995; Kashiwagi et al., 2005). However, it is very cumbersome to screen for root

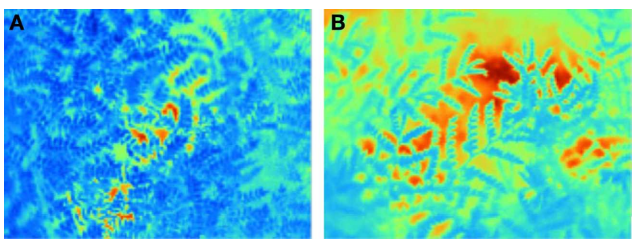

FIGURE 1 | Infra thermal camera images displaying the canopy temperature of a relatively (A) cooler canopy compared to a (B) warmer canopy.

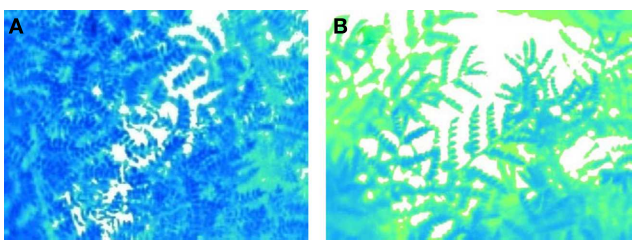

FIGURE 2 | Infra thermal camera images after removal of the background soil reflection and retaining the canopy image alone of a relatively (A) cooler canopy compared to a (B) warmer canopy.

traits under field conditions. To overcome this limitation, a cylinder culture system (using polyvinyl chloride (PVC) pipes 18 cm in diameter and 120 cm tall) has been developed that allows screening of large amounts of chickpea germplasm for root characteristics (root length density and rooting depth). With this system, the sampling efficiency can be improved dramatically up to about 25 profiles worker^{-1} day^{-1}, which is approximately 7.5 times faster than field sampling. These observations correlate well with the field observations ($r = 0.62$, $p < 0.05$) when cylinders are packed with Vertisols premixed to 70% field capacity soil moisture. Further, once the roots have been extracted, the root length can be measured quickly in a sophisticated image analysis system. Thus, platform scanners can each scan more than 150 samples per day, and the powerful image analysis software WinRhizo helps measure root length with a capacity of more than 500 images per day. This system is capable of providing reliable root phenotyping data for any large size populations. However, with the cylinder system, information cannot be obtained on root architecture or branching pattern. An acrylic root rhizobox method would be the ideal way to grow the plants in large populations and the image analysis systems could be applied directly to capture the image digitally and analyze it. Currently, the rhizobox is being optimizing at ICRISAT for both chickpeas (**Figure 4**) and pigeonpeas.

Recent work at ICRISAT has shown that the variation in root length density in the surface layer (15–30 cm depth) also matters and shown to contribute to the seed yield both under moderate to severe drought environments in Vertisols (Kashiwagi et al., 2006a; **Table 1**). This yield contributory effect was explained as a consequence of rapid absorption of soil water by the plants of a fraction of soil water which otherwise would have been lost due to evaporation. A wide range of diversity in rooting profile and

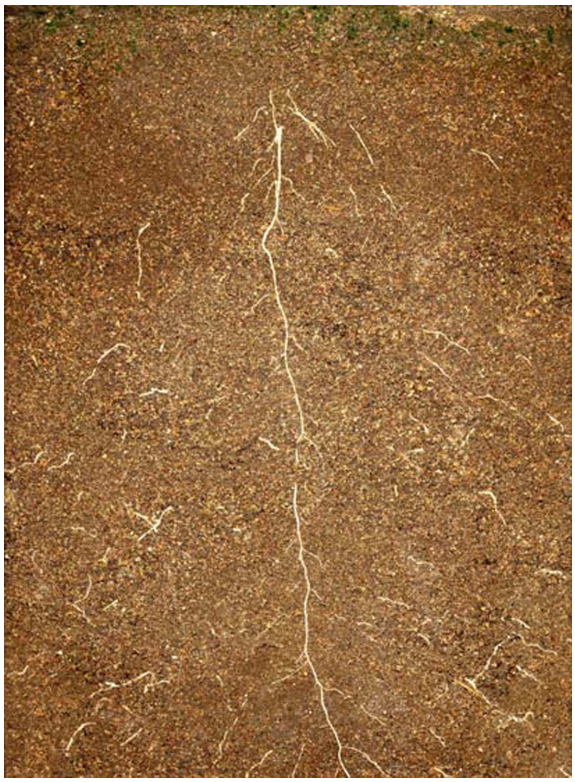

FIGURE 4 | Root morphology of 20 day old chickpea genotype Annigeri seen as a scanned image that was grown in a root box. The prominent tap root growth is completely visible while only part of the branches are visible.

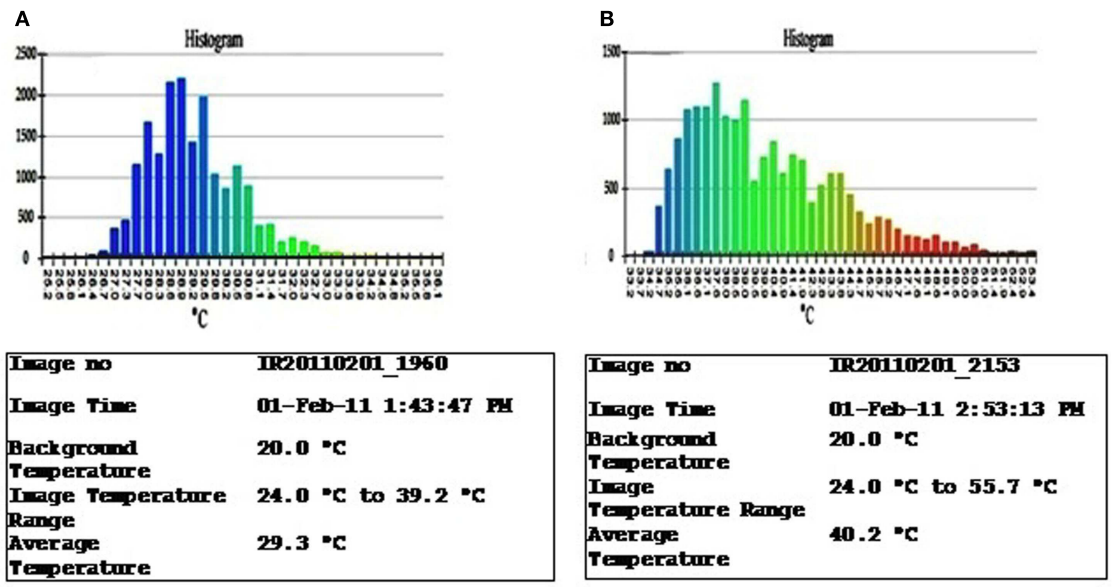

FIGURE 3 | Histograms and the details of the image displaying the extent canopy size under a class of temperature, of the Infra thermal camera images of a relatively (A) cooler canopy compared to a (B) warmer canopy.

Table 1 | Correlation coefficients among the root length densities (total and layer-wise) observed at 35 days after sowing and the seed yield of 12 cultivated chickpea genotypes grown in a Vertisol field during 2000–01 (moderate drought year) and 2001–02 (severe drought year) post-rainy seasons.

	RLD (0–15)	RLD (15–30)	RLD (30–45)	RLD (45–60)	RLD tot
2000–2001 FIELD TRIAL					
RLD 15–30	0.381				
RLD 30–45	−0.024	0.645*			
RLD 45–60	0.059	0.684*	0.935**		
RLD tot	0.532	0.883**	0.797**	0.838**	
YLD (g m^{-2})	0.344	0.699*	0.406	0.405	0.613*
2001–2002 FIELD TRIAL					
RLD 15–30	0.854**				
RLD 30–45	0.757**	0.785**			
RLD 45–60	0.577	0.528	0.819**		
RLD tot	0.943**	0.915**	0.909**	0.761**	
YLD (g m^{-2})	0.442	0.718**	0.779**	0.576*	0.659*

RLD 0–15 = Root length density at 0–15 cm soil depth.
RLD 15–30 = Root length density at 15–30 cm soil depth.
RLD 30–45 = Root length density at 30–45 cm soil depth.
RLD 45–60 = Root length density at 45–60 cm soil depth.
RLD tot = Average root length density at the 0–60 cm profile.
YLD (g m^{-2}) = Grain yield (g m^{-2}) at maturity.
**Significant at P = 0.05 and **Significant at P = 0.01.*

abundance has been noted in chickpeas: ICC 4958 and ICC 8261 have prolific and deeper roots, and ICC 1882 and ICC 283 have small and shallow roots (Kashiwagi et al., 2005). In pigeonpeas, a deeper rooting system is likely to have the advantage of sustaining better growth, even under medium and long-duration drought environments (Chauhan et al., 1992). In contrast, many high-yielding short-duration pigeonpea varieties that were developed to fit into sole cropping systems have shallow root systems and are unable to extract soil water effectively beyond 50 cm (Subbarao et al., 2000).

Water-use efficiency has been used to select for drought tolerance in many crops (Farquhar et al., 1982; Hubick et al., 1986; Wright et al., 1988, 1994). Although improved WUE under drought environments did not always result in better seed yield, it could improve biomass production (White et al., 1990). Phenotyping for WUE in chickpeas and pigeonpeas could be achieved by gravimetric methods in pot culture. In this approach, the pot-grown plants are covered with polythene bags to avoid direct evaporation and the pot weights are measured at the beginning and the end of the experiment to estimate the transpiration loss of each individual plant. The initial plant dry weight is measured at the beginning of the experiment using a different set of plants, and at the end of the experiment, the final plant dry weight is measured using some of the replicates (Krishnamurthy et al., 2007). WUE can be estimated using data on the amount of transpiration and the plant weight gain during the experiment. This method is already in use for groundnuts, and is simple and amenable to phenotyping of WUE in large-sized populations. Since this pot culture method does not permit natural root growth, the potential differences in WUE brought out by the deeper and shallower root systems of chickpea genotypes are expected to be masked. Certain improvements in the methodology to take into account differences in rooting depth are being tested, e.g., growing plants in deep cylinder systems. For pigeonpeas, the pot culture method has to be optimized because the root mass of this crop is expected to be much larger and deeper than that of chickpeas.

Carbon isotope discrimination ($\Delta^{13}C$) has been suggested as an indirect measure for WUE in many crops. Using this approach, Kashiwagi et al. (2006b) showed a clear relationship between WUE (obtained through a gravimetric method) and $\Delta^{13}C$ under soil water deficit conditions. In this method, only a very small quantity (a few mg) of dried plant sample (e.g., leaf) is needed for the analysis using a sophisticated mass-spectrometer, and the samples can be kept stored for a long time. Therefore, it can potentially cope with large-scale phenotyping. For pigeonpeas, the $\Delta^{13}C$ method would be more suitable because of the difficulties in estimating WUE gravimetrically using pot culture.

Osmotic adjustment could be increased to cope with the soil water deficit. It is the active accumulation of solutes in plant cells, as a result of which the water potential in the plant is decreased. OA has been shown to maintain photosynthesis and improve root growth and water extraction ability from the soil under drought conditions (Ludlow, 1980, 1987; Morgan and Condon, 1986). OA in chickpeas showed positive effects on seed yield under drought conditions (Morgan et al., 1991). Differences in OA observed in F_8 progenies and parents have been shown to vary from year to year and have not consistently benefited seed yield in chickpeas under terminal drought, either in Australian or Indian locations (Turner et al., 2007). However, OA enhanced the seed yield in pigeonpeas under drought by delaying leaf senescence and improving the remobilisation of assimilates from the stems and leaves (Flower and Ludlow, 1986, 1987).

Membrane stability has been considered to be an indicator for improving drought tolerance (Gaff, 1980). In most crops, once dehydration has exceeded a critical threshold level, membrane function collapses leading to the death of the plant. However, in some crops, the membrane can be reconstituted and becomes functional within hours of well-watered conditions being provided. This membrane stability could, therefore, be considered as an important trait to contribute to improving plant growth under drought (Gaff, 1980). However, a clear relationship between crop performance under drought conditions and membrane stability has not been reported. It should be well understood before opening it up to large-scale phenotyping.

PHENOTYPING TRAITS

Of the available phenotypic screens, it appears that options for drought tolerance/resistance breeding in chickpeas and pigeonpeas are limited at present to selection for early maturity (drought escape) and root traits (drought avoidance). Both of the traits are easy to score, moderate (root trait) to high (earliness) in heritability, and variation for these characteristics is controlled by a few genes. For example, a single major gene controls flowering in chickpeas (Or et al., 1999; Kumar and van Rheenen, 2000). These two traits can also be scored easily in segregating populations

to map QTLs associated with variation in flowering and root characteristics.

Like in chickpeas, earliness as a trait in pigeonpeas has also been used to select short or extra-short-duration lines that escape terminal drought, with a potential yield of about 2.5t ha^{-1} (Nam et al., 1993). A deeper rooting system would also be a promising trait to improve soil water uptake from the subsoil, thereby improving drought tolerance in pigeonpeas.

Polyvinyl chloride pipe-based phenotypic screens for root traits have been well documented and can be used to screen large numbers of chickpea germplasm/breeding populations (Kashiwagi et al., 2005, 2006a). With some modification, the PVC pipe-based phenotyping of root traits can also be applied to screening for root characteristics in pigeonpeas.

CONCLUSIONS

The core and mini core collections in chickpeas and pigeonpeas, representing over 80% of the diversity present in the entire collection, should be evaluated for traits associated with drought tolerance under terminal drought stress conditions. Chickpea and pigeonpea reference sets, selected on the basis of genotyping results of the composite collections (3,000 and 1,000 accessions respectively), should be evaluated for drought tolerance.

There is a need for further refinement of screening techniques and large-scale adoption of such techniques to select for traits associated with drought tolerance in breeding/mapping populations. There is also a need to saturate the mini core subset or reference set with an increased number of SSR and DArT markers to scan the whole genome and be used to detect marker-trait association using association genetics. The utility of leaf chlorophyll content as measured by Soil–Plant Analyses Development (SPAD) chlorophyll meter reading, WUE, OA, and leaf size and shape as a measure of drought tolerance need to be investigated further.

Early maturing pigeonpeas have a prolific but shallow root system. Consequently, there is a need to identify pigeonpea germplasm possessing early maturity and deep rooting. More attention is needed to understand marker-trait association in order to find PCR-based markers associated with drought tolerance to initiate marker-aided selection for traits associated with drought tolerance. Finally, there is a need to investigate the physiological basis of superior performance of pigeonpea hybrids under drought stress conditions.

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Phenotyping cowpeas for adaptation to drought

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Methods for phenotyping cowpeas for adaptation to drought are reviewed. Key factors involve achieving optimal time of flowering and cycle length, and appropriate morphology for different types of cultivars as they relate to their utilization for dry grain, hay, and fresh pea production. Strong resistance to vegetative-stage drought is available and should be incorporated. The extreme ability of extra-early erect cowpea cultivars to escape terminal drought should be exploited in zones with very short rainfall seasons. In zones with the possibility of limited rainfall in the middle of the growing season, resistance to mid-season drought, and the delayed-leaf-senescence trait can be valuable. Breeding for water-use efficiency, deeper rooting, and heat tolerance are discussed. Diseases and pests that influence adaptation to drought are considered. Resistance to the organism causing ashy stem blight disease should be incorporated because this disease can destroy cowpea seedlings under hot, dry soil conditions. The value of varietal intercrops with contrasting types of cowpea cultivars in enhancing adaptation to drought is described. Implications of cowpea/cereal rotations for cowpea breeding are discussed. Breeding strategies for enhancing cowpea adaptation to drought are described.

Keywords: cowpeas, drought resistance, drought adaptation, water-use efficiency, heat tolerance, leaf electrolyte leakage

INTRODUCTION

USES OF COWPEAS FOR FOOD AND FEED AS THEY RELATE TO CULTIVAR TYPES AND DROUGHT ADAPTATION

The various uses of cowpeas (*Vigna unguiculata* L. Walp.) as food and as feed for animals influence the types of cultivar that are needed. The developmental and morphological traits associated with these uses also influence the adaptation of the cultivars to drought. The main use of cowpeas as a food is as dry grains which have more resistance to terminal drought than either fresh peas or immature pods.

Cowpea grains have substantial quantities of protein (about 25%) and carbohydrate (about 64%), vitamins, and fiber. The amino acid and vitamin profiles of cowpea grain complement those of cereals. When one part of cowpea grain is combined with three parts of cereal grain it provides a near-complete food. In the subsistence farming areas where it mainly is grown, the main role of cowpeas is as a concentrated source of protein that is cheaper than fish, meat, poultry, or dairy products and combines well with cereal grains in the human diet.

In some cases, the pods of cowpeas are harvested when they are full sized and before they dry out. The pods are then shelled and the grains are cooked and eaten as fresh "southern peas." This practice has been important in the southeastern United States. Since 1985 the consumption of cowpeas as fresh southern peas has also become important in the semi-arid Sahelian zone of Africa in Senegal. The reason for this is that, with the introduction of extra-early cowpea varieties, southern peas became available during the "hungry period" of August and September. This period is just before the main harvests of pearl millet, sorghum, and dry grains of traditional varieties of cowpeas and peanuts, which begin in October. Estimates made in Senegal from 1994 to 1996 indicated that fresh southern peas commanded a price twice that of dry grains on a per seed basis. Marketing of fresh southern peas brings in much-needed cash during the hungry period and is done mainly by women. Estimates in the early 2000s indicated that about 30% of cowpea grains were consumed as fresh southern peas in Senegal. Substantial quantities of cowpeas were also being consumed as either fresh southern peas or immature pods in Niger. Consumption of cowpeas as fresh southern peas is probably increasing in the Sahelian zone as extra-early cowpea cultivars are being made available to farmers.

Immature pods of cowpea cultivars are consumed fresh in Kenya (African green beans), Trinidad (bodie beans), and Southeast Asia (yard-long beans). Bush-type cultivars with succulent pods bred for this particular use are available. The fresh leaves of cowpeas are consumed in sauces, especially in East Africa.

A major use of cowpeas in the Sahelian zone of Africa is as hay, after the pods have been harvested. Hay is particularly important in Niger where it had a cash value to farmers of about half of that of the grain in the 1990s. Hay is used to maintain draft animals during the long dry season in the Sahel and to fatten rams and goats in preparation for various festivals.

Cowpeas are particularly important as a rotation crop with cereals. They can enhance the fertility of the soil with respect to nitrogen and phosphate, thereby benefiting subsequent cereal crops. Certain cowpea lines can cause suicidal germination of the seeds of *Striga hermonthica*, which parasitizes pearl millet, sorghum, and maize. Certain lines can also suppress the populations of the nematode *Scutellonema cavenessi*, which is a major pest of pearl millet, sorghum, and peanut in the Sahel. Two successive

crops of pearl millet (Sauna III cultivar) can suppress the organism responsible for the ashy stem blight disease (*Macrophomina phaseolina*) that can attack cowpea seedlings growing in hot dry soils. These potential benefits of rotational systems involving sole crops of cowpeas, sole crops of cereal, and sole crops of other species, such as peanuts, have not been adequately quantified. In Africa, cowpeas are still often grown as an intercrop with cereals, although they are mainly grown as a sole crop in Senegal.

The guidelines for breeding cowpeas for adaptation to drought provided below are based on the assumption that, in the future, most cowpeas in the world will be grown as a sole crop in rotation with sole crops of cereals and some other species. The traits described that confer adaptation to drought in cowpeas as a sole crop, also are effective when cowpeas are grown as a component of a species intercrop.

CULTIVATED AREA AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

The cultivated area of cowpeas world-wide was estimated as being 14 million ha in 2000 (Singh et al., 2002) with the most production being in Africa in the Sudanian Savanna zone (especially in northern Nigeria) and the Sahelian zone (especially in southern Niger, central Mali, the Sudan, and Senegal). In addition, there was significant production in northeastern Brazil, eastern and southern Africa, and Southeast Asia. Most of the areas where cowpeas are grown are semi-arid, and often experience droughts ranging from being severe in the Sahelian zone to being moderate in the Sudanian Savanna zone, northeastern Brazil and East Africa during the long rainy season.

Average grain yields in 1999–2000 (in kg ha^{-1}) were 417 in Nigeria, 171 in Niger, 324 in Brazil, 220 in Mali, and 362 in Senegal. There is considerable opportunity to increase these yields. For example, under optimal conditions, cowpeas have produced 3,000 kg ha^{-1} in Senegal. Yield under optimal conditions can be estimated effectively for different cultivars and parts of the world by using a model. Grain yield of cowpeas in optimal conditions (Y) has been modeled by Hall (1999) using the following equation:

$$Y = \sum_{i=n}^{i=0} \mathrm{PPFD}_i \cdot \mathrm{GC}_i \cdot Q_i \cdot \mathrm{PG}_i$$

where PPFD is the flux density of photosynthetically active photons per day, GC is the ratio of plant cover to ground area on that day, Q is the efficiency for the conversion of intercepted photons to plant dry biomass, and PG is the ratio for the partitioning of plant biomass to grain. This equation applies over the period of days (n) when carbohydrate is being partitioned to grain. The number of days from the beginning of flowering to the time when 95% of the pods are mature provides an estimate of the value of n.

Examples of the use of this model are provided below based on measurements made in California and Senegal (Hall, 1999). The length of the reproductive period for cultivars growing in California was 50 days for the first flush of flowering and pod production. Due to long 15-h days and sunny conditions, PPFD can be as much as 50 mol photon m^{-2} (a conversion factor of 2 μmol photon J^{-1} can be used if the only data available are for daily solar irradiance). Under optimal conditions, GC should be close to 1.0. In California Q values for cowpeas of 450 mg dry matter mol photon^{-1} have been obtained. Under optimal conditions values for partitioning would be slightly less than the optimal apparent harvest index (HI; the ratio of grain yield to total shoot biomass at harvest), say, about 0.444. Putting these values into the model predicts that, with long sunny days, cowpeas can accumulate about 1,000 kg ha^{-1} of grain in 10 days. This would predict that during a 50-day period of grain filling under these optimal conditions, cowpeas could produce 5,000 kg ha^{-1}. Yields of this magnitude have been observed in large plot and large field conditions in California for crops that began flowering in 50 days and reached maturity in 100 days from sowing, although yields of 4,000 kg ha^{-1} are more common (Ehlers et al., 2000).

In Senegal, under tropical conditions with higher temperatures, these Californian cultivars develop faster and have a reproductive duration for the first flush of pods of only 25 days, the PPFD is less than 50 mol photon m^{-2} due to shorter days and cloudiness, and their grain yields were only 2,400 kg ha^{-1} (Hall and Patel, 1985). A cultivar that is well adapted to Senegal, "Mouride," has produced grain yields as high as 3,000 kg ha^{-1} and has a reproductive duration of about 35 days. These performances are approximately consistent with the model. The take-home message is that the yield potential of cowpeas is determined by the length of the reproductive period, which depends on the cultivar and temperature, the amount of sunlight during this period, which depends upon on the length of the day, the degree of cloudiness and the position of the sun in the sky, and the extent of production and partitioning of carbohydrate to grain.

GENETIC AND GENOMIC RESOURCES

In the early 2000s, the major cowpea germplasm collections were maintained by the International Institute of Tropical Agriculture (IITA; with 14,000 accessions), the United States Department of Agriculture (USDA; with 8,000 accessions), and the University of California, Riverside (UC-Riverside; with 5,000 accessions). The Istituto di Genetica Vegetale in Bari, Italy, held a collection of Mediterranean and African landraces (about 600 accessions). Several national programs in Africa (including Botswana, Burkina Faso, Ghana, Kenya, Nigeria, and Senegal), and the national programs in Brazil and India had substantial cowpea germplasm collections. In addition, IITA and the Botanical Research Institute in Pretoria, South Africa stored seeds of some wild relatives of cowpeas.

Ouédraogo et al. (2002) have developed a genetic linkage map for cowpeas that provides a basis for initiating marker-assisted selection (MAS). Further discussion of cowpea genetic and genomic resources can be found in Timko et al. (2007).

RESEARCH RELEVANT TO BREEDING COWPEAS FOR DRY, HOT ENVIRONMENTS

RESISTANCE TO VEGETATIVE-STAGE DROUGHT

Cowpeas were shown to have substantial resistance to vegetative-stage drought in California (Turk et al., 1980). In one treatment, seeds were sown into a dry soil profile with just enough water to permit germination and emergence. Plants survived for 43 days under very hot dry summer conditions with no rain but were

badly stunted. Most other crop plants would have been killed with this treatment. The stunted plants were irrigated on the 44th day and recovered rapidly, producing very high grain yields of 4,000 kg ha^{-1} by 107 days after sowing. A control treatment that had received optimal irrigations every week produced a similar grain yield during the same period. Subsequently, cowpeas have been observed to survive vegetative-stage droughts in the Sahelian zone that killed pearl millet and peanut plants growing in the same field (Hall, unpublished observations).

Survival of vegetative-stage droughts by cowpeas was associated with the maintenance of higher leaf water status than pearl millet (Petrie and Hall, 1992a,b,c). Cowpeas are fairly unique among crop plants in exhibiting very small changes in leaf water potential when subjected to drought and very little osmotic adjustment (Shackel and Hall, 1983). Cowpeas also have stomata that are very sensitive to soil drying, partially closing before any changes in leaf water potential were detected (Bates and Hall, 1981). When cowpea plants are subjected to drought in field conditions, their leaves do not usually wilt but tend to orient more vertically, tracking the sun in a manner that minimizes the interception of solar radiation (Shackel and Hall, 1979). These mechanisms contribute to the unique ability of cowpeas to survive extreme vegetative-stage droughts that kill most other crop plants.

A screening technique for survival of drought at the seedling-stage has been developed that uses a shallow soil layer in boxes (Singh and Matsui, 2002). When 190 diverse cowpea breeding lines from IITA were screened with this technique, only 22% were found to be susceptible to drought. This suggests that most current cowpea cultivars may have resistance to vegetative-stage drought. Inheritance studies indicated that the susceptibility was due to a single recessive gene. In another screening test it was shown that resistant cowpeas survived seedling-stage drought longer than all other species tested: lablab beans, bambara groundnuts, peanuts, pearl millet, sorghum, greengram, blackgram, maize, and soybeans.

There are two conditions where the resistance of cowpeas to vegetative-stage drought is not effective. First, when the organism causing the ashy stem blight disease (*M. phaseolina)* is present in the soil, resistance of cowpeas to vegetative-stage drought breaks down and the plants die. This disease organism is widespread and causes severe damage to cowpea seedlings growing in the hot, dry soil conditions that often occur in the Sahelian zone and in Botswana. Strong resistance to this disease was recently detected in cowpea germplasm (Ehlers, unpublished observations). Second, the lesser corn stalk borer (*Elasmopalpus lignosellus*) has attacked and killed young cowpea plants that were subjected to drought while plants in well-watered treatments were not killed. Varietal resistance to this pest is not yet available.

ESCAPING TERMINAL DROUGHT BY USING EXTRA-EARLY ERECT COWPEAS

Since1968, many years of droughts have occurred in the Sahelian zone, resulting in very short growing seasons. In response to this problem UC-Riverside and the Institut Sénégalais de Recherches Agricoles (ISRA) bred extra-early cowpea cultivars that had very short growth cycles. The ideotype involved combining vegetative-stage drought resistance with erect plant habit and early synchronous flowering beginning on low nodes on the main stem. These cultivars do not cover much ground during the vegetative-stage, and should be planted at close spacing (50 cm between rows and 25 cm between seeds). Close spacing also enhances the synchrony of pod production and earliness of harvest.

UC-Riverside bred the cultivar "Ein El Gazal" by crossing an erect early California cultivar that had resistance to vegetative-stage drought and an erect early line from Senegal bred by Djibril Sène. Lines were selected in California that had even earlier flowering than the parents. These lines were tested in the Sahelian zone at Louga, Senegal, in 1982. Line "UCR 1- 12-3" (subsequently called "Ein El Gazal") began flowering in 35 days and produced 1,091 kg ha^{-1} of dry grain by 55 days from sowing with a useful rainfall of only 181 mm and very hot conditions throughout the short season (Hall and Patel, 1985). Other crops grown in the Louga area in 1982, pearl millet, peanuts, and traditional cowpeas which had 90-day cycles, produced virtually no grain. In the same year at a wetter location (Bambey, Senegal, with 452 mm rain), "Ein El Gazal" produced 2,406 kg ha^{-1} of dry grain by 60 days from sowing (Hall and Patel, 1985) indicating it had high yield potential. It also performed well in the Sahelian zone of the Sudan (Hall and Patel, 1985) where it was released as a cultivar (Elawad and Hall, 2002). An extra-early erect cultivar bred in Senegal by ISRA, "Melakh," has resistance to two seed-borne diseases and cowpea aphid, and partial resistance to flower thrip. "Melakh" was released in Senegal in 1996 (Cisse et al., 1997). An early erect cultivar that matures in 60–65 days, "Vuli-1," was bred for cultivation during the short rains in Tanzania (Mligo and Singh, 2007).

"Ein El Gazal" and "Melakh" have three advantages: (i) they can survive vegetative-stage droughts; (ii) they can produce significant grain in locations and years when the rainy season is very short and all other crops produce little grain; and (iii) they produce fresh southern peas beginning about 45 days from sowing, which is during the hungry period in the Sahel. However, these extra-early erect cowpea cultivars have three disadvantages: (i) they must be sown at close spacing, which is not a major problem in Senegal where horse-drawn seeders are used; (ii) they produce very little hay; and (iii) they can be devastated by a mid-season drought (Thiaw et al., 1993) and some biotic stresses.

RESISTANCE TO MID-SEASON DROUGHT

Several approaches have been taken in breeding cowpea cultivars with greater resistance to mid-season drought than the extra-early erect cultivars. Line "58–57" was selected from a landrace growing around the Senegal River by Sène (1966). Among landraces it has a relatively short cycle, beginning flowering 41 days after sowing and reaching maturity in 75 days. It has a spreading habit and experiences sequential flowering, which may partially account for its resistance to mid-season drought. "58–57" is dual-purpose in that it can produce significant hay as well as grain. It should be sown at moderate spacing, such as with 50 cm between rows and 50 cm between seeds in the row.

"Mouride" was bred by ISRA by crossing "58–57" with an erect breeding line and was released in Senegal in 1992 (Cisse et al., 1995). It is semi-erect, and under well-watered conditions reaches maturity 65 days after sowing. It has substantial yield potential, having produced 3,000 kg ha^{-1} at Bambey, Senegal, and has

considerable ability to resist mid-season drought. The basis for this resistance is not known. "Mouride" also has resistance to two seed-borne diseases, the plant parasitic weed *Striga gesnerioides* and cowpea weevil. It should be sown at close spacing, such as with 50 cm between rows and 25 cm between seeds in the row.

Reproductive activity in cowpeas can involve two separate flushes of flowering with a period of about 1 week in between when no flowers are produced. Grain yield from the second flush strongly depends on the extent of plant death due to soil pathogens after the first flush of flowering and pod production is completed. A delayed-leaf-senescence (DLS) trait has been discovered that enables cowpeas to maintain a green canopy after the first flush of pods is mature and more consistently produce a second flush of flowers and pods (Gwathmey et al., 1992a). When genotypes with this trait were subjected to a mid-season drought during the first flowering period, they had the ability to survive and then produce a substantial second flush of pods and grain (Gwathmey and Hall, 1992). Early erect lines with the DLS trait were bred, selected and tested in Senegal. Under well-watered conditions they produced a first flush of 2,000 kg ha^{-1} of grain in about 65 days from sowing and then a second flush producing an additional 1,000 kg ha^{-1} of grain by 95 days from sowing (Hall et al., 1997). Cultivars with both earliness and the DLS trait may be most useful in the Sudanian Savanna, which usually has a longer rainy season than the Sahelian zone but occasional mid-season droughts.

The mechanism of the DLS trait is complex. It results in the accumulation of greater reserves of carbohydrates in the base of the stem (Gwathmey et al., 1992b) and probably also in the roots. The trait is most strongly expressed when an organism that causes the senescence of cowpeas during pod maturation is present in the soil. This organism is probably *Fusarium solani* f. sp. *phaseoli*. Presumably, roots with greater carbohydrate reserves are more resistant to this root rotting organism. Irrespective of the complexity of the trait, it has been consistently expressed by plants in soils where cowpeas have been grown in rotations for several years in many locations in California and also at Bambey in Senegal. The DLS trait can be selected effectively with advanced lines in appropriate field nurseries, and appears to have simple inheritance (Ismail et al., 2000).

Dual-purpose cowpea cultivars have been bred by B. B. Singh of IITA for use in the Sudanian Savanna zone (Dingkhun et al., 2006). These cultivars are semi-erect, and reach maturity in 85–95 days. They can produce 1,500–2,500 kg ha^{-1} grain and 3,000–5,000 kg ha^{-1} of hay when sown at moderately close spacing. The high hay production was associated with the ability to continue producing leaves after the grain had matured. Presumably these cultivars have the DLS trait.

VALUE OF VARIETAL INTERCROPS

Rainfall in the Sahelian zone has been so variable and droughts have been so extreme that a single type of cowpea cultivar may not adequately meet all of the needs of farmers. Consequently, it has been recommended that at least two types of cultivar be bred, and that farmers grow at least two types every year to enhance the chances that significant grain and hay production will be achieved every year (Hall, 2004a). The extra-early erect cultivars that have synchronous flowering and mature within 60 days from sowing, such as "Ein El Gazal," escape late-season drought and also have resistance to vegetative-stage drought. They provide useful food during the hungry period but are devastated by mid-season drought and also produce little hay. Spreading cultivars with sequential flowering and a medium-cycle maturing in 75 days, such as "58–57," have substantial resistance to mid-season drought and vegetative-stage drought. They produce useful quantities of both grain and hay, unless the growing season is very short, in which case they produce little grain. Also, they produce little grain during the hungry period. The most effective way for farmers to grow both types of cultivar may be in alternating rows as a varietal intercrop.

A comparison was made of varietal intercrops consisting of alternating rows of extra-early erect cultivars and medium-cycle spreading cultivars with sole crops of the same cultivars in the Sahelian zone of Senegal (Thiaw et al., 1993). The varietal intercrops produced more grain and hay under dry conditions with infertile soil, and were more stable than any of the sole crops of cowpeas that were tested. Some mechanisms for the beneficial effects of these varietal intercrops are as follows. In years when a mid-season drought occurs, the extra-early erect cultivar becomes stunted and the medium-cycle spreading cultivar compensates by growing into the space that is made available and produces much of the grain and hay yields of the intercrop. In years with a distinct, short rainy season, the extra-early erect cultivar produces abundant grain, while the medium-cycle spreading cultivar produces abundant hay for the intercrop but little grain. Farmers in the Sahel typically grow several cowpea cultivars but the author is not aware of any that have adopted the varietal intercrop system.

Cowpeas grown in a varietal intercrop would be rotated with sole crops of pearl millet and peanuts. For example, a 4-year rotation may be effective in parts of the Sahel in which cowpeas are followed by pearl millet, then peanuts, and then pearl millet. Thus, four equal-sized fields with staggered rotations would devote 50% of the land to producing pearl millet, the staple food crop, 25% to cowpeas, and 25% to peanuts.

WATER-USE EFFICIENCY

Increasing water-use efficiency (WUE), i.e., biomass production/crop water-use, has been proposed as a mechanism to enhance the adaptation of crops to drought. By measuring the stable carbon isotope composition of cowpea tissues, genotypic differences in transpiration efficiency (TE), i.e., photosynthetic rate/transpiration rate that were associated with genotypic differences in WUE have been detected (Ismail and Hall, 1992; Hall et al., 1994a). However, genetic selection experiments showed negative genetic correlations between grain yield and TE (and therefore WUE) for cowpeas growing under either well-watered or water-limited stored soil moisture conditions (Condon and Hall, 1997; Hall et al., 1997). These results may, in part, be explained by: (i) the negative genetic correlations that were observed between HI and TE; and (ii) the possibility that more open stomata are responsible for the decreases in TE and are resulting in enhanced photosynthesis and biomass production. In the water-limited environment, the lines with decreased TE but greater grain yields may also have had deeper rooting and greater soil water extraction.

Using selection for stable carbon isotope composition of plant tissues to decrease TE in cowpeas may provide a means to enhance grain yield under irrigated conditions in California. But there is no clear evidence that this approach would enhance drought adaptation in the Sahelian zone. TE was negatively correlated with grain yield for cowpeas grown on stored soil moisture in a subtropical zone in California. However, when a common set of cowpea genotypes was grown in California and in the tropical Sahelian zone of Senegal, there was no consistency in genotypic ranking for stable carbon isotope composition across these zones (Hall et al., 1994b). In Senegal there was a tendency for well adapted local cultivars to have carbon isotope compositions indicative of low TE.

DEEPER ROOTING

Adaptation to drought may be enhanced by breeding cowpeas with deeper rooting for a target production zone (TPZ) where the crop is grown under limited rainfall with substantial water available deep in the soil. A method has been developed for selecting stable lines of cowpeas for differences in rate of rooting (Robertson et al., 1985). The method consists of injecting a band of herbicide deep in the soil, sowing cowpea lines in row sections above the herbicide band, and scoring plants daily for the first sign of leaf herbicide symptoms as an indication that the roots had reached the herbicide band. In a separate experiment, genotypes that had showed herbicide symptoms earlier extracted more soil moisture deep in the profile. This method for detecting genotypic differences in rooting also has been shown to be effective in peanuts in Senegal (Khalfaoui and Havard, 1993). It is only effective with stable lines because the herbicide kills the plants.

The relatively small area of cowpeas grown in the Sahel during the dry season on deep alluvial soil next to rivers (the "*décrue*" system) might benefit from deeper rooting. The only water available to these plants is present in the soil at sowing. A breeding program for this environment might use the herbicide system to indirectly select for deeper rooting. Alternatively, a simpler indirect system could be used in which plants are sown at wide spacing (100 cm × 100 cm) in the fully moistened deep alluvial soil during the dry season, and then selected based upon the extent of total shoot biomass production. Presumably, genotypes that developed deeper, more effective root systems would also produce more shoot biomass.

Deeper roots may not be advantageous for the major area of cowpea production during the rainy season on the widespread sandy soils in the Sahel. Soil water depletion data have indicated that current cultivars can develop root systems as deep as 200 cm under these conditions. Cultivars that developed even deeper roots would not gain much additional water from these sandy soils where the field capacity is only about 8%, and only a few percentage points of soil water are available. Larger root systems have an additional cost to the plant, in terms of the carbohydrate and energy required for their construction and maintenance, a cost that must be more than repaid if the additional roots are to be of benefit to plant adaptation.

HEAT TOLERANCE

The semi-arid zones where a large area of cowpeas is grown also are hot. In subtropical areas with long days, it has been shown that high temperatures late at night disturb flower production and pollination, causing reductions in pod production (Hall, 2004b). Methods for breeding heat-tolerant cowpeas have been developed (Hall, 1992), involving selection for flower production and high pod set under hot night temperatures and long-day field or glasshouse conditions. These methods were used to breed a heat-tolerant cowpea cultivar for use in California (Ehlers et al., 2000). In addition, crosses were made between heat-tolerant lines developed in California and cultivars used in Ghana by K. O. Marfo. Lines were selected for heat tolerance in California and for other traits in Ghana. Two of the lines were released as cultivars in Ghana from this program (Padi et al., 2004a,b).

However, the value of the heat tolerance genes in tropical zones of Africa has not been established. Studies with pairs of lines with and without the heat tolerance genes showed the genes to be effective in California (Ismail and Hall, 1998) but of no benefit in Ghana and Senegal (Hall et al., 2002). The main reason for the latter result may be that the heat tolerance genes are much more effective under the long-day conditions that occur during the growing season in California, than under the shorter days that occur during the growing season in Ghana and Senegal (Ehlers and Hall, 1998). Another possible reason is that to be effective in tropical zones of Africa, the heat tolerance genes must be combined with other compatible genes. For example, some of the heat-tolerant lines bred in California produced many pods in Senegal but the pods did not develop due to biotic stresses.

A method has been developed that may be useful for selecting for heat tolerance under either short- or long-day conditions (Thiaw and Hall, 2004). Lines selected for low leaf electrolyte leakage under heat stress also had reproductive-stage heat tolerance. In addition, lines selected for reproductive-stage heat tolerance in long-day field conditions also had low leaf electrolyte leakage under heat stress. The leaf electrolyte leakage test can be conducted in a laboratory, with relatively simple equipment, using leaves from plants growing in field nurseries. However, it has not yet been determined whether this method can be used to select under short-day tropical conditions for plants that also exhibit heat tolerance during reproductive development under hot short-day tropical conditions.

In addition, incorporating heat tolerance during reproductive development was shown to result in a semi-dwarf habit (Ismail and Hall, 1998). While a semi-dwarf habit may be useful under intensive irrigated production in subtropical areas such as California (Ismail and Hall, 2000), it is not suitable for use under harsh rainfed conditions in semi-arid tropical environments where the expression of dwarfing is more extreme due to the more rapid plant development that occurs at higher temperatures. It has not been determined whether the association between the heat tolerance effects and dwarfing can be separated, or whether it is a pleiotropic effect of the same set of genes. Consequently, while the value of breeding for reproductive-stage heat tolerance has been established for cowpeas in subtropical long-day conditions, its value for tropical short-day environments is not clear.

BIOLOGICAL NITROGEN FIXATION

Usually, cowpeas are nodulated effectively when growing in field conditions. The author is not aware of a well-documented case

where cowpeas have responded to seed inoculation with *Rhizobia* spp. under field conditions in California (Hall and Frate, 1996) or elsewhere. State-wide average yields of cowpeas in California are high (they were 2,900 kg ha^{-1} in 1995), yet virtually no yield responses to nitrogen fertilization have been reported (Hall and Frate, 1996).

The negligible or small responses of cowpeas to soil fertilization with nitrogen may be due to its substantial biological nitrogen fixation, as has been observed in California (Elowad and Hall, 1987; Elowad et al., 1987) and Senegal (Mamadou Ndiaye, unpublished work of ISRA in the 1980s). Drought can substantially reduce biological fixation of cowpeas and cause plants to rely more on assimilated inorganic nitrogen (Elowad and Hall, 1987). This indicates that, for cowpeas, biological nitrogen fixation may be more sensitive to drought than is photosynthesis. However, this constraint does not appear to be large enough in cowpeas to justify the massive upstream plant breeding program that would likely be needed to enhance cowpea contributions to biological nitrogen fixation under drought.

METHODS FOR BREEDING COWPEAS FOR ADAPTATION TO DROUGHT

BREEDING STRATEGIES

A key first step is to determine the earliness of flowering, cycle length, and type of plant habit that are required in the TPZ for which the breeding program is responsible.

Optimal cycle lengths can be determined for cowpeas growing under rainfed conditions in semi-arid zones by using a hydrologic budget analysis. An effective way to do this has been described by Dancette and Hall (1979). With this method, a substantial set of rainfall data is needed for the TPZ covering at least 50 years. Also, estimates are needed of cowpea crop water-use coefficients, such as the values in Hall and Dancette (1978), and rooting depths, potential evapotranspiration, and the field capacity and lower limit of water availability in the soil. Hydrologic budgets are then determined for each of the 50-years of rainfall data to determine the maximum cowpea cycle length that would have been supported by providing the crop with at least 75% of its water requirement in that year. The 50 cycle lengths obtained for the 50-years of data are then examined, and the maximum cycle length that was predicted to be achieved in 40 out of the 50-years is chosen – this is the optimal cycle length for the TPZ. Cultivars with this cycle length are predicted to receive at least 75% of their maximal water requirements in 40 out of 50 years but suffer terminal drought of varying intensities in 10 of the years.

If the optimal cycle length is less than 75 days, then it will probably be necessary to develop a cultivar with synchronous flowering, which means it must be erect and sown at close spacing, and be early flowering to provide a reproductive period of adequate length. This type of cultivar would also be developed if the objective is to provide food during the hungry period, even in locations where rainfall supports a longer optimal cycle length. If the optimal cycle length is greater than 75 days, then more choices are possible, including the use of photoperiod-sensitive types for cases where the optimal cycle is longer than about 90 days. For grain production, erect types should be considered. For dual-purpose grain/hay types, either spreading types or semi-erect types with delayed-leaf-senescence (DLS) could be sought. For hay production, spreading types would be most consistent with the crop management methods currently being used in the Sahelian zone, such as in Niger.

Hydrologic budget analyses could also be used to determine the probability of mid-season droughts. Where such droughts occur frequently, consideration might be given to the use of either spreading types, semi-erect types such as "Mouride" or, if the optimal cycle length is long enough, early erect types with DLS. In many semi-arid environments varietal intercrops with contrasting types of cultivars may be useful.

TRIAL PLANNING

A key step in developing cultivars with enhanced adaptation to drought is to select for high average, stable yields in the TPZ. Initial field trials should be conducted with sole crop cowpeas grown on experiment stations throughout the TPZ. Separate trials should be conducted with sets of lines that have different cycle lengths and morphology. For example, one series of trials might be conducted with extremely extra-early (60-day cycle) erect lines, and another series with spreading 75-day cycle lines. Grain and hay yield should be determined for all entries, which will also provide the opportunity to calculate the apparent HI, a trait that may be useful in selection. A subset of the most effective lines would be chosen based on their performance in the experiment station trials. This small subset of lines would then be evaluated in farmer-managed, on-farm trials in the TPZ that include evaluations of grain quality by local people. Those lines that meet the needs of farmers and consumers, and that have high average, stable yields of grain, southern peas, and hay (depending on farmer and consumer needs and the type of cultivar being tested) would be candidates for release as cultivars.

WATER STRESS MANAGEMENT AND CHARACTERIZATION

Rainfall and potential evapotranspiration data, and in some cases pre-season and post-season soil moisture data should be taken at all trial sites so that hydrologic budget analyses can be performed to help in interpreting the data on plant performance. Ideally these analyses would be conducted for both the experiment station and on-farm trials.

PHENOTYPING TRAITS

With respect to drought adaptation, in addition to careful consideration of optimal phenology and morphology, it should be ensured that the available vegetative-stage drought resistance is present. The seedling screen involving a shallow soil layer in a box (Singh and Matsui, 2002) can be used to screen parents and, if necessary, segregating generations. Singh and Matsui (2002) and Muchero et al. (2008) have described some variation in this trait but it is not known whether it is worth pursuing, since some cowpea cultivars already have very strong resistance to vegetative-stage drought.

The robustness of cowpeas under hot dry soil conditions during the seedling and vegetative-stages could be enhanced considerably by incorporating the resistance to ashy stem blight (*M. phaseolina*) that recently has been discovered (Ehlers, unpublished observations).

The DLS trait may be useful, in some environments for enhancing resistance to mid-season droughts and/or enhancing hay quality. DLS can be incorporated using field nurseries that are naturally infested with *F. solani* f. sp. *phaseoli*. This organism may already be present in fields where cowpeas have been grown for several years, even where rotations have been practiced. Selection for DLS has been effective with lines in advanced generations (Ismail et al., 2000) but may not be effective with single plants. When selecting plants with DLS, only those with high pod set should be chosen because plants with little or no pod set, such as the occasional sterile plant, exhibit a false form of DLS that has no agronomic value.

In addition, high priority in the breeding program must be given to incorporating resistances to seed-borne diseases since these diseases are usually major problems confronting cowpea production. Genetic resistances are available in most cases but have not yet been incorporated into some current cultivars.

To reduce the need for applying pesticides, effort should be devoted to incorporating resistances to important insect pests, such as flower thrip, hairy caterpillar, pod borer, and various pod bugs that occur in the Sahelian and Sudanian Savanna zones. For some of these insects, strong sources of genetic resistance are not yet available.

Consideration should also be given to traits that benefit subsequent cereal crops grown in rotation, providing that these traits are relatively easy to incorporate. For example, in the Sahelian zone effort should be devoted to enhancing the resistance of cowpea cultivars to the nematode *S. cavenessi* both to improve cowpea performance in dry, infertile soils and to make it a more effective rotation crop with pearl millet, sorghum, and peanut which are susceptible to this nematode.

CONCLUSION

Due to the small number of active cowpea breeding programs in the world in relation to the large numbers of farmers growing this crop, the major effort in cowpea breeding must be on downstream programs that are devoted to cultivar development. These programs must focus on high priority constraints that can be solved at this time, such as by incorporating the resistances that are available to some of the major pests and diseases occurring in the TPZs.

With respect to drought adaptation, key factors involve achieving optimal time of flowering and cycle length, and appropriate morphology as they relate to the hydrologic budgets of TPZs and the different types of cultivars needed in relation to their utilization for dry grain, hay, and fresh pea production. The strong resistance to vegetative-stage drought that is available should be incorporated. The extreme ability of extra-early erect cowpea cultivars to escape terminal drought should be exploited in zones with very short rainfall seasons. In zones with the possibility of limited rainfall in the middle of the growing season, resistance to mid-season drought and the delayed-leaf-senescence trait can be valuable. Effective performance testing through field trials on experiment stations and by farmers is essential in breeding to enhance adaptation to drought and to encourage adoption of cultivars by farmers.

With respect to upstream research, emphasis should be given to developing MAS schemes for resistances to major pests and diseases. Cowpea breeders typically have to incorporate several resistances; methods that make possible the pyramiding of resistance genes would enhance the efficiency of their programs. A genetic linkage map that provides a basis for beginning this work has been developed (Ouédraogo et al., 2002). The strong vegetative-stage drought resistance of cowpeas might be studied with the objective of transferring it to other crop species using transgenic methods.

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Phenotypic approaches to drought in cassava: review

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Cassava is an important crop in Africa, Asia, Latin America, and the Caribbean. Cassava can be produced adequately in drought conditions making it the ideal food security crop in marginal environments. Although cassava can tolerate drought stress, it can be genetically improved to enhance productivity in such environments. Drought adaptation studies in over three decades in cassava have identified relevant mechanisms which have been explored in conventional breeding. Drought is a quantitative trait and its multigenic nature makes it very challenging to effectively manipulate and combine genes in breeding for rapid genetic gain and selection process. Cassava has a long growth cycle of 12–18 months which invariably contributes to a long breeding scheme for the crop. Modern breeding using advances in genomics and improved genotyping, is facilitating the dissection and genetic analysis of complex traits including drought tolerance, thus helping to better elucidate and understand the genetic basis of such traits. A beneficial goal of new innovative breeding strategies is to shorten the breeding cycle using minimized, efficient or fast phenotyping protocols. While high throughput genotyping have been achieved, this is rarely the case for phenotyping for drought adaptation. Some of the storage root phenotyping in cassava are often done very late in the evaluation cycle making selection process very slow. This paper highlights some modified traits suitable for early-growth phase phenotyping that may be used to reduce drought phenotyping cycle in cassava. Such modified traits can significantly complement the high throughput genotyping procedures to fast track breeding of improved drought tolerant varieties. The need for metabolite profiling, improved phenomics to take advantage of next generation sequencing technologies and high throughput phenotyping are basic steps for future direction to improve genetic gain and maximize speed for drought tolerance breeding.

Keywords: adaptation, drought tolerance, modern breeding, phenotyping, storage roots

INTRODUCTION

IMPORTANCE OF CASSAVA

Cassava (manioc, yuca, or mandioca; *Manihot esculenta* Crantz, Euphorbiaceae) is an important cash crop and food crop of resource-limited farmers in Africa, Asia, and Latin America and the Caribbean. The storage roots are utilized either fresh, as in the case of sweet cultivars low in cyanogenic glycosides, or after processing into dry products such as flour, starch, and animal feed in the case of bitter cultivars high in cyanogenic glycosides (Dufour, 1988; Essers, 1995; Balagopalan, 2002; Westby, 2002). Because of its relative high productivity under conditions of erratic rainfall and low-fertility soils, 250 million Africans depend on cassava as food, with more than 90% of the 117 million tons produced in sub-Saharan Africa (SSA) in 2007 being used for fresh consumption and processed food (Philips et al., 2006). A study by the International Food Policy Research Institute (IFPRI) predicts an overall 2.44% annual growth in the use of cassava as food in SSA, closely mirroring population growth, and a growth of 1.53% per annum in cassava for feed (Scott et al., 2000).

Cassava's ability to produce in marginal environments makes it the ideal food security crop in sub-Saharan Africa where it is a staple. It can be grown with minimal inputs, but gives considerably higher yields with fertilizers and good management. The crop is flexible as to the time of harvest and can be stored naturally for long periods by keeping the plants in the field with the roots in the soil. It has a remarkable ability to tolerate and recover from biotic and abiotic stresses. Cassava offers many different alternative uses as processed food, animal feed, starch, alcohol biofuel for vehicles etc. As countries develop, their demand for all these products increases dramatically. Although pruning the aerial parts of the plant 3 weeks before harvest can reduce deterioration of the roots (van Oirschot et al., 2000), their generally short shelf-life means that they have to be used immediately or processed into dry products. Therefore, cassava processing needs to be sited near to production fields, making it an ideal vehicle for

rural development through creating employment opportunities in the areas of cassava cultivation.

CULTIVATED AREA AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

Cassava is widely grown in tropical and sub-tropical Africa, Asia, and Latin America, between latitudes 30°N and 30°S, from sea level to above 2000 masl on marginal and highly-eroded low-fertility acidic soils, virtually without the application of agro-chemicals (El-Sharkawy, 1993, 2004; Ruppenthal et al., 1997). Africa is the largest producer of cassava with Asia as the second largest and then followed by the Americas (FAO, 2010).

Because of its metabolic efficiency under marginal conditions, cassava produces more energy per unit area than other crops under conditions of water stress and in poor soils (El-Sharkawy, 1993). By not having specific water-stress sensitive growth stages beyond storage root initiation, cassava can survive and be productive under conditions where other staple food crops, such as grain cereals and legumes, would rarely produce. Presumably, cassava originated in hot humid climates in the Amazonian lax forests (Allem, 2002). Yet it shows a high degree of tolerance to prolonged drought in areas with low and erratic precipitation of less than 600 mm annually, coupled with dry air and high temperatures (hence, high potential evapotranspiration), low fertility soils and high pest and disease pressure such as in Northeastern Brazil, the northern coast of Colombia, the coast of Peru, the Sahelian areas of West Africa, and drought-prone areas of East and Southern Africa, and parts of Thailand (El-Sharkawy, 1993). Particularly notable is cassava's recent expansion into the Sahelian parts of West Africa where its tolerance to various edaphoclimatic stresses gives an advantage over other staples (Romanoff and Lynam, 1992; El-Sharkawy, 2004).

Cassava has a high yield potential; when compared to maize, sorghum and rice in environments with no production constraints, it can match or exceed these crops in energy production per hectare (De Vries et al., 1967). Average annual cassava yields worldwide are 10 t ha^{-1} (fresh root; about 65% moisture content), ranging from 6 t ha^{-1} in Mozambique to 26 t ha^{-1}in India. Yields as high as 90 t ha^{-1} have been obtained in experimental trials of some improved cassava cultivars under near-optimum climatic conditions in Colombia (El-Sharkawy, 2005).

The gap between the potential yield and actual yields in farmers' fields is around 8-fold, suggesting that the highest potential of cassava production is far from being reached with traditional varieties, usually cultivated on marginal soils without inputs. Thus, a small increase in cassava yield in these marginal regions resulting from the use of improved drought-tolerant varieties would easily lead to an increase in global production. Many cassava genotypes have been identified that are very well adapted to drought and have been released in specific regions. However, it is necessary to understand the genetic and physiological traits that lie behind the mechanisms that make cassava a renowned drought-tolerant crop with the capacity for further progress, and for the application of these principles in breeding programmes for cassava and other crops.

GENETIC AND GENOMIC RESOURCES

There are several germplasm collections with hundreds to thousands of cassava accessions in the national programmes of Brazil, Mozambique, Nigeria, Tanzania, and Thailand. In addition, two International Agricultural Research Centers, Centro Internacional de Agricultura Tropical; International Center for Tropical Agriculture (CIAT) in Colombia and International Institute of Tropical Agriculture (IITA) with its headquarters in Nigeria, hold large collections. The collection at CIAT comprises more than 6000 accessions, and has been evaluated for pest and disease resistance and for novel starch quality traits (Bonierbale et al., 1995; Bellotti, 2002; CIAT, 2002). IITA's collection consists of nearly 2000 genotypes largely of West African origin. This germplasm has been systematically characterized and evaluated for disease response. Understanding the distribution of genetic diversity within and among individuals, populations, species, and genepools is crucial for the efficient management of germplasm collections.

Molecular markers are playing an increasing role in germplasm characterization. Beginning in the early 1990s, molecular and genomics tools were developed to elucidate the genetics of traits of economic value in cassava. Genetic markers developed for cassava include more than 3000 restriction fragment length polymorphism (RFLP), 800 simple sequence repeat (SSR), 120 random amplified polymorphic DNA (RAPD), and nine isoenzyme markers (Fregene et al., 1997; Mba et al., 2001; Okogbenin et al., 2006; Raji et al., 2009).

A total of a non-redundant set of 2146 SSRs comprised of 1675 curated from the genome and 471 curated from ESTs are now available (Ferguson et al., 2012b). The curated, paired SSR sets and associated information can be found at http://bioinformatics.iita.org/cassava_SSRs. SSR markers have also been employed to study the genetic diversity and structure in a large collection of local varieties from Africa and Latin America, the results of which have been deposited on the website of the Generation Challenge Programme (GCP) of the Consultative Group on International Agricultural Research (CGIAR) [1]. Data from a molecular diversity assessment of over 1000 varieties from seven countries in Southern, East and Central Africa are also available at the same site.

Many molecular genetic linkage maps have also been constructed for cassava. The first was constructed using an intraspecific F_1 cross and 612 RFLP, RAPD, SSR and isoenzyme markers (Fregene et al., 1997). The second map was constructed using 100 SSR markers and an S_1 family (Okogbenin et al., 2006). Recently new maps have been published (Chen et al., 2010; Kunkeaw et al., 2010, 2011; Sraphet et al., 2011; Whankaew et al., 2011). Molecular markers linked to resistance to cassava mosaic disease (CMD), cassava green mite (CGM), and cassava bacterial blight (CBB), β-carotene content, and early root yield have also been identified (Akano et al., 2002; Okogbenin and Fregene, 2002).

Diversity Array Technology (DArT) can be used to characterize several hundreds to thousands of polymorphisms in a timely,

[1] http://gcpcr.grinfo.net/index.php?offset=0&app=datasets&inc=search_simple&gcpcr_search_string=Cassava&orderby=&orderdesc=false&limit=10#search_results

cost-effective manner (Ferguson et al., 2012b). The first developed cassava DArT array had nearly 1000 polymorphic clones with a 99.8% reproducibility (Xia et al., 2005), offering a high-throughput marker screening system at a low cost.

Recently, the Generation Challenge Program (GCP) converted 1740 SNPs in cassava for use on the KASPar platform (LGC). Through the GCP IBM marker services (http://marlow.iplantcollaborative.org/marker-service). To date, 80,631 cassava ESTs have been deposited in GenBank (Lopez et al., 2004; Lokko et al., 2007; Sakurai et al., 2007; Ferguson et al., 2012a,b).

Four bacterial artificial chromosome (BAC) libraries have been generated in cassava for positional cloning and genome sequencing. The first library is from the CMD-resistant genotype TMS 3001 and it has a 5X genome coverage. The second library is from the whitefly-resistant genotype MECU72 with a 10X genome coverage, while the third was constructed from TME3, a CMD-resistant genotype, with an 11X genome coverage. The fourth library was constructed from AM560-2, a partially inbred genotype for physical mapping and sequencing of the cassava genome. These BAC resources are available from the Clemson University Genomics Institute (CUGI [2]). More than 2000 BAC ends have been sequenced in cassava and another 5000 are currently being sequenced.

A 22X genome sequence of cassava was recently completed via shotgun and a 454 Genome Sequencer FLX platform with long-read GS FLX Titanium chemistry. More than 61 million sequencing reads were generated and assembled into a draft genome that contains an estimated 95% of cassava genes. It is one of the first large genome projects to primarily use the 454 Life Sciences[3] long-read sequencing platform, which enabled both improved quality of the draft and its rapid generation. The annotated draft genome sequence is available at the United States Department of Energy Joint Genome Institute (DOE–JGI) Phytozome website[4].

IMPROVING CASSAVA ADAPTATION TO DROUGHT

Cassava's huge potential to produce well in marginal environment has made it a desirable and strategic crop for increasing food productivity by exploring the vast arable lands in the semi-arid and arid ecologies in the tropics. The wealth of genetic resources and the genetic diversity it offers has been deployed in the genetic improvement of cassava for drought tolerance. This has resulted in the identification of useful mechanisms associated with cassava's adaptation to drought. The mechanisms essentially combine dehydration avoidance, dehydration tolerance and those linked to optimum growth and metabolism. Several traits have been identified associated to these mechanism. Genetic improvement for drought adaptation in cassava is being enhanced by the increasing volume of genomic resources that are now available to breeding programmes. The ability to take maximum advantage of these genomic resources in modern breeding platforms essentially depends on improving capacity for drought phenotyping. Among the attributes for desirable drought phenotying traits in modern breeding are that it must be genetically associated with yield under stress, highly heritable, genetically variable, cheap, and fast to measure, stable within measurement period, and must not be associated with yield penalty under unstressed conditions. Modern breeding essentially entails combining genetic and genomic resources with good phenotyping protocols to efficiently breed for drought adapted varieties with speed and genetic again. This paper reviews drought adaptation studies in cassava, the critical phenotyping needs for modern breeding of drought tolerance and the future direction in terms of simple but advanced phenotyping methodologies that can further elucidate and enhance a better understanding of the mechanisms underlying drought adaptation in cassava.

PHENOTYPING FOR DROUGHT TOLERANCE IN CASSAVA

FIELD EXPERIMENTATION REQUIREMENTS

The key requirement for field trials to measure drought tolerance is to have appropriate water stress conditions. Achieving proper control over the field stress environment in order to assure the relevant drought test profile can be problematic. The common test criterion is yield when yield under stress is the target of the breeding programme. Yield under stress may be affected by the genetic makeup of yield potential and by specific genes affecting drought resistance. Estimating drought resistance in terms of the yield difference between potential and stressed growing conditions can isolate the two effects.

Well-designed and planned field experiments to determine relevant physiological traits are the most cost-effective way of evaluating drought tolerance in cassava. Ideal sites are semi-arid regions with more than 600 mm of rainfall distributed over 3 months of the year. Cassava requires 3 months of rainfall or irrigation for establishment of the crop, after which, tolerance to drought can be measured effectively. Sites in Colombia used by CIAT for the evaluation of drought tolerance include:

- Rio Hacha, Guajira Department: latitude: 11°32′40″N; longitude: 72°54′26″W.
- La Tatacoa desert (Villavieja), Huila Department: latitude: 3°13′22″N; longitude 75°13′21″W.

In Africa, IITA and national programmes use sites at:

- Hombolo, Tanzania.
- Kiboko and Kibwezi in Kenya.
- Minjibir (Kano State) in Nigeria.

The selected field should be flat, of well-drained and more or less homogenous soil. A simple randomized complete block design (RCBD) of 20 plant plots (4 × 5) and six replications can be used when planting material is abundant and not a limiting factor. Otherwise, single-row plots of six to eight plants can be used. It is preferable to have a higher number of replications and smaller plot sizes to reduce environmental effects. Where more than 200 genotypes are to be evaluated, it is preferable to divide the experiment into two or more individual experiments to limit the size of the field, and thereby limit environmental variation. Two treatments should be imposed: well-watered and water-stressed. The

[2]At Clemson University, Clemson, South Carolina, USA; http://www.genome.clemson.edu/
[3]http://www.454.com/
[4]www.phytozome.net/cassava

response to water stress of a genotype can then largely be assessed through differences in yield under the two treatments. In certain environments, a genotype with average yield potential, but little yield penalty under drought stress may be preferable to a genotype with high yield potential but a large yield penalty under drought stress. When response to late stage, long duration drought is being assessed, irrigation should be applied to both treatments for the first 3 months to get even plant establishment. Irrigation can then be withheld on one treatment 3 months after planting. If early drought is to be assessed irrigation should be withheld 2–3 weeks after planting.

Measurement of physiological traits normally begins at 3 months, at the onset of water stress and continue every month until harvest. Harvest related traits, for example harvest index (HI) and fresh root and foliage weight, are typically measured at 12 months after planting. Stomatal conductance and photosynthesis should be measured using field-portable gas exchange equipment, and the leaf canopy measured with a leaf canopy meter. Soil measurement is also very important to monitor stress levels. Soil cores are taken periodically at 0.3 m intervals within eight profiles from stressed and unstressed plots. The soil samples are immediately weighed and then oven dried at 70°C to a constant weight. Volumetric soil water content is often determined taking into consideration the soil bulk density at each layer. Simple equipment like the soil tensiometer or more sophisticated equipment such as the neutron probe can also be used to measure soil moisture content.

CONVENTIONAL TRAITS MEASURED IN DROUGHT ADAPTATION STUDIES OF CASSAVA

Pre-harvest traits are normally measured on a monthly basis beginning at three MAP and they include:

1. Number of primary stems.
2. Number of branching levels.
3. Length of primary and secondary stems.
4. Leaf retention using two methods: (1) percentage method (visual score), and (2) leaf scar method, a more quantitative measure of leaf retention.
5. Height of leafless stem.
6. Length and width of fully expanded leaf lobe.
7. Carbohydrate content of leaves, stems, and petiole.
8. Stomatal conductance and ABA content of leaves and stems.
9. Pest and disease incidence.

The traits measures at harvest at 12 MAP are:

1. Above-ground biomass.
2. Storage root fresh weight.
3. Number of storage roots.
4. Stem diameter.
5. Storage root dry matter (percentage).
6. Storage root starch content (percentage).

DROUGHT TOLERANCE RESPONSE IN CASSAVA

The physiological responses of cassava to water stress and possible mechanisms underlying the crop's tolerance to drought have been the subject of several studies (Connor and Cock, 1981; Connor and Palta, 1981; Cock, 1985; El-Sharkawy and Cock, 1987; Alves and Setter, 2000, 2004a; Ekanayake and Ginthinguri, 2000; Okogbenin et al., 2003; El-Sharkawy, 2004; Lenis et al., 2006). Various methodologies and mechanism have been reported for drought stress tolerance studies in cassava.

Stomatal conductance

Measurement of the stomatal control of water loss can be valuable in identifying desirable genotypes. In studies conducted on 10 cassava clones evaluated for drought tolerance at the IITA Minjibir station, the stomatal conductance of the lower leaf surface was measured using a porometer (Mk3, Delta-T Devices, Cambridge, England) and CO_2 fixation was measured with a leaf disc electrode (LD2, Hansatech, Norfolk, England). These studies showed that, while most clones increased stomatal conductance throughout the day, at 4 months after the last rains, clones TMS91934 and TMS84751 diminished their stomatal conductance after 14:00 h and 12:00 h, respectively (CIAT report, 1994). Apparently, these clones gave a better yield because they depleted the soil water more slowly. In such cases, optimizing crop water-use efficiency (WUE) is of greater importance than maximizing short-term growth—until the soil water is depleted, at which point growth is halted (El-Sharkawy, 1993).

Leaf formation and other growth parameters

The growth of cassava has been evaluated to assess the effect of drought stress on the crop's physiology with particular emphasis on leaf formation and other parameters such as plant height and number of active apices. In earlier work at IITA, Kano (Okogbenin et al., 1998), the proximity of an artificial lake to experimental station was used to set up different stress sites reflecting different water table depths. Five experimental sites representing water table depths over two seasons were used in drought tolerance studies at IITA's research station at Minjibir in Kano state in the Sudan savannah ecology. Experimental sites within 100 m of the lake were arbitrarily assigned to the high water table (HWT) section, while sites between 100 and 200 m from the lake were assigned to the intermediate water table (IWT) section. Field sites further than 200 m from the lake were assigned to the low water table (LWT) section. Nine varieties were evaluated in the first season, while eight clones were evaluated in the second season. The locations chosen for this study were sufficiently diverse to produce observable differential varietal responses to seasonal and site-specific moisture gradients at Minjibir. Data on different water table depths at various locations at Minjibir were available from previous studies (Ekanayake et al., 1996; Okogbenin et al., 1998, 1999) using the same sites. The varietal response differed with water table section, indicating differential adaptation responses to drought stress among varieties.

In the drought-stress study conducted at Minjibir, Nigeria (Okogbenin et al., 1998), differences in soil water content were established by planting two identical trials at different distances from an artificial lake, assuming that there were differences in the water table at the two sites. Soil water measurements taken using a neutron probe in 60 access tubes installed in all experimental

plots showing significant difference in relative water contents during the dry season as a result of the differences in water table caused by the proximity of the lake. This was especially so in the deeper layers of the soil profile until 22 weeks after the last rains. This illustrates an approach that could be used in the absence of irrigation facilities.

In this drought study at IITA, the number of newly formed leaves on an apex, the number of nodes, the number of active apices and the number of fallen leaves on one apex were measured at regular intervals, depending on the particular experiment. The number of fallen leaves was obtained by counting the number of bare nodes on one apex. Plant height was measured from the soil surface to the general height of the canopy.

Results suggest a strong influence of the water table on growth (as reflected in height and leaf formation) and leaf shedding of the 10 clones under examination. Plant growth represented by plant height measured at 3-week intervals starting ca 20 weeks after the rains was higher at the IWT sites when compared with data obtained from plants growing away from the lake (LWT sites); the differences increased with time after the rains. A similar effect of the depth of the water table was observed in the cumulative number of leaves formed per plant. Using the initial number of leaves per plant at the beginning of the dry season as a covariate, it was possible to detect statistically significant differences after 6–9 weeks. Plants growing closer to the lake were able to form more leaves than those at LWT sites.

The leaf area index (LAI) refers to the leaf area per unit area of ground. The maximum LAI in cassava ranges from 4 to 8, depending on the cultivar and the atmospheric and edaphic conditions prevailing during crop growth (Cock, 1985). At the onset of the dry period, the cassava crop reduces its leaf area by producing fewer and smaller leaves, and by shedding older leaves. The reduced leaf area in dry weather could be considered a means by which cassava reduces water loss by transpiration. However, reduction in leaf area during long periods of water stress also reduces the crop growth rate. The reduction is more pronounced in the shoots than in the roots, particularly in varieties with vigorous vegetative growth. Upon recovery from water stress, cassava rapidly regenerates new leaves and the LAI of previously stressed cassava plants becomes higher than in non-stressed plants (El-Sharkawy and Cock, 1987).

Dry periods also cause a decline in LAI. After a decline during the dry season, and followed by a second rainy season, leaf area may increase a second time, but may not be as high as in the first season (Osiru et al., 1995). Selection for higher top weight and hence higher LAI should favor high root yield, since there is an optimum relationship between root yield and LAI. Drought resistance determines the base yield under stress, while recovery prescribes the upper potential after stress. Rapid production of new leaves in the recovery phase with the commencement of rainfall toward the end of the growth cycle (Ekanayake, 1993) could stimulate greater accumulation of assimilates in the roots of highly vigorous varieties such as TMS90257, TMS91934, TMS50207, and TMS30572 after the dry season and just before harvest.

Leaf area density (LAD) is the integral of LAI over time. LAD is calculated by multiplying LAI with the time (in days or weeks) during which the leaf area is photosynthetically functional. Good examples of long-LAD varieties developed at IITA are TMS91934 and TMS4(2)1425 (Osiru et al., 1995).

Water-use efficiency

WUE has been used to evaluate drought tolerance in cassava. Regarding the water extraction capacity of the different clones in situations of water availability in the IITA study described above, those with higher yields in the LWT site showed a tendency to extract less water from the deeper layers of the soil (120–180 cm) during the first 24 weeks of measurements. TMS90853 extracted 64%, 51%, and 49% of the available moisture at 10, 150, and 180 cm, respectively in the soil profile. TMS50207 extracted 60%, 57%, and 35% at the same respective depths. However, under water stress, TMS90853 had higher top growth than TMS50207, which explains the high amount of water extracted at deeper levels by TMS90853. The clones that extracted higher percentages of the water from deeper soil layers (TMS4(2)1425 and TMS84751) had the lowest root yield, showing poor WUE. However, TMS91934 and TMS84751 formed too many leaves in at the LWT site and also shed a high number of leaves at the same location, suggesting inefficient utilization of the little water available in the soil profile. Under stress, TMS30572 showed a reduced yield of 39% and 44% of shoot and root weight, respectively (Okogbenin et al., 2003).

Leaf scars and leaf life

Cassava adapts to water shortage by reducing its leaf canopy (Connor et al., 1981; El-Sharkawy and Cock, 1987) to reduce water use. Hence, leaf shedding is an effective adaptation mechanism as a response to moisture stress. In the drought experiment at IITA, one of the youngest leaves (not unfolded length approximately 1 cm) per plant of all sample plants was regularly (generally once a month) tagged with a label on which the clone, plant number, date of labeling, and replication number were coded. The tagged leaves that had fallen were collected every week, enabling the life of individual leaves formed at different plant ages to be calculated.

Leaves dropped as a percentage of leaves formed increased from HWT to LWT sites. Thus, it may be desirable to breed and select for better leaf retention when developing varieties adapted to dry areas. The reduction in leaf canopy could not be attributed solely to observed leaf fall since, at the IWT (moderately stressed) site, more leaves were shed than at the LWT site. Plants at sites supporting vigorous growth were more likely to develop a very dense leaf canopy. Mutual shading of leaves limits leaf life and accelerates leaf senescence in such plants (Rosas et al., 1976). This may be responsible in part for the high rate of leaf shed at IWT compared with LWT sites. However, genetic variation for leaf scars was minimal amongst varieties. Because vigorous clones were more likely to shed more leaves and vice versa, the number of leaf scars was, as expected, strongly associated with the number of leaves formed.

Leaf photosynthesis

The use of leaf photosynthesis as a selection criterion in cassava improvement programmes might be difficult to handle when

evaluating large breeding populations. Despite this, canopy-scale photosynthesis, which can be evaluated by measuring crop biomass growth rate, should be included at least in the evaluation and selection of parental materials. Its use should be combined with other important yield-related traits, particularly a relatively high (>0.5) harvest index (HI; Kawano, 1990, 2003, a large root sink using root number per plant as an indicator (Cock et al., 1979), and longer leaf life, i.e., greater leaf retention and duration over the growth cycle (El-Sharkawy, 2004; Lenis et al., 2006). Recent advances in molecular biology and the development of more precise techniques and equipment will further enhance and accelerate the elucidation of the fundamental mechanisms underlying photosynthetic potential and associated beneficial traits and their controlling genes.

Breeding at CIAT, while diversifying the genetic base, has incorporated many such accessions for their useful plant traits. Outstanding among the accessions used is the clone MBRA12. This exhibits high leaf photosynthesis when grown outdoors in pots or in the field in a mid-altitude warm climate and high yield coupled with resistance to mites (Byrne et al., 1982). Other accessions of Brazilian origin, MBRA383 and MBRA191 that ranked highly in this group of clones, were also reported to be among the highest ranked clones (fourth and fifth, respectively, among 33 evaluated) (El-Sharkawy, 2004).

Storage root and shoot harvest

In the IITA experiments described earlier, the internal samples were collected at harvest. The plants were separated into leaves, stems, original stem cuttings and storage roots, and bulked per plot. The root fresh weight was measured and the HI was calculated by expressing the root yield as a fraction of the total biomass.

At harvest (12 MAP), the root yield of certain varieties at the severely stressed site (LWT) approached that of the less stressed sites (IWT and HWT). El-Sharkawy (1993) reported that leaves of plants growing in highly stressed environment tend to have higher stomatal conductance than leaves of similar ages in unstressed plants. Varieties TMS63397, TMS50395, TMS84751, and TMS4(2)1425 did not show much differences in final root yield among the different water table sections (Okogbenin et al., 2003).

Varieties with a good top weight tended to produce a good top yield. Previously work (Connor et al., 1981) suggests that vigorous genotypes produce better under stress than less vigorous types. Therefore, a variety with optimal leafiness under good conditions is required for the attainment of a high yield in both high- and low-stress conditions, (Ekanayake and Ginthinguri, 2000). The relative reduction in yield caused by water stress was used to assess the relationship between drought resistance and yield performance.

Average fresh shoot yield was higher at the IWT site than at the LWT site (**Figure 1**; Okogbenin et al., 2003). The reduction in crop growth was more pronounced in the shoots than in the roots, particularly in varieties with vigorous vegetative growth. Results revealed a 37% reduction in fresh root yield, compared with a 22% reduction in fresh root yield from the IWT site to the most severely stressed LWT site. A highly significant non-linear

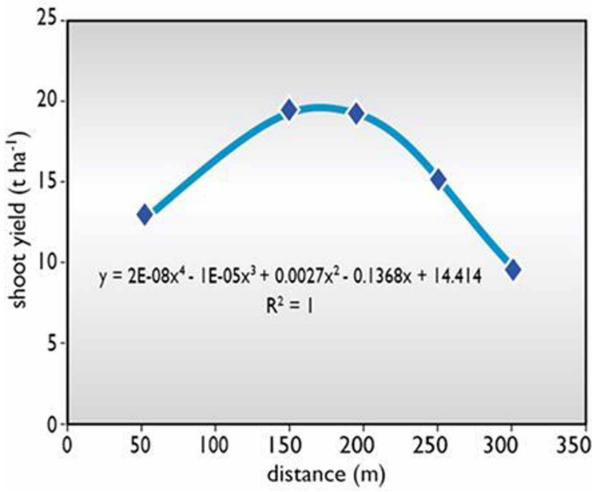

FIGURE 1 | The relationship between fresh shoot yield and water table depth as a function of field location (distance from lake) at IITA's research station at Minjibir, Kano, Nigeria. Source: redrawn from Okogbenin et al. (2003).

relationship was observed between fresh shoot yield and water table depth (**Figure 2**). Fresh shoot yield is a parameter of economic importance in dry ecological zones where animal feed supply is critical during the dry season. Therefore, varieties that produce abundant foliage are desirable as a source of feed.

In summary, conditions at the IWT site were more conducive to cassava growth than those at the HWT or LWT sites. Total plant biomass was higher at the IWT site (41 t ha^{-1}) than at the LWT (29 t ha^{-1}) and HWT (24 t ha^{-1}) sites (Okogbenin et al., 2003). The seemingly poor growth observed at HWT may have been caused by waterlogged conditions (due to inadequate drainage) that persisted in the first 3 months of rainfall, thus interfering with initial plant growth and development at this location. Compared with results at the IWT site, decreases at the LWT site were 23% for leaves formed, 19% for leaf scars, 22% for root yield and 37% for shoot yield. Again, compared with the IWT site, decreases at the HWT site were 25% for leaves formed, 27% for leaf scars, 50% for root yield and 32% for shoot yield. The greater reductions at the HWT site are in agreement with earlier findings that cassava tolerates stress from drought better than from waterlogging (Lahai et al., 1999). Severe drought stress at the LWT site caused a reduction in growth in this section of the field.

Fibrous root measurements

Evaluation for deep fibrous root system for drought measurement is relatively difficult in cassava. Cassava roots could be as long as 2 m in the ground and could help cassava to have access to deep water layers and may be deployed by the crop as escape mechanism from to evade water stress. Studies were conducted at Ibadan with the objective of identifying differences in the fibrous root system of 10 IITA genotypes, using early root growth as a selection criterion for adaptation to drought. This revealed large genotypic differences in fibrous root weight and root length as measured 2–5 weeks after planting (WAP) (CIAT report, 1994).

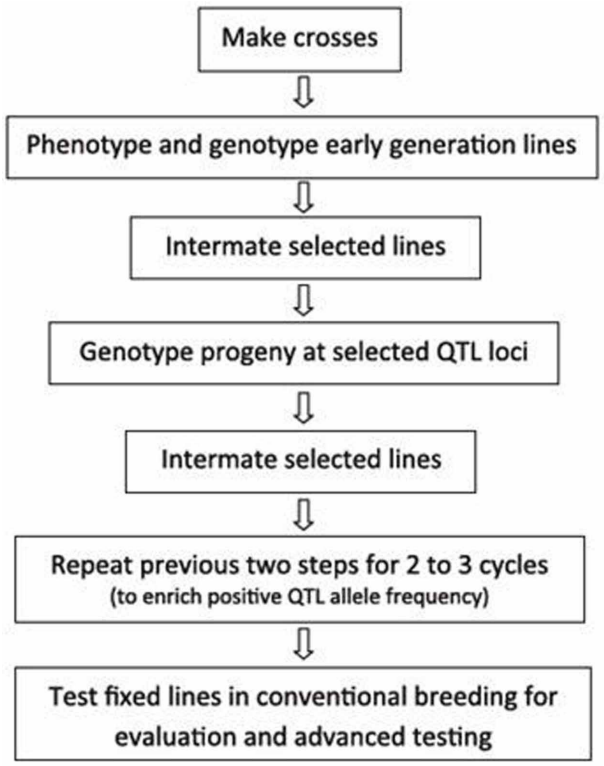

FIGURE 2 | MARS scheme (adapted from Integrated Breeding Platform—Generation Challenge Programme) (Ferguson et al., 2012b).

Water stress responses

Several reviews of cassava's tolerance to water stress have been produced (El-Sharkawy, 2004; Setter and Fregene, 2007). These reviews reveal that the principal mechanisms that may control tolerance to drought in cassava include its sensitivity and response to changes in atmospheric humidity and soil water status El-Sharkawy and Cock, 1984. Thus, once exposed to dry air and/or dry soils, cassava leaves partially close their stomata thereby restricting water loss. They are also capable of partially retaining their photosynthetic capacity under prolonged water shortage. Considerable variation has been observed in leaf conductance and this parameter seems to be useful to preselect sources of germplasm conferring adaptation to prolonged dry periods [Empresa Brasileira de Pesquisa Agropecuária, Brazilian Agricultural Research Corporation (Embrapa, 1992; Iglesias et al., 1995)].

Cassava is capable of forming deep rooting systems (below 2 m soil depth) that allow the crop to extract storage water when available (El-Sharkawy, 2004). Although cassava has sparse fine root systems compared with other crops such as cereals and tropical grasses, it is capable of penetrating into deeper soil layers.

Cassava conserves water under extended stress by reducing light interception, achieved through a reduced leaf canopy via restricted formation of new leaves, production of leaves of a smaller size, drooping of the leaves ("heliotropic response"), and leaf fall (Porto, 1983; El-Sharkawy and Cock, 1987; Calatayud et al., 2000; Alves and Setter, 2004b). Although a reduction in leaf area conserves water, it would also lead to a reduction in total biomass and yield (Connor and Cock, 1981; El-Sharkawy and Cock, 1987; Connor et al., 1981; Porto, 1983; El-Sharkawy et al., 1992; El-Sharkawy and Cadavid, 2002). However, upon rewatering, cassava can recover rapidly by forming new leaves. This increases light interception and canopy photosynthesis, thus compensating for previous losses in biomass, particularly root yield.

Osmoregulation under extended water stress

One means of increasing drought tolerance is by accumulation of osmotically active solutes, so that turgor and turgor-dependent processes may be maintained during episodes of dry down. In some plant species, osmotic adjustment (OA) allows cell enlargement and plant growth under high water stress, and allows stomata to remain partially open and CO_2 assimilation to continue at low water potentials that would otherwise be inhibitory (Pugnaire et al., 1994). However, the extent of OA has been found to be quite limited in cassava's tolerance to drought. In studies with potted greenhouse-grown cassava, Alves (1998) found that the largest increases in solutes after a few days of water deficit occurred in the youngest and folded (i.e., expanding) leaves, with only about 0.14 MPa OA increase in mature leaves, pointing to a limited effect of OA in the latter leaves (Alves and Setter, 2004a). Such studies need to be carried out on field-grown plants subjected to gradual, prolonged water stress to ensure that they extrapolate to field conditions.

Abscisic acid accumulation

Under drought, changes in the biosynthesis, content and distribution of plant growth regulators such as abscisic acid (ABA) within plant organs and tissues—particularly in roots, leaves, and buds—may play an important role in sensing changes in both soil water and atmospheric humidity, and in controlling stomatal movements, leaf formation and extension, root growth and bud dormancy. They may also be involved in other biological functions such as the expression of dehydrin and other proteins that are thought to stabilize macromolecular structure (Alves and Setter, 2000, 2004a).

Studies by Alves and Setter (2000) showed that five cassava varieties rapidly accumulated large amounts of ABA coincident with the cessation of leaf expansion growth and transpiration. The high ABA readings were almost completely reversed to control levels after 1 day of rewatering. This rapid return to control levels corresponded with a rapid recovery of leaf area growth rates. A substantial proportion of the variation in ABA concentration in cassava correlated with genotype, suggesting that genetic variation for the trait might be found in cassava.

Critical period for drought tolerance

In general, for cassava grown in a range of environmental conditions, there is a positive correlation between the total biomass and storage root biomass. However, during growth, there are distinct developmental phases. During the first 3 months, cassava accumulates dry matter more in the leaves than the stems and

tuberous roots. After the third month, more is accumulated in the roots than the rest of the plant (Ghosh et al., 1988).

Connor et al. (1981) reported that, when rainfall was withheld from cassava for 10 weeks commencing 12 weeks after planting, tuber yield was reduced by 32% compared to the control. Oliveira et al. (1982) imposed a water deficit for 2 months during successive 2-month periods from the first up to the 11th month after planting (MAP) and they found that a critical period for cassava root yield extended from the first to the fifth MAP. This period corresponds to the stages of root initiation and bulking. Water stress in this period reduced storage root yield by 60%. A similar conclusion was reached by Porto et al. (1989), who evaluated cassava grown in a lysimeter with water-stress conditions imposed over a 100-day period with no water, starting at three and six MAP. They reported that the accumulation of total dry matter as well as root dry weight was reduced more by water stress beginning at three than at six MAP. Thus, the more severe effect corresponded to stress during the period of rapid leaf growth and bulking rather than the later period of bulking.

MODERN BREEDING STRATEGIES FOR DROUGHT TOLERANCE

Current objectives for breeding for drought tolerance in several cassava breeding programmes include: (1) characterization of germplasm for tolerance to extended water shortages, either natural or imposed, and to low-fertility soils; (2) characterization of germplasm for vigor under early drought (within the first 3 months); (3) study of leaf photosynthetic potential in relation to productivity under various edaphoclimatic conditions; and (4) identification of other plant traits that might be of use in cassava improvement. Breeding substantially until recently has been based on classical approach.

Selection of parental materials for tolerance to water stress and infertile soils has resulted in breeding improved germplasm adapted to both stress environments. The International Fund for Agricultural Development (IFAD) has supported a long-term project for selection in different semi-arid environments in Northeastern Brazil—where the greatest genetic diversity of cassava germplasm for adaptation to drought is found—and distribution of the elite germplasm throughout Africa. Prior to screening for drought tolerance, it is important to incorporate CMD resistance for Africa as a whole, as well as cassava brown streak disease (CBSD) field resistance for Eastern and Southern Africa. Otherwise, the effects of these diseases can mask the plant's response to drought.

The immense diversity of environmental components in major drought-prone areas of the world poses difficulties in planning specific crosses and in selecting breeding materials that will suit the specific ecological conditions peculiar to a site. A systematic and uniform characterization of the pertinent environment factors for important drought-affected areas would guide researchers in formulating breeding objectives and procedures, and greatly accelerate the impact of breeding programmes. CIAT and other breeding programmes in Latin America and the Caribbean have used this systematic approach. Adopting appropriate selection criteria is very important. They should include rapid, inexpensive and simple methodologies, and should be based on physiological interaction of drought with crop growth and yield. Any criteria based on a variety's ability to maintain a high water status and efficient water use would clearly relate to productivity. By diagnosing environmental factors prevailing in different drought-prone areas, the breeder is in a better position to incorporate specific drought-resistance mechanisms and recovery capabilities into breeding populations.

In Nigeria which is the world largest producer of cassava, its national research center for cassava, the National Root Crops Research Institute (NRCRI) is massively screening elite germplasm to identify genotypic responses to drought that can selectively be hybridized and recombined to develop an array of genotypes adapted to the various needs of drought-prone areas. The selection of progenies at drought-prone sites is critical for the identification of genotypes that perform well under water stress.

In previous breeding activities at IITA, three factors were considered in relation to drought tolerance: (1) the timing and length of water stress; (2) yield under different water regimes, or at different sites; and (3) drought reactions scored for vegetative growth during the dry season. This form of multicriterion comparison has provided more meaningful interpretations of varietal differences in agronomic performance related to drought than comparison of absolute yields. Varieties with both drought resistance and good recovery ability are key requirements for stable performance in areas with a longer rainy season or those with a bimodal rainfall pattern that are also prone to occasional prolonged drought periods.

Given the vast array of opportunities of molecular resources being generated and available to cassava, molecular breeding is now rapidly evolving for the crop. From the initial molecular breeding initiatives supported by the GCP since 2003, many breeding programmes have developed capacity to deploy molecular tools. The BMGF in 2012 is supporting a Cornell University led consortium to use of genomic selection in Africa to fast-track cassava breeding and is in the process generating and using huge assembly of sequence data (http://www.nextgencassava.org/). These developments are rapidly changing the landscape of breeding in Africa and globally. These rapid changes generally require faster phenotyping protocols and efficient genomic selection tools to increase genetic gain and expedite product delivery of drought tolerance products. Some of the modern breeding strategies require minimal phenotyping or early and fast phenotyping protocols.

Breeding for complex traits is expensive due to the need for highly replicated phenotyping trials over several environments. This justifies the quest for a MAB approach that increases precision of selection and reduces the requirement for phenotyping. MARS is a MAB strategy for forward breeding of genes and QTLs for relatively complex traits (Ribaut and Betran, 1999; Ragot et al., 2000; Eathington, 2005; Crosbie et al., 2006) such a drought tolerance. It is a genotype construction process that increases the frequency of beneficial alleles and aids the development of genotypes with the best haplotype combination at selected loci in the genome. A typical MARS scheme is illustrated in **Figure 2**. Under the GCP—Cassava Challenge Initiative, African breeding programs have initiated MARS for drought tolerance breeding in cassava. SSR and SNP markers are used to identify QTLs and then

to identify important allele combinations through three cycles of selection, which is only then which is only then followed by phenotyping.

Genomic Selection is an alternative approach well suited to complex traits (Meuwissen et al., 2001). This approach depends on high-throughput genotyping and novel statistical methods. GS uses all marker data as predictors of performance, thus enabling the selection for multiple loci of small genetic effect (Jannink et al., 2010). Essentially, breeding populations are extensively genotyped (using next generation sequencing technologies) to give full genome coverage and phenotyped to create models that calculate genomic estimates of breeding values (GEBVs) which are used to select candidate parents. These values can then be used for selection within a breeding population, without the need for phenotypic evaluation. This new breeding approach have strong significant benefits in breeding for drought tolerance and quantitative traits in highly heterozygous species as cassava (Heffner et al., 2009; Jannink et al., 2010).

Genome wide selection (GWS) is a strategy found suitable for complex traits controlled by many QTLs and with a low h^2. GWS can be implemented in the same way as MARS except that all individuals are genotyped with a large number of markers (Ferguson et al., 2012b). Genome wide selection (Meuwissen et al., 2001) determines prediction of performance based on as many loci as possible (unlimited number) without QTL mapping. In GWS, trait values are predicted from a weighted index calculated for each marker. Simulation studies have indicated that across different numbers of QTL (20, 40, and 100) and levels of h^2, responses to genome wide selection were 18–43% larger than the corresponding responses to MARS (Bernardo and Yu, 2007).

KEY EARLY-GROWTH PHASE PHENOTYPING METHODOLOGY FOR MOLECULAR BREEDING

A key focus for modern breeding is the need to rapidly make genetic advances and reduce the breeding scheme by efficiently stack traits using both molecular tools and efficient phenotyping strategies. This is more challenging for complex traits and especially for those that are often evaluated very late in the growth cycle. While a good number of traits often evaluated at harvest periods are strong drought tolerance determinants, their late measurements make them undesirable to meet the objectives of modern breeding that seeks a fast screening procedure and quick systematic elimination to reduce population sizes that are measured late in breeding scheme. Modified traits that have key predictive power to estimate yield and adaptation potential in drought prone environments and which can easily be assessed early in the growth cycle are target traits of interests for modern breeding. Recently three modified traits have proved very useful based on their recent application in drought phenotyping within a 7–8 month evaluation cycle in contrast to a 12–18 month cycle that typically applies under drought stress ecologies.

BULKING AT 7 MAP

Bulking in cassava refers to the swelling or thickening of the storage roots as they are filled with excess assimilates after the plant might have satisfied the needs for vegetative growth. Early bulking has been used as concept to described early maturing cassava or early-ready cassava varieties that are harvestable at 7–8 months. The food security role of cassava in averting famine has necessitated the need for early-ready varieties in contrast to late yielding varieties. However, early bulking has been a trait mainly evaluated mainly in humid agro-ecologies where conditions are rather optimal for growth.

In the dry ecology, drought imposes slow crop development that makes harvest of cassava to extend beyond 12 months and sometimes between 15 and 18 MAP. Early bulking is therefore seldom considered as a measurable trait in marginal environment. The need to use as an early screening procedure has rapidly become important given the need to accelerate phenotyping for assessing productivity in drought prone environments. Early bulking in dry ecologies is rather implied to identify good bulkers under stress rather than identifying early maturing varieties. Thus evaluating early bulking for drought tolerance is used to select good varieties potential good yield at 12 MAP. So it is a fast screening method to select for yield under drought tolerance *per se*. This have been applied in recent studies for drought adaptation.

A study of early bulking was conducted in the guinea savannah (Olasanmi, 2010). Dry season could vary from 4 to 6 months in the savanna zone requiring cassava varieties to have good adaptation for drought tolerance to enhance good productivity in these ecologies as well. Some pre-selected cassava genotypes with good bulking (**Figure 3**) were evaluated by NRCRI in 2010 and 2011 at Otobi (derived savanna) (**Table 1**). They were evaluated for early bulking in terms of root yield and other related parameters at 7 months after planting. The objective was to test the hypothesis that early bulkers could be used as a identify potentially good productive genotypes at 12 months in regions were drought stress could be a severe limitation to productivity.

In the study a set of 33 early bulking cassava genotypes and two check varieties (TMS 30572 and TMS 98/0505) were evaluated for yield at 7 and 12 MAP. A new parameter which was used to assess tolerance is the relative increase in yield from 7 MAP to 12 MAP. Fresh root yield and other yield related attributes of the early bulking cassava genotypes evaluated at Otobi are shown in **Table 1**. The average yield at 7 MAP was 10 t ha^{-1} at Otobi in the derived savanna while it 21.2 t ha^{-1} at Otobi. Results indicate that genotypes that were good bulking at 7 MAP relatively maintained good yields at 12 MAP. At Otobi, about 81% of the

FIGURE 3 | Good bulking genotypes (at 7 months after planting) developed in the Cassava breeding programme at National Root Crops Research Institute, Umudike, Nigeria. (A) Early bulking genotype with big sized commercial roots at 7 MAP. **(B)** Early bulking genotype with moderate sized commercial roots at 7 MAP.

Table 1 | Fresh root yield and other yield related attributes of early bulking cassava genotypes at two harvest dates at Otobi, Nigeria (source Olasanmi, 2010).

Genotype	7 MAP		12 MAP		Yield increase	% Yield increase
	FRY (t/ha)	PI	FRY (t/ha)	HI		
COB-4-52	2.33	0.16	10.51	0.41	8.18	350.6
COB-6-31	5.67	0.19	20.61	0.39	14.94	263.7
COB-5-86	9.96	0.32	35.86	0.40	25.90	260.2
COB-4-79	4.78	0.29	15.56	0.40	10.78	225.6
COB-1-139	9.31	0.34	30.11	0.45	20.81	223.6
COB-5-17	9.47	0.24	29.67	0.32	20.20	213.4
COB-7-180	7.59	0.19	22.41	0.30	14.82	195.3
COB-5-28	6.68	0.32	19.52	0.38	12.85	192.4
COB-5-44	9.78	0.22	28.33	0.24	18.56	189.8
COB-5-4	10.65	0.38	29.20	0.47	18.56	174.3
COB-4-100	10.56	0.29	27.66	0.37	17.11	162.0
COB-6-41	7.61	0.20	19.00	0.23	11.39	149.6
COB-7-197	13.93	0.30	33.85	0.36	19.92	143.0
COB-7-25	20.00	0.33	47.29	0.46	27.29	136.4
COB-5-53	6.35	0.37	14.31	0.45	7.96	125.5
COB-1-103	9.86	0.32	21.88	0.43	12.02	121.9
COB-4-75	13.68	0.36	26.81	0.44	13.13	96.0
COB-5-24	7.11	0.31	13.79	0.33	6.68	94.0
COB-4-77	11.13	0.33	21.59	0.43	10.46	93.9
COB-5-104	6.39	0.24	12.25	0.33	5.86	91.6
COB-4-74	8.79	0.28	16.78	0.42	7.99	91.0
COB-6-19	9.78	0.25	17.83	0.46	8.05	82.3
COB-5-12	10.65	0.29	18.87	0.34	8.22	77.2
COB-1-163	14.00	0.28	24.69	0.34	10.69	76.3
COB-5-57	11.25	0.25	19.77	0.28	8.52	75.8
COB-4-27	10.11	0.34	17.39	0.38	7.29	72.1
TMS 98/0505	11.24	0.21	18.51	0.27	7.26	64.6
COB-5-48	7.64	0.24	11.67	0.27	4.03	52.8
COB-5-11	11.46	0.28	17.46	0.36	6.00	52.4
COB-6-4	12.54	0.24	18.95	0.28	6.42	51.2
COB-5-61	12.07	0.32	16.20	0.33	4.13	34.3
TMS 30572	14.71	0.27	18.90	0.31	4.19	28.4
COB-5-36	9.25	0.24	11.45	0.22	2.20	23.8
COB-6-1	14.56	0.33	12.50	0.30	−2.06	−14.1

MAP, months after planting; FRY, fresh root yield; HI, harvest index; PI, partioning index.

genotypes having about 20 t ha^{-1} or more at 12 MAP had 9–10 t ha^{-1} at 7 MAP. The results therefore shows that early bulking could be used as a useful parameter to screen for productivity at 12 MAP. Such genotypes are likely to maximize available moisture for bulking to improve yields. These materials are being planned for further test in semi-arid zones (Sudan and Sahel savannas). Yield differences between 7 MAP and 12 MAP tend to indicate that 18 (54.5%) of the genotypes based on the results were very good bulking materials with less than 55% root yield increase at 12 MAP over root yield at 7 MAP. Genotypes with good bulking at 7 MAP and without highly extended yield increase are considered more drought tolerant. The results obtained showed that 7-month bulking assessment in ecologies with high drought stress could be used as a good trait to rapidly screen for drought tolerance under modern breeding. Efforts to screen for bulking at 5 MAP for productivity potential at 12 MAP are underway.

PARTITIONING INDEX

Harvest index (HI) is the ratio of economic yield to that of biomass yield of a crop and is typically measured at 12 MAP. Molecular breeding essentially requires rapid screening methodology that necessitates quick prediction for good partioning to estimate yield potential. HI at 12 MAP is rather late in the growth cycle and makes it not readily ideal for early screening of breeding populations for drought adaptation in the cassava breeding scheme.

The ability to estimate quick partitioning of assimates at the early growth phase is therefore considered more desirable. Recent initiatives to assess partioning index at the early stages have been explored to improve rapid screening for good yield. Duque (2012) examined 45 diverse cassava genotypes representing a range of reported drought tolerances from among collections at CIAT and Embrapa. The studies were done on potted plants so that water supply in well-watered and water-stressed treatments could be controlled. Partioning index which is the ratio of the storage root weight as a fraction of the total plant biomass at 4–5 months was correlated with harvest index at 12 MAP. The correlation between storage root mass and the partitioning ratio of storage root biomass:total plant biomass was found high, especially underwater stress (Duque, 2012). The study showed that the best genotypes maintain a robust developmental programme that sustains storage root growth in the face of water stress, whereas poorer genotypes allow storage root growth to suffer at the expense of other growing plant organs. In terms of phenotyping strategies, the study suggests that evaluation of biomass partitioning ratio at an early stage of storage root development could be a useful indicator of a genotype's tendency to favor storage root growth when resources are limited by water stress.

Findings in a study reported by Olasanmi (2010) has also shown good correlation between partioning index at (7 MAP) and harvest index (12 MAP) for drought tolerant genotypes and might be useful as a critical screening method for preliminary selection for drought adaptation evaluation. Results obtained in the study (at Umudike—humid ecology; and Otobi—Guinea savanna) indicated that genotypes that had a PI of 0.3 tended to produce better and maintained good HI at 12 MAP. In the study, the difference between PI and HI among three classes of cassava (early bulkers, medium bulker and late bulkers) at two harvesting age (7 and 12 months after planting) were significant. The difference was widest for the late bulkers than the other two classes (**Table 2**). Medium cassava bulkers are those that are intermediate between the early and late types. Due to late bulking, the PI does not necessarily correlate with HI at 12 MAP for this group. PI therefore tend to predict stronger for early bulking genotypes as shown by the results (Olasanmi, 2010), The implication for late bulkers in dry ecologies is that due to drought effect, it may likely attain maturity very late often well beyond 12 MAP for good and reasonable yield to be attained. The use of PI could rapidly allow breeders to cut down on the population and thus accelerate rapid

Table 2 | Average harvest index (HI) among different bulking rate groups of cassava at two locations in Nigeria.

Location	Late bulkers		Medium bulkers		Early bulkers	
	7 MAP	12 MAP	7 MAP	12 MAP	7 MAP	12 MAP
Umudike	0.32	0.59	0.42	0.59	0.45	0.57
Otobi	0.28	0.38	0.28	0.35	0.29	0.29

	Difference between PI and HI between the two harvesting age		
	Late bulkers	Medium bulkers	Early bulkers
Umudike	0.27	0.17	0.12
Otobi	0.10	0.07	0.00

selection thus reducing the breeding scheme through shortened phenotyping regimes.

STEM STARCH CONTENT

Cassava is vegetatively propagated. The size and quality of stem are of fundamental importance for high yields (Eke-Okoro et al., 2001). Differences in weight of stem cuttings result in differences in food reserve (Okeke, 1998), and it is on this that the initial growth of the plant depends implying basically that stem weight or starch are associated to the establishment phase of cassava in the field. This has recently been explored for drought tolerance phenotyping. The establishment phase of cassava is critical to rapid adaptation of cassava and has been hypothesized as more critical under water stress either at the initiation of the growth phase or during prolonged stress when food reserves are mobilized to sustain metabolic activities.

Given that water stress diminishes photosynthetic carbon fixation, and yet cassava can retain the ability to resume growth after long drought periods, it has been hypothesized that carbohydrate storage reserves in cassava's thick robust stems might provide a supply of carbohydrate to sustain meristems and other respiring organs during prolonged stress. (Duque, 2012) studied found that reserves in leaf blades were limited and these reserves were depleted rapidly during stress (**Figure 4**). In contrast, stems and storage roots maintained a relatively high starch content per organ from treatment initiation to the final harvest. Total nonstructural carbohydrate (TNC) content per plant was maintained in storage roots through the entirety of the experiment, while the stem became a source of slowly remobilized starch during stress. The amount of starch stored in stems was considerable, representing about 35% of the TNC in the plant at stress initiation (T_0), and 6% of total plant dry mass. These data suggest that this pool of TNC reserves is important in sustaining meristems and other respiring organs during prolonged stress. Duque (2012) showed that cassava stems accumulate starch (**Figure 5**) gradually over a 45-day period of growth after seedling establishment, in advance of storage root bulking (**Table 3**). In studies with 15 diverse genotypes, fresh root biomass production under stress correlated with the extent to which a genotype accumulated starch in its stems. Collectively, these studies suggest that the extent to which stems accumulate starch in advance of water stress could be a valuable trait for drought tolerance.

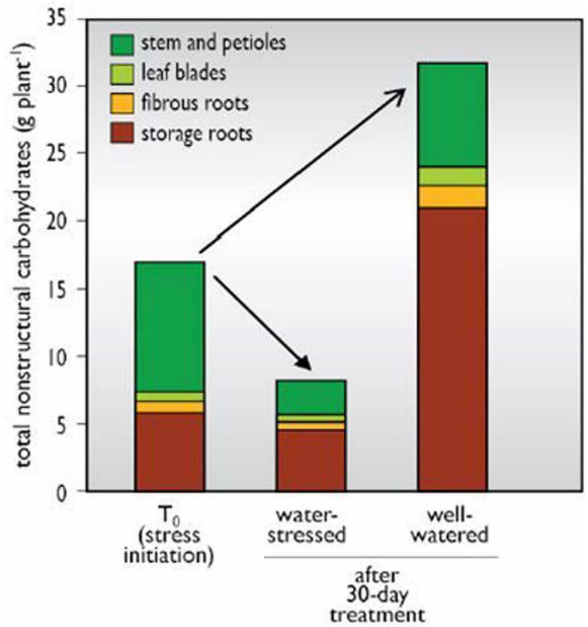

FIGURE 4 | Accumulation of total non-structural carbohydrates in cassava plant parts during initial growth and during a 40 subsequent period of water-stressed or well-watered conditions. Source: (Duque, 2012).

FUTURE DIRECTION: PHENOMICS AND HIGH THROUGHPUT PHENOTYING

Phenomics is the systematic study of phenotypes on a genome-wide scale and defines phenotypic features across multiple levels of expression. It involves a large-scale phenotypic data collection and analysis, and thus enables the characterization of phenotypes in a rigorous and efficient way, to link traits with the associated genes. Phenomics is normally conducted by running multiple phenotypic assays on a large set of genotypes. Phenotypic parameters cover morphological measures (plant height), dynamic measures (metabolism) and molecular measures (transcript profiles). Developing strong capacity for cassava phenomics is essential to high throughput phenotyping to close the gap between plant physiology and genetics (Furbank, 2009).

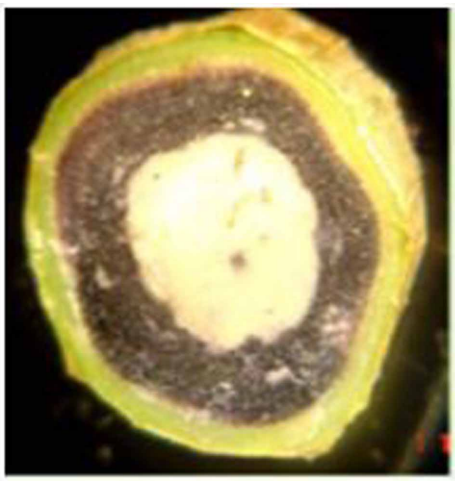

FIGURE 5 | Starch in cassava stems—remobilized during stress (staining with iodine). Source: Duque, 2012.

Table 3 | Carbohydrate accumulation in the initial growth period after seedling establishment of cassava in the Corpoica (Corporación Colombiana de Investigación Agropecuaria; Colombian Corporation for Agricultural Research) field sites at Turipana and El Guamo in Colombia. An average of 15 genotypes is shown (source: Duque, 2012).

	Carbohydrate (μmol g^{-1} dry weight)			
	Leaf blade		Stems	
Days from plant establishment	Total sugars	Starch	Total sugars	Starch
0	75	15	121	293
15	78	14	178	332
30	79	19	192	659
45	101	5	239	1016

High-throughput phenotyping technologies will be particularly important for studies of drought tolerance.

High throughput phenotyping for target and correlated traits is increasingly becoming important in crops. However, there are still many important traits that are difficult to evaluate. Phenomics technologies could bring new approaches to address these challenges to efficiently identify superior genotypes and train prediction models. Improved methods are required needed for high-throughput collection of diverse phenotypic measures, in the field and controlled environments. Some of the high throughput technologies used in drought studies in several crops include imaging systems, remote sensing, canopy spectral reflectance (for water use efficiency), etc. Cassava can immensely benefit from the use of such technologies.

Information about the physiological changes in response to drought over time is vital to identification and characterization of the different drought-tolerance mechanisms. This has been demonstrated in many crops based on high throughput technologies. For example, Image-based phenotyping offers a way to capture and extract morphological and developmental phenotype data, through non-destructive close-range or remote-sensing technologies. Remote sensing, an increasingly powerful tool has long been used in an attempt to measure the water status of individual plants or canopies (Blum et al., 1982). The most frequently used technique is thermal infrared imaging, or infrared thermography (IRT), to measure the leaf or canopy temperature which is drought parameter related to the extent of stomatal opening and evaporative cooling often measured (Balota et al., 2008). The use of thermal cameras for canopy temperature measurement offer a key benefit compared with temperature sensors (thermometers) as a facility for spatial resolution. It thus allows more precise measurements in a fraction of the time needed to perform several replicate readings per plot than an infrared thermometer, which is prone to error due to changing environmental conditions between measurements. In addition, a large number of plots in a field trial can be imaged at the same time, ideally allowing a comparison of differences in canopy temperature among genotypes without the need for normalization to determine the absolute leaf temperature (Jones et al., 2009).

Non-destructive imaging techniques allow a temporal resolution and monitoring of the same plants throughout the experiment. The development of good image systems that avoid destructive sampling will be very critical for root and tuber crops like cassava when storage root development is critical for assessment of drought on growth and development in the crop's growth cycle. The Combination of high throughput technologies have the huge potential to increase the power of data analysis. For example the combination of color and thermal imaging, has been indicated to increase the information and precision of leaf temperature measurements compared with thermal imaging alone (Berger et al., 2010). Although cassava have yet to significantly deploy high throughput technologies in drought studies, it is expected that this will change as cassava phenomics improve.

Despite the array of data characterizing water deficit responses that may relate to dehydration tolerance, there is still little understanding as to which responses, whether at the gene or cellular level, are actually adaptive in nature and truly critical for or central to tolerance (Bray, 2002). Metabolite profiling offers strong opportunities to remedy these gaps. Some of the most important responses of a plant against drought stress are associated with the accumulation of minerals (Samarah et al., 2004) and the enhanced synthesis of osmoprotectants, osmolytes, anti oxidants, or compatible solutes, which are part of normal metabolism. The accumulation of these compounds helps the stressed cells in water retention (Hare et al., 1998; Setter, 2012) and in the maintenance of the structural integrity of the cell membranes (Conroy et al., 1988). Metabolic profiles have the potential to uncover a cascade of biochemical regulation strategies that may be explored to enhance drought tolerance in crops (Setter, 2012). Mass spectrometry (MS) and Nuclear magnetic resonance (NMR) spectroscopy are used to identify and to quantify metabolites. Nuclear magnetic resonance (NMR) spectroscopy can be used to monitor and quantify

the degree of metabolic impact induced by drought or other environmental disturbances (Bligny and Douce, 2001; Charlton et al., 2008), since NMR can bring "high-throughput" spectroscopic/structural information on a wide range of metabolites simultaneously with high analytical precision.

Phenomic datasets can be large and complex and appropriate management systems are required to enhance analysis. The power of phenomics is largely expected to be enhanced when datasets are combined and correlated across different studies. Considering the complexity of both drought and plant responses to drought, trait dissection effected by high-throughput phenotyping provides strong process to understand plant responses to drought, and its genetic basis for effective application to improve crop performance and yield under a variety of drought conditions in crops (Berger et al., 2010).

In cassava, drought phenotyping has with morpho-physiological and agronomic traits that does not integratively provide sufficient understanding to drought tolerance in this crop. The increasing genomic resources arising from the use of next generation sequencing technologies and GBS in cassava implies that the quantum of genotypic data or information being generated can only be meaningfully analyzed and applied by improving capacity in phenomics. High throughput phenotyping will be required to complement molecular tools for rapid genetic gain for drought tolerance in a fast track breeding scheme.

CONCLUSIONS

A review of the literature on drought tolerance in cassava reveals the physiological basis of drought tolerance in cassava and its integration with agronomic traits. Some traits are not easily phenotyped. For example, a deeper root system provides access to more soil water for the crop during drought. Basically, breeding and selection based on root system evaluation have not been well explored, and simple methods to evaluate root systems have yet to be developed in cassava.

A good number of the conventional traits typically used to assess drought adaptation, though relevant have limitations especially for those that are measured late in the growth cycle which makes current efforts to reduce the breeding cycle a challenge. The use of molecular tools in breeding is designed to efficiently select for genes for rapid genetic advances in the breeding (especially for complex traits). The strength and beneficial aspect of molecular tools lies in fast tracking the development of varieties that maximize gene combinations for complex traits. Molecular breeding thus require strategies that not only support fast rapid phenotyping protocols but minimal phenotyping. In such scenario, many of the current traits are not well suited to the modern breeding paradigms. Therefore the need to identify more efficient and rapid and or simple phenotyping protocols are expected to increase. Drought adaptation traits that may easily be used to assess productivity at early growth phase may be a quick strategy to accelerate drought tolerance selection in modern breeding for cassava. Physiological traits such as stomatal conductance and leaf photosynthesis that are easily measured will continue to be favored traits in modern breeding.

Complementary phenotyping strategies such as metabolite profiling used in combination with conventional cassava drought phenotyping traits will further enhance our understanding of drought tolerance in cassava. While rapid advances in high throughput genotyping has been achieved, much have yet to be done for phenotyping for cassava. The power to detect useful genes and understand the metabolic pathways of drought tolerance can only be efficiently dissected by complementing it with high through phenotyping that enhances quality of phenotypic data both in precision and accuracy. Developing strong phenomic capacity for drought tolerance in cassava is therefore crucial to the process. Significant progress in drought tolerance breeding will largely depend on how quickly this capacity is developed.

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Phenotyping bananas for drought resistance

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Drought has emerged as one of the major constraints in banana production. Its effects are pronounced substantially in the tropics and sub-tropics of the world due to climate change. Bananas are quite sensitive to drought; however, genotypes with "B" genome are more tolerant to abiotic stresses than those solely based on "A" genome. In particular, bananas with "ABB" genomes are more tolerant to drought and other abiotic stresses than other genotypes. A good phenotyping plan is a prerequisite for any improvement program for targeted traits. In the present article, known drought tolerant traits of other crop plants are validated in bananas with different genomic backgrounds and presented. Since, banana is recalcitrant to breeding, strategies for making hybrids between different genomic backgrounds are also discussed. Stomatal conductance, cell membrane stability (CMS), leaf emergence rate, rate of leaf senescence, RWC, and bunch yield under soil moisture deficit stress are some of the traits associated with drought tolerance. Among these stress bunch yield under drought should be given top priority for phenotyping. In the light of recently released *Musa* genome draft sequence, the molecular breeders may have interest in developing molecular markers for drought resistance.

Keywords: bananas, breeding, bunch yield, drought stress, phenotype, RWC

INTRODUCTION

IMPORTANCE OF BANANAS

Bananas (refers to banana, plantain, and cooking bananas) are one of the earliest crop plants to have been domesticated. Originally, they were adapted from the humid tropics to broad subtropical climatic conditions. Bananas are one of the most important, but undervalued, food crops in the world. Bananas provide a staple food for millions of people; particularly plantains have remained a staple food of many ethnic groups in Africa, an area where the green revolution has had little influence. Bananas are considered an important food security crop, providing a cheap and easily produced source of energy. In addition, they are rich in certain minerals and in vitamins A, C, and B6. It has been estimated that the highest consumption rates are on the island of New Guinea and in the Great Lakes region of East Africa, where bananas form a large proportion of the diet and consumption amounts to 200–250 kg person^{-1} year^{-1} whereas in Europe and North America consumption is approximately 15–16 kg person^{-1} year^{-1} (INIBAP, 1992). Bananas are consumed in various forms, and consumption methods have evolved and been refined by humans over time. They are eaten raw, cooked, baked, steamed, or fermented. In many places, the whole plant is exploited with uses for the leaves, pseudostem, medicinally rich plant sap or fiber. Thus bananas and plantains are grown for specific purposes apart from the edible fruit and have become interwoven with the culture and livelihoods of human society. Although today bananas and plantains are best known as a food crop, almost every part of the plant can be used in one way or another.

CULTIVATED AREA AND YIELD PERFORMANCE UNDER OPTIMAL CONDITIONS

Basically, bananas have occupied the status of commercial crop. Traditional banana growers, with the exception of a few large companies, are responsible for most production world-wide. Bananas and plantains (*Musa* spp.) are grown in more than a hundred tropical and subtropical countries and provide staple food for hundreds of millions of people. Bananas and plantains are grown in about 130 countries around the world, exhibiting a spectacular production of 122.85 million tons (FAOSTAT, 2012). India alone produces 26.20 million tons on an area of 0.70 million ha and contributes to 21.30 percent of global production (2007–2008). India is the largest producer in the world, followed by China, The Philippines, Brazil, and Ecuador. Around 87% of all the bananas grown worldwide are produced by small-scale farmers for home consumption or for sale in local and regional markets, while the remaining 13%, mainly dessert bananas, are traded internationally. Dessert bananas are also grown commercially in the subtropics and in Mediterranean climates including Israel and other East Asian countries, for internal consumption or local export (FAOSTAT, 2008). More than two-thirds of the bananas grown in the world for export are irrigated (Stover and Simmonds, 1987).

DROUGHT RESISTANCE

Water limitation is a major problem for global agriculture, permanently affecting 28% of the world's soils with almost half of all soils intermittently limited because of shallowness, poor water holding capacity, and other factors (Dudal, 1977). Drought in

agriculture is "shortage of water in the root zone, resulting in decreased crop yield" (Salekdeh et al., 2009). Drought tolerance consists of drought avoidance and/or dehydration tolerance that are ultimately measured by the reproductive success of the species (Taylor et al., 2007). Drought avoidance strategies in plants include deep rooting, conservative use of available water to ensure grain filling is completed, and lifecycle modifications to match rainfall. Dehydration tolerance involves the plants' ability to partially dehydrate but remain viable and grow again when rainfall resumes.

The effect of drought on plants is complex and plants respond with many protective adaptations. Drought causes the plant to suffer from dehydration and overheating of its cells and tissues. Hence, drought resistance of the plant includes the ability to withstand dehydration and ability to withstand overheating (heat-resistant). High heat-resistance is not always linked with high drought-resistant and there is no universal mechanism of adaptation of plants to drought. Drought-resistance is a property which is formed and developed in the process of ontogenesis and is based on the whole preceding phylogeny of the plant. Based on the above observation, Henckel (1964) defined drought resistance as follows. "Drought-resistant plants are those which in the process of ontogenesis are able to adapt to the effect of drought and which can normally grow, develop, and reproduce under drought conditions because of a number of properties acquired in the process of evolution under the influence of environment."

Drought is one of the important abiotic constraints restricting banana cultivation and its further adoption into non-conventional growing areas. Breeding for drought alone has not been focussed among any of the global banana breeding programs but it has been an essential trait considered along with other important ones like Fusarium wilt (race 1, 2, and 4), Sigatoka leaf spot (*M. fijiensis*, *M. eumusae*, and *M. musicola*), etc. Recent issues of climate change have warranted the need for the development of commercial banana varieties suited for less water environments. In this perspective the strengths and weaknesses in the banana crop for breeding drought tolerant genotypes has been discussed below with emphasis on genetic resources, drought tolerance, compatibility, outcome of breeding programs, etc. In this article explanation is placed largely on the basis of experiments performed in India.

BANANAS PLANT WATER RELATIONS

Bananas pose challenge to physiologists to measure indicators of water deficits, due to the presence of large air pockets within the leaves, and laticifers containing latex within the leaves, fruit, and corm that hinder the use of standard methods of measuring water relations (Turner and Thomas, 1998). Milburn et al. (1990), Kallarackal et al. (1990), Turner and Thomas (1998), subsequently demonstrated different methods to measure a series of physiological indications in relation to drought tolerance, *viz.*, of water potential, the volumetric (relative leaf water content), or thermodynamic tissue water status (leaf water, osmotic, and pressure potentials) of a laticiferous plant like the banana. The method described by Milburn et al. (1990), which is based on measurements of the refractive index of exuded latex, was preferred and its reliability subsequently confirmed by Thomas and Turner (2001). The water potential of well-watered plants was found to cycle diurnally within the remarkably narrow range of 0 to −0.35 MPa. In fact, the rate of extension of the youngest leaf may be the most sensitive indicator of plant water status (Kallarackal et al., 1990), providing it is not too hot (Thomas and Turner, 1998). Under hot, arid conditions, leaf folding is not considered to be a reliable plant-based indicator of when to irrigate (Thomas and Turner, 1998).

Banana production constraints are dominated largely by biotic and abiotic stresses. However, while research on biotic stresses has drawn sufficient attention worldwide, abiotic stresses have gone unnoticed. Among the abiotic stresses, drought, salinity and heat are the most important. Drought has rarely been addressed in the past, but is gaining importance in the face of depleting natural resources. The results of successful cultivation, especially of the water loving Cavendish clones, in drought prone areas with protected irrigation have provided the required momentum to perform research on drought in bananas. In subtropical and semi-arid banana cultivation zones, where rainy days are limited and there is an uneven distribution of rainfall, new crop management practices in terms of varieties selected, soil improvement (in terms of physical properties and nutrient enrichment), water management, etc. are being adopted. Although a large amount of research has been carried out on tropics including water management, drip irrigation, and fertigation, work on evaluation of banana and plantain varieties under conditions of water deficit is still very limited, as is the availability of related information. Probable reasons could be that most genebanks and breeding programs actively involved in germplasm evaluation and development are located in the humid tropics and ample rainfall. Moreover, creating large-scale drought conditions for a crop like bananas that is large and of long duration (12–20 months), presents many practical difficulties.

Screening germplasm for the drought has been initiated in some breeding programs such as that of the International Institute for Tropical Agriculture (IITA), Nigeria, NRCB, India and the Centro de Investigación Científica del Yucatán (CICY), Mexico. IITA has planted a large amount of germplasm in semi-arid zones of Uganda. The material that is being screened for drought tolerance includes landraces, East-African highland bananas, plantains and their triploid and tetraploid hybrids[1]. Similarly, NRCB is located in the dry tropics and is maintaining and evaluating a total of 340 core accessions for response to various biotic stresses, male, and female fertility, compatibility with other groups and subgroups, and seed setting ability. NRCB has screened 112 genotypes from a core collection of 340 accessions in response to water deficit conditions. Systematic screening of a wide range of germplasm for specific traits like leaf water retention capacity (LWRC) has also been attempted (Ravi and Uma, 2009). Observations on the response of various genotypes to water deficit under field conditions and their amenability for improvement through classical breeding are presented in **Table 1**.

Bananas, being a commercial crop in the tropical and subtropical region of the world, are prone to their growth and productivity

[1]http://www.iita.org/cms/details/banana_project_details.aspx?articleid=228&zoneid=308

Table 1 | General observations on germplasm performance under water deficit conditions and note on their breeding behavior (Anon, 1999, 2000, 2004, 2006, 2007; Uma and Sathiamoorthy, 2002; Uma et al., 2002).

Genomic group	Subgroup or status	Genotypes (varieties/types)	Reaction to water deficit	Breeding behavior	General remarks
AA	Wild	*M. acuminata* ssp Burmannica *M. acuminata* ssp burmannicoides *M. acuminata* ssp malaccensis *M. acuminata* ssp zebrina	Highly susceptible	Male and female fertile	Widely used donors for biotic stress tolerance genes
BB	Wild	Types Athiakol, Manohar, Bacharia Malbhog	Susceptible	Male and female fertile	Not used in breeding program due to BSV being integrated into the host genome
		Types Attikol, Elavazhai	Less tolerant	Male and female fertile	Not used in breeding program due to BSV being integrated into the host genome
		Types Bhimkol	Moderately tolerant	Male and female fertile	Not used in breeding program due to BSV being integrated into the host genome
		M. balbisiana type Andaman	Tolerant	Male and female fertile	Not used in breeding program due to BSV being integrated into the host genome
AAA	Unique	Thellachakkarakeli	Moderately tolerant	Female fertile	Elite cultivars due to quality fruits. Produces average bunch even under water deficit conditions
	Cavendish	Grand Naine, Robusta, Dwarf Cavendish, Williams	Highly susceptible	Female fertile	Complete failure of crop
		Red banana and Green red banana	Susceptible	Moderately female fertile	Complete failure of crop
	Ney Poovan	Ney Poovan, Nattu Poovan, Njali Poovan	Tolerant	Reduced female fertile	Produces bunch even under water deficit
AAB	Mysore	Mysore, Poovan, Champa	Moderately tolerant	Female fertile	Produces bunch even under water deficit
	Pome	Prata	Susceptible	Female fertile	Fruits fail to fill and central core becomes conspicuous
		Small fruited varieties such as Pacha, Ladies Finger, Mannan, Krishnavazhai, Malai Kali	Susceptible	Female fertile	Fails to develop but under normal conditions sets seeds unlike counterparts with bigger fruits
ABB	Pisang Awak	Karpuravalli, Ankur-II, Gauria, Chinia, Bankela, Udhayam	Tolerant	Female fertile	Reduction in number of hands but retains finger size; sets seeds even under water deficit
	Monthan	Kachkel, Yengu Bontha, Bankeli, Pidi Monthan, Lamby	Moderately tolerant	Male fertile Female sterile	Reduction in number of hands and size of the fruit
		Ash Monthan	Tolerant	Male fertile Female sterile	Imposition of drought at flowering even with 3–4 green leaves produces normal bunch
ABB	Bluggoe	Birbutia, Bersain, Beula, Kothia, Chakia, Gauria, Nepali Kallu Monthan, Sakkai	Moderately tolerant	Male and female fertile	Bunches develop even with water stress; a hardy group of plants
ABB	Unique	Bangrier, Kanchikela	Moderately tolerant	Male and female fertile	Yield stability over the years; less reduction in yield; sets seeds even under water deficit, but has poor germination

being adversely affected by water stress. In traditional banana growing areas, long-term drought is not common, even though it is as potential an abiotic stress as short dry seasons. Inherent crop-based problems like being a long duration crop (10–12 months) make drought a potential threat in bananas. In addition, the high leaf area index (LAI) and shallow root system makes the banana plant extremely susceptible to water shortage (Robinson, 1996). Consequently the plant requires supplementary irrigations during dry periods to prevent reductions in yield and fruit quality. Some of the work carried out with bananas has been reviewed below, indirectly throwing some light on the crop's reaction to water deficit and field performance.

EVAPOTRANSPIRATION FROM BANANA PLANTATIONS

Precise information on the amount of irrigation to be applied is usually lacking, although a few experiments have been reported based on available water in the soil at field capacity. Depending on the prevailing climatic conditions, estimates of the annual evapotranspiration of the banana plant range from 1200 to 2690 mm (Robinson and Alberts, 1986). The water requirements of drip-irrigated bananas grown under semiarid conditions on a Mollisol or on an Ultisol with transient dry periods were determined. Using Class A pan factors that ranged from 0.25 to 1.25, it was found that all yield components for the plant crop and two ratoon crops were significantly improved with an increase in water application (Goenaga and Irizarry, 1998). Young et al. (1985) reported similar results when banana plants were irrigated according to pan factor treatments that ranged from 0.2 to 1.8. The water requirement of bananas in the humid tropics has been reported to be about 1–1.4 times the class A Pan evaporation (Stover and Simmonds, 1987). In a large-scale plantation in Honduras, plants were irrigated when the soil moisture tension (as recorded by tensiometers) exceeded −0.02 MPa at 15 cm and 30 cm (Stover and Simmonds, 1987).

BANANA GENOTYPIC VARIATION FOR DROUGHT RESISTANCE

Banana plants are very sensitive to soil water deficit, as shown in numerous field experiments (Robinson, 1996). Banana leaves remain highly hydrated, even under drought (Shamueli, 1953; Turner and Thomas, 1998) indicating that the closure of stomata caused by soil water deficits is likely to be linked to a signal from the roots rather than a water deficit in the leaves (Turner, 2003). In a split root experiment, Thomas (1995) observed that drying part of the root system had no effect on leaf water status but did close the stomata. Severing the roots on the dry side caused the stomata to reopen. These observations support the view that the roots produce a signal that is transported to the leaves. This mechanism conserves the plant's water, but reduces carbon assimilation and productivity. From this point of view, study of root volume and structure may be less important. However, it is well-established that drought tolerant plants possess deep root systems. Root length density (Ld, measured in cm cm^{-3}) and specific root length (Lw, measured in m g^{-1}) are quantitative features of the architecture of root systems. Ld quantifies the capacity of the root system to explore the soil volume, and a high Ld means that the roots absorb more of the nutrients in a volume of soil, especially those nutrients that diffuse to the root surface. Banana and plantain roots have a Ld of about 1 cm cm^{-3} (Irizarry et al., 1981), which is similar to the root systems of trees. In contrast, Turner (2005) reported that herbaceous species have a Ld in the surface layers of the soil, of 4–50 cm cm^{-3}. Therefore it is worth studying Ld, an important trait linked to drought stress in the banana root system, as described by Blomme et al. (2005).

In an experiment with cv. Williams has grown under subtropical seasonal conditions, plants that were well-watered in spring and autumn exhibited a high transpiration rate, especially with a normal summer. Whereas in extreme conditions of winter or a very hot summer, an internal stress developed within the plant, which reduced the transpiration rate in both situations. The evaporative demand exceeded the water absorption potential as reflected in decreased transpiration and stomatal conductance (Robinson and Bower, 1988).

However, there are not many reports on the impact of water stress at different growth phases on yield and yield parameters. In a field experiment conducted at the NRCB farm, water stress was imposed on plants under drip irrigation by withholding water for 1 month at flowering. This decreased the bunch weight by 42.07, 25.0, and 18.83 percent in cvs Robusta, Karpuravalli, and Rasthali, respectively. When water stress of 1 month's duration was imposed 30 days after flowering, the bunch weight was reduced by 18.83, 27.66, and 11.25 percent, and when imposed 60 days after flowering by 25.0, 16.84, and 16.47 percent, respectively in the three cultivars. Among all the three cultivars tested, Robusta was the most sensitive. The maximum reduction in fruit length (11–14 percent) and circumference (5.75–16 percent) was observed at harvest when water stress was imposed at flowering (Anon, 2008).

Banana cv. Williams in which bunch emergence occurred during a period of soil water stress ($\Psi s = -0.5$ MPa) showed maturity bronzing at harvest, had shorter fruits and reduced green life (−29 percentage), and exhibited longer duration of fruit filling (Daniells et al., 1987). It has also been observed in bananas that growth and yields decreased drastically when the intervals between watering increased and when the soil moisture fell below 66 percent of the total available soil moisture (Robinson and Bower, 1988). Because of the tissue morphology, bananas require a certain amount of available soil moisture for normal development and growth. This high water requirement is the result of a large leaf area used for transpiration. It has been shown that the transpiration of banana plants in full sunlight is approximately 40–50 mg H_2O dm^{-2} min^{-1} (Shamueli, 1953; Morello, 1954; Tai, 1977). According to previous calculations, the daily water uptake by cv. Dominico-Harton, a plantain with a constant leaf area of 14 m^2 was estimated to be 26L in sunny weather, 17L in semi-cloudy weather, and 10L in cloudy weather. In a commercial plantation with a density of 1500 plants per hectare and a LAI of 2.1, water requirements were observed to be approximately 1170 m^3, 765 m^3, and 450 m^3 in sunny, semi-cloudy, and cloudy weather, respectively. However, in practice, 150 mm of precipitation per month was reported to be sufficient to cover the water requirements of cv. Dominico-Harton (Belalcázar et al., 1990). In general, shortening of the irrigation interval with the same amount of water through a pulse system reduces the water tension in the upper soil layer,

diminishes the soil temperature, encourages shallower rooting and reduces leaching of nitrates (Lahav and Kalmar, 1981). This practice is more important for the humid tropics and semi-arid areas.

Bananas are no longer considered as a single crop commodity owing to their vast diversity in terms of ploidy (2×, 3×, and 4×) and genomic constitution (AA, AAA, BB, AB, AAB, ABB, ABBB, etc.). Present day bananas have derived from two major ancestors *M. acuminata* contributing the A genome and *M. balbisiana* contributing the B genome. In nature, *M. acuminata* and its subspecies are considered as slender and delicate plants nurtured under shade and conducive environmental conditions, while *M. balbisiana* has diversified and being domesticated under harsh weather conditions, and is often resistant to many abiotic stresses including drought and extremes of temperature. On the other hand, banana cultivars containing the B genome being more resistant to abiotic stress than those solely based on the A genome. For instance, in Egypt where banana genetic diversity is higher than the rest of the region, the traditional AAB and ABB varieties cultivated in rural areas proved to be more resistant or tolerant to drought than the Cavendish ones (De Langhe, 2002). Another is the "Sugar" ABB Pisang Awak variety grown in Oman where it is shown to be well adapted to dryness at the Agriculture research station of Salalah (De Langhe, 2002). There are very few ABB dessert varieties showing good palatability and high productivity in the natural germplasm. Therefore, the triploid breeding strategy offers good future prospects through the combination of edible AB cultivars with wild *balbisiana* to create new productive dessert ABB varieties, palatable and tolerant to drought and cold temperatures.

Musa genotypes have exhibited differences in stomatal sensitivity based on the age of the leaf, and modulated by environmental factors such as irradiance, vapor pressure deficit (VPD), and soil-plant-water relations. On the basis of leaf conductance measurements, Ekanayake et al. (1994) identified tolerant ABB cultivars ("Fougamou" and "Bluggoe") and sensitive genotypes ("Bobby Tannap" AAB and one of its hybrids TMP × 582–4) for transient dry conditions. In a pot study Thomas et al. (1998) compared the effects of environmental variables on leaf gas exchange processes (including transpiration) of three cultivars differing in their genomic constitution ("Williams" AAA; "Lady Finger" AAB; "Bluggoe" ABB). They found that, as the saturation deficit of the air was increased (from 1.5 to 5.7 kPa), both stomatal conductance and net photosynthesis declined linearly. Since increasing proportions of the B genome reduced this sensitivity to the dryness of the air and increased the instantaneous water use efficiency of the leaf, Thomas et al. (1998) concluded that the B genome contributes to drought tolerance in *Musa* spp.

Musa genotypes have different inbuilt mechanisms for resistance to drought stress. Research has been carried out on the effect of water deficit on commercial cultivars by a number of workers (Cayón et al., 1998), diploid *acuminata* clones (Ismail et al., 2000; Shamsuddin et al., 2000), and Cavendish clones (Eckstein and Robinson, 1995; Ramcharan et al., 1995; Orjeda and Suarez Sanchez, 1998; Thomas et al., 1998). However, there are very few reports on reactions to drought across the genotypes and their differential physiological, biochemical, and agronomic expression (Garcia and Manzanilla, 1994; Bananuka et al., 1999; Wagner et al., 2000; Abeywickrama and Weerasinghe, 2002; Ravi and Uma, 2009). Cultivars that demonstrated small reductions in gas exchange and leaf area and maintained the high water retention capacity and assimilation rate showed more resistance to drought stress (Bananuka et al., 1999).

Water stress induces oxidative damage and protective mechanisms differ among banana cultivars (Chai et al., 2005). Correlations between stomatal conductance, transpiration, and photosynthesis in water-stressed plants are well documented (Kallarackal et al., 1990). Twenty-four diploids (AA) were phenotyped for drought tolerant traits. A wide variation in chlorophyll content was found among the 24 diploid (AA) banana genotypes. In this group, Anaikomban recorded the highest chlorophyll a, chlorophyll b, and total chlorophyll content among all the genotypes tested and Hatidat, Kanaibansi, Siguzani, and Namarai recorded the lowest chlorophyll content (Anon, 2008).

METHODOLOGY

Existing banana improvement programs have used only a fraction of the genetic diversity concealed in the wild and edible *Musa* species (*M. balbisiana and M nagensium*) (Lusty, 2005). Studies have been preliminary and neither exhaustive nor conclusive in terms of methodology, parameters, and research conditions. Much scope is left for future work to further refine the procedures and methodologies to be followed in the field as well as under controlled conditions.

BREEDING STRATEGY

Bananas require frequent irrigation to avoid significant crop losses, especially during dry periods. Bananas, originally a tropical fruit crop, have reached the sub-tropics and even semi-arid regions owing to their adaptations and to growers' perseverance to manage the crop at the small expense of yield and quality. Modified or improvized agricultural practices like drip or fertigation over natural irrigation and flooding, and adaptation of banana varieties with a capacity to tolerate water deficits to a certain extent have allowed successful banana cultivation in non-conventional zones. However, breeding bananas for drought tolerance is an important alternative strategy to combat production constraints such as dwindling water resources.

Drought tolerance in bananas is the ability to survive under water scarcity during various stages of crop growth, without significant yield reductions. However, in nature, drought tolerance is always at the cost of yield and quality. Water scarcity can be overcome by cultural management or through genetic improvement. The latter is a long-term solution that can reach the poor grower and allow expansion of the crop to marginal lands. The technology to be developed has to be a robust and reproducible, with easy application in the field. The protocol for developing such technologies needs background information on various aspects of the crop in question—bananas—and a standard procedure. Earlier works on drought tolerance in bananas and drought as a trait have, for various reasons, been the subject of little research.

Drought is a complex environmental factor that is varied over a location and time frame. This makes it difficult for the researcher to create a standard for drought when the crop is challenged

under field conditions. These realtime unpredictable situations are entirely different from screening for drought under controlled conditions. Therefore, breeding for the targeted environment with specific to the phenological stage will pave the success in breeding.

Reports in many other crops have suggested that the response to drought is a complex trait controlled by a number of genes. Drought seldom occurs by itself. In natural situations, drought is always coupled with high temperature stress and often with soil salinity. Working on a single mechanism to tolerate drought alone does not offer a solution; it needs to be researched with a multiple trait perspective. It is more complex in a crop like bananas because: (1) it is a long duration crop ranging from 12 to 18 months according to cultivar; and (2) it has three to four critical periods of crop growth spread over the 12–18 months, namely the juvenile stage, flower bud differentiation, shooting, and finally bunch maturity.

INHERENT PROBLEMS IN BANANA BREEDING

Most important of all, bananas have their own crop-based inherent problems for breeding, being a sterile crop (male and/or female), exhibiting polyploidy, and with most of the commercial varieties being sterile triploids and vegetatively propagated.

Another factor from the breeder's point of view is that bananas are no longer considered as a single crop commodity, since they have broad user applications. There are more than 30 varieties under cultivation in various parts of the globe. Each variety has its own breeding constraints. In the following section, breeding is addressed by the group and subgroup of economic significance. Present day commercial varieties of bananas and plantains are the evolutionary derivatives of crosses within and between two ancestral diploid species, the *M. acuminata* (AA) and *M. balbisiana* (BB) contributing A and B genomes, respectively (Simmonds, 1962). Although the involvement of other wild species such as *M. textilis* and *M. schizocarpa* has been proven (Carreel, 1994), a major role has been played only by *M. acuminata* and *M. balbisiana*. In conjunction with chromosome restitution, crosses have led to the development of autoploids and homogenomic hybrids, and alloploids and heterogenomic hybrids. Ploidy and genomic configurations played a vital role, leading to the development of the major groups being: diploids (AA, BB, and AB); triploids (AAA, AAB, and ABB); and tetraploids (AAAA, AAAB, AABB, and ABBB). Of these, commercial varieties fall into the genomic categories AAA, AB, AAB, and ABB. However, the cultivars grouped in the same genomic category could be very diverse (Simmonds, 1962; Stover and Simmonds, 1987) and hence, classified as subgroups. They consist of clones with similar morphological traits, having arisen from a single core clone through somatic mutations. Accordingly the classification of bananas is depicted in **Figure 1**.

Bananas and plantains are peculiar crops owing to their morphology. A sound breeding strategy needs background information on various fundamental aspects. In bananas, differentiation of the vegetative phase into the reproductive phase occurs with the completion of the emergence of all leaves. The number of leaves is a predetermined factor and ranges from 35 to 72, depending on the variety. The number of days taken to complete leaf emergence depends on the phyllochron values, which in turn depend on the prevailing climatic conditions. The normal range is from 6 to 16 months in *Eumusa* and can be as long as 50 months in some *Ensete* species at higher altitudes. Cessation of the vegetative phase is marked by the emergence of a flag leaf. On average, triploid commercial varieties take 8–12 months to complete the vegetative phase.

The flower axis start from the heart and pushes upward through the pseudostem. Generally, female or pistillate flowers are formed first on the flowering axis, followed by male or staminate flowers. Occasionally, the formation of perfect flowers in between the male and female phase is also noticed in some varieties. Flowers are borne on a cushion-like structure, arranged spirally and spatially along the axis. They are biseriate in nature and subtended or covered by a bract. Two or three bracts lift at a time and lifting starts from late evening to early morning. Female flowers become receptive in the early morning before the sun gets

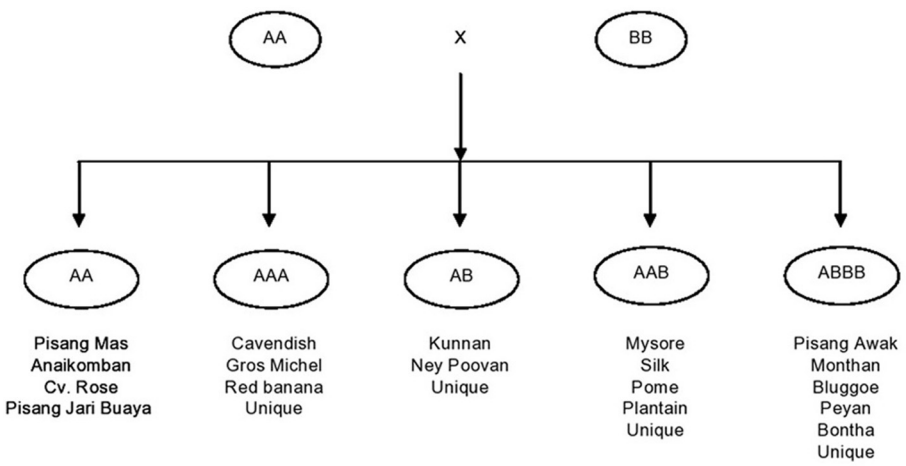

FIGURE 1 | Development of banana cultivars with the various major genomic groups.

too hot. Anthesis have also started in the evening, and mature viable pollen is ready and available in the morning for crossing. In some genotypes, pollen germination was noticed before anther dehiscence. In such cases the time of crossing need to be carefully adjusted to achieve better results.

Pollen from the pollen parents is collected along with the flower. The pollen is squeezed out of the pollen sac using the thumbnail and is spread onto the receptive sticky stigma to effect pollination. The bunch is then covered with a muslin bag to prevent unwanted pollination. The ovary starts enlarging and develops into a fruit. In general, bananas are parthenocarpic, and fruits develop mainly from the ovary wall. This phenomenon does not need the stimulus of pollination.

Fruits are carefully collected after full maturity and allowed to ripen. Seeds are extracted either manually or mechanically. Seeds are freed from the adhering pulp and soaked in fresh water for 4–5 days, changing the water daily. On the fifth or sixth day, seeds are either sown in seed pans with sterile soil and coco pith mixture for better water retention. Seed germination is a highly variable factor depending on the variety and parental combination, but it ranges from 2 to 10%. The germination time varies from seven to 120 days. During this period, care should be taken to prevent seeds being eaten by ants and squirrels, or rotting due to soil-borne or water-borne fungi. When the seeds have germinated and two to three leaves emerged, the seedlings are shifted from pans to polybags with a red soil/sand/coco pith mixture. After 2 months, the plants become physiologically mature enough for field planting. Labeling is important at every stage.

Embryo culture and embryo rescue

Germination and successful regeneration of seed progeny is as low as <1 percent. Complementing seed germination with embryo culture can enhance the regeneration rate by 30–50 percent (Rowe and Rosales, 1996; Tenkouano, 2006). A protocol to improve germination through embryo rescue has not yet been well documented. However, Ortiz et al. (1995) have successfully extracted 55–60 percent mature embryos (instead of fully mature embryos at fruit maturity) and cultured them on half strength Murashige and Skoog (MS) medium with modified hormonal concentrations and under continuous light.

BREEDING SCHEMES

Diploid breeding

Diploid breeding is vital to banana improvement programs, offering various advantages including a vast genetic background, the occurrence of high levels of male and female fertility, low levels of heterozygosity (which reduce the time to develop homozygous lines), easy genetic manipulation, and ease of study. Selected diploids, especially those exhibiting drought tolerance, are intercrossed to develop superior diploids, followed by selection for progenies exhibiting combined traits of drought tolerance and agronomic superiority.

Triploid–diploid breeding

The success of the triploid by diploid crosses depends on: (1) female fertility of the triploid; (2) the number of functional, fertile female gametes; and (3) inclusion of the B genome in either of the parents, and more specifically the triploid. The improved diploids are used to develop 4× and 2× progenies from 3× to 2× crosses. Alternatively 2× − 2× crosses may also result in 3× hybrids through unilateral sexual polyploidisation, where the parents produce either 2n pollen or a 2n egg (Tenkouano, 2006). Although some of the 4× progenies exhibit traits of interest, their female fertile nature results in the presence of seeds in the pulp, reducing consumer preference. This is overcome by crossing them with improved drought resistant diploid parents to derive sterile triploids (3×). Triploids are always superior to tetraploids in terms of sterility, reduced crop duration, optimum tree geometry, and better leaf retention. However, the choice of parents should allow capitalization on heterosis and pyramiding of genes of interest (Tenkouano, 2001). Although a number of breeding schemes can theoretically be conceptualized and attempted, the genomic and ploidy diversity in bananas makes the situation complex (Vuylsteke et al., 1997). The early success of the above mentioned breeding schemes makes them more realistic and practical, keeping in mind the complex nature of the drought tolerance trait.

Ploidy and genome analysis of progenies

Wide arrays of genotypes are observed in segregating population as a result of the variable ploidy and genomic status of the parents. Early analysis of ploidy and the genome is necessary to evaluate progenies for their basic purposes as dessert, cooking, or beer bananas. This is facilitated by the use of precise, non-destructive, faster and less labor-intensive flow-cytometry (Dolezel et al., 1994). For genome analysis, A- and B-specific markers have been developed and used (Pillay et al., 2000; Nwakanma et al., 2002). It has advantages over Simmonds' scoring system (Simmonds, 1966), by not relying only on morphotaxonomic traits and by being applicable at any stage of plant growth (Tenkouano, 2001). Genome-specific markers have been successfully employed in several breeding programs.

TRIAL PLANNING

Pot studies

Irrespective of whether it is the parent genotype or the hybrid progenies that are to be evaluated for drought, uniform planting material is a prerequisite. Being clonally propagated, *in vitro* culture offers bananas the best way to have uniform plants in sufficient numbers. Secondary hardened plants with 5–6 healthy leaves and a minimum of 4–5 major roots are selected for planting in 70–80 kg capacity concrete pots. The pots are filled with equal amounts of soil, sand, and compost. Fertilizer, NPK (15:15:15) is applied in doses of 20 g per plant before the induction of water stress The plants are irrigated regularly (on alternate days or by drip irrigation) until they are ready for imposition of the stress treatment. They are grown in a glasshouse or the phytotron where near-natural conditions are simulated. The stress period imposed through withholding of irrigation must be a minimum of 3–4 weeks to allow expression of the potential to adapt to the drought environment.

Field studies

For the field trial, uniform size suckers of recommended weight (1.5–2.0 kg) should be planted or tissue culture plants with 4–6

healthy leaves and 4–5 primary cord roots should be taken to achieve uniformity in growth and development (Ravi and Uma, 2011). Care should be taken to undertake this field trial in soil that is uniform in physical structure and fertility.

In the case of hybrid progenies, individual plantlets developed through embryo germination are planted in the field after 3 months under various hardening treatments. This is a pre-evaluation plot used for preliminary evaluations. Suckers obtained are multiplied *in vitro* for the production of 20–25 plants. From 10 uniform plants selected for screening against drought under controlled conditions. The actual number of plants required depends on the trait that is going to be studied and the methodology of the study. If the sampling technique is destructive, then more plants have to be made available when designing the experiment. During the crop growth period, side suckers should not be allowed to develop. One follower sucker should be allowed only after shooting. The plot should be weed free, and mechanical intercultural operations should be kept at a minimum to avoid root damage.

Water stress management and characterization

For evaluating any genotypes under field conditions, it is important to maintain cultural practices that are recommended in particular agro-climatic conditions as being most suitable for normal growth of the plant. The duration of stress is an important factor. Since the crop growth period extends over more than a year, the test accessions need to be protected from natural rainfall. For this purpose, a rainout shelter must be erected in the field and irrigated provided by a controlled irrigation system (drip/micro irrigation in the root zone). The size of the rainout shelters has to be determined according to the number of accessions and their maximum height. Estimating drought resistance in terms of the yield difference between potential (optimal) and stress conditions can differentiate genotype performance (Blum, www.plantstress.com).

As mentioned earlier, the stress intensity and phenological stage have to be defined based on the target environment. Though the bananas are sensitive to water stress, the most critical stage is the floral primordial initiation stage (Robinson and Alberts, 1986) than vegetative and fruit development stage. Water status measurements based on soil or root properties are more closely associated with leaf gas exchange than conventional techniques for measuring leaf water status (Turner and Thomas, 1998). The use of plant morphological characteristics in assessing plant water status, such as the rate of emergence of the youngest leaf also should not be ignored (Turner and Thomas, 1998). Thus, during the treatment period, soil matric potential monitored along while measuring leaf gas exchange parameters. The banana plant develops severe water deficit symptoms, when soil moisture reaches at *ca*. Fifty-five to sixty percentage of available soil moisture and then stressed plants must be given normal irrigation until the end of the harvest. To assess the effect of soil moisture deficit stress on juvenile vegetative stage, floral primordial initiation (PI) stage, flowering and bunch development on yield and yield parameters, separate experiment is to be laid out for each phenological stage. In banana cv. "Williams" early juvenile vegetative stage is insensitive to drought and 4–5 months after planting (coincides with PI stage) is sensitive to drought stress (Robinson and Alberts, 1986).

In places where a rainout shelter facility is not available, then the experiment has to be conducted in an arid zone where irrigation should be provided artificially. All the above parameters can be measured in field-grown plants, where the main concern is the overall effect of water stress on crop yield.

WATER AND PLANT WATER STRATEGY

In general, it is agreed that crop drought resistance is a major factor in the stabilization of crop performance in drought-prone environments. Drought resistance is now considered by breeders and molecular biologists to be a valid breeding trait. However, there is a serious lack of conceptualization, direction, and protocol for measuring drought resistance (Blum, www.plantstress.com). Tests for drought resistance must be performed with whole plants and/or plant communities (Blum, www.plantstress.com). Three major characteristics that contribute to genetic variation for drought resistance are: (1) maintenance of a high plant water content and delayed symptoms of water deficit such as wilting; (2) maintenance of plant function at a low water status; and (3) recovery of hydration and function from a low plant water status. The following methods accommodate the above points.

Phenotyping traits

Worldwide there is great interest in improving the drought tolerance of crop plants. Although it is known that drought adaptive traits are complex and multigenic, understanding of their physiological and genetic basis is incomplete, making specific genetic targets rare. Genetic improvement of drought resistance in crop plants require identification of relevant drought resistance mechanisms and the development of a suitable methodology for their measurement in the screening of germplasm or breeding population (Blum and Ebercon, 1981).

Plant growth. Plant growth is the real measurement because the whole plant is involved in the comparison among treatments. Besides plant height, pseudostem girth, phyllochron and leaf emergence rate, the following growth parameters should be measured.

Plant growth analyses are performed as follows. Five plants are to be harvested at fortnightly/monthly and divided into their respective parts and dried at 70°C in hot air oven for 48 h, giving dry weight (W). The area of each leaf was calculated from the formula (Turner, 1972).

1. Leaf area $(A) = 0.83\ (l\ \mathrm{b})$ where l = length of lamina in cms and b = breadth of lamina at its widest point (**Figure 2**) (Summerville, 1944).

From these data four factors were calculated as follows:

2. Leaf Area Index *(L)*: area of leaf (A) per unit area of land (dimensionless).
3. Leaf Area Ratio (F): area of leaf (A) per unit of total plant dry weight $(\mathrm{m}^{-2}\ \mathrm{g}^{-1})$.
4. Relative Growth Rate (R): $(\ln W2 - \ln W1)/(t2 - t1)$, where W, and W, are plant dry weights at times t, and t, respectively.

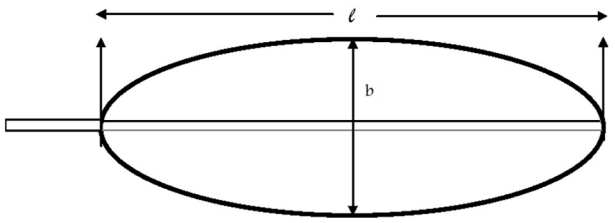

FIGURE 2 | Diagram representing the banana leaf for measuring lamina length (*l*) and width (*b*).

5. Net Assimilation Rate (E): from (1/A). (DW/dt), where W is the dry weight of the plant at time t and A is the leaf area ($g\,m^{-2}day^{-1}$).
6. Specific leaf weight (SLW) is calculated as leaf area/leaf weight.
7. The leaf emergence rate (LER) is a useful index of the vegetative development rate of a banana plant and is closely related to temperature. The leaves emerged during the experimental period are noted in both control and treated plants. The total number of fully opened leaves produced during the experimental period should be calculated on a weekly basis.

Allometric relationships developed for highland banana (Nyombi et al., 2009) can be adopted in all other environments by evaluating different phenological stages for use in growth assessment, understanding banana crop physiology, and yield prediction. An allometric relation is one whereby one measured parameter is a good estimate of other unmeasured parameter in the same organism. The authors derived the following equation to derive various growth parameters for highland bananas. Total plant leaf area (TLA) was estimated as the product of the measured middle leaf area (MLA) and the number of functional leaves. MLA was estimated as MLA (m^2) $= -0.404 + 0.381$ height (m) $+ 0.411$ girth (m). The allometric relationship between aboveground biomass (AGB in kg DM) and girth (cm) during the vegetative phase followed a power function, AGB $= 0.0001$ (girth) 2.35 ($R^2 = 0.99$), but followed exponential functions at flowering, AGB $= 0.325$ e0. 036 (girth) ($R^2 = 0.79$) and at harvest, AGB $= 0.069$ e0.068 (girth) ($R^2 = 0.96$). Girth at flowering was a good parameter for predicting yields with $R^2 = 0.7$ (cv. Mbwazirume) and $R^2 = 0.57$ (cv. Kisansa) obtained between actual and predicted bunch weights. This article shows that the allometric relationship can be derived for different banana cultivars in different agroclimatic zones for developing banana growth models, which can help breeders and agronomists to further exploit the crop's potential.

Plant water status

Relative water content. The relative water content (RWC) is the ratio of water present in the leaf disc at the time of sampling to that present in the excised disc after it has been fully dehydrated. The result is expressed as a percentage. A concern about this technique is the absorption of water into the intracellular spaces in the floating discs (Milburn et al., 1990). The values of RWC obtained in the study by Turner and Lahav (1983) revealed absorption of water into the intracellular spaces not to be a dominant factor. Therefore, this parameter needs to be considered to assess drought resistance traits. The third youngest leaf is taken for this measurement. Twenty leaf discs (12 mm dia) are extracted with a cork borer, half of the number from each half of the middle place of the lamina and weighed immediately, taking care to minimize water loss from the fresh sample. Discs float on deionized water containing $CaSO_4$ for 6 h at 25°C, and then weighed to determine the turgid weight. The leaf discs are placed between two layers of tissue paper with a 500 g weight placed on it for 1 min before weighing (Weatherly, 1950; Barrs, 1968). The leaf discs are then oven dried at 105°C for 24 h. RWC is calculated as follows:

$$\text{RWC} = 100\left(D_f - D_d\right) / \left(D_t - D_d\right)$$

where, D_f, D_t, and D_d are the fresh, turgid, and dry weights, respectively. The RWC data recorded in banana genotypes with different genomic background is presented in **Table 2**. Where, Drought stress was imposed at 6-months-old plants for 3 weeks and the soil matric potential reached at −0.60 MPa at the end of the stress period.

Leaf water retention capacity. Assessment of the rate of water loss from excised leaves or plants has shown some promise for differentiating drought resistance of wheat cultivars (Bayles et al., 1937; Sandhu and Laude, 1958; Salim et al., 1969; Dedio, 1975; Clarke and McCaig, 1982). A similar technique was applied by Bananuka et al. (1999) to assess drought stress resistance in bananas. The principle behind this technique is that drought stressed or hardened plants retain more water than unstressed plants and, when the stress is imposed across many different genotypes, the tolerant genotypes exhibit a greater capacity for water retention in the leaf tissue. The difference in LWRC may be due to the tightness of stomatal closure (Kirkham et al., 1980) or to other causes such as cuticular resistance to water loss (Clarke and McCaig, 1982). The third fully matured leaves from the top is sampled for measuring LWRC. Leaves (or strip of leaves) are excised and put under a polyethylene cover to avoid losing moisture and immediately weighed to give the fresh weight. They are left in a chamber at a temperature of 30–35°C and a RH of 50–60 percent for 24 and 48 h and weighed again. The leaves are also weighed after oven drying at 80°C for 24 h. Then the leaf water is calculated for 24 and 48 h by subtracting the oven dried leaves and expressed in terms of percentage of leaf water present at 24 and 48 h, as follows:

$$\text{Leaf water retention capacity}\,(\%) = \left(\text{fresh weight} - \text{dry weight}\right) / \text{fresh weight} \times 100$$

The LWRC, as a proxy for drought resistant or water-use efficiency, should be treated very cautiously (Turner et al., 2007). For large-scale field screening this method can be very well adopted (Ravi and Uma, 2009) and must be validated for yield under irrigated and stress plots so that drought resistance/susceptibility can be quantified.

Table 2 | Relative water content (RWC) of drought stressed banana genotypes.

Genotypes with genome background with ploidy level	0 DAT		7 DAT		14 DAT		21 DAT		28 DAT	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Paghalapahad wild (BB)	93.2	90.8	94.4	79.3	81.0	67.3	76.3	69.3	78.7	71.7
Athiakol (BB)	74.3	68.2	86.5	80.4	78.9	70.7	85.2	63.8	87.6	66.2
Karpuravalli (ABB)	81.0	83.3	79.7	71.2	79.3	70.4	87.2	61.9	79.5	64.3
Peyan (ABB)	81.2	77.0	81.2	69.8	87.6	68.4	76.2	62.6	78.6	65.0
Kothia (ABB)	97.5	92.0	87.5	69.0	83.5	68.5	85.6	62.8	88.0	65.2
Vennutu Mannan (ABB)	82.8	81	82.7	70.23	85.0	78.2	90.9	83.6	93.2	86.0
Saba (ABB)	81.2	78.5	89.7	79.8	85.7	75.7	77.3	84.5	79.6	66.9
Monthan (ABB)	76.3	78.9	81.1	78.2	78.9	76.3	85.6	86.7	87.9	69.1
Nendran (AAB)	77.0	79.8	78.2	73.9	81.4	71.5	84.3	71.2	86.6	73.6
Poovan (AAB)	80.1	88.8	93.3	71	81.7	76.9	84.7	75.9	87.0	68.3
Chinali (AAB)	83.2	79.6	88.6	72.3	75.9	68.6	86.9	71.8	89.2	74.1
Rasthali (AAB)	86.5	80.8	81.7	72.6	79.8	60.8	86.9	68.2	89.2	70.6
Jwari Bale (AAA)	81.4	81.2	81.23	62.4	89.0	70.0	87.7	56.2	90.1	68.6
Ney Poovan (AB)	82.4	85.8	88.4	69.2	79.0	69.0	87.4	63.8	79.8	66.2
Robusta (AAA)	90.2	88.0	83.56	67.9	80.4	71.4	83.0	56.4	85.4	68.8
Red Banana (AAA)	87.7	78.8	81.32	76.1	76.9	77.9	88.7	52.6	91.1	65.0
Pisang Jari Buaya (AA)	60.6	53.5	85.14	70.1	83.4	64	85.6	50	87.9	62.4
Calcutta 4 (AA)	75.9	75.9	81.21	71.1	79.3	64.4	84.9	69.6	77.2	72
X	81.81	80.11	84.75	72.47	81.48	70.56	84.69	67.27	85.37	69.11
T	NS		*		*		*		*	
V	*		*		*		*		*	
T × *V*	NS		*		*		*		*	
CV%	10.68		10.6		13.31		9.64		10.72	

Drought stress was imposed in 6-month-old plants.

Source: Ravi and Uma (2012).

**Significance at 5% level of CD. NS, Non significant; T, Treatment; V, Genotype; T1, control; T2, stress; DAT, Days after treatment.*

*The relative water content (RWC) of banana genotypes significantly varied between treatments after the second week of stress imposition (**Table 2**). After 14 DAT the RWC in T2 maintained between 70.56 and 69.11. In irrigated control RWC maintained > 80.*

Plant function

Leaf gas exchange. Leaf gas exchange or the rate of extrusion of the leaf is a more sensitive method for determining the response of banana plants to water deficit. A strong association exists between soil water status and leaf gas exchange (Turner and Thomas, 1998). This parameter can be measured with the portable photosynthesis measuring system, e.g., an infrared gas analyzer (IRGA). The third youngest leaf is used, with a minimum of three measurements for each leaf. The middle or distal quarter of the third youngest leaf is used for the measurement.

Quantification of photosynthetic pigments. Drought affects photosystem II more than photosystem I in the photosynthetic mechanism. They become uncoupled, resulting in free, high-energy electrons in the leaf. The uncoupled electron transport leads to photooxidation of chlorophyll and loss of photosynthetic capacity. The chlorophyll content is measured from the third youngest leaf lamina, sampling from both sides of the middle portion of the leaf from three plants, using three replications for each treatment. Banana leaf discs of 40–50 mg (3–4 leaf discs with a diameter of 10 mm) in fresh weight can be extracted in 10 mL of dimethyl sulphoxide (DMSO) in a glass test tube covered with aluminium foil and kept in an oven at 65°C for 4 h (Hiscox and Israelstam, 1979). Tubes are withdrawn and the temperature brought down around 25°C. Leaving the sample over night under dark in the room temperature (23–25°C) ensures complete extraction of pigments and in DMSO chlorophyll degradation is negligible. The optical density (OD) of the extract is read on a spectrophotometer at 645 nm and 663 nm and chlorophyll a (chl a) and chlorophyll b (chl b) concentrations (in $\mu g\ ml^{-1}$) are calculated using the formula given by Arnon (1949):

$$\text{Chl a} = 12.7\text{D } 663 - 2.69\ \text{D}645$$

$$\text{Chl b} = 22.9\ \text{D}645 - 4.68\ \text{D}663$$

Cell membrane stability. Tissue tolerance may be exhibited and measured in any of the tissue's physiological or metabolic functions. It is a process specific since different physiological

processes may show tolerance or susceptibility (Blum, 1979). A valid and functional drought tolerance test should therefore relate to integrated plant responses at low plant organization level (i.e., tissue growth), or a single attribute related to the basic facets of cellular or tissue responses to stress (Blum, 1981). A critical role of cell membrane stability (CMS) under conditions of moisture stress as a major component of drought tolerance (Bewley, 1979). The rate of injury to cell membranes by drought is estimated through measurement of electrolyte leakage from the cells (electrical conductivity). For drought tolerance, the method is based on dehydration of leaf discs in PEG solution and subsequent measure of electrical conductivity of aqueous medium.

Banana genotypes are to be grown free from diseases and nutrient deficiencies. In two sets of plants per genotype, one set of plants is subject to soil moisture deficit stress by withholding irrigation and other will be irrigated. Twenty leaf discs (1.2 cm diameter) are to be taken from 3 to 4 plants per replication from third fully matured young leaf. The leaf discs are to be placed in 100 cm^3 flask and washed 2–3 times with deionized distilled water. For desiccation treatment (T) leaf discs are to be submerged/float in 30 cm^3 of 30% of PEG 6000 solution for 24 h at 10°C and for control (C) incubate the leaf discs with deionized distilled water. After incubation in PEG leaf discs are to be quickly washed three times with deionized distilled water. Both desiccated and control samples are to be immersed in 30 cm^3 of deionized distilled water for 24 h at 10°C. The flask contents are then warmed to 25°C, shaken and electrical conductivity is measured using an Electrical Conductivity Meter. Following conductivity measurement, the leaf tissues are to be autoclaved for 15 min and again electrical conductivity is measured at 25°C. Sufficient replications are to be maintained to satisfy the statistical analysis.

CMS of leaf tissues is calculated as the percentage injury using the following equation.

Percentage injury $= 1 - [1 - (T_1/T_2)\,/1 - (C_1/C_2)] \times 100$, where T and C refer to mean of treatment and controls, respectively, and the subscripts 1 and 2 refer to initial and final conductivities, respectively.

Yield and yield parameters. Any stress effect ultimately has to be evaluated in terms of economic yield. Therefore, drought stress imposed on the third and fifth month after planting and at flowering for a period of 4 weeks has to be evaluated in terms of yield. In the **Table 3**, bunch yield of different banana genotypes under drought stress (imposed at 6-month-old plants for 3 weeks and soil matric potential reached −0.6 MPa at the end of stress period) is presented.

The bunch weight of different genotypes under irrigated and stress environment analyses in SAS 9.2 Proc Glm model for Tukey test (**Table 3**). Among the tested banana genotypes Saba (ABB), Monthan (ABB), and Vennutu Mannan (ABB) recorded higher bunch yield under soil moisture deficit stress. These genotypes also recorded higher bunch yield under irrigated environment. The interaction plot for yield (**Figure 3**) also revealed the same.

Yield components such as the number of hands, the number of fingers, the rate of finger growth (in terms of length and circumference) must be compared in stressed and

Table 3 | Effect of drought stress on banana bunch weight (*V* × *T*).

Genotype treatment	LS mean	Group	Bunch weight (Kg)
V7	T1	20.33	A
V5	T1	16.66	AB
V6	T1	15.19	ABC
V8	T1	14.16	BCD
V7	T2	13.87	BCDE
V2	T1	13.00	BCDEF
V8	T2	11.38	BCDEFG
V10	T1	11.00	BCDEFG
V1	T1	11.00	BCDEFG
V3	T1	10.67	CDEFG
V6	T2	10.58	CDEFG
V11	T1	9.67	CDEFGH
V12	T1	9.67	CDEFGH
V3	T2	9.33	CDEFGHI
V10	T2	9.27	DEFGHI
V9	T1	9.17	DEFGHI
V1	T2	9.00	BCDEFGHI
V15	T1	8.83	DEFGHI
V5	T2	8.67	DEFGHI
V14	T1	8.17	EFGHI
V11	T2	8.17	EFGHI
V16	T1	8.00	FGHI
V4	T1	7.83	FGHI
V9	T2	7.00	GHI
V17	T1	6.83	GHI
V2	T2	6.50	FGHI
V16	T2	6.33	GHI
V4	T2	6.33	GHI
V14	T2	6.17	GHI
V13	T1	6.00	GHI
V15	T2	6.00	GHI
V18	T1	5.90	GHI
V13	T2	4.50	HI
V18	T2	3.75	HI
V12	T2	3.50	I
V17	T2	3.50	HI

Paghalapahad wild (BB)	V1
Athiakol (BB)	V2
Karpuravalli (ABB)	V3
Peyan (ABB)	V4
Kothia (ABB)	V5
Vennuttu mannan (ABB)	V6
Saba (ABB)	V7
Monthan (ABB)	V8
Nendran (AAB)	V9
Poovan (AAB)	V10
Chinali (AAB)	V11
Rasthali (AAB)	V12
Jwari bale (AAA)	V13
Ney Poovan (AB)	V14
Robusta (AAA)	V15
Red banana (AAA)	V16
Pisang Jari buaya (AA)	V17
Calcutta 4 (AA)	V18

Source: Ravi and Uma (2012).

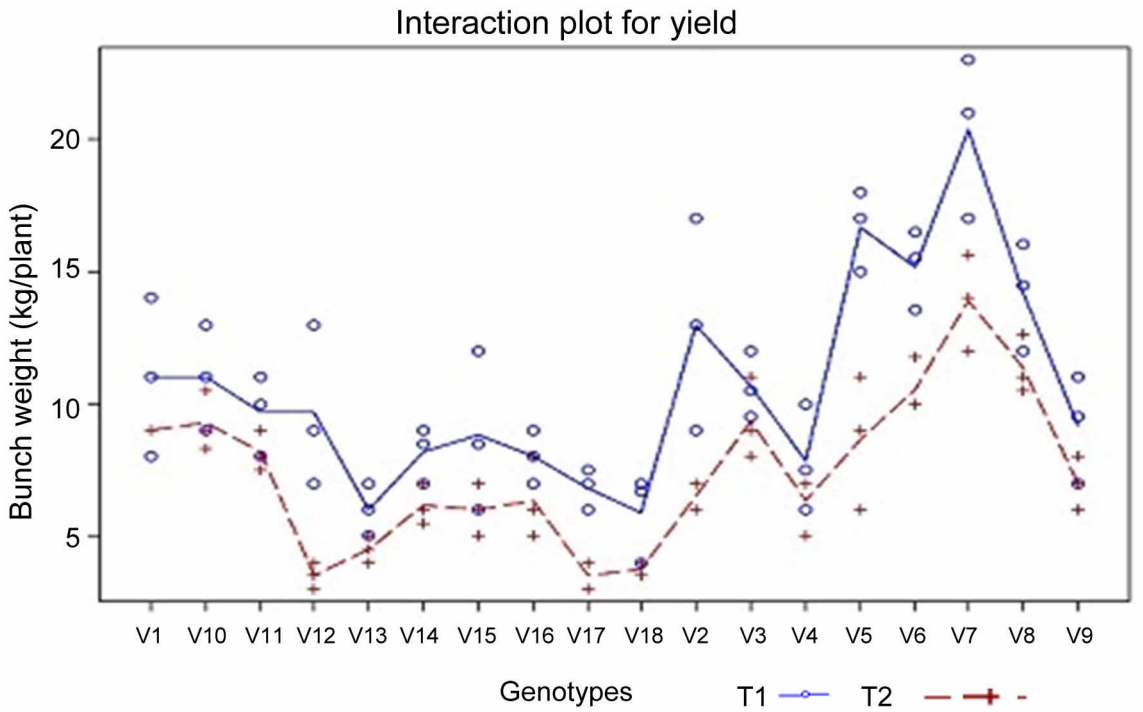

FIGURE 3 | Interaction plot for yield (bunch weigh in kg; $n = 3$) under different treatments (TRE), i.e., irrigated (T1-blue solid line) and drought stress (T2-red dotted line; soil moisture deficit stress imposed at six month old plants for three weeks and soil matric potential reached −0.6 MPa at the end of stress period) condition in different banana genotypes (VAR); V1—Paghalapahad Wild (BB); V10—Poovan (AAB); V11—Chinali (AAB); V12—Rasthali (AAB); V13—Jwari Bale (AAA); V14—Ney Poovan (AB); V15—Robusta (AAA); V16—Red Banana (AAA); V17—Pisang Jari Buaya (AA); V18—Calcutta 4 (AA); V2—Athikol (BB); V3—Karpuravalli (ABB); V4—Peyan (ABB); V5—Kothia (ABB); V6—Vennuttu Mannan (ABB); V7—Saba (ABB); V8—Monthan (ABB) and V9—Nendran (AAB). Source: Ravi and Uma (2012).

unstressed plants. At harvest, the individual finger weight difference can be calculated for the stressed and unstressed plants as:

$$\text{Geometric mean yield (GM)} = (Y_s \times Y_w)/2$$

where $Y_s =$ genotypic performance under stress and $Y_w =$ genotypic performance under well-watered condition.

Drought susceptibility index. The drought susceptibility index (DSI) is measured following Fischer and Maurer (1978):

$$S = [1 - (Y_s/Y_w)]/DII$$

where, $DII = (1 - X_d/X_p)$, and X_d and X_p are the mean experiment yield of all genotypes grown under drought stress and well watered regimes, respectively. In addition to yield and yield parameters, the traits measured in the preliminary evaluation also have to be taken into consideration for correlating with economic yield. For large numbers of genotypes (>25), the preliminary screening has to be done in the greenhouse or under a protected structure using plant growth analyses, plant-water relations, and plant functions. Drought tolerant plants identified thus must then be evaluated in the field. During field evaluation, stress can be imposed at different phenological stages.

CONCLUSIONS

Water limitation is a universal problem for agriculture. Bananas and plantains are a staple food for developing countries and a high value crop in others. Increased production of bananas and plantains with limited resources, especially water, is a priority. This can be achieved through improved production technologies and varietal improvement for water-limited environments. Improvement of varieties through conventional and novel approaches relies on identification of traits conferring drought tolerance. Phenotyping bananas for drought resistance, in germplasm available with the national agricultural research systems across the world, is to be prioritized to identify useful accessions for the banana improvement program. Many published literatures support on soil water status is linked to leaf gas exchange parameters and rate of emergence of the youngest leaf. Measurement of the fraction of transpirable soil water (FTSW) is laborious in banana but valuable information can be generated as this variable can be used in much the same way that "leaf nitrogen (N) content" is used instead of "applied N fertilizer" as the independent variable in studies on the impact of N fertilizer on leaf photosynthetic rates (Peng et al., 1995). In general, any variation in experimental conditions during the imposition of water stress should be avoided. Care should be taken to control all other factors except the stress to be imposed,

which is slightly difficult to manipulate. Cultivation and management are to be practiced as recommended for that test location. The stress has to be imposed for 3–4 weeks. Local drought tolerant varieties (based on their field performance) should be used as controls. The impact of the stress should be studied upto the final harvest. Any plant measurements must be corroborated with measurements of the soil moisture status. A controlled environment such as a rainout shelter is a preferred facility for conducting field level experiments in a long duration crop like bananas. It is an established fact that in bananas root signals plays a very significant role in recognizing the soil moisture deficit stress. The morphology, geometry, and quantitative nature of banana roots (root number, length, diameter, root mass, and RLD) merit study in relation to drought tolerance.

There is a great interest evinced among researchers in improving the drought tolerance of crop plants across the world. Presently, researchers are able to elucidate gene functions and mechanisms to regulate major plant traits through genomics, epigenomics, transcriptomics, proteomics, and metabolomics. To reap the benefit of recent molecular tools, good phenotyping is warranted. In the light of *Musa* genome sequence information available to all the researchers from July 2012, a quantum jump of molecular work toward the banana improvement program is contemplated.

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