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ABIOTIC STRESS: MOLECULAR GENETICS AND GENOMICS

Topic Editors
Mukesh Jain, Rohini Garg
and Rajeev K. Varshney

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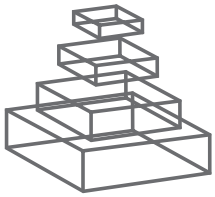

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ABIOTIC STRESS: MOLECULAR GENETICS AND GENOMICS

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Abiotic stresses are the major cause that limits productivity of crop plants worldwide. Plants have developed intricate machinery to respond and adapt over these adverse environmental conditions both at physiological and molecular levels. Due to increasing problems of abiotic stresses, plant biotechnologists and breeders need to employ new approaches to improve abiotic stress tolerance in crop plants. Although current research has divulged several key genes, gene regulatory networks and quantitative trait loci that mediate plant responses to various abiotic stresses, the comprehensive understanding of this complex trait is still not available. This e-book is focused on molecular genetics and genomics approaches to understand the plant response/adaptation to various abiotic stresses. It includes different types of articles (original research, method, opinion and review) that provide current insights into different aspects of plant responses and adaptation to abiotic stresses.

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Molecular genetics and genomics of abiotic stress responses

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Abiotic stresses are the major causes that limit productivity of crop plants worldwide. Plants have developed intricate machinery to respond and adapt over these adverse environmental conditions both at physiological and molecular levels. Due to increasing abiotic stress constraints, plant biotechnologists and breeders need to devise and employ new approaches to improve abiotic stress tolerance in crop plants. Although the current research has divulged several key genes, gene regulatory networks and quantitative trait loci (QTLs) that mediate plant responses to various abiotic stresses, the comprehensive understanding of this complex trait is still not available. With an objective to understand the plant response/adaptation to various abiotic stresses, a special issue was planned for the journal. The current research topic "Abiotic Stress: Molecular Genetics and Genomics" has a combination of primary research articles, perspective, opinion and review work, written by authorities in their respective fields. These articles provide novel insights and detailed overviews on the current knowledge into different aspects of plant responses and adaptation to abiotic stresses.

The perspective article by Henry (2014) presents genomic strategies for development of climate resilient crop varieties to ensure food security. The discovery of genomic variations and genes associated with climate adaptation found in wild relatives of crop plants via whole-genome resequencing may be directly relevant for implementing breeding approaches to develop environmentally adapted crops. In terms of understanding allelic variations, Roorkiwal et al. (2014) report allele diversity for 10 abiotic stress-responsive genes in the reference set chickpea representing the diversity of global chickpea germplasm. Detailed analysis provides haplotype network as well as estimates on genetic diversity for candidate genes in the germplasm collection. The next article by Deshmukh et al. (2014) highlights the importance of integration of various omics approaches for abiotic stress tolerance in model legume crop, soybean. Significant genomic advances have been made for abiotic stress tolerance in soybean in terms of availability of molecular markers, QTL mapping, genome-wide association studies (GWAS), genomic selection (GS) strategies, and transcriptome profiling. It has been suggested that combining QTL mapping based on GWAS along with transcriptome profiling can provide a valuable approach to identify candidate genes involved in desired trait(s) (Deshmukh et al., 2014). It has been realized that studies in other omics branches like proteomics, metabolomics and ionomics and their integration with genomics are equally important and should be part of future research to understand abiotic stress responses.

Two review articles (Golldack et al., 2014; Nakashima et al., 2014) provide important insights into signaling mechanism and transcriptional regulatory network, and their cross-talk in various abiotic stress responses. Both of these articles highlight the central role of transcription factors (TFs) in abiotic stress response and tolerance mechanisms. Other molecular signaling components, such as mitogen activated protein kinases (MAPKs), reactive oxygen species (ROS) and lipid-derived pathways have also been implicated in plant adaptation to environmental adversity (Golldack et al., 2014). In addition, the crucial role of β-catenin-like armadillo (ARM) proteins in abiotic stress responses has also been anticipated (Sharma et al., 2014). The study of these proteins can provide novel insights into the regulation of abiotic stress responses. Nakashima et al. (2014) suggested that TFs function in crosstalk among various abiotic stress responses and are being utilized to improve abiotic stress tolerance in different crops. However, it is important to examine the molecular effects of overexpression of TFs in addition to stress tolerance, because their overexpression may affect other signaling pathways too. The combing/pyramiding of transgenes for different stresses through molecular breeding can provide superior lines with improved stress tolerance in plants.

Calcium ions play a pivotal role in several signal transduction cascades in plants especially abiotic stress signaling. Calcineurin B-Like proteins (CBLs) function as calcium sensors and modulate the activity of CBL-Interacting Protein Kinases (CIPKs). The CBL-CIPK network helps maintaining proper ion balances during abiotic stresses. The CBL and CIPK homologs are present in all green lineages and phylogenomic analysis suggests their expansion from a single CBL-CIPK pair present in the ancestor of modern plants and algae (Kleist et al., 2014). The conservation of NAF domain and yeast two-hybrid results pointed the presence of physically and functionally connected CBL-CIPK network in plants. It is intriguing to analyze the precise role of CBL-CIPK pairs in abiotic stress responses. Virus-induced gene silencing (VIGS) has emerged as an efficient and robust tool for gene function analysis in plants. Ramegowda et al. (2014) provide an elegant overview of the usage of VIGS in different crop species. The article covers recent advances, limitations and future

prospects for characterization of abiotic stress related genes and understanding abiotic stress tolerance mechanism. Sengupta and Majumder (2014) addressed the mechanical-functional trade-off in plant vasculature, which can have an adaptive value under abiotic stress conditions. The authors have also provided physiological and genomic basis of abiotic stress tolerance and new possibilities for bridging physiology and genomics for crop improvement.

In summary, the articles presented here emphasize the involvement of a variety of genes/pathways and regulatory networks in abiotic stress responses. The broad-range of articles involving genomics and breeding approaches deepen our existing knowledge about this complex trait. Further, despite the existing comprehensive knowledge in this area, many questions still remain unaddressed. With the climate change threat, depletion of natural resources and ever increasing global population, sustainable and higher crop production is greatly needed. Therefore, there is an urgent need to employ various approaches and their integration to understand the molecular basis of abiotic stress response and adaptation for the development of stress-tolerant crop varieties.

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Genomics strategies for germplasm characterization and the development of climate resilient crops

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Food security requires the development and deployment of crop varieties resilient to climate variation and change. The study of variations in the genome of wild plant populations can be used to guide crop improvement. Genome variation found in wild crop relatives may be directly relevant to the breeding of environmentally adapted and climate resilient crops. Analysis of the genomes of populations growing in contrasting environments will reveal the genes subject to natural selection in adaptation to climate variations. Whole genome sequencing of these populations should define the numbers and types of genes associated with climate adaptation. This strategy is facilitated by recent advances in sequencing technologies. Wild relatives of rice and barley have been used to assess these approaches. This strategy is most easily applied to species for which a high quality reference genome sequence is available and where populations of wild relatives can be found growing in diverse environments or across environmental gradients.

Keywords: genomics, evolution, climate adaptation, crops, wild crop relatives

NEED TO ADAPT CROPS TO NEW AND CHANGING ENVIRONMENTS AND THE ROLE OF GENOMICS

Agriculture needs significant increases in productivity to satisfy the expected growth in demand for food in the next few decades. The impact of climate variability and climate change on agricultural productivity is likely to be a major constraint to achieving increased food production. This makes the development of crop genotypes with resilience to climate change an important strategy for food security. Innovations in crop improvement based upon application of advanced genomics tools may be a way to address this need. The delivery of these technologies will require significant efforts in coordinated development and delivery of improved germplasm (Lybbert et al., 2013). Genomics allows resources available for crop adaptation to environmental stress to be characterized and utilized (Bansal et al., 2013). An evolutionary perspective may assist in the effective application of the power of genomic tools to the development of climate resilient crops adapted to a changing environment.

GENOMIC ANALYSIS OF CROP EVOLUTION AND ADAPTATION TO CLIMATE CHANGE

Crop evolution has been relatively rapid under human selection over the last 10,000 years of agriculture. However, it is built on a very much longer period of evolution of wild crop relatives and the plant groups from which they are sourced. Understanding the processes and history of crop domestication and the evolution of related wild species provides critical knowledge to guide the development of crop varieties that are resilient to climate change in the future.

Analysis of wild plant populations provides evidence of factors contributing to success in periods of climate change. For example, hybridization between species may be an advantage in adapting to rapid climate change by providing new genetic combinations to cope with new environmental circumstances. Closely related species that can hybridize are more likely to survive than highly divergent species that cannot hybridize (Becker et al., 2013). Analysis of the genetics of populations growing across environmental gradients or from contrasting environments may be used to identify how plant populations adapt to climate under natural selection (Cronin et al., 2007). Sampling of populations at the same time over a long period of time can also be used to monitor adaptation to climate change but few sites have been sampled in the past in a way that allows this type of analysis to be conducted. Establishment of long term experiments of this type would be of great value. Recent dramatic improvements in genome analysis tools due to rapid advances in DNA sequencing technology make feasible research that should deliver much greater understanding of the relationships between wild and domesticated plant populations (Henry, 2012, 2013).

Recent fossil evidence suggests early diversification of groups of crop wild relatives such as the grasses (Prasad et al., 2011). The climate resilience of domesticated rice populations may be related to their evolutionary history. For example, expansion of the range of climates to which crops are adapted will require the transfer of genes from wild populations adapted to new environments or the use of novel genes. Crop species are derived for many different flowering plant groups but many are from a small number of families (e.g., Poaceae and Fabaceae). Crop plants have many traits that reflect the environments in which they evolved prior to domestication. Humans have collected plants for food for a long period of time prior to domestication of plants and the establishment of agriculture in the last 10,000 years. Pre-domestication use of plants by humans or natural variants that suit domestication (Ishii et al., 2013) may have also impacted upon some plant populations but domestication has usually resulted in significant genetic alteration

of plants to suit human production in agriculture and food uses (Jin et al., 2008).

CHOICE OF SPECIES FOR CLIMATE RESILIENT AGRICULTURE

Domesticated crop species are few in number compared to the total number of land plant species (Henry, 2010). A small number of plant species that have been adapted to wide scale production account for a large part of the energy and protein in human diets. These have become the key crops contributing to global food security. A larger number of species have been domesticated for more limited local production in specific regions. Some of these could be considered for adaptation to a wider range of environments.

Genomics tools provide new options for accelerated domestication of new species to allow adaptation of agriculture to climate change (Shapter et al., 2013).

MONO-PHYLETIC AND POLYPHYLETIC DOMESTICATION

Domestication may have been a single genetic event with all the domesticated plants being descendent from the same wild parents or have involved a few or many independent domestication events with many wild plants contributing to the domesticated gene pool. This understanding may provide the opportunity to repeat the domestication of important crop species from a different or more diverse gene pool. Genome analysis may be used to guide this process.

CENTERS OF ORIGIN

The center of origin of a crop species is the region from which the species is believed to have been domesticated. These are the environments that the crop plant was originally best adapted to survive at the time of domestication. Domestication from a different population selected by genome analysis may provide an opportunity to develop genotypes adapted to a new environment.

CENTERS OF DIVERSITY

Genome analysis allows rapid identification of geographic centers of genome diversity. The center of diversity of a crop species is the region displaying the greatest genetic diversity of the crop species or its wild relatives. This may be distinct from the center of origin as plant species may have been domesticated in areas that are not those including the greatest diversity. Identification of these locations may provide new and diverse germplasm and define new environments for production of the crop now or in the future. Asian rice (*Oryza sativa*) was probably domesticated in China from wild *O. rufipogon*. The A genome clade of wild rice relatives is now considered to be most diverse further south with a center of diversity in New Guinea, Australia, and Indonesia. These locations may prove to be good sources of novel germplasm for rice improvement. Species from more temperate regions could be used to adapt rice to production in cooler climates.

PRIMARY, SECONDARY, AND TERTIARY GENE POOLS

The gene pools of crop species may be considered at several levels. Genomic analysis may have value at all of these levels. The primary gene pool is the gene pool of the plant found in domestication and usually the species from which the crop was domesticated. The primary gene pool includes those plants that are available for direct use in genetic improvement of the species. The secondary gene pool may include more diverse material from other species that can be accessed but with a greater degree of difficulty. This often includes other species in the same or a related genus (Dillon et al., 2007). The tertiary gene pool is a wider group of plants from which genes can be accessed but only with significant difficulty (e.g., plants in the family outside the genus that can be accessed as a source of new genes but only with technological intervention). Understanding the genetic basis of domestication and the issues associated with access of genes from more difficult (or distant) relatives facilitates their use in crop improvement and in the domestication of new species to adapt agriculture to climate change (Malory et al., 2011). These analyses are more powerful at the whole genome level.

ADVANCES IN GENOMICS OF CROPS

Advances in DNA sequencing in the last few years have resulted in genomic sequence data becoming more readily available (Edwards et al., 2012). Major efforts have been made to produce reference genome sequences for key species. This allows rapid analysis of sequence variation within species. However, *de novo* assembly of sequence data may be necessary to detect all differences and advances in sequencing technology to make this routinely possible with large plant genomes will be a significant advance.

Analysis of the genomes of plants growing along environmental gradients may provide a greater understanding of how plants adapt to climate variation under natural selection (Cronin et al., 2007; Fitzgerald et al., 2011; Shapter et al., 2012).

GENOMIC ANALYSIS OF GENETIC RESOURCES

Analysis of the genomes of plant genetic resources will become a key tool to enable their utilization in crop improvement for climate adaptation. Targeting of genetic resources from environments that match the one being breed for is an important strategy. Large scale sequencing of accessions in plant germplasm collections will provide a platform to enable these approaches (Henry, 2013).

Increased utilization of wild crop relatives will remain a major strategy for adaptation of crops to the environmental factors associated with climate change. Many crop wild relatives remain poorly collected and are not yet represent well in seed banks. Climate change and human development risk loss of this genetic diversity making accelerated collection of crop wild relatives urgent. Rice illustrates this challenge. The closest wild relatives of rice are those from the A genome clade from which rice was domesticated (Vaughan et al., 2006). Recent research has identified two possible new species in this group that represent important new sources of diversity for rice improvement (Sotowa et al., 2013). Rice wild relatives from some regions such as Africa (Wambugu et al., 2013) and Australia (Henry et al., 2010) are poorly known.

ANALYSIS OF NATURAL POPULATIONS AS A GUIDE TO IMPROVEMENT OF CROPS FOR AGRICULTURAL PRODUCTION

The analysis of populations of wild relatives of barley (Cronin et al., 2007; Fitzgerald et al., 2011) and rice (Fitzgerald et al., 2011; Shapter et al., 2012) indicate the potential value of genome analysis of these populations to support efforts to develop crop varieties adapted to new climates.

In these studies, wild plants were collected from diverse environments or along a sharp environmental gradient. Sampling of the same population over time as the climate changes could be simulated by this strategy. In only a few cases we can access samples that have been sampled from the same population over a significant period of time. Key findings were that adaptation to hotter or dryer environments was associated with increased diversity of biotic stress genes. Coping with abiotic stress may be confounded by overriding associated changes in the biotic environment (Fitzgerald et al., 2011).

REMOVING THE CONSTRAINT OF END USE QUALITY ON RAPID CROP ADAPTATION TO CLIMATE

Productivity gains in crop production require elimination of constraints to utilization of more diverse germplasm. In some species the requirements of end uses are a major limitation. Market requirements for specific food or processing attributes that are complex or not well understood at the genetic level can greatly hamper attempts to use diverse adapted germplasm. Genomics tools that allow these traits to be readily selected for in breeding will assist by removing these as constraints to rapid climate adaptation (Henry, 2014). Food quality traits are often associated with human selection in domestication. They are often relatively simply controlled genetically because of their relatively recent and brief evolution under human selection in the last 10,000 years or less. Improved understanding these genes can be targeted as achievable steps toward removing a major constraint on climate adaptation.

AVOIDING SELECTION THAT REDUCES CLIMATIC RESILIENCE

Human selection for quality may result in loss of environmental adaptation. Fragrance in rice is highly attractive to humans and adds significant value to rice. The sequencing of the rice genome allowed the identification of the genetic basis of this trait (Bradbury et al., 2005) due to the gene being flanked by closely linked known markers (Qingsheng et al., 2003). The gene responsible is an aldehyde dehydrogenase (Bradbury et al., 2008) the activity of which is lost in fragrant genotypes. The loss of the gene reduces the ability of the plant to cope with salt stress (Fitzgerald et al., 2010). Whole genome understanding of genes responsible for quality (Kharabian-Masouleh et al., 2012) will allow their relationship to abiotic stress tolerance genes to be carefully evaluated. Very attractive traits like fragrance may require strategies such as selection of compensating abiotic stress tolerance genes to counteract the deleterious effects of the quality gene.

DURABLE PEST AND DISEASE RESISTANCE IN A CHANGING CLIMATE

The breeding of crops to cope with new pests and diseases will be a key strategy to allow plants to cope with new climates. Genes from wild populations will continue to be a major option but this may need to be complemented by the use of novel transgenes or genetic modifications.

ROLE OF CONTINUING TECHNOLOGY ADVANCES

Technology advances will continue to be critical. Ultimately we need to be able to access whole genome information on all crop species and their wild relatives to be effective in rapid crop adaptation to climate. Ongoing developments in the chemistry of DNA sequencing and in information technology hardware and software will be required to allow these very large amounts of information to be captured and managed.

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β-catenin in plants and animals: common players but different pathways

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INTRODUCTION

A key node in number of essential cellular processes in eukaryotes, Armadillo was originally characterized in Drosophila as the component of Wingless/Wnt signal transduction pathway (Nusslein-Volhard and Wieschaus, 1980). β-catenin is the mammalian homolog of Armadillo playing dual role in structural and transcriptional regulation during embryonic development (Conacci-Sorrell et al., 2002). Even though initially characterized in animals, members of the Armadillo proteins are also known to exist in non-animals including slime mold (*Dictyostelium discoideum*) and plants (Wang et al., 1998; Barelle et al., 2006; Veses et al., 2009). The existence of Armadillo repeat family of proteins across species suggests ancient evolutionary origin and functional conservation of these proteins in multicellular organisms (Coates, 2003). The intricate role of β-catenin raises several doubts about the mechanism by which it mediates interaction with diverse partner proteins using common interface, and how this interaction influences adhesion and transcription?

The ARM family proteins have been identified with multiple functional domains in more than one species. Genome-wide studies in plants have shown the existence of large number of Armadillo homologs in *Physcomitrella patens*, Arabidopsis and *Oryza sativa* (Mudgil et al., 2004; Sharma et al., 2014). One assumption is that, Armadillo family being evolutionary conserved, perform similar role in all organisms. However, the existence of multigene Armadillo family with various subfamilies indicate novel species specific functions of these proteins in plants. Several recent studies have made known the function of numerous ARM proteins in Arabidopsis and rice. Apart from their analogous role in regulation of gene expression and developmental processes, various proteins were discovered to be predominantly involved in plant stress responses.

Thus, an intriguing and important question remains as in what way the similar effector proteins of Wnt pathway function and how similar canonical response is prevented or exist in plants. Recent progress in studies of ARM proteins in plants has suggested some possible answers to this question. However, the Wnt signaling mechanism regulated by ARM repeat proteins is still unknown. Regarding this, many underscoring questions are just beginning to emerge that remains to be answered.

Wnt SIGNALING—DEVELOPMENTAL REGULATION IN PLANTS AND ANIMALS

Wnt proteins are one of the foremost signaling molecule essential for cell polarity, embryonic development and the determination of cell fate in metazoa (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Logan and Nusse, 2004). A combination of molecular and genetic studies has provided evidences for how Wnt1, Wnt3a, and Wnt8 specifically induce the activation of "canonical β-catenin" pathway in animals (Du et al., 1995; Shimizu et al., 1997; Kuhl et al., 2000). However, no evidence for a Wnt, Frizzled (Fz) and low-density-lipoprotein-related protein receptors has been obtained in plants. Despite this, few homologs of proteins, which act as negative regulator of Wnt signaling has been unveiled in plants. Based on BLAST searches, the serine/threonine kinase GSK-3 (glycogen synthase kinase-3), CK1 (casein kinase 1) and APC (Adenomatous polyposis coli), which together form a destruction complex to stimulate degradation of β-catenin in animals were found to be conserved in plants (**Figure 1**) (Li et al., 2001). This has been proven in animals that activity of GSK3/CK1 complex is inhibited in response to Wnt signal perception at the cell surface to relieve its inhibitory effects on downstream β-catenin (He et al., 2004; Tamai et al., 2004; Nusse, 2005). The conservation of β-catenin destruction complex in plants points toward novel targets and modulation of Wnt signaling.

POTENTIAL "Wnt-LIKE" SIGNALING FUNCTIONS FOR PLANT ARM FAMILY PROTEINS

Arabidopsis comprises a multigene SHAGGY-related protein kinase (ASK) gene family, which is 70% identical to glycogen synthase kinase-3 from mammals, (Bourouis et al., 1990; Siegfried et al., 1990; Woodgett, 1990) classified into four distinct subfamilies (Jonak and Hirt, 2002). In the past few years, significant progress has been made in understanding how GSK3s perform their diverse functions in plants. The diverged biological functions of these members in signal transduction, cell patterning, cytokinesis and determination of cell fate has been established and credited to their diversity within plants (Dornelas et al., 1998).

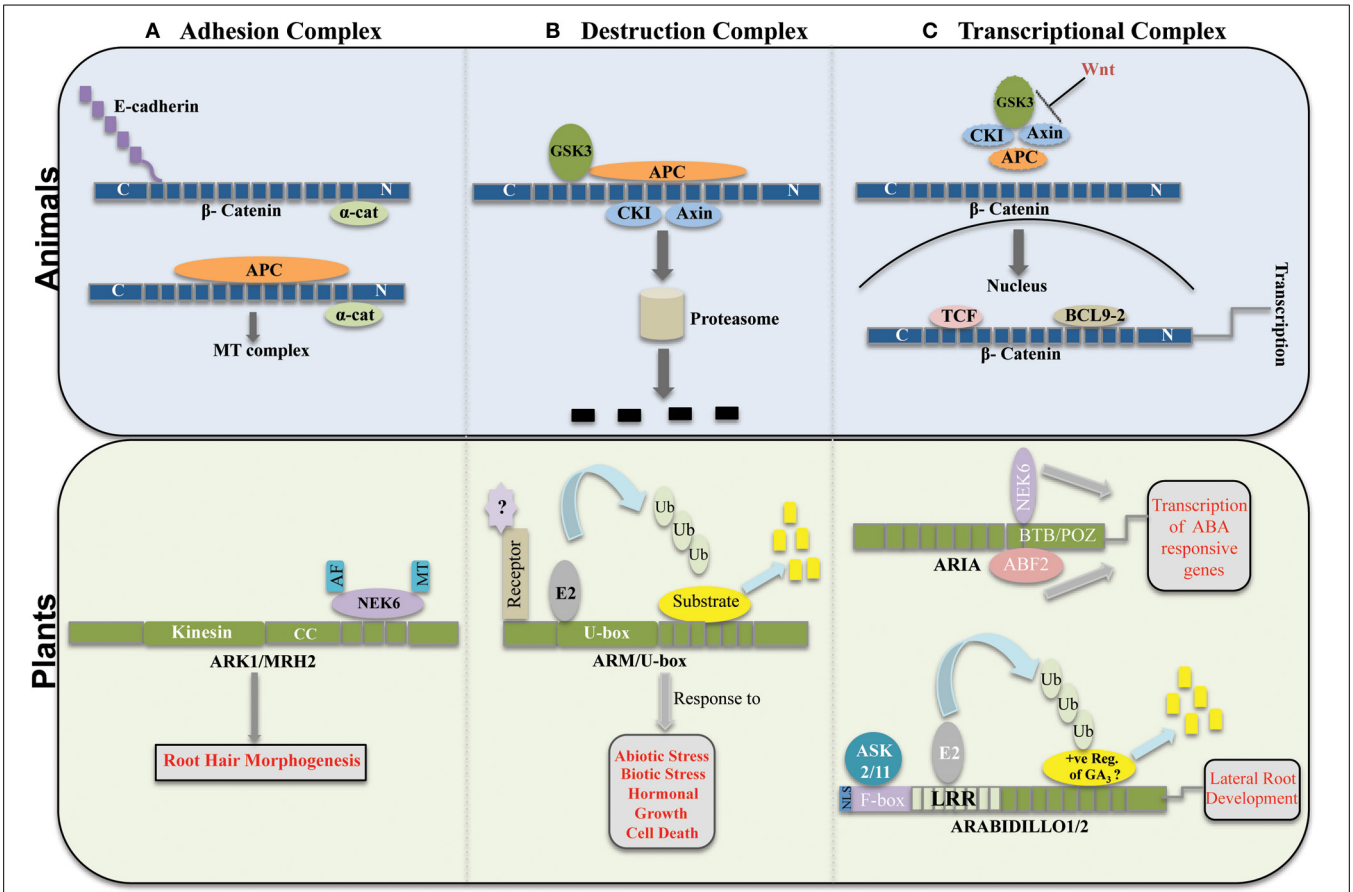

FIGURE 1 | Functional comparison of β-cat like-ARM repeats protein in plants and animals. (A) Adhesion Complex: β-catenin in animals binds cytoplasmic tail of cadherin to link it with α-catenin. Additionally, β-catenin together with APC interacts with microtubule complexes. In plants, ARK1/MRH2 (ARM repeat kinesin1/morphogenesis of root hair 1) interacts with NEK6 (NIMA-related protein kinase 6) to mediate root epidermal cell morphogenesis. CC represent coiled coil domain. **(B)** Destruction Complex: β-catenin is targeted for proteasomal degradation by a GSK3, APC, CKI, and Axin complex in the cytoplasm. Similarly in plants, ARM/U-box proteins, in response to various stimuli target substrate protein for proteasomal degradation. **(C)** Transcriptional Complex: Wnt signals inhibits the destruction complex, free β-catenin enters the nucleus where it links with the transcriptional regulators to activate transcription of target genes. In plants, ARIA an ARM protein with BTB/POZ domain binds with ABF2 and NEK6 transcription factors to stimulate transcription of ABA responsive genes. Additionally, ARABIDILLO1/2 interacts with ASK2/11 through their F-box domain to mediate degradation of possibly a positive regulator of GA3 signaling to promote transcription of genes related to lateral root development.

Most of the plants GSKs are found to be involved in brassinosteroid signaling and salt stress response (Dornelas et al., 2000; Kim et al., 2009). Brassinosteroids (BRs) are plant hormones, which signal through a plasma membrane localized receptor kinase BRI1. BRI1 interacts with BAK1 (BRI1 associated receptor kinase 1) to mediate plant steroid signaling (Nam and Li, 2002). BES1 has been identified as a suppressor of BRI1, which in turn is negatively regulated by a kinase BIN2 (Yin et al., 2002). Interestingly, the BR signaling pathway mechanism is analogous to the Wnt signaling pathway. In the proposed model, BIN2 which shares sequence homology with GSK-3 (Li and Nam, 2002), phosphorylate and destabilize its substrate BES-1. In response to brassinosteroids, BES-1 is stabilized and accumulates in the nucleus to activate target gene expression (Yin et al., 2002).

It is important to note that both BES-1 and β-catenin does not share homology at the protein sequence level. Similarly, BRI1 and Wnt are the two different receptors and does not belong to the same family (He et al., 2002; Yin et al., 2002; Zhao et al., 2002). However, it will be interesting to know if any of the protein in multigene Armadillo family in plants, gets regulated in the same manner or it is simply the way in which the pathway is conserved.

Meanwhile, several lines of evidence suggest the role of Wnt signaling proteins i.e., Armadillo repeats containing proteins in the developmental regulation in both animals and plants (Amador et al., 2001). p120ctn is an Armadillo repeat protein identified as a component of E-cadherin-catenin cell adhesion complex (Daniel et al., 2002). The signaling and cell adhesion co-factor p120ctn is the only known binding partner for Kaiso, a novel BTB/POZ domain zinc finger transcription factor (Daniel et al., 2002). Another possible candidate mediating interaction within actin and microtubule filaments in plants is ARK/MRH2 kinesin (ARM repeat kinesin/Morphogenesis of

root hair). ARK/MRH2 interacts with NIMA-related protein kinase NEK6, to regulate epidermal cell morphogenesis by modulating microtubule dynamics (Sakai et al., 2008).

In relation to this, Arabidopsis (AT5G13060) and rice (LOC_Os05G33050) also possess homologous proteins comprising ARM repeats and a BTB/POZ domain (**Figure 1**). The Arabidopsis BTB/POZ ARM protein also known as ABAP1 has been shown to be involved in DNA replication and gene transcription controls (Masuda et al., 2008).

Arabidillo-1/-2 and *Oryzadillo* are the closest homolog of β-catenin in Arabidopsis and *Oryza sativa* respectively, consisting of an F-box motif near their N-terminal, and several presumed sites for GSK-3 phosphorylation (Gagne et al., 2002; Kuroda et al., 2002; Coates, 2003). Remarkably, Arabidillo's are closest to the β-catenin homolog in Dictyostelium' Aar protein that consists of an F-box domain and is required for the differentiation and expression of prespore specific genes (Grimson et al., 2000). Besides, analogous to animals, physical interaction of Arabidillo-1/-2 proteins through their F-box domain with ASKs (SHAGGY-like protein kinase) lead to the formation of SCF complexes that target various substrates for ubiquitn/26S proteasome–mediated proteolysis has been proven in plants (Changjun et al., 2010). This suggest an evolutionary conservation of signal transduction pathway elements and their site of action within animals and plants.

BEYOND Wnt SIGNALING: ROLE OF PLANT ARM PROTEINS

Exposure to abiotic and biotic stress results in alteration of cellular homeostasis in plants. The first response to stress factors, is to activate the signal transduction pathways that stimulate cell defense and adaptive mechanisms. Ubiquitination is a unique protein degradation mechanism utilized by plants to effectively degrade detrimental cellular proteins and components specific to these stress signalings. A majority of U-box E3 ubiquitin ligase encoding ARM proteins related to biotic and abiotic stress have been identified in plants. We can certainly anticipate new insight into the molecular mechanism of plant β-catenin-like proteins function in the context of abiotic stress signals.

There are 41 and 47 predicted U-box/ARM proteins in the genome of Arabidopsis and rice respectively (Mudgil et al., 2004; Sharma et al., 2014). A few of them have been functionally characterized in Arabidopsis. Many of these proteins have now been linked to specific stress and hormonal responses.

A biological role for the U-box/ARM protein *AtPUB9* has been proposed in ABA (Abscisic acid) signaling (Samuel et al., 2008). In Arabidopsis, ATPUB18 and ATPUB19 are the two homologous proteins. Molecular analysis of *AtPUB19* showed that it is upregulated in response to drought, salt, cold and ABA (Liu et al., 2011). In the consecutive year, role of *ATPUB18* as a negative regulator has been put forward in ABA-mediated stomatal closure and drought responses (Seo et al., 2012). A different homologous pair of PUB proteins, AtPUB22 and 23 have been shown to play a combinatory role in the negative regulation of drought stress (Cho et al., 2008; Seo et al., 2012). A closely related ortholog of *ATPUB22/23* in *Capsicum annum* known as *CaPUB1* was found to be highly inducible in response to various abiotic stresses such as drought, cold and salt (Cho et al., 2006).

Another report suggested the role of AtCHIP, an Arabidopsis U-box/ARM protein in response to extreme temperature conditions. Subsequently, AtCHIP was reported to be involved in the ABA stress signaling pathway by mediating interaction with protein phosphatase 2A (Yan et al., 2003). In rice, SPL11 was identified as a U-box containg ARM protein that functions as a negative regulator in the control of cell death and pathogen defense (Zeng et al., 2004). The Arabidopsis ortholog of SPL11, ATPUB13 is a functionally conserved protein regulating plant defense, cell death and flowering time (Li et al., 2012a,b). In *Nicotiana*, two U-box/ARM proteins NtCMPG1 and tobacco ACRE276 and their functional homolog in Arabidopsis, AtPUB17 has been implicated as positive mediators of plant defense and stress signaling (Gonzalez-Lamothe et al., 2006; Yang et al., 2006). Apart from this, expression analysis in rice has confirmed many of the ARM proteins without any associated domain to be differentially regulated under abiotic stress conditions suggesting a role of ARM repeats in the stress regulation (Sharma et al., 2014).

On the basis of facts described above, it can be concluded that animal and plant ARM repeat proteins share many resemblances. Therefore, it is possible that at least some transcription effectors involved in Wnt signaling are evolutionary conserved. These elements include nuclear accumulation in response to extracellular signal, phosphorylation and degradation. Apart from the common response, plants possess specific signaling pathways mediated by ARM proteins. In plants, ubiquitination is critically involved in the function of ARM proteins. The proliferation of β-catenin-like ARM proteins in plants suggest their significance in the regulation of diverse biological fuctions in them. Further study of these proteins in plants would contribute to our understanding of the molecular factors involved in response to abiotic stress.

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Tolerance to drought and salt stress in plants: unraveling the signaling networks

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Tolerance of plants to abiotic stressors such as drought and salinity is triggered by complex multicomponent signaling pathways to restore cellular homeostasis and promote survival. Major plant transcription factor families such as bZIP, NAC, AP2/ERF, and MYB orchestrate regulatory networks underlying abiotic stress tolerance. Sucrose non-fermenting 1-related protein kinase 2 and mitogen-activated protein kinase pathways contribute to initiation of stress adaptive downstream responses and promote plant growth and development. As a convergent point of multiple abiotic cues, cellular effects of environmental stresses are not only imbalances of ionic and osmotic homeostasis but also impaired photosynthesis, cellular energy depletion, and redox imbalances. Recent evidence of regulatory systems that link sensing and signaling of environmental conditions and the intracellular redox status have shed light on interfaces of stress and energy signaling. ROS (reactive oxygen species) cause severe cellular damage by peroxidation and de-esterification of membrane-lipids, however, current models also define a pivotal signaling function of ROS in triggering tolerance against stress. Recent research advances suggest and support a regulatory role of ROS in the cross talks of stress triggered hormonal signaling such as the abscisic acid pathway and endogenously induced redox and metabolite signals. Here, we discuss and review the versatile molecular convergence in the abiotic stress responsive signaling networks in the context of ROS and lipid-derived signals and the specific role of stomatal signaling.

Keywords: transcription factor, *Arabidopsis*, lipid signaling, ROS, drought, MAP kinase

INTRODUCTION

Survival of plants under adverse environmental conditions relies on integration of stress adaptive metabolic and structural changes into endogenous developmental programs. Abiotic environmental factors such as drought and salinity are significant plant stressors with major impact on plant development and productivity thus causing serious agricultural yield losses (Flowers, 2004; Godfray et al., 2010; Tester and Langridge, 2010; Agarwal et al., 2013). The complex regulatory processes of plant drought and salt adaptation involve control of water flux and cellular osmotic adjustment via biosynthesis of osmoprotectants (Hasegawa et al., 2000; Flowers, 2004; Munns, 2005; Ashraf and Akram, 2009; Agarwal et al., 2013). Salinity induced imbalance of cellular ion homeostasis is coped with regulated ion influx and efflux at the plasma membrane and vacuolar ion sequestration (Hasegawa et al., 2000). Significantly, drought and salinity have additionally major detrimental impacts on the cellular energy supply and redox homeostasis that are balanced by global re-programming of plant primary metabolism and altered cellular architecture (Chen et al., 2005; Baena-González et al., 2007; Jaspers and Kangasjärvi, 2010; Miller et al., 2010; Zhu et al., 2010). In this review we focus on recent advances in understanding cellular signaling networks of biotechnological relevance in plant drought and salt adaptation. Here, we focus on induced rather than intrinsic tolerance mechanisms and do not explicitly distinguish between stress survival and tolerance. Known research findings on hormonal signal perception and transduction were integrated in the context of plant signaling networks under drought and salinity. We particularly aimed on reviewing links of drought and salt induced signal transduction to plant hormonal pathways, metabolism, energy supply and developmental processes.

PLANT HORMONES: PIVOTAL ROLES IN PLANT STRESS SIGNALING

Plant hormones function as central integrators that link and reprogram the complex developmental and stress adaptive signaling cascades. The phytohormone abscisic acid (ABA) functions as a key regulator in the activation of plant cellular adaptation to drought and salinity and has a pivotal function as a growth inhibitor (Cutler et al., 2010; Raghavendra et al., 2010; Weiner et al., 2010). Additionally, the view of function of ABA as a linking hub of environmental adaptation and primary metabolism is increasingly emerging. Intriguingly, ABA triggers both transcriptional reprogramming of cellular mechanisms of abiotic stress adaptation and transcriptional changes in carbohydrate and lipid metabolism indicating function of ABA at the interface of plant stress response and cellular primary metabolism (Seki et al., 2002; Li et al., 2006; Hey et al., 2010).

Abscisic acid signals are perceived by different cellular receptors and a concept of activation of specific cellular ABA responses by perception in the distinct cellular compartments is currently emerging. The nucleocytoplasmic receptors PYR/PYL/RCARs

(PYRABACTIN RESISTANCE/ PYRABACTIN RESISTANCE-LIKE/REGULATORY COMPONENT OF ABA RECEPTORS) bind ABA and inhibit type 2C protein phosphatases (PP2Cs) such as ABI1 and ABI2 (Ma et al., 2009; Park et al., 2009). Inactivation of PP2Cs activates accumulation of active SNF1-RELATED PROTEIN KINASES (SnRK2s; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). The SnRK2s regulate ABA-responsive transcription factors including AREB/ABFs [ABA-RESPONSIVE PROMOTER ELEMENTS (ABREs) BINDING FACTORS (ABFs)] and activate ABA-responsive genes and ABA-responsive physiological processes (Umezawa et al., 2009; Vlad et al., 2009). Recently, function of plasma membrane-localized G protein-coupled receptor-type G proteins (GTGs) as ABA receptor in *Arabidopsis* has been shown (Pandey et al., 2009). Binding of ABA by GTG1/GTG2 and ABA hyposensitivity of GTG1/GTG2 *Arabidopsis* loss of function mutants supported a function of GTG1 and GTG2 as membrane-localized ABA receptors (Pandey et al., 2009). Extending the concept of involvement of GTG1 and GTG2 in ABA signaling, a role of the proteins in growth and development of *Arabidopsis* seedlings and in pollen tube growth by function as voltage-dependent anion channels has been reported (Jaffé et al., 2012). Thus, linking and dynamic integration of GTG1 and GTG2 in cellular ABA signaling and developmental regulation seems likely. Intriguingly, evidence for a third pathway of ABA perception has been emerging with the H subunit of Mg-chelatase (CHLH/ABAR). Integration of CHLH/ABAR in the cellular ABA signaling cascade as a chloroplastic ABA receptor and by plastid-to-nucleus retrograde signaling via the ABA responsive nucleocytoplasmic transcription repressor WRKY40 has been reported (Shen et al., 2006; Shang et al., 2010; Du et al., 2012). These findings strongly suggest contribution of a chloroplast-localized pathway to modulate cellular ABA signaling (Shen et al., 2006; Shang et al., 2010; Du et al., 2012).

Currently, increasing evidence has been emerging for modulation of ABA-mediated environmental signaling by interaction and competition with hormonal key regulators of plant cellular developmental and metabolic signaling. The complex and divergent endogenous and exogenous signals perceived by plant cells during development and environmental adversity are linked and integrated by distinct and interactive hormonal pathways. Particularly, convergence and functional modulation of ABA signaling by the plant growth regulating phytohormones gibberellic acid (GA) has a key regulatory function in the plant cellular network of stress and developmental signaling (Golldack et al., 2013). According to accepted concepts, in *Arabidopsis* GA signaling is mediated by binding of GA to GID1a/b/c that are GA receptor orthologs of the rice GA receptor gene *OsGID1* (*GA INSENSITIVE DWARF 1*; Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006; Feng et al., 2008). GA responsive GRAS [for GA Insensitive (GAI), REPRESSOR of *ga1-3* (RGA), SCARECROW (SCR)] transcription factors function as major regulators in plant GA-controlled development. Cellular accumulation of the GRAS protein subgroup of DELLA proteins (GAI, RGA, RGL1, RGL2, RGL3) represses GA signaling and restrains growth and development (Cheng et al., 2004; Tyler et al., 2004; Yu et al., 2004). Interaction of DELLA proteins with the GA receptor GID1 induces degradation of the DELLA proteins and activates the function of GA (Cheng et al., 2004; Tyler et al., 2004; Yu et al., 2004). GA signals mediate binding of DELLA proteins to GID1 that is followed by conformational conversion of DELLA proteins. The modified DELLAs are recognized by the the F-box protein SLEEPY1 (SLY1) in *Arabidopsis* (Silverstone et al., 2001, 2007; Fu et al., 2002; Sasaki et al., 2003; Dill et al., 2004). Subsequently, DELLAs are polyubiquitinated by the SCFSLY1/GID2 ubiquitin E3 ligase complex and degraded via the 26S proteasome pathway (Silverstone et al., 2001; Fu et al., 2002; Sasaki et al., 2003; Dill et al., 2004).

A linking function of DELLA proteins at the interface of ABA-mediated abiotic stress responses and GA-controlled developmental signaling has been supported by modified salt tolerance of the quadruple DELLA mutant with functional losses of *rga, gai, rgl1*, and *rgl2* (Achard et al., 2006). Interestingly, the RING-H2 zinc finger factor *XERICO* regulates tolerance to drought and ABA biosynthesis in *Arabidopsis* (Ko et al., 2006). In addition, XERICO is a transcriptional downstream target of DELLA proteins indicating function of XERICO as a node of plant abiotic stress responses and development by linking GA and ABA signaling pathways (Ko et al., 2006; Zentella et al., 2007; Ariizumi et al., 2013).

Recently, interesting evidence has been also provided for a convergence and crosstalk of GA and ABA signaling with the developmental regulator jasmonate in plant responses to drought. Jasmonates are membrane-lipid derived metabolites that originate from linolenic acid and have signaling functions in plant growth and biotic stress responses (e.g., Wasternack, 2007; Wasternack and Hause, 2013). Drought-induced transcriptional regulation of the rice JA receptor protein *OsCOI1a* (CORONATINE INSENSITIVE 1) and of key regulators of JA signaling *OsJAZ* (jasmonic acid ZIM-domain proteins) indicate significant integration of JA metabolism and signaling in plant abiotic stress responses (Du et al., 2013a; Lee et al., 2013). Importantly, expression of the DELLA protein RGL3 responds to JA, and additionally RGL3 interacts with JAZ proteins (Wild et al., 2012). These recent research advances emphasize function of DELLAs as an interface of ABA, GA and jasmonic acid signaling and suggest pivotal functional involvement of lipid-derived signaling in abiotic stress responses (**Figure 1**).

MAJOR PLANT TRANSCRIPTION FACTOR FAMILIES: KEY PLAYERS IN THE REGULATORY NETWORKS UNDERLYING PLANT RESPONSES TO ABIOTIC STRESS

Comprehensive research on diverse abiotic stress responsive transcription factors shed light on the cellular mechanisms defining plant environmental adaptation (Golldack et al., 2011). Significantly, the majority of ABA-regulated genes share the conserved ABA-responsive *cis* element (ABRE; Yamaguchi-Shinozaki and Shinozaki, 2005, 2006). Besides the AREB/ABF (ABA-responsive element binding protein/ABRE-binding factor) family, the DREB/CBF subfamily of the AP2/ERF transcription factors has a central function in regulating plant adaptation to adversity via ABA dependent and independent pathways (Yamaguchi-Shinozaki and Shinozaki, 2005, 2006). Significant evidence for a linking function of DREB/CBF in integrating environmentally derived signals and plant development was early provided

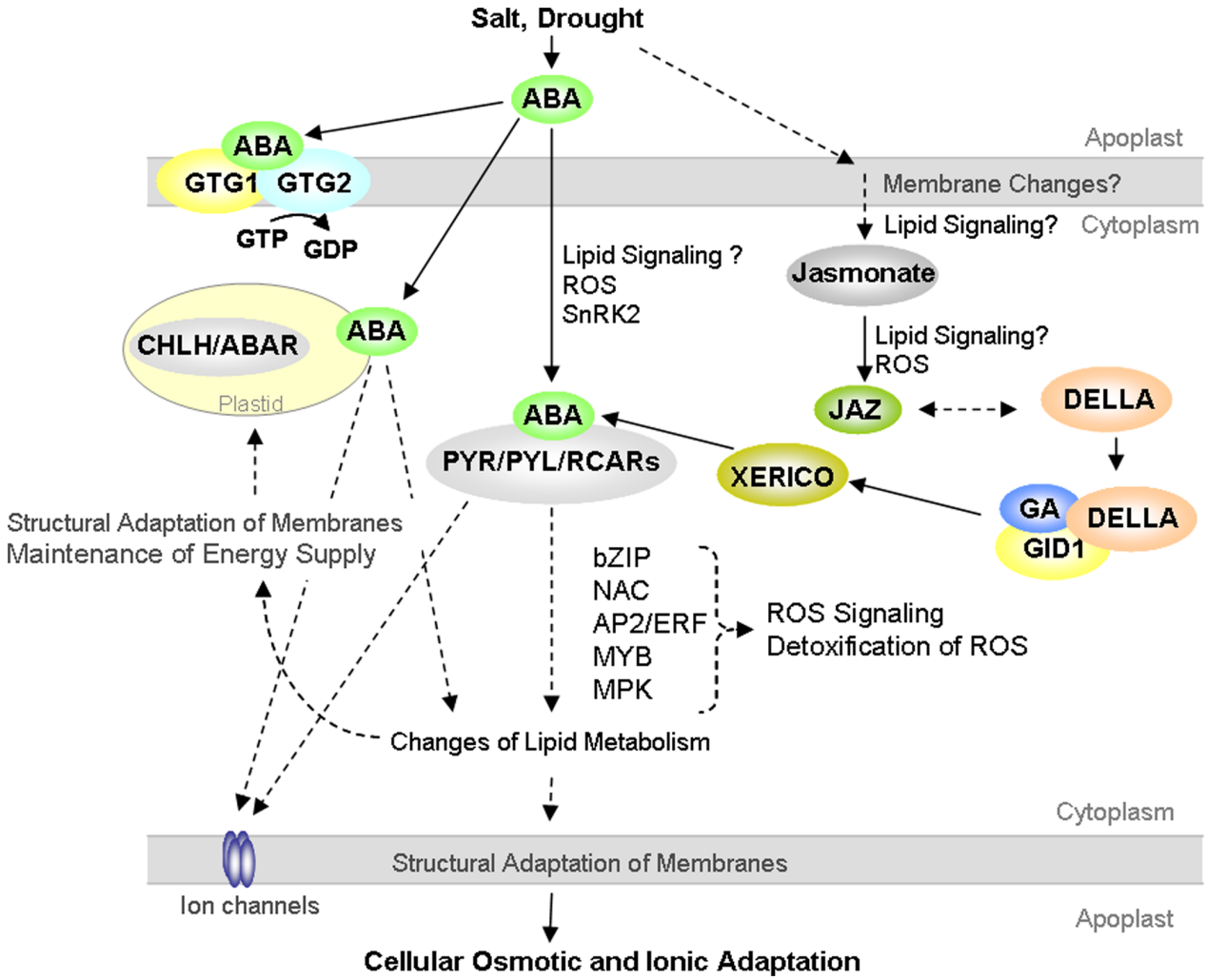

FIGURE 1 | Proposed model on crosstalk of abscisic acid (ABA), gibberellic acid (GA), and jasmonate signaling in plant cellular responses to the abiotic stressors drought and salt. Hypothesized links are illustrated with dashed lines. The lines and arrows illustrate pathways that are not shown and described in detail. Compare text for details.

by DREB/CBF overexpressing *Arabidopsis* with increased tolerance to drought, salt, and cold that was counterbalanced by serious developmental defects (Kasuga et al., 1999). Supporting this functional connection, cold responsive CBF1 regulated GA biosynthesis and accumulation of the DELLA protein RGA thus suggesting integration of AP2/ERF in abiotic stress signaling and GA-regulated plant development (Achard et al., 2008). The bZIP-type AREB/ABF transcription factors AREB1, AREB2, and AREB3 target cooperatively ABRE-dependent gene expression via a suggested interaction with the sucrose non-fermenting 1-related protein kinase 2 (SnRK2) protein kinase SRK2D/SnRK2.2 (Yoshida et al., 2010). In addition, the *Arabidopsis* transcription factor *bZIP24* controls reprogramming of a broad array of salinity dependent and developmental gene expression indicating a pivotal role of the factor in maintaining plant development under conditions of adversity (Yang et al., 2009).

The view of an integrative function of many transcription factors in linking and balancing related or seemingly unrelated cellular responses is further supported by other drought and salt responsive transcription factors. Intriguingly, the picture is increasingly emerging that plant signaling does not function as independent and paralleled pathways but cellular crosstalks and hubs within the signaling network exist. The view is increasingly emerging that stress adaptive signaling is tightly linked to the cellular primary metabolism, energy supply and developmental processes. Thus, the tomato *NAC*-type (*NAM, ATAF1,2, CUC2*) transcription factor *SlNAC1* was responsive to multiple abiotic and biotic stresses (Ma et al., 2013). Regulation of the factor by ABA, methyl jasmonate, gibberellin, and ethylene indicates a node role of the factor in diverse signal transduction pathways in tomato (Ma et al., 2013). The ABA-responsive NAC-transcription factor *VNI2* (*VND-INTERACTING1)* is a repressor of xylem vessel formation and has additional functions in leaf

aging thus integrating plant senescence to ABA signaling (Yang et al., 2011). As another example, the *NAC* transcription factor *ANAC042* (*JUB1, JUNGBRUNNEN 1*) links leaf senescence to hyperosmotic salinity response and is involved in H_2O_2 signaling (Wu et al., 2012). Over-expression of the drought and ABA responsive rice NAC-type transcription factor *OsNAC10* allowed identification of NAC dependent target genes that included AP2 and WRKY-type transcription factors (Jeong et al., 2010). These findings strongly indicate a hub role of NAC transcription factors in stress relevant hierarchic regulatory pathways.

Drought and ABA-responsive NAC factors are likely to control and link subclusters of cellular stress adaptation processes under control of diverse subsets of specific transcription factors such as members of the AP2 and WRKY families. Thus, hypersensitivity to drought of an *Arabidopsis WRKY63* loss of function mutant was related to reduced ABA sensitivity in guard cells indicating specific control of abiotic stress adaptation by this WRKY transcription factor (Ren et al., 2010). ABA and salt responsive *Arabidopsis WRKY33* downstream targets genes with functions in detoxification of reactive oxygen species (ROS) such as glutathione *S*-transferase *GSTU11*, peroxidases, and lipoxygenase *LOX1* (Jiang and Deyholos, 2009). According to the involvement of WRKY33 in osmotic stress responses, ROS detoxification and ROS scavenging, a role of WRKY controlled cellular ROS levels in abiotic stress signaling seems likely. Extending and supplementing this concept, the WRKY-type transcription factor *ThWRKY4* from *Tamarix hispida* controls cellular accumulation of ROS via regulating expression and activity of antioxidant genes such as superoxide dismutase and peroxidase (Zheng et al., 2013). Modified tolerance of *ThWRKY4* overexpressing plants to salt and oxidative stress was referred to *ThWRKY4*-mediated cellular protection against toxic ROS levels (Zheng et al., 2013). Accordingly, an involvement of WRKY in linking osmotic and oxidative stress defense as well as in ROS mediated signaling crosstalks is suggested.

Another crucial and undervalued mechanism of plant adaptation to drought and salinity is the maintenance of cell wall development and generation of the extracellular matrix in terms of plant development and of protection against water loss. Intriguingly, transcriptional expression of the *Arabidopsis* R2R3-MYB transcription factor *AtMYB41* was induced by drought, salt, and ABA (Cominelli et al., 2008; Lippold et al., 2009). Modified drought sensitivity of *AtMYB41* overexpressing *Arabidopsis* was linked to lipid metabolism, cell wall expansion, and cuticle deposition demonstrating a key function of *AtMYB41* in plant drought protection and survival via primary lipid metabolism and cuticle formation (Cominelli et al., 2008). Recently, function of *AtMYB41* was also linked to primary carbon metabolism indicating a relationship between cuticle deposition, plant tolerance against desiccation as well as cellular lipid and carbon metabolism (Cominelli et al., 2008; Lippold et al., 2009). The salt-responsive rice R2R3-type MYB transcription factor *OsMPS* (*MULTIPASS*) targets genes with function in biosynthesis of phytohormones and of the cell-wall (Schmidt et al., 2013a). These recent research advances highlight the importance of a functional plant extracellular matrix and of cuticular polymer biosynthesis for plant salt and drought adaptation. Accordingly, a key function of stress responsive transcription factors in integrating cuticle formation in the cellular primary metabolism in response to environmental adversity is supported and likely.

LIPIDS: STILL AN ENIGMA IN ABIOTIC STRESS ADAPTATION AND STRESS DERIVED SIGNALING?

Plant adaptation to a changing water and ionic status in the surrounding environment requires rapid and sensitive sensing of the stress situation and stress induced signaling. A crucial and existential challenge for plant cells is the maintenance of integrity of cellular membranes both at the plasma membrane and of the enodomembranes. Thus, plants ensure homeostasis of metabolism and cellular energy supply. Additionally, increasing evidence for pivotal involvement of lipid-derived signaling in primary sensing of environmental changes and in triggering and regulating cellular hormonal signaling cascades has been emerging (**Figure 1**). Interestingly, vice versa ABA transcriptionally downstream targets lipid metabolism and lipid transfer proteins suggesting tight interaction of ABA-dependent signaling and lipid metabolic pathways to maintain structure and function of cellular membranes (Seki et al., 2002; Li et al., 2006). Thus, ABA-triggered modification of primary lipid metabolism contributes unequivocally to stress adaptive reorganization of membranes and to the maintenance of cellular energy supply under abiotic stress conditions and limitation in water supply. Increased transpirational water loss of *Arabidopsis* mutants with a functional knock out of *LTP3* (*Lipid Transfer Protein 3*) suggests lipid-based adaptive changes of membranes and the plant cuticle to regulate water loss and transpiration under drought (Guo et al., 2013).

Drought-induced changes of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) contents in the chloroplast envelope and in thylakoid membranes in cowpea (*Vigna unguiculata*) have been suggested to stabilize and maintain lamellar bilayer structure and thus the function of chloroplasts under drought stress (Torres-Franklin et al., 2007). In support of these findings, changes of MGDG in the drought tolerant resurrection plant *Craterostigma plantagineum* during desiccation are likely to contribute to membrane stabilization and to the maintenance of photosynthetic energy supply (Gasulla et al., 2013). The *Arabidopsis* cold-responsive *SFR2* (*SENSITIVE TO FREEZING 2*) mediates removal of monogalactolipids from the chloroplast envelope membrane and stabilizes membranes during freezing indicating that structural re-shaping of chloroplast membranes is an essential and general mechanism of plant cellular dehydration responses (Moellering et al., 2010).

Next to strong evidences for a fundamental importance of lipid mediated re-organization of cellular membranes to cope with changes in the plant water status, also comprehensive evidence for functions of lipid signaling in plant drought and salt responses has been emerging. In rice, levels of PIP2 (phosphatidylinositol bisphosphate), PA (phosphatidic acid), and DGPP (diacylglycerolpyrophosphate) increased upon salt stress (Darwish et al., 2009). Based on these findings involvement of phospholipase C and diacylglycerol kinase in salt stress induced signaling has been hypothesized (Darwish et al., 2009). Function of phospholipase C was linked to ABA signaling and stomatal regulation indicating a functional role of phosphoinositides in

guard cell signaling (Hunt et al., 2003; Mills et al., 2004). The inositol phosphate myo-inositol hexakisphosphate (InsP6) has a role as an ABA-responsive signaling molecule that regulates stomatal closure via cellular calcium and the plasma membrane potassium conductance (Lemtiri-Chlieh et al., 2003). Phosphoinositides have key roles in regulating membrane peripheral signaling proteins and influence the activity of integral proteins and ion channels (Suh et al., 2006; Falkenburger et al., 2010). Importantly, work on inhibitors of phosphoinositide-dependent phospholipases C (PI-PLCs) in *Arabidopsis* has provided considerable insight in the drought stress related lipid signaling by identifying links of phosphoinositides to the DREB2 pathway (Djafi et al., 2013).

A role of lipid-derived messengers in ABA signaling was also evident by ACBP1 (acyl-CoA-binding protein 1) regulated expression of PHOSPHOLIPASE Dα1 (PLDα1; Du et al., 2013b). PHOSPHOLIPASE Dα1 has a function in the biosynthesis of the ABA regulating lipid messenger PA indicating that modulation of cellular lipid profiles is essential for regulation of abiotic stress related ABA signaling (Du et al., 2013b; Jia et al., 2013; Lu et al., 2013).

SnRK2 AND MAPK: ANOTHER CHAPTER IN PLANT ABIOTIC STRESS SIGNALING

Protein kinases of diverse types and families are central integrators of plant abiotic stress signaling that link cellular metabolic signaling to stress adaptive physiological processes as regulation of ionic and osmotic homeostasis and to concerted changes of ROS in stressed plant cells (**Figure 1**). Accepted models emphasize hub functions of yeast sucrose non-fermenting 1 (SNF1) serine-threonine protein kinase, homologous mammalian AMP-activated protein kinase (AMPK) and plant SnRKs [Snf (sucrose non-fermenting)-1-related protein kinases] in the cellular carbon and energy metabolism (Halford and Hey, 2009). In plants, SnRK1 subgroup kinases have reported functions in metabolic signaling and development (Zhang et al., 2001; Halford et al., 2003). Considerable insight into protein kinase functions in plant abiotic stress adaptation has been provided by elucidation of the SOS pathway with central functions in maintenance and regulation of ion homeostasis under salt stress. Intriguingly, the SnRK3 SOS2-like (Salt Overly Sensitive3) protein kinases interact with SOS3-like calcium-binding proteins to activate the plasma membrane Na^+/H^+ antiporter SOS1 via the SOS pathway (Chinnusamy et al., 2004; Du et al., 2011). Recent research highlights direct interaction of SnRK2.8 and the ABA responsive NAC (NAM/ATAF1/2/CUC2) transcription factor NTL6 indicating integration of a SnRK2-type kinase in the ABA controlled cellular framework of abiotic stress adaptation (Kim et al., 2012). Extending these findings, in rice, the SnRK2 kinase *SAPK4* links regulation of ion homeostasis to scavenging of ROS thus suggesting interaction of ionic and oxidative stress signaling pathways in plant adaptation to adversity (Diédhiou et al., 2008). Consistent with these findings, a node function of SnRK2-type kinases in ABA signaling and ROS generation has been elucidated in stomatal guard cells. The ABA responsive SnRK2 *OST1* (*OPEN STOMATA 1*) regulates stomatal closure by modulating the cellular production of H_2O_2 via NADPH oxidases (Sirichandra et al., 2009; Vlad et al., 2009). *Arabidopsis OST1* mutants provided evidence for a role of *OST1* in the regulation of inward K^+ channels, Ca^{2+} -permeable channels and the slow anion channel *SLAC1* thus supporting a hub function of *OST1* in linking ABA, ion channels and NADPH oxidases in the regulation of stomatal apertures in guard cells (Sirichandra et al., 2009; Vlad et al., 2009; Acharya et al., 2013). As a fascinating finding, the *Arabidopsis snrk2.2/2.3/2.6* triple-mutant with decreased sensitivity to ABA allowed identification of SnRK2 phosphorylation targets that included proteins with functions in chloroplasts, in signal transduction and in the regulation of flowering (Wang et al., 2013). These research advances provide insights in SnRK2-mediated regulatory crosstalks and interactions of developmental, metabolic and stress adaptive processes in the plant cellular signaling framework.

Recent advances on mitogen-activated protein kinase (MAPK) mediated signal transduction cascades have provided another pivotal understanding of the integration of physiological and cellular responses to environmental adversity. MAPK cascades functionally link MAP3Ks (MAP2K kinase) serine/threonine kinases, MAP2K (MAPK kinase) dual-specificity kinases and MAPK serine/threonine kinases (Colcombet and Hirt, 2008). As an accepted concept of functional importance in abiotic stress adaptation, involvement of MAPKs in drought and salt adaptation have been reported for wide ranging plant species such as rice, *Arabidopsis* to alfalfa *SIMK* and *SIMKK* (Kiegerl et al., 2000; Ning et al., 2010; Yu et al., 2010). Recent research highlights a central role of *Arabidopsis* MKK4 in the osmotic stress response by regulation of MPK3 activity, accumulation of ROS and targeting the ABA biosynthetic process via *NCED3* (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3*; Kim et al., 2011). Several studies indicated a hub function of MPK6 as another member of the MAPK cascade in linking of osmotic stress responses to ROS and oxidative bursts. Thus, recent research has identified abiotic stress induced ROS accumulation under control of MPK6, MKK1, and MKKK20 supporting a dynamic control of the signaling component ROS by MPK6 and other components of the MAPK pathway (Xing et al., 2008; Kim et al., 2012).

Novel findings uncover links of the MAPK cascade to cellular lipid transfer processes indicating a coupling of MAP-type kinases to stress adaptive changes of membranes, intracellular membrane trafficking or probably to stress-dependent lipid signaling. Thus, recent research advances proved direct regulation of MPK6 mediated phosphorylation of the plasma membrane Na^+/H^+ antiporter *SOS1* by NaCl and by PA supporting relationships of lipids to MAPK signaling in plant salt stress responses (Yu et al., 2010). Integration of MPK6 in differential signaling pathways has been additionally reported by interaction of MPK6 with the *Arabidopsis* C2H2-type zinc finger protein *ZAT6* that functions both in plant developmental processes and in osmotic stress responses (Liu et al., 2013b). In several recent studies, emphasis has been placed on detailed characterization of co-regulation and interaction of the MAP kinase pathway and ROS signaling within the cellular signaling framework thus further strengthening the understanding of MAP kinase as a hub in signaling under environmental adversity. In rice, the salt responsive MAPK cascade is linked to ROS signaling by the transcription factor *SERF1* (*salt-responsive*

ERF1; Schmidt et al., 2013b). Cotton MAPK *GhMPK16* is functionally involved in pathogen resistance, drought tolerance and ROS accumulation indicating a role of *GhMPK16* as an interface between biotic and abiotic stress signaling (Shi et al., 2011).

ROS SIGNALING IN PLANTS UNDER DROUGHT AND SALT STRESS

Current concepts emphasize a central function of cellular ROS as a signaling interface in plant drought and salt adaptation hat links stress signals to regulation of metabolism and the cellular energy balance (**Figure 1**). Significantly, environmental adversity such as drought and salinity impairs cellular ionic and osmotic homeostasis but additionally compromises photosynthesis, cellular energy depletion, and redox imbalances (e.g., Baena-González et al., 2007; Abogadallah, 2010; Jaspers and Kangasjärvi, 2010; Miller et al., 2010; Zhu et al., 2010). Excess generation and accumulation of ROS such as superoxide, hydrogen peroxide and nitric oxide cause oxidative damages in the apoplastic compartment and damages of cellular membranes by lipid peroxidation and have an extensive impact on ion homeostasis by interfering ion fluxes (Baier et al., 2005). Excess ROS amounts are particularly scavenged by antioxidant metabolites such as ascorbate, glutathione, tocopherols and by ROS detoxifying enzymes as superoxide dismutase, ascorbate peroxidase, and catalase (Mittler, 2002; Neill et al., 2002). Current models emphasize a dual regulatory function of ROS as a signaling molecule in plant drought and osmotic stress tolerance by sensing the cellular redox state and in retrograde signaling. Studies on transcription factors of the WRKY and basic-helix-loop helix types enhanced the understanding of crosstalks of osmotic and oxidative stress responsive signaling pathways significantly. Thus, *Arabidopsis WRKY33* responds to osmotic and oxidative stresses (Miller et al., 2008). Regulatory function of *bHLH92* and *WRKY33* in ROS detoxification by targeting peroxidases and glutathione-*S*-transferases suggested a function of the transcription factors in linking ROS scavenging to osmotic and oxidative stress induced signaling (Miller et al., 2008; Jiang and Deyholos, 2009; Jiang et al., 2009). Recent research advances linked the regulation of *Arabidopsis* salt and osmotic stress tolerance to ROS-responsive *WRKY15* and mitochondrial retrograde signaling (Vanderauwera et al., 2012). Another recent advance in understanding the importance of ROS in plant salt responses was the discovery of a coupled function of plastid heme oxygenases and ROS production in salt acclimation (Xie et al., 2011). These findings strongly suggest involvement of the chloroplast to nucleus signaling pathway in plant salt adaptation (Xie et al., 2011). Additionally, work on cross-species expression of a SUMO conjugating enzyme has provided considerable insight into the links of ROS, ABA dependent signaling and the sumoylation pathway in plant salt and drought tolerance (Karan and Subudhi, 2012). Functional relation of the maize bZIP transcription factor *ABP9*, glutamate carboxypeptidase *AMP1*, and the ankyrin-repeat protein ITN1 to ABA signaling, ROS generation and ROS scavenging further support interaction and correlation of ABA and ROS related pathways as signaling nodes in plant adaptation to drought and salt (Sakamoto et al., 2008; Zhang et al., 2011; Shi et al., 2013).

THE SPECIFIC FUNCTION OF STOMATAL SIGNALING IN PLANT DROUGHT AND SALT TOLERANCE

Constant dynamic regulation of stomatal aperture is obligatory for successful adaptation of plants to abiotic stresses. Prevention of excess water loss via transpiration depends on reliable adjustment of stomatal closure to environmental adversity. Hence, elucidation of sensing and signaling in stomatal guard cells has been attracting particular attention to understand regulation of stomatal conductance under conditions of drought and salinity. As another example, in maize mutants of the E3 ubiquitin ligase *ZmRFP1*, enhanced drought tolerance and decreased ROS accumulation indicated linked regulation of stomatal closure and ROS scavenging (Liu et al., 2013a). The *Arabidopsis* plasma membrane receptor kinase, *GHR1* (*GUARD CELL HYDROGEN PEROXIDE-RESISTANT1*) linked ABA and H_2O_2 signaling in stomatal closure (Hua et al., 2012). In addition, *GHR1* regulated an S-type anion channel suggesting a node function of this receptor kinase in ion homeostasis, ABA and H_2O_2 mediated signaling pathways in guard cells (Hua et al., 2012).

As aforementioned, the SnRK2 protein kinase *OST1* (*SnRK2 OPEN STOMATA 1*) is a central regulator of stomatal aperture and links guard cell movement to the ABA signaling network (Sirichandra et al., 2009). *OST1* targets NADPH oxidases, inward K^+ channels, Ca^{2+} -permeable channels and the slow anion channel *SLAC1* in stomatal guard cells (Sirichandra et al., 2009; Vlad et al., 2009; Acharya et al., 2013). In addition, the SnRK2 protein kinase *OST1* also targets voltage-dependent quickly activating anion channels of the R-/QUAC-type in guard cells (Imes et al., 2013). These data suggest coordinated control of SLAC1-mediated transport of chloride and nitrate and QUAC1-mediated transport of malate in the same ABA signaling pathway (Imes et al., 2013). Recently, the finding of direct dephosphorylation of SLAC1 by the PP2C (protein phosphatase 2C) *ABI1* provided interesting evidence for a specific alternative regulatory mechanism of the anion channel SLAC1 (Brandt et al., 2012).

Recent research uncovered co-regulation of ABA-induced stomatal closure, guard cell H^+-ATPase and Mg-chelatase H subunit (*CHLH*; Tsuzuki et al., 2013). CHLH/ABAR is involved in the chlorophyll biosynthetic process and a function of CHLH/ABAR as a chloroplastic ABA receptor via plastid-to-nucleus retrograde ABA signaling has been suggested (Shen et al., 2006; Shang et al., 2010; Du et al., 2012). In *Arabidopsis*, functional mutation of CHLH affected phosphorylation of H^+-ATPase and blue light dependent stomatal regulation (Tsuzuki et al., 2013). These findings validate importance of *CHLH* in linking the ABA signaling network to the regulation of ionic homeostasis and blue light responses in guard cells and plant drought tolerance (Tsuzuki et al., 2013). Interestingly, ABA-dependent regulation of stomatal closure responds to mutation of the phosphate transporter *PHO1* and the vacuolar H^+-ATPase subunit A (Zimmerli et al., 2012; Zhang et al., 2013). Again, these results support interaction and co-regulation of ion homeostasis in guard cells via ion transport, ABA signaling, and regulation of stomatal aperture (Zimmerli et al., 2012; Zhang et al., 2013). Intriguingly, the transporter *ZIFL1* (*Induced Facilitator-Like 1*) mediates potassium fluxes and has a dual function in

regulating both cellular auxin transport and stomatal closure (Remy et al., 2013).

In conclusion, recent research advances have elucidated a molecular cellular signaling network for the understanding how plants control and regulate adaptation to the abiotic stresses drought and salinity. Essentially, molecular signaling components in plant adaptation to environmental adversity have been connected to hub transcription factors, MAPK pathways, ROS and lipid-derived pathways. Importantly, it is expected that further and perspective advances in the network modeling of cellular abiotic stress signaling will provide new and efficient strategies for improving environmental tolerance in crops.

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The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat

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Drought negatively impacts plant growth and the productivity of crops around the world. Understanding the molecular mechanisms in the drought response is important for improvement of drought tolerance using molecular techniques. In plants, abscisic acid (ABA) is accumulated under osmotic stress conditions caused by drought, and has a key role in stress responses and tolerance. Comprehensive molecular analyses have shown that ABA regulates the expression of many genes under osmotic stress conditions, and the ABA-responsive element (ABRE) is the major *cis*-element for ABA-responsive gene expression. Transcription factors (TFs) are master regulators of gene expression. ABRE-binding protein and ABRE-binding factor TFs control gene expression in an ABA-dependent manner. SNF1-related protein kinases 2, group A 2C-type protein phosphatases, and ABA receptors were shown to control the ABA signaling pathway. ABA-independent signaling pathways such as dehydration-responsive element-binding protein TFs and NAC TFs are also involved in stress responses including drought, heat, and cold. Recent studies have suggested that there are interactions between the major ABA signaling pathway and other signaling factors in stress responses. The important roles of these TFs in crosstalk among abiotic stress responses will be discussed. Control of ABA or stress signaling factor expression can improve tolerance to environmental stresses. Recent studies using crops have shown that stress-specific overexpression of TFs improves drought tolerance and grain yield compared with controls in the field.

Keywords: ABA, transcription factor, signal transduction, abiotic stress, drought

INTRODUCTION

The world population is expected to reach nine billion by 2050. Considering this population increase, crop yields need to be improved by 40% in areas where drought is likely to occur by 2025 (Pennisi, 2008). In addition, frequent occurrences of drought and abnormal weather events have lately been observed all over the world. Drought negatively impacts plant growth and crop production (Bray et al., 2000). Almost every year, some region of the earth is hit by drought, damaging crops, and disrupting agricultural production. Severe drought affected the central and south of the US Corn Belt during 2012 (Edmeades, 2013). Drought also causes great damage to the production of other crops such as rice, wheat, and soybean. The southern states of Brazil, which account for 40% of the soybean production by the second leading producer worldwide, lost more than 20% of their production because of drought during the 2003/2004 and 2004/2005 seasons (Polizel et al., 2011). The development of stress-tolerant crops will be significantly advantageous in areas where such stresses occur frequently. Recently, some progress has been made toward identification of stress-related genes potentially capable of increasing the tolerance of plants to abiotic stress. Understanding the molecular mechanisms in the drought response is important to improve drought tolerance using molecular techniques. ABA accumulates under osmotic stress caused by drought, but also by other water limiting conditions, and plays an important role in stress responses and tolerance in plants (reviewed in Finkelstein et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Nakashima et al., 2009b; **Figure 1**). Molecular studies have revealed that ABA-independent gene expression is also important in stress tolerance in plants (**Figure 1**). In this review, we summarize some of the most important TFs in drought responses and discuss their regulatory networks and crosstalk in abiotic stress responses. By applying current knowledge of stress-regulated TFs and their target genes, improvement of drought stress tolerance is in progress in various crops using transgenic technology.

Abbreviations: ABA, abscisic acid; ABF, ABRE-binding factor; ABRE, ABA-responsive element; AP2, APETALA 2; AREB, ABRE-binding protein; bZIP, basic leucine zipper; CBF, CRT binding factor; CE, coupling element; DRE, dehydration-responsive element; DREB, DRE-binding protein; DRIP, DREB2A-interacting protein; CRT, C-repeat; ERF, ethylene-responsive element binding factor; GWAS, genome-wide association study; NAC, NAM, ATAF, and CUC; PP2C, 2C-type protein phosphatase; PYL, PYR1-like; PYR, pyrabactin resistance; QTL, quantitative trait locus; RCAR, regulatory component of ABA receptor; SNAC, stress-responsive NAC; SnRK2, SNF1-related protein kinase 2; TF, transcription factor.

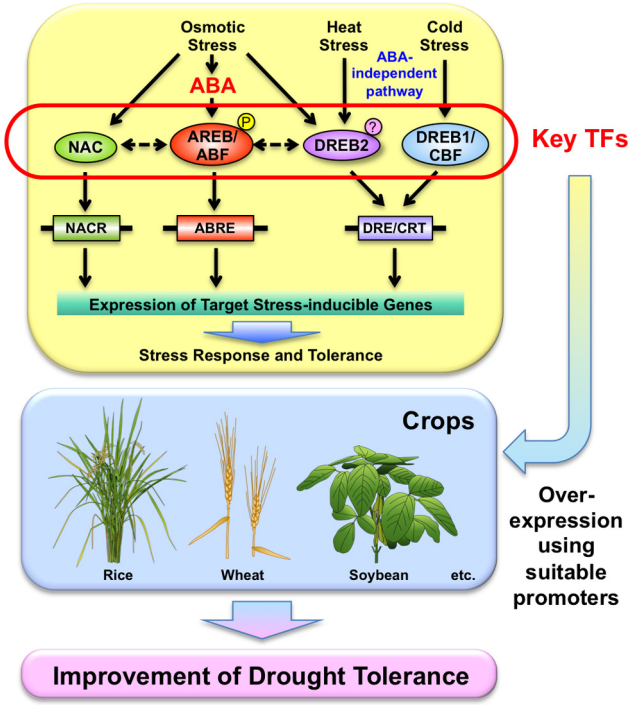

FIGURE 1 | Utilization of transcription factors (TFs) involved in stress-responsive pathways in stress responses for the improvement of drought tolerance of crops. Usage of suitable promoters might be necessary to control their expression.

AREB/ABF TFs FOR ABA-DEPENDENT GENE EXPRESSION

The promoter regions of ABA-responsive genes contain a conserved *cis*-element, named the ABRE (PyACGTGG/TC), which controls gene expression (**Figure 1**). Studies have revealed that expression of ABA-responsive genes requires more than one ABRE or a combination of an ABRE and a CE for a functional promoter (reviewed in Fujita et al., 2011, 2013; Nakashima and Yamaguchi-Shinozaki, 2013). Comprehensive and molecular analyses showed that ABA regulates the expression of many genes under osmotic stress conditions, and that the ABRE is the major *cis*-element for ABA-responsive gene expression (Maruyama et al., 2012). AREB/ABFs are bZIP TFs that regulate ABA-dependent gene expression, acting as major TFs under abiotic stress conditions in *Arabidopsis* (reviewed in Fujita et al., 2011, 2013; **Figure 1**). Among the nine members of the AREB/ABF TF family identified in *Arabidopsis*, AREB1/ABF2 has been reported to control ABA signaling and environmental stress responses during the vegetative growth stage. The AREB/ABF TFs are induced by abiotic stress and their transcriptional activities are controlled by ABA-dependent phosphorylation. ABA is required for full activation of AREB1 (Fujita et al., 2005; Yoshida et al., 2010) and its activity is regulated by the ABA-dependent phosphorylation of multiple sites within conserved domains (Furihata et al., 2006). Transgenic *Arabidopsis* plants overexpressing deleted and active forms of AREB1 showed enhanced drought tolerance and ABA hypersensitivity (Fujita et al., 2005). Overexpression of *AREB1* also improved drought tolerance in rice and soybean (Oh et al., 2005; Barbosa et al., 2013). Progress in understanding ABA perception and signal transduction has been made recently (reviewed in Cutler et al., 2010; Raghavendra et al., 2010; Umezawa et al., 2010; Weiner et al., 2010; Nakashima and Yamaguchi-Shinozaki, 2013). It was revealed that SnRK2, group A PP2Cs, and RCAR/PYR/PYL ABA receptors control the ABA signaling pathway including AREB/ABFs in land plants (reviewed in Umezawa et al., 2010; Miyakawa et al., 2013; Nakashima and Yamaguchi-Shinozaki, 2013). The phosphorylation of AREB/ABFs by SnRK2s is critical in the ABA-dependent signaling network (Fujita et al., 2009; Nakashima et al., 2009a; Umezawa et al., 2013). Recent studies have indicated that group A PP2Cs evolved early in land plants as key regulators of intrinsic desiccation tolerance, such as in the moss *Physcomitrella patens* (Komatsu et al., 2013). Perception and signaling factors such as PYL4 can also be used to improve stress tolerance (Pizzio et al., 2013).

DREB1/CBF TFs FOR COLD-RESPONSIVE GENE EXPRESSION TO IMPROVE DROUGHT TOLERANCE

Analysis of the promoter regions of genes showing ABA-independent expression in stress responses and tolerance has shown a *cis*-element with the sequence A/GCCGAC, designated the DRE/CRT (**Figure 1**). Two groups of AP2/ERF TFs were identified as DREB; DREB1/CBF and DREB2 in *Arabidopsis* (Liu et al., 1998). DREB1/CBF TFs specifically interact with the DRE/CRT and control the expression of a large number of stress-responsive genes in *Arabidopsis*. Improvements in tolerance to drought, salinity and freezing stresses have been reported in transgenic *Arabidopsis* overexpressing DREB1/CBF TFs, although their constitutive expression causes growth defects (Liu et al., 1998; Kasuga et al., 1999). However, overexpression of *DREB1* under the control of the *Arabidopsis* stress-responsive *RD29A* promoter improved stress tolerance in *Arabidopsis* without growth defects (Kasuga et al., 1999). Cold-inducible *DREB1/CBF* genes have also been isolated from a number of plant species, such as maize, oilseed rape, rye (*Secale cereale*), rice, tomato, and wheat (*Triticum aestivum*; reviewed in Mizoi et al., 2012). Interestingly, the major QTLs for tolerance to frost in *Arabidopsis*, diploid wheat (*T. monococcum*) and barley map to *DREB1/CBF* genes, and the expression levels of *DREB1/CBF* genes are correlated with frost tolerance (Vágújfalvi et al., 2003; Alonso-Blanco et al., 2005; Francia et al., 2007; Knox et al., 2008). Thus, the function of the DREB1/CBF regulon in the regulation of cold stress responses is widely conserved in angiosperms. Overexpression of DREB/CBF TFs has been reported to enhance drought tolerance in transgenic crops including chrysanthemum (Hong et al., 2006), peanut (Bhatnagar-Mathur et al., 2007, Bhatnagar-Mathur et al., 2013), potato (Behnam et al., 2007; Iwaki et al., 2013), rice (Oh et al., 2005; Ito et al., 2006; Datta et al., 2012), soybean (Polizel et al., 2011; de Paiva Rolla et al., 2013), tobacco (Kasuga et al., 2004), tomato (Hsieh et al., 2002a,b), and wheat (Pellegrineschi et al., 2004; Saint Pierre et al., 2012). For example, rice DREB1/CBF-type TFs involved in cold-responsive gene expression also conferred improved tolerance to drought in transgenic rice (Ito et al., 2006). The rice *DREB1/CBF*-type genes, *OsDREB1A* and *OsDREB1B*, are induced by cold stress. Transgenic *Arabidopsis* and rice plants overexpressing rice *OsDREB1* or *Arabidopsis DREB1* genes showed

improved tolerance to drought, high-salt and cold stresses but defective growth under normal growth conditions. Elevated contents of osmoprotectants including free proline and soluble sugars were detected in the transgenic rice. These results indicate that the *DREB1/CBF* regulon is conserved in rice, and that DREB1/CBF-type genes may be useful for improvement of tolerance to different environmental stresses in various kinds of transgenic monocot plants as well as dicot plants.

DREB2 TFs FOR OSMOTIC- AND HEAT-RESPONSIVE GENE EXPRESSION TO IMPROVE DROUGHT TOLERANCE

The *DREB2* gene encoding a DRE/CRT-binding protein is induced by osmotic stress (Liu et al., 1998; **Figure 1**). However, transgenic plants overexpressing *DREB2A* did not show any changes in phenotype. Domain analysis of DREB2A using *Arabidopsis* protoplasts showed that deletion of the central region makes DREB2A constitutively active (DREB2Aca), indicating that this region contains a negative regulatory domain (NRD; Sakuma et al., 2006a). Overexpression of *DREB2Aca* induced growth defects, up-regulation of stress-inducible genes, and enhanced drought tolerance (Sakuma et al., 2006a). Stress-inducible overexpression of *DREB2ca* improved drought tolerance in *Arabidopsis* and soybean without growth defects (Sakuma et al., 2006a; Engels et al., 2013). The NRD region of DREB2A is required for regulation of DREB2A protein stability. As mentioned above, overexpression of *DREB1A* improves freezing and dehydration stress tolerance in transgenic plants. By contrast, overexpression of *DREB2Aca* improves dehydration stress tolerance but only slightly improves freezing stress tolerance in transgenic plants. Integrated analysis of transcripts and metabolites was conducted to see the difference in the downstream gene products of DREB1A and DREB2A in *Arabidopsis* (Maruyama et al., 2009). Microarray analysis indicated that the downstream gene products of DREB1A and those of DREB2A have similar putative functions, but the expression of genes for carbohydrate metabolism in *DREB1A* and *DREB2A* transgenic plants is very different. Under dehydration and cold conditions, expression of genes for starch-degradation, sucrose metabolism and sugar alcohol synthesis changes dynamically. As a result, many kinds of mono-, di-, and trisaccharides, and sugar alcohols accumulate in plants. Overexpression of *DREB1A* caused similar changes in these metabolic processes, and these changes might improve dehydration and freezing stress tolerance in transgenic plants. By contrast, overexpression of *DREB2Aca* did not increase the level of these metabolites in transgenic plants. In addition, degradation of DREB2A is mediated by DRIPs, which are C3HC4 RING domain-containing proteins. DRIPs bind to DREB2A and function as E3 ubiquitin ligases mediating ubiquitination of DREB2A (Qin et al., 2008). Overexpression of *DREB2Aca* also induced expression of genes related to heat shock stress and improved thermotolerance in transgenic plants (Sakuma et al., 2006b). These results indicate that DREB2s function in both dehydration and heat shock stress responses. DREB2-type proteins have been isolated from a number of other plant species such as barley, rice, sunflower, maize, and wheat (Mizoi et al., 2012). GmDREB2A;2 is a DREB2A ortholog in soybean (Mizoi et al., 2013), but there are differences between DREB2A and GmDREB2A;2 in the NRD sequence. The effects on gene expression in transgenic plants overexpressing *GmDREB2A;2* are different from those in transgenic plants overexpressing *DREB2A*. This suggests that specialization in DREB2 regulons has occurred, although their basic functions are conserved between *Arabidopsis* and soybean. Recently, GWAS of ZmDREB2 and natural variations in the drought tolerance of maize (*Zea mays*) indicated that natural variation in the promoter region of *ZmDREB2.7* contributes to drought tolerance in maize (Liu et al., 2013). The favorable *ZmDREB2.7* allele may be a good resource for improving drought tolerance in maize. Recent studies suggest that DREB2 has important functions in drought tolerance, and that it can be used for improvement of drought tolerance in crops.

NAC TFs FOR DROUGHT-RESPONSIVE GENE EXPRESSION TO IMPROVE DROUGHT TOLERANCE

NAM, ATAF, and CUC TF proteins are plant-specific TFs. More than 100 *NAC* genes have been identified in *Arabidopsis* and rice (reviewed in Nakashima et al., 2012). Phylogenetic analyses indicate that six groups were established in an ancient moss. NAC TFs have a variety of important functions in development and stress responses. The genes in the SNAC group have important roles in the control of environmental stress tolerance (reviewed in Nakashima et al., 2012; **Figure 1**), and can bind to the NACR (NAC recognition sequence; CACG core). Stress-responsive *Arabidopsis SNAC* genes such as *RD26* and *ATAF1*, and rice *SNAC* genes such as *SNAC1*, *OsNAC6*/*SNAC2*, and *OsNAC5* can improve drought and/or high-salt stress tolerance when overexpressed (Tran et al., 2004; Hu et al., 2006; Nakashima et al., 2007; Takasaki et al., 2010; reviewed in Nakashima et al., 2012). Stress-responsive overexpression of *NACs* utilizing rice stress-responsive *LIP9*, *OsNAC6*, or *OsHox24* promoters is effective in inducing stress tolerance without the inhibitory effects of NAC on plant growth (Nakashima et al., 2007, 2012, 2014; Takasaki et al., 2010). Recent studies have suggested that the root-specific promoter *RCc3* is useful for the overexpression of *SNACs* such as *SNAC1* and *OsNAC10* to enhance the abiotic stress tolerance of rice in field conditions (Jeong et al., 2010, 2013; Redillas et al., 2012). These results indicate that SNACs have important roles in the control of abiotic stress responses and tolerance and that it is possible to improve stress tolerance by overexpressing SNACs using suitable promoters in the field. The many kinds of drought-responsive or tissue/organ-specific promoters reported for roots and stomata might be effective tools to control the expression of drought-responsive factors that cause growth defects at the right time and right position (Nakashima et al., 2007, 2014; Rai et al., 2009; Wu et al., 2009; Xiao et al., 2009; Yi et al., 2010; Ganguly et al., 2011; Yang and Xiong, 2011; Bang et al., 2013; Rusconi et al., 2013).

INTERACTIONS BETWEEN MULTIPLE TFs IN DROUGHT RESPONSES

Evidence for interaction between the AREB/ABFs and DREB/CBFs has been reported. The DRE/CRT motif in the promoters of drought-responsive genes is a binding region for an ABA-independent DREB/CBF TF and functions as a CE for ABRE in ABA-dependent gene expression (Narusaka et al., 2003). Lee et al. (2010) showed that the DREB1A/CBF3, DREB2A,

and DREB2C proteins interact physically with AREB/ABF proteins. These data suggest crosstalk between elements of the ABA-dependent and -independent response pathways. Moreover, interactions in the signaling pathways have also been indicated. Kim et al. (2011) reported that an ABRE promoter sequence, AREB/ABF TFs, and SnRK2s are involved in expression of the *DREB2A* gene under osmotic stress conditions, suggesting complex interaction between the AREB and DREB regulons at the gene expression level as well as the protein level.

Interaction between the AREB/ABFs and NACs has also been indicated at the gene expression level. Jensen et al. (2013) reported that *Arabidopsis* SNAC TF ATAF1 directly regulates the ABA biosynthetic gene *NCED3* in *Arabidopsis*, suggesting that SNAC TFs may regulate ABA-dependent gene expression of ABRE regulons. On the other hand, the promoters of *SNAC* genes contain ABRE sequences (Nakashima et al., 2012). Recently, Xu et al. (2013) reported that *Arabidopsis* ANAC096 cooperates with AREB/ABF factors (ABF2/AREB1 and ABF4/AREB2) in dehydration and osmotic stress responses. These results indicate complex interaction between the AREB/ABF and NAC regulons.

Finally, interaction between DREB/CBFs and other kinds of AP2/ERFs at the gene expression level has also been suggested. Cheng et al. (2013) reported that the *Arabidopsis* ERF1 regulates gene expression by binding to two kinds of *cis*-elements, the GCC box and DRE/CRT, in response to different stress signals. ERF1 is an upstream TF in both ethylene and jasmonate signaling and is involved in resistance to pathogens. Their results suggested that ERF1 bound to the GCC box but not the DRE/CRT in response to biotic stress, and to the DRE/CRT under abiotic stress. These results suggest that ERF1 may integrate ethylene, jasmonate, and ABA signaling and play an important role in biotic and abiotic stress responses.

CONCLUSION

Molecular analysis has suggested that drought-responsive TFs such as DREB1/CBF, DREB2, AREB/ABF, and NAC TFs function in drought responses and tolerance (**Figure 1**). These TFs also function in crosstalk in abiotic stress responses, such as drought, cold, and heat. As mentioned above, these factors can be used to improve drought tolerance in a variety of crops. Our group has utilized these key TFs for the improvement of drought tolerance in crops including rice, wheat, and soybean in collaboration with international and domestic institutes (Pellegrineschi et al., 2004; Hong et al., 2006; Behnam et al., 2007; Bhatnagar-Mathur et al., 2007; Polizel et al., 2011; Datta et al., 2012; Ishizaki et al., 2012; Saint Pierre et al., 2012; Barbosa et al., 2013; Bhatnagar-Mathur et al., 2013; de Paiva Rolla et al., 2013; Engels et al., 2013; Iwaki et al., 2013). Some results using crops including rice and peanut have shown that stress-specific overexpression of *DREB1A* improves drought tolerance and grain yield compared with controls in the field (Datta et al., 2012; Bhatnagar-Mathur et al., 2013). These results suggest that overexpression of key TFs under the control of suitable promoters can improve stress tolerance, although the regulatory network in the plant response is complex in water limiting environments (**Figure 1**). Since TFs function in balanced crosstalk in abiotic stress responses, overexpression of a certain TF may affect other signaling pathways. Thus, we should examine the molecular effects of overexpressing TFs in addition to conducting stress tolerance assays. In addition, the effects of a transgene may depend on the genetic background of the species or cultivar used for transformation. Furthermore, since the degree of drought varies in actual fields (strength, timing, and period of stress, complex stresses such as drought with heat stress etc.), the effect of a transgene may differ depending on environmental conditions. Continuous field experiments might be necessary to see the effects of transgene-encoded TFs in the field using a variety of genotypes and environments. Recently, QTL analyses have revealed novel genes involved in drought resistance. *DEEPER ROOTING 1* (*DRO1*), a QTL controlling root growth angle in rice, was cloned and characterized (Uga et al., 2013). This study revealed that changes in root system architecture can improve drought avoidance. Other drought resistant QTLs have also been reported in rice. Multiple QTLs were reported in the rice mega-variety IR64 that enhance the yield under drought conditions (Swamy et al., 2013). Combinations/pyramiding of transgenic plants and QTL drought resistant varieties by marker-assist selection (MAS) may promote drought tolerance.

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Physiological and genomic basis of mechanical-functional trade-off in plant vasculature

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Some areas in plant abiotic stress research are not frequently addressed by genomic and molecular tools. One such area is the cross reaction of gravitational force with upward capillary pull of water and the mechanical-functional trade-off in plant vasculature. Although frost, drought and flooding stress greatly impact these physiological processes and consequently plant performance, the genomic and molecular basis of such trade-off is only sporadically addressed and so is its adaptive value. Embolism resistance is an important multiple stress- opposition trait and do offer scopes for critical insight to unravel and modify the input of living cells in the process and their biotechnological intervention may be of great importance. Vascular plants employ different physiological strategies to cope with embolism and variation is observed across the kingdom. The genomic resources in this area have started to emerge and open up possibilities of synthesis, validation and utilization of the new knowledge-base. This review article assesses the research till date on this issue and discusses new possibilities for bridging physiology and genomics of a plant, and foresees its implementation in crop science.

Keywords: embolism, cavitation, xylem, drought, freezing, mechanical stress

INTRODUCTION

A green plant is unique in its hydraulic architecture. Hydraulic conductivity of the xylem is closely linked to the minimum leaf area, which it must supply with water and nutrients for survival. Hydraulic conductivity, as quantified by Zimmermann (1974), is generally measured as leaf specific conductivity (flow rate per unit pressure gradient) divided by the leaf area supplied by the xylem pipeline segment. This measure is a key for quick evaluation of pressure gradients within a plant. Modeling the functional and natural architecture of plant water flow pipeline takes more traits in consideration than merely the physical attributes of a mechanical pump. The contribution of living cells and more specifically, genes and proteins, for maintenance of the "green pump" remains largely unaddressed.

Several theories have been proposed to explain ascent of sap. The operation of the green pump is simple yet elegant and is best described by the Cohesion-Tension Theory (CTT) (Dixon, 1914) but also synthesized from the work of many scientists over the last few decades. Besides physical explanations, the living parenchyma cells around xylem were originally proposed to be of importance by Bose (1923) in his pulsation theory. Later, the living xylem parenchyma cells indeed proved of high importance for the continuous ascent of sap.

The major governing factors are the physical properties of aqueous solution, means of transport and xylem anatomy, consideration of all of which makes the "sap conducting system" comparable to basic hydraulic systems such as pumps and irrigations in household or human blood vasculature. Components of such system are mainly (i) a driving force, (ii) a pipeline system, (iii) a reservoir and other regulating factors. To establish a soil-water-atmosphere continuum, an uninterrupted "water network" is necessary, which is built in the plant where transpirational evaporation is the driving force (**Figure 1A**). The evaporation of water from the porous green tissue surface creates a capillary pull in the water menisci (**Figure 1Ai**) and a curvature is induced in them, which is sufficient to support a huge water column against gravity in the stem and root vascular cylinder (**Figure 1Aii**). The water reservoir is the soil, wherefrom the root draws its supply (**Figure 1Aiii**). The empirical Jurin law says that a menisci radius of 0.12 μm can support a column of 120 m (Zimmermann, 1983). The pull creates sub-atmospheric pressure in the xylem vessels. As the height of a plant increases, the water potential drops, and it is expected that leaves, twigs and upper extremities will display a 10–1000 times drop of pressure (**Figure 1A**, Tyree and Sperry, 1989). Sixty five percentage of the water potential drop occurs in tree trunk xylem, with a 20% contribution from root and 14% from leaves (Tyree and Sperry, 1989). This explains why big tree trunks can survive severe localized damages near the base.

PLANT ARCHITECTURE AND THE GREEN PUMP

Architecture of a plant is defined by its height, girth, woodiness, root system design and shoot disposition. Such architecture varies across the plant kingdom, along which varies the plants' hydraulic nature. Secondary thickening is a major player that governs the green pump. It has been shown that root pressure plays little or no part in maintenance of this column in woody plants. Severing the root may not hamper upward movement of water, if there is a direct supply to the vessels; however leaves are necessary. Even the best vacuum pump is able to pull water to not more than 10.4 m, considering that a Sequoia tree may have to pull water up to 100 m. However, in the monocots, root pressure is considered to be a major player of sap pull.

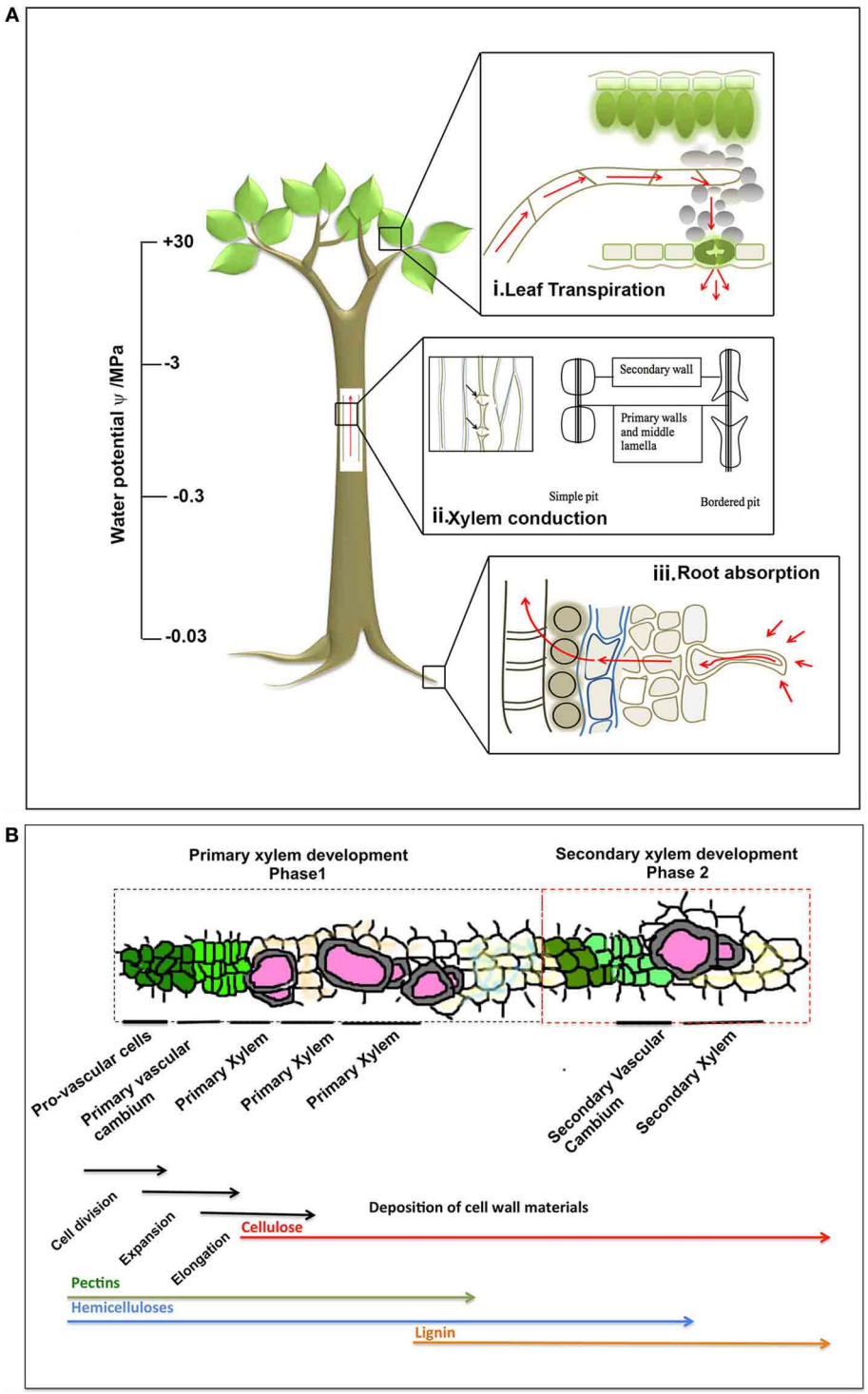

FIGURE 1 | (A) The soil-plant-air continuum functioning in maintenance of water transport column. The plant root takes up water from soil, and the water column is maintained continuous along the xylem. The continuity across the xylem vessel is maintained by several intrinsic physical properties of water, input from the adjoining living cells and transpirational pool. The rough estimate of pressure along the vascular cylinder is presented in the scale bar (image not to actual scale). **(B)** A schematic of xylogenesis, adapted and modified from Hertzberg et al., 2001. The two phases of xylem development (primary and secondary); and the tissues involved in the process are shown within respective dotted boxes. The biological processes (cell division, expansion, elongation, deposition of cell wall) involved are shown by black arrows, under corresponding tissue types. The cell wall materials that are deposited are also shown under corresponding tissue types during xylogenesis. The order of such differentiation may be traced from left to right in the figure, though their actual time frame may differ from species to species.

Considering the physical properties of green-pump, cavitation and embolism are major threats to the water column in xylem and subsequently, to survival, across the kingdom. To successfully transport water and minerals from soil to leaf, existing pressure in xylem conduits needs to remain sub-atmospheric (negative), in contrast to animal system where long distance transport is actively under positive pressure. The molecular property of cohesion gives a high strength to water. Ultrapure water confined to tubes of very small bore will need a tension comparable to the strength needed to break steel columns of the same diameter. Cohesion imparts strength comparable to solid wires in a water column. The vice is: once air is introduced in such system, the column will snap apart. To prevent such snapping, xylem properties play an important role.

PHYSIOLOGY OF XYLOGENESIS: THE BIPHASIC DEVELOPMENT IN XYLEM

The biphasic development of xylem in plants is critical to understand the hydraulic architecture as well as the air-water-soil continuum (**Figure 1B**). Procambium develops into xylem precursor cells that eventually differentiate into xylem fiber cells, xylem parenchyma, and tracheary elements, consisting of vessels and tracheids in the first phase. The second phase deposits secondary xylem walls onto the primary xylem walls (Fukuda, 1997; De Boer and Volkov, 2003), derived from vascular cambium and made of cellulose microfibrils impregnated with lignin, structural proteins, hemicellulose and pectin (**Figure 1B**, Ye, 2002; Fukuda, 2004; Yokoyama and Nishitani, 2006). Prior to secondary development, the tracheary components elongate and with the advent of secondary wall deposition, the cellular components in the living tracheid undergo programmed cell death (Fukuda, 2004) living only the hollow pipeline (Fukuda, 1997; Zhang et al., 2011) composed of vessels interconnected by pits (De Boer and Volkov, 2003; Choat and Pittermann, 2009). The paired pits are often bordered (**Figure 1A**); from secondary deposition forming two overarched secondary walls, in between which a fine pit membrane with small pores persist. Pit membranes are made up of meshes of polysaccharide (Tyree and Zimmermann, 2002; Pérez-Donoso et al., 2010) and allow axial passage of water and small molecules. Besides, they act as safety protection against spread of air seeds (Tyree and Zimmermann, 2002; De Boer and Volkov, 2003; Choat et al., 2008; Pérez-Donoso et al., 2010).

PHYSIOLOGY OF CAVITATION

The negative pressure in the xylem may descend low enough to make the water metastable. To achieve non-disrupted flow in such system, water must remain liquid below its vapor pressure. This metastable state induces nucleation of vaporization, or cavitation. Cavitation is the introduction of air spaces into the continuous water column and under physical metastable state water is prone to form air bubbles easily. Introduced in a xylem lumen, air cavities rupture the water column and in its worst, block the transport of water and minerals to the leaf. This blockage is known as "embolism" and may lead the plant to a lethal fate.

Cavitation is known to occur in plants frequently. Paradoxically, occurrence of cavitation is the strongest support for CTT. It is only natural to observe cavitation if water is under such negative pressure. The root vessels of field grown, well watered maize plants have been known to embolize daily and then refill. Vessels that were filled by dawn may embolize at mid-afternoon and by sunset they are again refilled (McCully et al., 1998). When transpiration rate is high and water scarcity is at bay, trees display cavitation, which means that embolism can well be induced by water stress. Large metaxylem vessels show a higher rate of embolism, and evidence suggest that water stress-induced embolism is of the frequent most sort (Tyree and Sperry, 1989). It is a prerequisite for cavitation that some vessels are embolized to start with; which is met by bubbles introduced in some of the vessels by mechanical damage, harbivory and insect attack.

STRESS-INDUCED EMBOLISM IN PLANTS

Both abiotic and biotic stresses can induce embolism in a plant. Drought and frost—induced embolisms are most prevalent, while mechanical stress and pathogen-induced damage are often the primary inducers.

Desert plants and dry-season crops are most threatened by drought-induced embolism. Air-seeding increases during drought as the sap pressure becomes increasingly negative due to high suction. The evaporation from leaf surface increases and the porous conduit wall may release air inside the functional conduits. They behave as nucleation centers and cause the sap pressure to increase to atmospheric level. The bubble is then likely to start an embolism that fills up the diameter of conduit, as the surrounding water is pulled up by transpiration.

Interconduit pit membranes with nano-scale pores normally restrict passage of air bubble from affected to functional conduits but at a high pressure difference they fail to stop the propagation. The rate of this propagation is important to measure the cavitation resistance in a plant.

Freezing is another cause of embolism, specially in woody temperate species. Freeze-thaw cycles may lead to 100% loss of water transport due to embolism in some species (Scholander et al., 1961). The primary governing factor in damage intensity seems to be the mean diameter of the conduits. Smaller vessel diameters are more vulnerable to damage.

Frost-induced air seeding is caused by segregation of gas by ice. There is a certain amount of salting out from the sap during freezing of sap, and if the salts are not able to move through the walls, they raise the osmotic pressure of remaining solution (Sevanto et al., 2012). This embolism can be more severe if there is functional drought prevailing. Freezing-induced embolism is a primary stress in forests where seasonal freeze-thaw is observed. Herbaceous plants, on the other hand, hardly survive freezing and are mostly at threat from drought-induced embolism.

Vascular wilt pathogens can wipe out entire crop. It is known that vascular pathogens induce water stress in their hosts; but can embolism be a cause of such stress? All vascular wilt pathogens break into rigid secondary xylem walls to enter the vessels as well as the pit membranes. Generally vascular wilt pathogens or their spores and conidia are too large to pass through pit membrane pores (Mollenhauer and Hopkins, 1974; Choat et al., 2003, 2004; Qin et al., 2008). Even when they manage to break into the vessel the milieu is not friendly. The microenvironment of xylem

pipeline is nutritionally very poor and the pathogens surviving in xylem niche are not too many in number. It is speculated that they prefer this environment to minimize competition. Nevertheless, fungal and bacterial pathogens can extract the little amount of ions and nutrients available in the xylem stream and are able to break through and digest secondary wood to leech nutrition from living cells. Doing so, they weaken the pressurized cell wall and their infestation within the dead pipeline makes the water stream reactive and prone to cavitation. They may as well block the vessels and pit membranes, occluding parts of functional conduit network.

There is also an internal mechanical stress associated with ascent of sap. The high negative tension within the xylem pipeline causes an inward pool. Depending on the sapwood elasticity, there is a daily diameter change of tree trunk correlated to transpiration and daylight. In Scots pine, Perämäki et al. (2001) described daily changes in the sapwood diameter. The pull causes pressure on a stem surface element directed toward the center of the stem and the tracheal structure resists the movement of the surface element. The mechanical strength of the tracheary wall and its composition is, hence, an important factor in maintaining normal xylem activity as is the plasticity of pit membrane structure and composition.

VULNERABILITY OF XYLEM TO CAVITATION

Xylem seems to be vulnerable to cavitation in many different ways. This vulnerability can vary depending on the species, season, and availability, state and temperature of water. Broadly, the vulnerability of plants to cavitation is often plotted on xylem vulnerability curves, which is a function of decline in xylem hydraulic conductivity due to increasingly negative xylem pressure. Such declines are typically expressed relative to the maximum decline possible as the Percentage Loss of Conductivity (PLC). Comparisons of the vulnerability to cavitation among species are made using the xylem pressure at 50% loss of conductivity (P_{50}) with the traditional plotting of vulnerability curve (Meinzer and McCulloh, 2013). There remain controversies related to the techniques used for measurement of vulnerability described elsewhere in details (McElrone et al., 2012; Cochard et al., 2013; Wheeler et al., 2013).

The vulnerability curve for a number of tree species, as put forward by Tyree et al. (1999) shows a typical exponential shape, indicating that sub-zero pressure is a direct inducer of cavitation. This makes cavitation a regular process and necessitates a resistance mechanism in plants. It has also been claimed that cavitation is rapidly repaired by a miraculous mechanism (Holbrook and Zwieniecki, 1999) known as "refilling." We can thus categorize cavitation resistance under two proposed mechanisms; one, by refilling the air bubbles efficiently; and two, by modulating pit membrane properties. The possible genetic controls of both are worthy of discussion.

CAVITATION RESITANCE BY REFILLING: A QUESTIONABLE TRAIT

The removal of air seeds from lumen to turn a non-functional vessel to functional is known as refilling. The idea, though widely observed, recently was confronted with a serious doubt voiced by the plant hydraulic scientists. The long-established experimental procedure that has been followed to measure cavitation has been pronounced faulty (Sperry, 2013). It has been claimed that the standard procedure of xylem hydraulic conductivity measurement, by excising the stem under water to avoid air aspiration in the open conduits, is not a valid observation procedure. It has been suggested that in many species, significant amount of cavitation is introduced even when the stem is cut under water. The consequences of this artifact on previous datasets were significant, as it may be reflected in all vulnerability to cavitation curves obtained in other species for a long period of time; and perturb our analysis of refilled vessels.

However debatable the issue may be, recent high resolution and real-time imaging studies (Holbrook et al., 2001; Windt et al., 2006; Scheenen et al., 2007; Brodersen et al., 2010) also satisfy the requirements of the hypothesis that plant has some kind of resistance strategies to protect itself from embolism. It has been proposed that plants have an osmotically driven embolism repair mechanism and existing rehydration pathways through the xylem. The mechanisms were predicted to be largely of two types: (i) "novel" refilling, a refilling mechanism without "positive root pressures, even when xylem pressures are still substantially negative"; (ii) root pressure aiding the refilling of vessels raising the pressure inside vessels near atmospheric (Salleo et al., 1996; Holbrook and Zwieniecki, 1999; Tyree et al., 1999; Hacke and Sperry, 2003; Stiller et al., 2005). The first type is common among woody dicots whereas evidence of the second type is common among annual herbaceous species.

GENETIC CONTROL OF REFILLING MECHANISM

Bay leaf tree, *Laurus nobilis* is an aromatic shrub in which mechanism of refilling is proposed to be linked to starch to sugar conversion. Reserve carbohydrate depletion from xylem parenchyma induces phloem unloading in a radial manner via ray parenchyma (Salleo et al., 2009; Nardini et al., 2011). Xylem-phloem solute exchange has been found to occur along both symplastic and apoplastic paths (Van Bel, 1990). It has been hypothesized that solutes might move radially along the ray cell walls, enter the embolized xylem conduits and increase the solute concentration of the residual water within them, thus promoting xylem refilling by altering osmoticum. The role of xylem parenchyma in refilling is significant. Lianas, shrubs and vine fibers are often observed to have living protoplasts and starch granules (Fahn and Leshem, 1963; Brodersen et al., 2010). Repeated cycles of embolism and repair are correlated to cyclic depletion of starch in xylem during drought (Salleo et al., 2009; Secchi et al., 2011). Debatably, repeated cycles of embolism formation and repair may disable the refilling mechanism and ultimately lead to carbon starvation (Sala et al., 2010, 2012; McDowell, 2011). The hydrolyzed starch movement from xylem is yet unresolved.

Water stressed *Populus trichocarpa* plants revealed an upregulation of ion transporters, aquaporins, and carbon metabolism related genes (Secchi et al., 2011; Secchi and Zwieniecki, 2012). A putative sucrose-cation co-transporter may aid the refilling process as suggested by the chemical profiling of vessel lumen. Grapevine refilling petioles show strong upregulation of carbon metabolism and aquaporin expression (Perrone et al., 2012).

A basic assumption is made that in dicots, to enhance refilling ability trait, one might target carbohydrate metabolizing genes in a localized manner to improve sucrose release. Sucrose may be used as an osmoticum inside non-functional lumens or may be used as energy currency. Localization of increased aquaporins (PIPs and TIPs) within axial parenchyma surrounding conduits may prove important. It is now proved by imaging studies (Brodersen et al., 2010) that living cells play a central role in embolism refilling and restoring transport, and by further prevention of air seed and pathogen by sealing off conduits with tyloses. Further detailed work is needed to identify the stress signals that mediate talk between xylem vessels and parenchyma.

In monocots, root pressure is the most important mechanism for refilling reported till date. Grasses exhibit root pressure more often, and with the increase of plant height the basal root pressure increases (Cao et al., 2012). Monocots do not exhibit secondary thickening and ray cells thus the osmoticum and sucrose transport theory do not apply to monocots (Andre, 1998). Selection for root pressure in these species solves the embolism repair problem and negates the need for carbohydrate transport along the pathway common in woody angiosperms (Brodersen et al., 2013). However, Stiller et al. (2005) showed the presence of "novel" refilling in rice in presence of high negative pressure and suggested that in upland or low-rainfed rice this mechanism can serve side by side of a positive root pressure. Root pressure may involve a stronger mechanical tissue, and whether or not any trade-off between safety and efficiency is involved is unclear. Study of more vascular function mutants in monocot crops may resolve the genes involved in this process.

GENOMIC PERSPECTIVE: GENES, PROTEINS AND MODELS IMPLICATED IN REFILLING

The battle with cavitation is fought either with efficient refilling or fine structural modulation of pit membrane and strength of vascular cylinder wall. The genomic, transcriptomic and proteomic studies may thus come under two broad sections: genomic basis of refilling and genomic basic of mechanical strength (**Figure 2A**).

GENOMIC BASIS OF REFILLING

The process of refilling or repair of embolism requires pumping water in an air-filled cavity. Physically this will require an empty or air-filled vessel, functional neighbor vessels, a source of energy to drive the refilling and a source of water to refill. In the previous sections, the physical and physiological components of embolism repair have been discussed in detail. However, a reductionist biologist looks further beyond for the possible identities of molecular candidates that repair the non-functional vessel. It is hypothesized that refilling is a result of an intricate interaction of xylem parenchyma, (even possibly phloem), vessel wall chemistry, and the composition and flexibility of pit membranes (Holbrook and Zwieniecki, 1999). The signals that are sensed when embolism occurs and the cascades that follow the primary signal transduction event, involve interconnected molecular regulators; that has been subject of several studies. The most recent model of refilling puts forward a role of sugar signaling in embolism sensing and refilling mechanism, the involved gene families being Aquaporins, Sucrose transporters and enzymes related to starch breakdown, Alpha and Beta Amylase (Secchi and Zwieniecki, 2010).

AQUAPORINS

Aquaporins are conservedly implicated in the refilling process of angiosperms and gymnosperms from the very beginning. The refilling of vessels in *Populus trichocarpa* is accompanied by selective upregulation of PIPs (Plasma Membrane Intrinsic Proteins). Secchi et al. (2011) proposed that the sensing of embolism and accomplishment of refilling is mediated by sugar signals, specifically sucrose. According to their proposed model, when a vessel is filled with air, free passage of sucrose to the vessel lumen is hindered, and the sucrose molecules are deposited on vessel wall. This, with a positive feedback loop generate a cascade of high starch to sucrose conversion (Bucci et al., 2003; Salleo et al., 2004; Regier et al., 2009). The increased sucrose pool would be maintained by upregulation of amylases and sugar transporters. Secchi et al. (2011) showed a distinct upregulation in aquaporins and sucrose transporter (*PtSuc 2.1*) in air injected or artificially high osmotica-treated vessels. *Ptsuc2.1* shows a high homology to walnut sucrose transporter, which, on upregulation is able to relieve freeze-thaw induced embolism (Decourteix et al., 2006). The increased sucrose and the upregulation of aquaporins are correlated spatially and temporally, but connections are difficult to establish. The model hence proposed is schematically represented in **Figure 2B**. Almeida-Rodriguez et al. (2011) showed a gene expression profile of 33 Aquaporins in fine roots of hybrid poplar saplings and compared light and high transpiration induced vascular hydraulics physiology with respect to Aquaporin expression. Dynamic changes were observed in expression pattern of at least 11 aquaporins from poplar; and some of them were localized in the root tissue. In Arabidopsis, Postaire et al. (2010) showed that, hydraulic conductivity of excised rosettes and roots are correlated wih expression of aquaporins. AtPIP1; 2, AtPIP2;1, and AtPIP2;6 are the most highly expressed PIP genes in the Arabidopsis rosette (Alexandersson et al., 2005) and under long night, AtPIP1;2 knockout plants loose 21% hydraulic conductivity in the rosette(Postaire et al., 2010). The disturbed hydraulics phenotype is a genetic dissection of the direct relation between aquaporin expression and plant water transport; although there may be components other than Aquaporin that may serve an important role (Sack and Holbrook, 2006; Heinen et al., 2009). It has been shown in hybrid poplar *Populus trichocarpa* × *deltoides,* increasing evaporation from leaf surface and perturbed hydraulics is correlated with high aquaporin expression (Plavcová et al., 2013). In common grapevine, *Vitis vinifera L.* (cv Chardonnay) inhibitors of aquaporin-mediated transport greatly affects both leaf hydraulic conductance and stomatal conductance (Pou et al., 2013). Of 23–28 Aquaporin isoforms in grapevine, a subset including VvPIP2;2, VvTIP1;1 plays important role during early water stress, while VvPIP2;1, VvPIP2;3, VvTIP2;1 are highly expressed during recovery(Pou et al., 2013). In Maize roots, radial water transport are diurnally regulated by proteins from the PIP2 group (Lopez et al., 2003). It is evident, though, that not all aquaporins participate in the refilling process. The sugar signal initiation is one important component; as originally described by Secchi et al. (2011) and must induce embolism-related aquaporin isoforms.

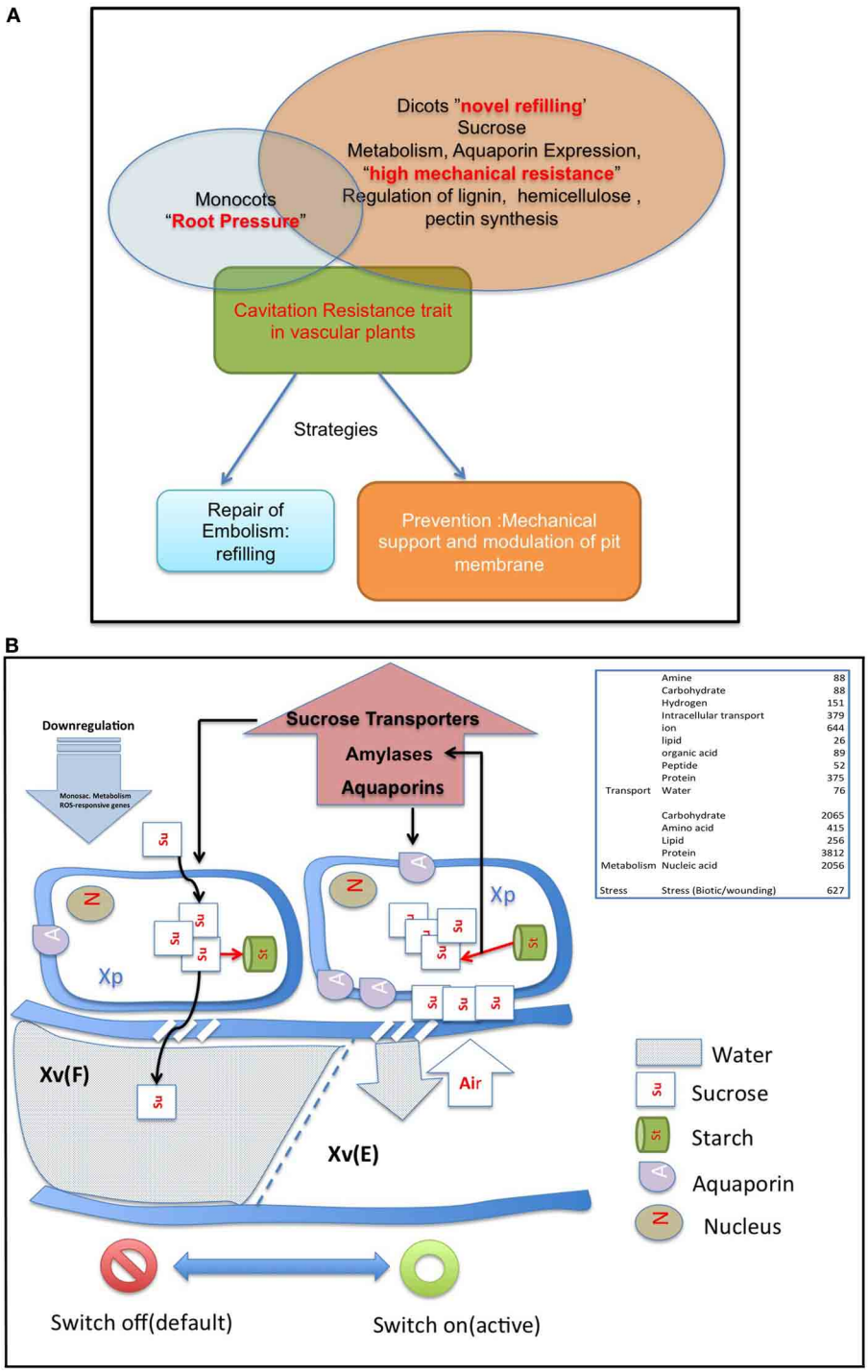

FIGURE 2 | (A) The strategies of vascular plant in a battle against embolism. Monocots often employ root pressure, while dicots employ novel refilling mechanism, and mechanical resistance to resist cavitation. There is no clear demarcation between the strategies employed by the two groups, and the strategies may overlap. **(B)** The sugar sensing model of embolism refilling process, modified from Secchi et al. (2011). For detail explanations of the model, refer text and Secchi et al. (2011). Briefly, when vessels are filled and functional, a default "switch off" mode is active. Sucrose is continuously transported from accompanying xylem parenchyma cells into the vessels. Cavitation induces a "switch on" mode of sensing. When a vessel is filled with air, free passage of sucrose to the vessel lumen is hindered, and the sucrose molecules are deposited on vessel wall. This, with a positive feedback loop generates a cascade of high starch to sucrose conversion (Bucci et al., 2003; Salleo et al., 2004; Regier et al., 2009). The increased sucrose pool would be maintained by upregulation of amylases and sugar transporters. The genes up/downregulated during the sensing process are mentioned in the figure. Abbreviations used: Xv(F), Xylem Vessel Filled; Xv(E), Xylem Vessel Embolized; Xp, Xylem Parenchyma. Other abbreviations are explained in the figure.

The transcriptomic studies show that a very high number of Carbohydrate Metabolism related genes were upregulated during embolism (Secchi et al., 2011). Upregulation of the disaccharide metabolism gene group was observed, along with downregulation of monosaccharide metabolism; that suggests an accumulation of sucrose pool on the vessel wall (Secchi et al., 2011). Further upregulation of ion transporters and downregulation of carbohydrate transporters build up an osmoticum inside the cell to facilitate efflux of water. **Figure 2B** (inset) shows a summary of the number of gene categories showing differential expression during embolism (Secchi et al., 2011). The energy required for the pumping in comes from starch hydrolysis and one can presume, xylem specific isoforms of aquaporin, Starch synthetase and sucrose transporters will be highly expressed during refilling in plants. For critical evaluation of the model parameters, and its feasibility across the plant kingdom we extracted all aquaporin gene sequences from Arabidopsis and the Arabidopsis homologs of *Populus trichocarpa* sucrose transporters and amylases implicated in embolism Secchi et al., 2009, 2011; Secchi and Zwieniecki, 2010, 2012, 2013, 2014. The accession numbers of the fetched Arabidopsis genes are presented in **Tables 1A,B**. We subjected the gene sequences to protein-protein interaction network interaction analysis in String software in Expasy, without suggested functional neighbors (Szklarczyk et al., 2010). Generated interaction network for Arabidopsis gene subsets (mentioned in **Table 1**) clearly shows three interaction network clusters, connected to each other (**Figure 3**), the middle cluster (termed 'a' in **Figure 3**) shows evidenced network of PIPs as well as a RD28, dehydration stress related protein. Two other clusters (b and c in **Figure 3**) exhibit sucrose transporters and NIPs. Amylases form an un-joined node (d in **Figure 3**). We further localized the genes in Arabidopsis publicly available transcriptome analysis database in different tissues and observed shared enrichment in root endodermis, cortex and stele using e-northern (**Figure 4A**, Toufighi et al., 2005). A co-expression profile (**Figure 4B**) was obtained using string software, and the common n-mers present in the genes to induce a co-expression in certain tissues has been analyzed using promomer tool (**Figure 4C**; **Table 2**, Supplementary Table 1, Toufighi et al., 2005). Many of the enriched *cis*-elements contribute to dehydration and sugar stress. Overall, the genomic and transcriptomic data and candidate-gene based data emphasizes the high probability of sugar sensing of embolism. Secchi and Zwieniecki (2014) also showed that in hybrid poplar, downregulation of PIP1 delimits the recovery of the plant from water-stress-induced embolism, and thus is probably manages the vulnerability of xylem in negative pressure under control condition. The sugar content in the plant tissue strengthens the view further (Secchi and Zwieniecki, 2014).

TRANSCRIPTION FACTORS

The corregulation of sugar metabolism and water transport pathways require a complex transcriptional switch. Indeed, a large number of transcription factors control the refilling process, and they may regulate the diurnal pattern, the temporal accuracy and spatial distribution of the pathways involved. The role of TFs is shared; However, a look at the *cis* elements of pathway components may elucidate the nature of such sharing. The transcription factors important for xylogenesis and probably embolism are: AP2/EREBP, bZIP, C3HHD-ZIPIII, NAC, MYB, bHLH, WRKY, AP2/ERF, WRKY, HD, AUX/IAA, ARF, ZF, AP2, MYC, (Arabidopsis); HD-ZIPIII, MYB, MADS, and LIM in *Populus*, MYB and Hap5a in Pine and HRT in *Hordeum* (Dharmawardhana et al., 2010). With the onset of genomic approaches, much more intensive analysis have been made possible. In a comprehensive genome-wide transcriptome analysis of *P. trichocarpa*, with snapshots from each elongating internode from a sapling stage (Internode1 through Internode11) a large number of differential representation of transcription factors have been obtained (Dharmawardhana et al., 2010). No less than 1800 transcription factors were readily detectable in at least one growth phase, of which, 439 are differentially regulated during xylogenesis (Dharmawardhana et al., 2010); some of which are represented in **Table 3**. Another study identified 588 differentially changed transcripts during shoot organogenesis in *Populus* (Bao et al., 2009, 2013). While the refilling process is majorly governed by sugar and dehydration signaling, NAC and Myb TF families remain singularly important in both xylem maturation and lignin biosynthesis. Aspects of xylogenesis that may be linked with mechanical-functional trade-off of vascular bundle revolve around lignin. There have been studies on genomics and transcriptomics of xylogenesis and secondary wood formation; however the genes responsible to maintain integrity of the vascular cylinder are not clearly known. In Supplementary Table 2, a comparative snapshot of some selected transcripts and emanating studies revealing the xylogenesis transcriptome in gymnosperms and angiosperms is provided. Several recent studies address the genomics of xylogenesis excellently; some of which are summarized in **Table 4**.

CAVITATION RESISTANCE INTRODUCED BY PIT MEMBRANE

The major key of cavitation resistance is pit membrane adaptation. To survive, ultrastructure of pit membrane needs to balance between minimizing vascular resistance and limiting invasion by pathogen and microbes. While the first is favored by thin and highly porous membrane, the later needs thick membrane and narrower pores. This calls for a trade-off between water transport function and biotic invasion resistance.

The thickness range of the pit membranes in the angiosperms is very broad, almost 70–1900 nm and so are the diameter of the pores (10–225 nm). Species with thicker pit membrane and smaller pores prevent seeding and embolism more successfully and thus may represent the group of species which has higher drought resistance.

Pit membrane porosity is not the only determinant of air bubble propagation among conduits. The other factor which serve equally important role is the contact angle between pit membrane and air water interface. This particular property is a direct function of pit membrane composition. The more hydrophobic the membranes are the more the contact angle and subsequently lower the pressure needed for air-seeding. Additionally, high lignin content, though required for mechanical strength, interrupt with the hydrogeling of pectins. Pectic substances can swell

Table 1A | Genes, families and members important in refilling experimentally reported in *Populus trichocarpa*.

Gene families	Specific genes				
	Family	**Subfamily**	**Gene name**	**JGIv2.0 annotation name**	**Arabidopsis homologs**
Aquaporins	PIP (Plasma Intrinsic Protein)	PoptrPIP1	PoptrPIP1.1	POPTR_0008s06580	For analysis, the entire aquaporin family of Arabidopsis has been used instead of only specific homologs, refer to **Table 1B**.
			PoptrPIP1.2	POPTR_0003s12870	
			PoptrPIP1.3	POPTR_0010s19930	
			PoptrPIP1.4	POPTR_0006s09920	
			PoptrPIP1.5	POPTR_0016s12070	
		PoptrPIP2	PoptrPIP2.1	POPTR_0006s09910	
			PoptrPIP2.2	POPTR_0009s13890	
			PoptrPIP2.3	POPTR_0004s18240	
			PoptrPIP2.4	POPTR_0016s09090	
			PoptrPIP2.5	POPTR_0010s22950	
			PoptrPIP2.6	POPTR_0006s12980	
			PoptrPIP2.7	POPTR_0008s03950	
			PoptrPIP2.8	POPTR_0009s01940	
Alpha-beta amylases	Alpha-amylase	PoptrAMY	PtAMY1	POPTR_0515s00220	AT4G25000
			PtAMY2	POPTR_0002s01570	AT1G76130
			PtAMY3	POPTR_0010s10300	AT1G69830
	Beta amylase	PoptrBMY	PtBMY1a	POPTR_0008s17420	AT3G23920
			PtBMY1b	POPTR_0001s11000	AT3G23920
			PtBMY2	POPTR_0003s10570	AT5G45300
			PtBMY3	POPTR_0008s20870	AT5G18670
			PtBMY4	POPTR_0003s08360	AT2G02860
			PtBMY5	POPTR_0017s06840	AT1G09960
Sucrose transporters	Sucrose transporter		PtSUC2.1	POPTR_0019s11560	AT5G55700
			PtSUT1.2	POPTR_0013s11950	AT4G15210
			PtSUT2.a	POPTR_0008s14750	AT1G22710

Gene ID data compiled from Secchi et al. (2011); TAIR and phytozome public database.

or shrink in presence or absence of water and thus they control the porosity of membranes. Polygalacturonase mutants in *Arabidopsis* showed a higher P_{50} value (−2.25MPa), suggesting a role for pectins in vulnerability to cavitation (Tixier et al., 2013). Mechanically stronger pit membranes thus may resist stretching and expansion of pore membranes indicating a compromise in function. Water stress has been reported to exhibit a direct relation to low lignin synthesis (Donaldson, 2002; Alvarez et al., 2008) although it is not known whether this low lignin help the water transport better.

SUGGESTED GENETIC BASIS OF CAVITATION RESISTANCE BY PIT MEMBRANE MODULATION AND MECHANICAL SUPPORT

Identification of genes and proteins behind the structural and mechanical controls of pit membrane formation has not progressed so far as repair mechanism of embolism is concerned. Genetic aspects of plant hydraulics are little studied, since most of the xylem studies are done in woody trees and study of herbaceous crops is rather scant. It is hard to obtain mutants in trees as the generation time is high, and the study process is long and laborious. Also, hydraulics in plants is not a simple structural or functional trait but is a complex physiological phenomenon. Figuring out the multitrait control switch of this function is thus difficult.

CAN LIGNIN BIOSYNTHESIS BE CONSIDERED AS A CONTROL SWITCH?

Among the living cell processes that may take active part in controlling hydraulics, lignin biosynthesis is a major candidate and highly deciphered. In chemical nature, it is a polymer of phenylpropanoid compounds synthesized through a complex biosynthetic route (**Figure 5**; Hertzberg et al., 2001; Vanholme et al., 2010). Luckily enough, the genes on the metabolic grid are sequenced in plants like *Arabidopsis* and *Populus*, which is helpful to understand their modulation under stress. Till date, both biotic and abiotic stressors have been implicated in modulation of lignin biosynthesis, as well as seasonal, developmental and

Table 1B | The entire aquaporin family in Arabidopsis extracted from TAIR.

Gene family name	Accession	TIGR Protein Type
Delta tonoplast integral protein family	At1g31880	Major intrinsic protein, putative
	At1g80760	Nodulin-like protein
	At1g73190	Tonoplast intrinsic protein, alpha (alpha-TIP)
	At2g45960	Aquaporin (plasma membrane intrinsic protein 1B)
	AT3g06100	Putative major intrinsic protein
	AT5g47450	Membrane channel protein-like; aquaporin (tonoplast intrinsic protein)-like
	AT3g53420	Plasma membrane intrinsic protein 2a
	At2g36830	Putative aquaporin (tonoplast intrinsic protein gamma)
	At2g37170	Aquaporin (plasma membrane intrinsic protein 2B)
	At2g37180	Aquaporin (plasma membrane intrinsic protein 2C)
	AT4g35100	Plasma membrane intrinsic protein (SIMIP)
	At2g29870	Putative aquaporin (plasma membrane intrinsic protein)
	At1g01620	Plasma membrane intrinsic protein 1c, putative
	AT3g61430	Plasma membrane intrinsic protein 1a
	AT3g54820	Aquaporin/MIP–like protein
	At1g17810	Tonoplast intrinsic protein, putative
	AT3g47440	Aquaporin-like protein
	At2g16850	Putative aquaporin (plasma membrane intrinsic protein)
	At2g39010	Putative aquaporin (water channel protein)
	AT3g16240	Delta tonoplast integral protein (delta-TIP)
	At1g52180	Aquaporin, putative
	AT4g23400	Water channel–like protein
	At2g25810	Putative aquaporin (tonoplast intrinsic protein)
	AT4g00430	Probable plasma membrane intrinsic protein 1c
	AT5g37810	Membrane integral protein (MIP)–like
	AT5g37820	Membrane integral protein (MIP)–like
	AT4g17340	Membrane channel like protein
	AT4g10380	Major intrinsic protein (MIP)–like

varietal changes (Anterola and Lewis, 2002; Zhong and Ye, 2009). Representing a large share of non-fossil organic carbon in biosphere, lignification provides mechanical support and defends the plant against pests and pathogens. The mechanical support, further, is mostly linked to xylem vessels and hydraulics.

Lignin is made from monolignols (hydroxy-cinnamyl alcohol), sinapyl alcohol, coniferyl alchol, and *p*-coumaryl alcohol in a smaller quantity. The complex metabolic grid and the transcriptional switches are described in details elsewhere (Hertzberg et al., 2001). The major metabolic pathway channeling into this grid is phenylpropanoid pathways through phenylalanine (Phe). Phe, synthesized in plastid through shikimic acid biosynthesis pathway, eventually generates p-coumaric acid by the activity Phenylalanine Ammonia-Lyase (PAL) and Cinnamate 4-Hydroxylase (C4H). *p*-coumaric acid empties itself into the lignin biosynthesis grid to result into three kinds of lignin units; guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units. Gymnosperm lignin polymer is majorly composed of G and H units, angiosperms show G and S units and H is elevated in compressed softwood and grasses (Boerjan et al., 2003).

There are stresses in nature that change plant lignin content. For example, lignin amount in *Picea abies* is predicted to correlate positively with annual average temperature (Gindl et al., 2000). Temperate monocots as well show an increase of lignin in response to increasing temperature (Ford et al., 1979). In *Triticum aestivum*, 2°C chilling stress decreases leaf lignin but increases in root is observed (Olenichenko and Zagoskina, 2005). Curiously, some studies have shown that although no changes in the levels of lignin or its precursors were observed in plants maintained at low temperatures, there was an increase in related enzyme activities as well as an increase in gene expression. Cold acclimatization in Rhododendron shows upregulation of C3H, a cytochrome P450-dependent monooxygenase without further functional characterization (El Kayal et al., 2006). It has been argued that expression of *C3H* could result in changes in the composition of lignin, altering the stiffness of the cell wall albeit without a definitive proof. The basal part of the maize roots show a growth reduction and low plasticity of cell wall associated with upregulation of two genes in lignin grid (Fan et al., 2006) in response to drought. The increase of free lignin precursors in the xylem sap and reduced anionic peroxidase activity in maize has been associated with low lignin synthesis in drought (Alvarez et al., 2008). It is possible that

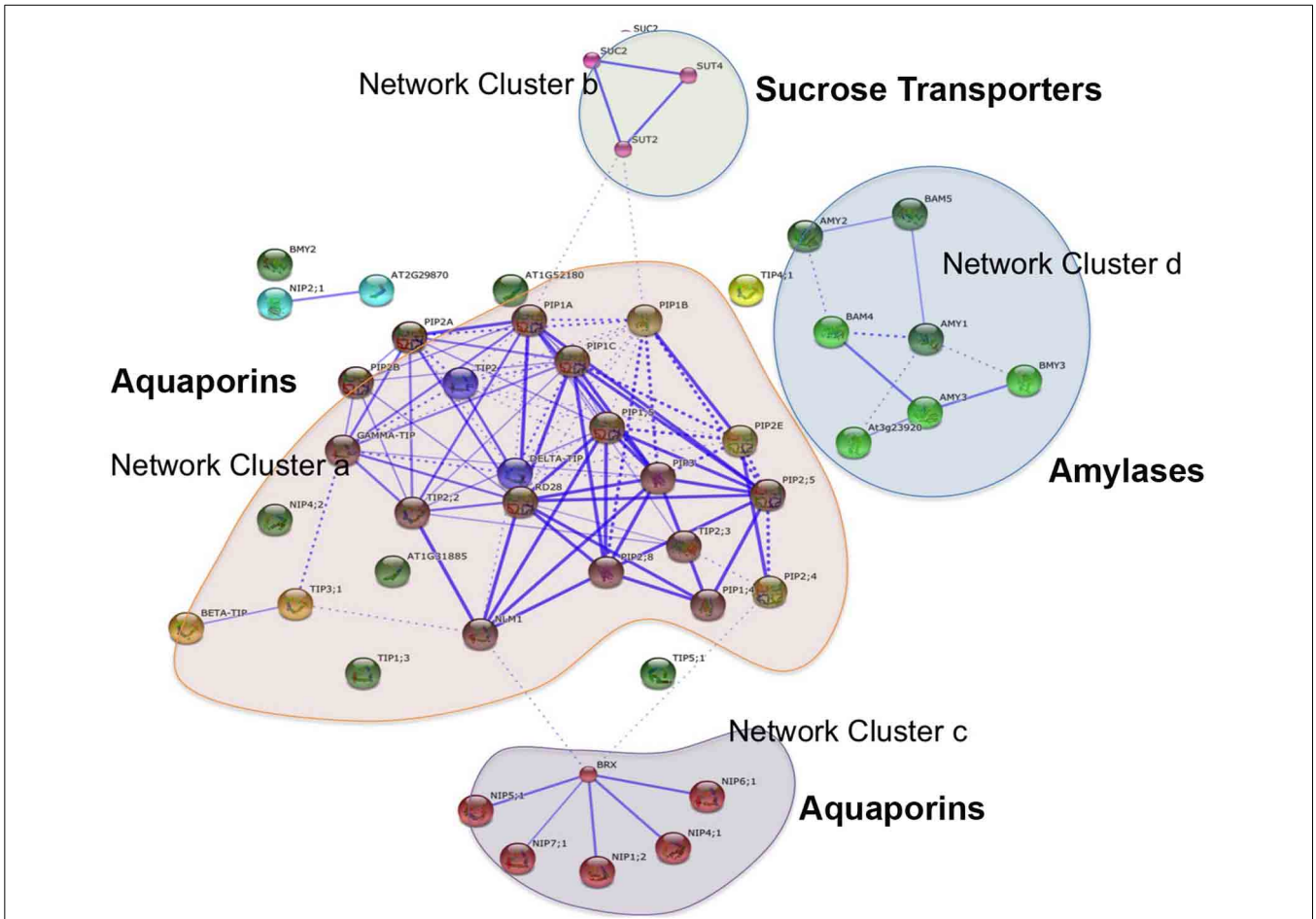

FIGURE 3 | The protein-protein interaction network of Arabidopsis sucrose transporters, amylases and aquaporins, generated using String database. Thicker lines indicate stronger reaction (Szklarczyk et al., 2010).

reducing lignin may directly affect the vascular tissue, encouraging water transport, lowering air seeding and increasing cavitation resistance; however it is not known what share of reduced lignin actually amount to stem vasculature, water column support and pit membrane plasticity.

BIOTECHNOLOGICAL MODIFICATION OF LIGNIN METABOLISM

With the advancement of genomic data, it is now possible to map the genetic changes which may influence hydraulic architecture. However, the model systems are questionable. Among the woody plant species, the genome of poplar has been sequenced; and the lignin biosynthesis network is fully characterized in *Arabidopsis* and rice. It is expected that change in lignin content may result differently in herbaceous and woody plants. There are controversial results obtained so far. In free-standing transgenic poplar trees, a 20–40% reduction in lignin content was associated with increased xylem vulnerability to embolism, shoot dieback and mortality (Voelker et al., 2011). Similarly the severe inhibition of cell wall lignification produced trees with a collapsed xylem phenotype, resulting in compromised vascular integrity, and displayed reduced hydraulic conductivity and a greater susceptibility to wall failure and cavitation (Coleman et al., 2008). A study on the xylem traits of 316 angiosperm trees in Yunnan, and their correlations with climatic factors claimed that wood density and stem hydraulic traits are independent variables (Zhang et al., 2013).

A weak pipeline and less lignification compromises vascular integrity as observed from the above results. On the other hand, low lignin helps to increase the plasticity of the pit membrane pectin. Thus compromising lignin quantity may have serious impact on strength of the vascular cylinder; on the other hand, it may increase the pit membrane hydrophilic property and may offer resistance toward cavitation.

Lately, *Arabidopsis* has been taken in as a model for secondary tissue development, although it lacks formation of secondary wood. Tixier et al. (2013) argued that *Arabidopsis* might be as well considered to be a model of xylem hydraulics. They regarded the inflorescence stem of *A. thaliana* as a model for xylem hydraulics despite its herbaceous habit, as it has been shown previously that the inflorescence stem achieves secondary growth (Altamura et al., 2001; Ko et al., 2004), allows long-distance water transport

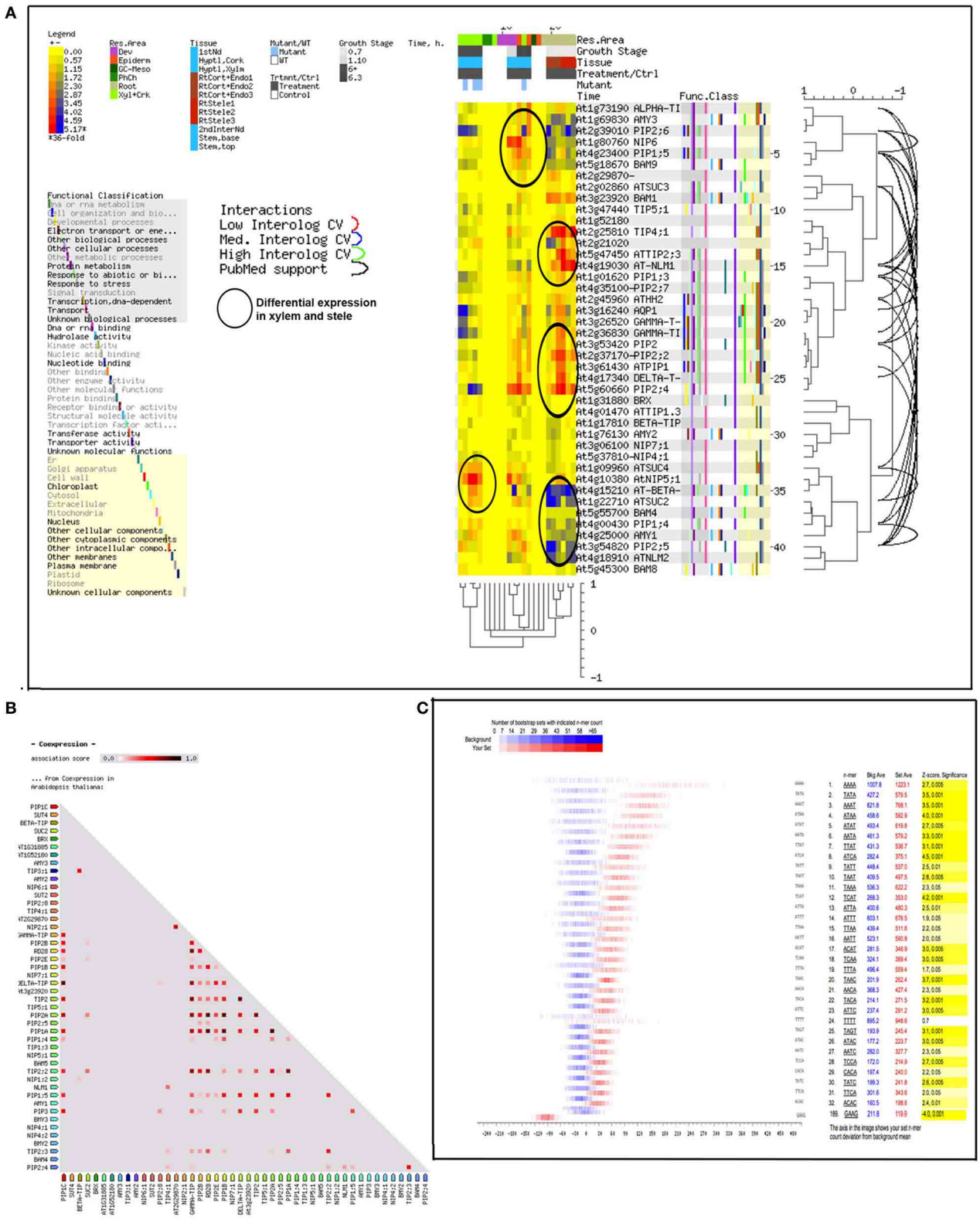

FIGURE 4 | (A) Localization of the genes from **Tables 1**, **2** in various Arabidopsis tissue, from public microarray databases, and e-northern tool at Botany Array Resource (Toufighi et al., 2005). **(B)** Co-expression profile of the genes in Arabidopsis (Szklarczyk et al., 2010). **(C)** Distribution of relevant n-mers in the promoters of the above genes. That may induce shared expression. The results are generated using String and Promomer tools in Botany Array Resource (Toufighi et al., 2005). A tabulated form of the results are presented in Supplementary Table 5.

Table 2 | Representative common n-mer details over represented in the embolism with respective transcription factors and their probable roles.

n-mers	Z-score	Regulation mode	Probable role	Consensus matches to n-mer in the PLACE 25.0.1 database
AAAT**	3.5	Positive	Dehydration responsive	Matched AAAT at offset 4 in CACTAAATTGTCAC 14BPATERD1: "14 bp region" (from −599 to −566) necessary for expression of erd1 (early responsive to dehydration) in dehydrated Arabidopsis
ATAA**	4.0	Positive	Sugar responsive	Matched ATAA at offset 2 in ACATAAAATAAAAAAAGGCA −314MOTIFZMSBE1: located between −314 and −295 region of maize (Z.m.) Sbe1 gene promoter; critical positive cis element; important for the high-level, sugar-responsive expression of the Sbe1 gene in maize endosperm cells; recognized by nuclear protein
ATAT**	2.7	Positive/negative	MADS domain	Matched [AT][AT][AT][AT] at offset 5 in TTDCCWWWWWWGGHAA AGAMOUSATCONSENSUS: binding consensus sequence of Arabidopsis (A.t.) AGAMOUS MADS domain
AATA	3.3	Positive	Sugar-responsive	Matched AATA at offset 6 in ACATAAAATAAAAAAAGGCA −314MOTIFZMSBE1: Located between −314 and −295 region of maize (Z.m.) Sbe1 gene promoter; critical positive cis element; important for the high-level, sugar-responsive expression of the Sbe1 gene in maize endosperm cells; recognized by nuclear protein
TTAT	3.1	Positive	Sugar responsive, binding activity to Myb core	Matched AATA at offset 6 in ACATAAAATAAAAAAAGGCA −314MOTIFZMSBE1: located between −314 and −295 region of maize (Z.m.) Sbe1 gene promoter; critical positive cis element; important for the high-level, sugar-responsive expression of the Sbe1 gene in maize endosperm cells; recognized by nuclear protein; matched TATT at offset 2 in TTTATTTACCAAACGGTAACATC23BPUASNSCYCB1: "23 bp UAS (Upstream activating sequence)" found in the promoter of *Nicotiana sylvestris* (N.s.) CycB1 gene; located between −386 and −409; contains a 5 bp element identical to the MYB binding core (ACGT); required for M-phase-specific expression; binds protein complexes in a cell cycle-regulated manner
ATCA**	4.5	Positive/negative	MADS domain, homeobox binding domain	Matched [AT][AT][ACGT][ACGT] at offset 8 in NTTDCCWWWWWNNGGWAAN AGL1ATCONSENSUS: binding consensus sequence of Arabidopsis (A.t.) AGL1 (AGAMOUS-like 1); AGL1 contains MADS domain; see S000339; AGL20 is a MADS domain gene from Arabidopsis that is activated in shoot apical meristem during the transition to flowering; AGL20 is also regulated by the Gibberellin pathway; complex regulatory net works involving several MADS-genes underlie development of vegetative structures
GAAG**	4.0	Positive	ABA-responsive, MADS	Matched GAAG at offset 6 in ATGTACGAAGC ABAREG2: motif related to ABA regulation; gene: sunflower helianthinin; transacting factor: bZIP? Matched [ACGT][ACGT][AT][ACGT] at offset 0 in NNWNCCAWWWWTRGWWAN AGL2ATCONSENSUS: binding consensus sequence of Arabidopsis (A.t.) AGL2 (AGAMOUS-like 2); AGL2 contains MADS domain; AGL2 binds DNA as a dimer
CGAA	2.4	Positive	ABA-responsive	Matched CGAA at offset 5 in ATGTACGAAGC ABAREG2: motif related to ABA regulation; gene: sunflower helianthinin; transacting factor: bZIP?

*An html table for all n-mers is presented in Supplementary Table 1. **denotes overrepresentation.*

from the roots to the aerial parts of plant, and experience gravity and other mechanical perturbations (Telewski, 2006). There are distinct similarities between woody dicots and *Arabidopsis* inflorescence stems with respect to vessel length and diameter as well as presence of simple perforation plates and border (Sperry et al., 2005; Hacke et al., 2006; Schweingruber, 2006; Wheeler et al., 2007; Christman and Sperry, 2010). It has a genetic potential to develop ray cells and rayless wood is observed in juvenile trees

Table 3 | Some representative transcription factors in *Populus trichocarpa* Xylem Maturation (Dharmawardhana et al., 2010).

WRKY family transcription factor
DRE binding protein (DREB1A)
Ethylene responsive element binding factor
Putative AP2 domain transcription factor
Ethylene responsive element binding factor 4 (aterf4,9)
Homeodomain–like protein.1
Auxin response transcription factor (ARF1,9)
WRKY family transcription factor
ATPAO4 (POLYAMINE OXIDASE 4); amine oxidase
Ethylene-responsive transcriptional coactivator
Lateral root primordia (LRP1)
Transcription factor TINY, putative
MADS-box protein
Putative CCCH-type zinc finger protein
bHLH protein/contains helix-loop-helix DNA binding motif
Zinc finger protein Zat12
WRKY family transcription factor
BEL1-like homeobox 4 protein (BLH4)
TINY-like protein
Myb family transcription factor
Putative squamosa-promoter binding protein
Putative transcription factor/similar to transcription factor SF3
ES43 like protein/ES43 protein
AP2 domain protein RAP2.1
Abscisic acid responsive elements-binding factor (ABF3)
bHLH protein/contains helix-loop-helix DNA binding motif
Myb family transcription factor
CCAAT-binding transcription factor subunit A (CBF-A)

Table 4 | Representative transcriptome studies in literature.

Xylogenesis	Embolism	Lignin biosynthesis
Li et al., 2013	Secchi et al., 2011	Hertzberg et al., 2001
Carvalho et al., 2013		Zhong et al., 2011
Pesquet et al., 2005		Lu et al., 2005
Li et al., 2012		Schrader et al., 2004
Dharmawardhana et al., 2010		
Karpinska et al., 2004		
Bao et al., 2009		
Rengel et al., 2009		
Mishima et al., 2014		
Plavcová et al., 2013		
Zhong et al., 2011		

(Carlquist, 2009; Dulin and Kirchoff, 2010). Having *Arabidopsis* as a full proof model for woodiness may open numerous possibilities. The best among them are study of environmental stresses on hydraulic characters. A number of mutants can be generated and screened in *Arabidopsis* with deviant safety vs. efficiency phenotype with little effort. The *Arabidopsis thaliana* irregular xylem 4 phenotype (*irx4*) a mutant for cinnamoyl-CoA reductase 1 (*CCR1*) gene, has provided us with valuable insight in the role of lignin reduction and associated phenotypic changes in vasculature. As reported by Jones (2001), near-half decrease of lignin component with no associated change in cellulose or hemicellulose content gives the plant an aberrant vascular phenotype. Most of the cell interior is filled up with expanded cell wall and the xylem vessels collapse. Abnormal lignin gives the cell wall a weak ultrastructure and less structural integrity (Jones et al., 2001; Patten et al., 2005). Later it has been claimed that by modulating the *CCR* gene, *irx4* mutant has obtained a delayed albeit normal pattern of lignification program (Laskar et al., 2006). It thus has to be borne in mind that not only the content but the spatio-temporal pattern of lignin deposition may change the xylem ultrastructure and change the safety-efficiency trade-off limit.

There are a few transcriptional control switches in lignin production which can be used in modification of vascular conductance. Modulation of co-ordinate expression of cellulose and lignin in rice is an important study regarding such transgene opportunities. Expression of the *Arabidopsis SHN2* gene (Aharoni et al., 2004) under a constitutive promoter in rice alters its lignocellulosic properties along with introduction of drought resistance and enhanced water use efficiency (Karaba, 2007). The *Arabidopsis* SHINE/WAX INDUCER (SHN/WIN) transcription factor belongs to the AP2/ERF TF family, and besides wax regulation, control drought tolerance in *Arabidopsis* (Aharoni et al., 2004; Broun et al., 2004; Kannangara et al., 2007). Expression analysis of cell wall biosynthetic genes and their putative transcriptional regulators shows that moderated lignocellulose coordinated regulation of the cellulose and lignin pathways which decreases lignin but compensates mechanical strength by increasing cellulose. All the processes ascribed to master control switch SHN may be directed toward evolution of land plants; waxy cover to lignin synthesis for erect disposition and water transport. However, no xylem irregularities are seen in this mutant (Aharoni et al., 2004).

As the best studied pathway related to secondary cell wall formation, lignin biosynthesis should offer the best metabolic grid that can be tweaked in plants to genetically understand mechanical functional trade-off and resistance to cavitation. General reduction of PAL (Phenylalanine ammonia lyase, E.C. 4.3.1.5) activities in developing plants may be one possible point of interest. PAL is a "metabolic branch- point" where Phe is directed toward either lignins or proteins (Rubery and Fosket, 1969). However, according to Anterola et al. (1999, 2002) and other such studies there are other pathways originating from pentose phosphate or glycolysis that may directly end into lignin biosynthesis and PAL may not serve as rate limiting step at all. Cinnamate 4-hydroxylase (C4H) is another candidate that has been downregulated with decrease in overall lignin content, however, with no effect on vascular integrity or function (Fahrendorf and Dixon, 1993; Nedelkina et al., 1999). *p*-Coumarate-3-hydroxylase (C3H) in *Arabidopsis* (CYP98A3) may be necessary and rate-limiting step in the monolignol pathway (Schoch et al., 2001). Its expression is correlated with the onset of lignification and a mutant line results in dwarfed phenotype with reduced lignin (Schoch

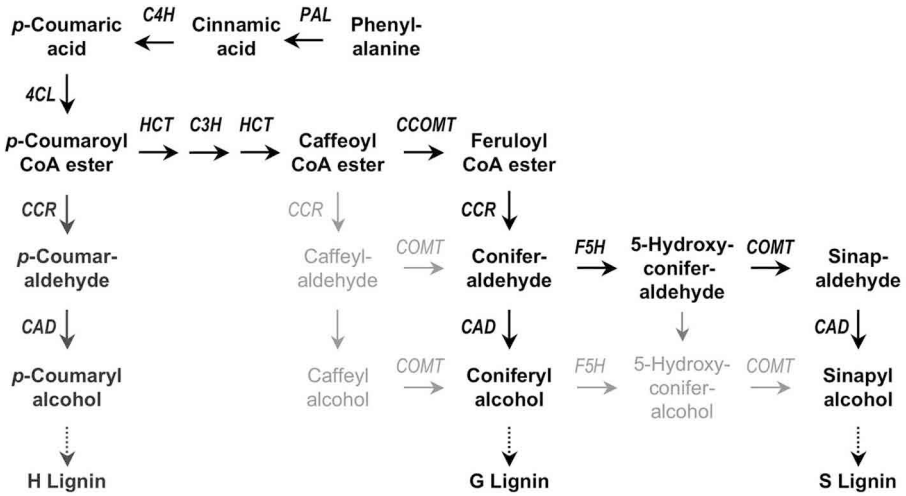

FIGURE 5 | Simplified scheme for monolignol synthesis. The main pathway in dicotyledonous plants is highlighted in black, involving phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate CoA ligase (*4CL*), *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase (HCT), *p*-coumarate 3-hydroxylase (*C3H*), caffeoyl-CoA *O*-methyltransferase (*CCOMT*), hydroxycinnamyl-CoA reductase (*CCR*), ferulate 5-hydroxylase (*F5H*), caffeate *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (*CAD*). Alternate pathways are in light gray. H subunits are only minor lignin components in dicots. Adapted from Quentin et al. (2009).

et al., 2001). Cinnamoyl CoA O-methyltransferase (CCOMT), 4-coumarate:CoA ligase (4CL), cinnamoyl CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD) isoforms are downstream pathways in monolignol formation, and their relation to vascular integrity are yet to establish, though phenotypes associated with their mutations are tall/dwarf stature, altered lignin composition, and reduced mechanical support. Conclusive data are yet to be obtained from these studies.

CONCLUSION

Hydraulic safety margin in a plant is clearly driven by its phylogenetic origin. Conifers have developed minimal hydraulic resistance which is a necessity for water transport through short unicellular tracheids. The unique torus-margo anatomy of the conifer pit membrane let them adaptively overpower multicellular vessels in angiosperms in certain cases. Conifer stems are proposed to have larger hydraulic safety margins when compared with most angiosperm stems (Meinzer et al., 2009; Choat et al., 2012; Johnson et al., 2012) although it is also suggested that they recover poorly from drought-induced embolism (Brodribb et al., 2010). The refilling mechanisms vary greatly between monocots and dicots and herbaceous and woody plants. Resistance to cavitation is thus closely related to many factors: such as nature of the mechanical tissue, the vasculature, the height of the plant, the systematic position of the plant, developmental stage and stresses the plant must face. It can be further emphasized that though, in certain dicots a trade-off within the water transport ability and mechanical strength (efficiency vs. safety) has been observed, the genomic factors which may control the trade-off are not identified till date completely; and the observation is far from universal. The two major physiological phenomena which seem to be linked to embolism resistance are lignification and solute transport between xylem parenchyma, vessel and phloem. The genes and proteins behind these physiological traits are many, and even the obtained transgenic plants and mutants have only been scantily characterized. The effects of assembly of the components are poorly understood and the models proposed do not address all plant families universally. Overall, although a phylogenetic trend is observed among the plants for the evolutionary establishment of hydraulic safety margins, the mechanisms behind have not been understood enough till date to predict the molecular basis and evolution in genomic scale. However, the best metabolic pathway to offer advantageous biotechnological outputs appears to be the lignin synthesis network, which should be assessed by mutant screening as well as by tissue specific overexpression studies in the plant. In case of monocots, drought-induced root- specific overexpression may be of advantage in generating better crops, as root pressure seems to be the major regulator. Crop biotechnology is largely benefitted when the gene pool and their interaction behind a biological process is better known. Overexpressing aquaporins along with the sugar sensing network under a dehydration-responsive promoter could be a formidable strategy to prevent embolism-induced wilting. An approach toward modulation of lignin biosynthesis grid regulation may yield better woody, or even herbaceous crops. The overwhelming knowledge emanating from transcriptomic and genomic studies build the platform where biologists can attempt crop modification for such complex traits as vascular integrity and water transport, without or marginally limiting other beneficial traits, in near future.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00224/abstract

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Integrating omic approaches for abiotic stress tolerance in soybean

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Soybean production is greatly influenced by abiotic stresses imposed by environmental factors such as drought, water submergence, salt, and heavy metals. A thorough understanding of plant response to abiotic stress at the molecular level is a prerequisite for its effective management. The molecular mechanism of stress tolerance is complex and requires information at the omic level to understand it effectively. In this regard, enormous progress has been made in the omics field in the areas of genomics, transcriptomics, and proteomics. The emerging field of ionomics is also being employed for investigating abiotic stress tolerance in soybean. Omic approaches generate a huge amount of data, and adequate advancements in computational tools have been achieved for effective analysis. However, the integration of omic-scale information to address complex genetics and physiological questions is still a challenge. In this review, we have described advances in omic tools in the view of conventional and modern approaches being used to dissect abiotic stress tolerance in soybean. Emphasis was given to approaches such as quantitative trait loci (QTL) mapping, genome-wide association studies (GWAS), and genomic selection (GS). Comparative genomics and candidate gene approaches are also discussed considering identification of potential genomic loci, genes, and biochemical pathways involved in stress tolerance mechanism in soybean. This review also provides a comprehensive catalog of available online omic resources for soybean and its effective utilization. We have also addressed the significance of phenomics in the integrated approaches and recognized high-throughput multi-dimensional phenotyping as a major limiting factor for the improvement of abiotic stress tolerance in soybean.

Keywords: abiotic stress tolerance, soybean, genomics, proteomics, transcriptomics, ionomics, phenomics

INTRODUCTION

Soybean is the most important legume crop which provides sources of oil and protein for human as well as for livestock. Soybean also enhances soil fertility because of the symbiotic nitrogen fixing ability. Soybean contributed to more than 50% of globally consumed edible oil (SoyStats, 2013[1]). Apart from the consumption, soybean oil is being considered as a future source of fuel and efforts are being made to improve soy-diesel production (Candeia et al., 2009). Soybean protein-based bio-degradable materials are also being considered as an alternative for plastics (Song et al., 2011). Soybean products are gaining attention because of its pharmaceutical attributes such as anti-cancerous properties (Ko et al., 2013). Such diverse uses of soybean make it a more widely desired crop plant and are rapidly increasing its demand. In this regard, soybean yield improvement has been achieved by 1.3% per year (Ray et al., 2013). However, the increasing global population will need double the current food production by the year 2050 and at the current rate it can achieve only ∼55% (Ray et al., 2013). It may be more difficult to produce sufficient yield with the changing climate. Therefore soybean yield prediction must consider the ongoing challenges of extreme weather such as drought, flood, heat, cold, frost, and possible UV stress.

Abiotic stresses are the most challenging of all major constraints in crop production. Soybean production is not only influenced by environmental factors, such as drought, water submergence, salt, and heavy metals, but it also faces challenges to get adapted in non-traditional areas. This demands extensive breeding for the development of local cultivars (Tanksley and Nelson, 1996; Grainger and Rajcan, 2013). Direct selection for yield stability based on multi-location trials has been traditionally used for the development of varieties adapted to adverse environmental conditions. This approach is more difficult for abiotic stress related traits because of low heritability and highly influenced by environmental conditions (Manavalan et al., 2009). Direct selection is also a time-consuming and labor intensive process. Strategic marker-assisted breeding can efficiently accelerate the development of tolerant cultivars; however, it also necessitates knowledge about genomic loci governing the traits and the availability of tightly linked molecular markers (Xu et al., 2012). Molecular marker development has been accelerated with the availability of sequenced genomes and organelles in crop plants (Singh et al., 2010; Sonah et al., 2011a; Tomar et al., 2014).

Marker-assisted breeding has become sophisticated with the availability of complete soybean genome sequence due to

[1]Available online at: http://www.soystats.com (Accessed December 10, 2013).

subsequent development of locus-specific molecular markers (Schmutz et al., 2010; Song et al., 2010). Genome-wide high density markers availability also facilitates the haplotype analysis and identification of different alleles for agronomical important traits (Tardivel et al., 2014). Marker-assisted breeding has been carried-out mostly for simple traits governed by a single, or at most a few loci (Shi et al., 2009; Jun et al., 2012). Marker-assisted breeding also suffers due to undesired genetic drag (Tanksley and Nelson, 1996; Shi et al., 2009). The genetic background of the recurrent parent also plays an important role in the phenotypic expression of newly introgressed gene(s) mostly because of the complex epistatic interaction (Palloix et al., 2009). In the case of multiple complex traits, epistatic interaction is more unpredictable and it is hard to develop a strategic breeding plan until unless solid information is available about the molecular mechanisms involved in the trait development. Recent technological development in genomics provides tremendous power to predict genetic factors, their evolution, distribution, and interactions at great extent (Morrell et al., 2011; Sonah et al., 2011b). Genetic engineering is the most advanced approach that has been used for the genetic improvement of soybean. Genetically modified (GM) soybean crops for insect-resistance and herbicide-tolerance has covered most of the cultivated area in the world (Carpenter, 2010). Although, GM soybean has proven to be very successful, it raises ethical controversies, and it is available only for few traits (Carpenter, 2010). Integration of multi-disciplinary knowledge is required to design future soybean varieties with ideal plant types providing high and stable yield in adverse climatic conditions. In this context, a detailed review was made to evaluate progress achieved in different omic approaches and to highlight future perspectives for its effective exploration toward the development of abiotic stress tolerant soybean cultivars.

OMICS APPROACHES IN THE TECHNOLOGICAL ERA

Plant molecular biology aims to study cellular processes, their genetic control, and interactions with environmental changes. Such a multi-dimensional and detailed investigation requires large-scale experiments involving entire genetic, structural, or functional components. These large scale studies are called "omics." Major components of omics include genomics, transcriptomics, proteomics, and metabolomics (**Figure 1**). These omics approaches are routinely used in various research disciplines of crop plants, including soybean. Omics approaches have improved very rapidly during the last decade as technology advances. Subsequently, high-throughput data developed by omic experiments require extensive computational resources for storage and analysis. Thus, several online databases, analysis servers, and omics platforms have been developed. Omics is getting broader coverage and it is anticipated that several new omic fields will evolve in near future.

GENOMICS ADVANCES FOR ABIOTIC STRESS TOLERANCE IN SOYBEAN

MOLECULAR MARKER RESOURCES

Genomic applications in soybean have become more standard with the availability of whole genome sequence (WGS) (Schmutz et al., 2010). The WGS provided the basis for the development of thousands of simple sequence repeat (SSR) markers and millions of single nucleotide polymorphism (SNP) markers (Song et al., 2010; Sonah et al., 2013). Recent developments in next generation sequencing (NGS) technologies make sequencing-based genotyping cost effective and efficient. Three main complexity reduction methods, namely Reduced Representation Libraries (RRLs), Restriction site Associated DNA (RAD) sequencing, and Genotyping-by-Sequencing (GBS) are being routinely used. Among these, GBS is gaining more attention because of its simplified and cost effective methodology (Elshire et al., 2011; Sonah et al., 2012). The GBS approach has been successfully used in several crop species (Poland and Rife, 2012). Recently, GBS methodology has been improved and streamlined for soybean (Sonah et al., 2013). However, sequencing-based genotyping methods require computational expertise and significant time for data analysis. This restricts its use in marker-assisted breeding where timely selection is very important. GBS will be widely used in the future with an increasing number of software packages and computational pipelines (Sonah et al., 2013).

Technological advances have also provided a high-throughput, reliable, and quick array-based genotyping platforms. The SNP array development require initial information about SNPs, fortunately, information about millions of SNPs is already available in the public domain (**Table 1**). The Illumina Infinium array (SoySNP50K iSelect BeadChip) for ∼50,000 SNPs has been successfully developed and used for the genotyping of several soybean plant introduction (PI) lines (Song et al., 2013). Technological advances beyond this make it possible to re-sequence hundreds of lines in a cost effective manner and has started a new era of genotyping by re-sequencing (Lam et al., 2010; Li et al., 2013; Xu et al., 2013). Now, the challenge for plant biologists is how to effectively use these resources for marker-assisted applications.

QTL MAPPING FOR ABIOTIC STRESS TOLERANCE IN SOYBEAN

Genetic fingerprinting, linkage mapping, and quantitative trait loci (QTL) mapping are marker based applications that have become more sophisticated with the availability of different genotyping platforms (**Table 1**). Consequently, several efforts have been made to identify QTL for abiotic stress tolerance in soybean (Table S1). QTL studies have identified thousands of QTL spanning the entire genome (www.soykb.org, www.soybase.org). This is due to the complex inheritance of abiotic stress tolerance which has identified unstable QTL across different environments. Further utilization of QTL information for marker-assisted breeding or candidate gene identification has become difficult due to this complexity. Statistical tools such as "Meta-QTL analysis" have been advanced that compile QTL data from different studies together on the same linkage map for identification of precise QTL region (Deshmukh et al., 2012; Sosnowski et al., 2012). Several efforts have been performed to identify meta-QTL for different agronomical and quantitative traits in soybean (**Table 2**). Meta-analysis studies are still required exclusively for abiotic traits.

GENOME-WIDE ASSOCIATION STUDIES (GWAS) IN SOYBEAN

QTL mapping using bi-parental populations has limitations because of restricted allelic diversity and genomic resolution.

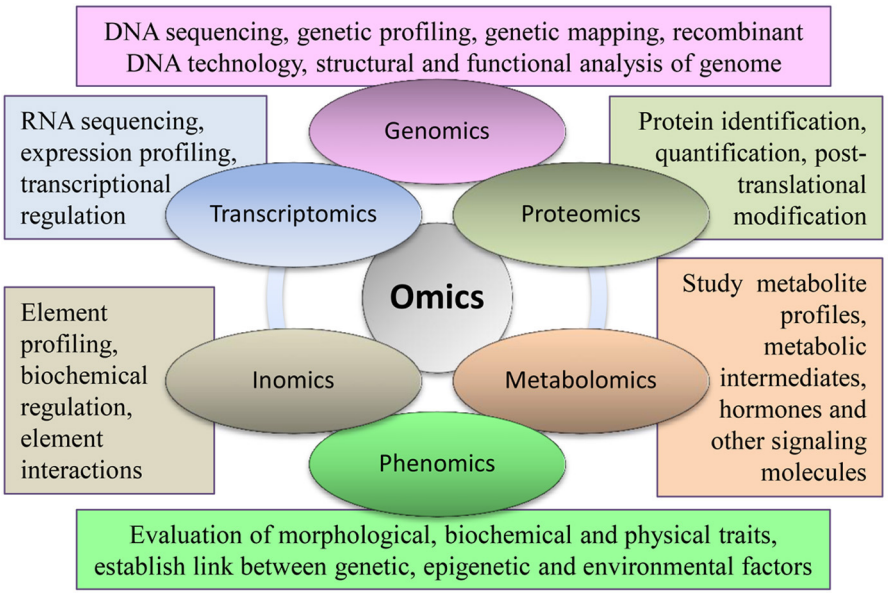

FIGURE 1 | Important branches of omics with their major components being used in different integrated approaches in soybean.

Table 1 | List of significant studies performed to develop SNP markers and subsequent genotyping using different technological platforms in soybean.

Sr. No	Genotyping platform/Approach	Genotypes	SNPs	References
1	Illumina GoldenGate assay	3 RIL mapping populations	384	Hyten et al., 2008
2	Illumina Infinium SoySNP6K BeadChip	92 RILs	5376	Akond et al., 2013
3	Illumina genome analyzer/Reduced Representation Libraries (RRLs)	5 diverse genotypes	14,550	Varala et al., 2011
4	Illumina GoldenGate assay	3 RIL mapping populations	1536	Hyten et al., 2010b; Vuong et al., 2010
5	Illumina genome analyzer /RRLs	444 RILs	25,047	Hyten et al., 2010a
6	Illumina GAIIx/Genotyping by sequencing (GBS)	8 diverse genotypes	10,120	Sonah et al., 2013
7	Illumina Genome Analyzer II/whole genome re-sequencing	17 wild and 14 cultivated	2,05,614	Lam et al., 2010
8	Illumina Genome Analyzer II/whole genome re-sequencing	25 diverse genotypes	51,02,244	Li et al., 2013
9	Illumina genome analyzer/RRLs	Parental lines of mapping population	39,022	Wu et al., 2010
10	Illumina Infinium BeadChip	96 each of landraces, elite cultivars and wild accessions	52,041	Song et al., 2013

The allelic diversity can be increased to some extent by using multi-parental crosses. Recently, Multi-parent Advanced Generation Inter-Cross populations (MAGIC) has been used to identify QTL for blast and bacterial blight resistance, salinity and submergence tolerance, and grain quality traits in rice (Bandillo et al., 2013). Such multi-parental populations has mapping resolution limitations since it depends on meiotic events (crossing-over) (Kover et al., 2009). In contrast, the genome-wide association study (GWAS) approach provides opportunities to explore the tremendous allelic diversity existing in natural soybean germplasm. Mapping resolution of GWAS is also higher since millions of crossing events have been accumulated in the germplasm during evolution.

GWAS is routinely being used in many plant species, but only a few studies have been reported in soybean (Table S2). These studies were performed with limited markers and genotypes. GWAS in soybean is lagging behind compared to maize, mostly because of the slow linkage disequilibrium (LD) decay (Hyten et al., 2007; Mamidi et al., 2011). Another serious problem is the confounding population structure since it may cause spurious associations leading to an increased false-discovery rate (FDR). Studies that involve case-control phenotypes (binary) carefully relate the cases and controls to minimize confounding effects.

Table 2 | Meta-QTL studies performed for different traits in soybean.

Sr. No	Trait	Meta QTL	QTL compiled	Studies compiled	References
1	Soybean cyst nematode resistance	7	62	17	Guo et al., 2006
2	Soybean cyst nematode resistance	16	151	19	Zhang et al., 2010
3	Seed oil content	20	121	22	Qi et al., 2011b
4	Seed oil content	25	130	39	Qi et al., 2011a
5	100-seed weight	17	65	12	Zhao-Ming et al., 2009
6	100-seed weight	15	117	13	Sun et al., 2012a
7	Fungal disease resistance	23	107	23	Wang et al., 2010
8	Insect resistance	20	81	–	Jing et al., 2009
9	Seed protein content	23	107	29	Zhao-Ming et al., 2011
10	Plant height	12	93	13	Sun et al., 2012b
11	Phosphorus efficiency	29	96	–	Huang et al., 2011
12	Growth stages	9	98	10	Qiong et al., 2009

GWAS for quantitative traits like abiotic stress tolerance are predictable to be affected by a confounding population. Different models have been developed for population stratification and spurious allelic associations like MLM and CMLM which takes into account the population structure and kinship. Recently, GWAS for *Sclerotinia sclerotiorum* resistance was performed using 7864 SNPs in soybean (Bastien et al., 2014). The study provided details of a probable marker requirement and methodologies involving population stratification for effective GWAS (Bastien et al., 2014). Development in statistical tools, genotyping methods, and studies involving larger sets of genotypes will definitely improve GWAS power in soybean.

GENOMIC SELECTION (GS) IN SOYBEAN

Marker-assisted breeding for simple Mendelian traits are easy and effective, but it can be problematic for the complex traits such as abiotic stresses that are generally polygenic. Even major QTLs can explain only a small fraction of phenotypic variation and may show unexpected trait expression in new genetic backgrounds because of epistatic interactions. These limitations can be effectively addressed by the use of an approach called "Genomic-selection" (GS). GS is relatively simple, more reliable, and a more powerful approach where breeding values of lines are predicted using their phenotypes and marker genotypes (Heffner et al., 2009). GS is more effective since it uses all marker information simultaneously to develop a prediction model avoiding biased marker effects (Heffner et al., 2009). GS captures small-effect QTL that governs most of the variation including epistatic interaction effects.

An overview of research articles regarding GS published during last decade showed exponential growth within recent years (Figure S1). The increasing popularity of GS among plant as well as animal breeders is mostly because of the reduced cost of genotyping. Currently, GS is being used for breeding in several different crops (Table S3). In soybean, efforts have been made to evaluate GS using different models. A GS study in soybean has used 126 recombinant inbred lines and 80 SSR markers to predict primary embryogenesis capacity which is a highly polygenic trait (Hu et al., 2011). In this report, high correlation ($r^2 = 0.78$) has been observed among the genomic estimated breeding value (GEBV) and the phenotypic value. Another study published recently using 288 cultivars and 79 SSR markers, found a correlation coefficient of 0.90 among the GEBV and the phenotypic value (Shu et al., 2012). Both the reports have shown high accuracy of prediction but only with a few markers and genotypes. Predicting the accuracy of GS will need more investigations involving high-throughput genotyping of larger populations evaluated across different environments.

Accuracy of GS largely depends on genetic × environmental (G × E) interaction but most of the studies focused only on an estimation of the main effect for each marker. These multi-environmental trials are of prime importance for plant breeding not only to study G × E but especially to increase the number of breeding cycles per year. The challenge for GS is to get accurate GEBV in respect to the G × E effect. Considering environmental effects is not new for plant breeders and most statistical models used for multi-location trials do reflect G × E (Hammer et al., 2006). It is also more common in QTL mapping studies where QTL × environment interaction evaluations were utilized to estimate QTL effect.

Improved factorial regression models have been proposed recently for GS that consider stress covariates derived from daily weather data (Heslot et al., 2014). This model has shown increased accuracy by 11.1% for predicting GEBV in unobserved environments where weather data is available (Heslot et al., 2014). This study suggests possible utilization of phenotypic data and historical data of weather conditions accumulated over decades in different soybean breeding programs. Similar information can be used for abiotic stress tolerance improvement in soybean.

COMBINING MARKER-ASSISTED BREEDING WITH GENOMIC SELECTION

Molecular marker genotyping is a common requirement for QTL mapping, GWAS, and GS and can be the basis for combining these approaches (**Figure 2**). Most of the GS studies have used recombinant inbred line (RIL) populations to train the prediction model (Table S3). Therefore, GS and QTL mapping can be performed simultaneously. A set of diverse cultivars can be used for GWAS and GS all together (Table S3). In the marker-assisted breeding, introgression of QTL or GWAS loci to well adapted cultivar

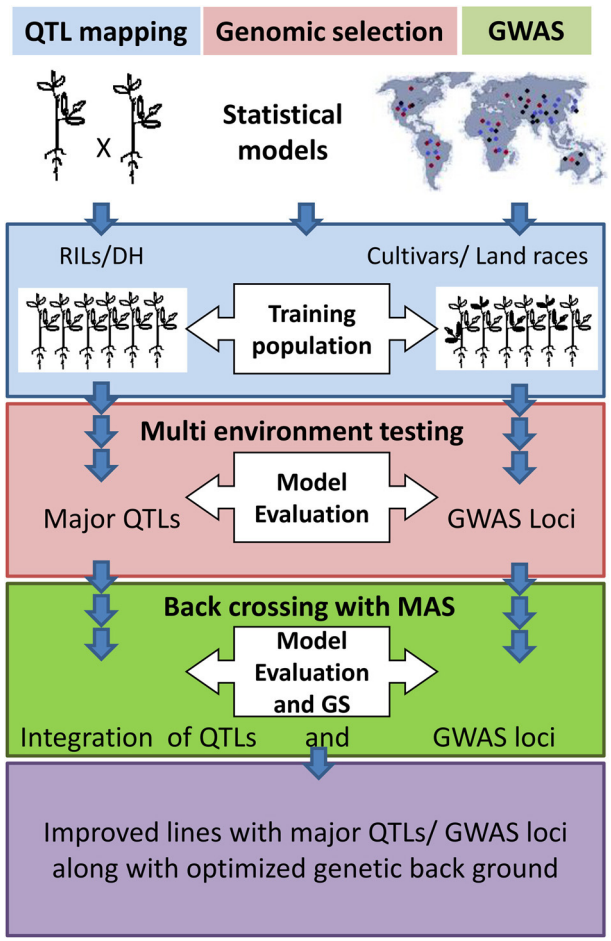

FIGURE 2 | Combined approach of QTL mapping/Genome-wide association study (GWAS) and Genomic selection (GS).

is performed. The donor line (for QTL or GWAS loci) may be wild or low yielding line. Therefore, several cycles of backcrossing are performed to retain the genetic background of the recipient parent (the adapted cultivar) except for the QTL/GWAS loci which represent the donor background. Nevertheless, GS does not provide control over the genetic background and this may be problematic when the donor is not an adapted line. In addition, GS cannot guarantee for major QTL which are already known. Therefore, information about QTL/GWAS loci should be incorporated with GS models so that the balance of genetic background can be made along with maximum gain of breeding value.

TRANSCRIPTOME PROFILING FOR ABIOTIC STRESS TOLERANCE

Plants, including soybean, responses to external environments is very complex. A wide range of defense mechanisms are activated that increases plant tolerance against adverse conditions in order to avoid damage imposed by abiotic stresses. The first step toward stress response is stress signal recognition and subsequent molecular, biochemical, and physiological responses activated through signal transduction (Komatsu et al., 2009; Ge et al., 2010; Le et al., 2012). Understanding such responses is very important for effective management of abiotic stress. Transcriptome profiling provides an opportunity to investigate plant response regulation and to identify genes involved in stress tolerance mechanisms. Earlier, approaches using expressed sequence tags (ESTs) sequencing along with several techniques, such as suppression subtractive hybridization (SSH), have been extensively used for transcriptome profiling of soybean under abiotic stress conditions (Clement et al., 2008). In addition, information of ESTs have been used to develop spotted microarrays (O'Rourke et al., 2007). These techniques are efficient but do not ensure analysis of entire genes in the soybean genome. Several high-throughput techniques have been developed for transcriptome analysis due to the advancement in sequencing technology and the availability of the whole soybean genome sequence, (Libault et al., 2010; Schmutz et al., 2010; Cheng et al., 2013). These platforms have been extensively used for transcriptome profiling to uplift abiotic stress tolerance mechanisms in soybean (**Table 3**).

Microarray is a high-throughput technology where thousands of probes representing different genes are hybridized with RNA samples. Using the hybridization signal level, gene expression is calculated. The Affymetrix GeneChip representing 61K probe sets is routinely being used for transcriptome profiling of soybean under different abiotic stresses (Haerizadeh et al., 2011; Le et al., 2012). The normalized expression data generated using the Affymetrix GeneChip can be used to compare soybean experiments performed across the world. An expression database has been developed to globally explore public and proprietary expression data (www.genevestigator.com). The microarray data represents various tissues, developmental stages, and environmental conditions (**Table 3**). Effective analysis of such tremendous data using sequence homology and functional annotation will be helpful to understand biological processes.

RNA-Seq, AN ADVANCED APPROACH FOR TRANSCRIPTOME PROFILING

Cost effective and high-throughput sequencing technologies make it possible to analyze transcriptomes by sequencing, known as RNA-seq. The RNA-seq approach has several advances over the microarray technology where available genomic information is used to design probe sets. However, RNA-seq does not require gene information and is capable of identifying novel transcripts that were previously unknown and also provides opportunities to analyze non-coding RNAs. The relative accuracy of microarrays and RNA-Seq has been evaluated using proteomics and it has been shown that RNA-Seq provides a better estimate of absolute expression levels (Fu et al., 2009). Applications of RNA-seq can be expanded further with an increased understanding of molecular regulations. For instance, RNA-seq is being used for transcription start site mapping, strand-specific measurements, gene fusion detection, small RNA characterization, and detection of alternative splicing events (Ozsolak and Milos, 2010).

RNA-Seq has been performed to investigate seven tissues and seven stages in seed development in soybean (Severin et al., 2010). This effort has generated an expression atlas for soybean genes which serves as a useful resource. The tissue specific expression pattern of genes is helpful in understanding regulation and tissue specific function.

Table 3 | Major transcriptomic analysis for the abiotic stress tolerance in soybean using different technological platforms.

Sr. No.	Trait/tissue	Platform	DEG*	Key points	References
1	Soybean root development/root tips and non-meristematic tissue	Affymetrix chips containing 37,500 probe sets	9148	Resource of novel target genes for further studies involving root development and biology	Haerizadeh et al., 2011
2	Iron stress/root from isogenic lines	Custom array containing 9728 cDNAs	48	Genes involved in DNA repair and RNA stability were induced	O'Rourke et al., 2007
3	Drought stress at late developmental stages/V6 and R2 stages under drought and control	61 K Affymetrix Soybean Array GeneChip	3276 for V6 3270 for R2	Expression of many *GmNAC* and hormone-related genes was altered by drought in V6 and/or R2 leaves	Le et al., 2012
4	Herbicide resistance/plant under atrazine and bentazon stress	cDNA microarray with 36,760 different cDNA clones	6646	Expression of genes related to cell recovery, such ribosomal components	Zhu et al., 2009
5	Saline-alkaline stress tolerance/NaCl and $NaHCO_3$ treatments	AffymetrixSoybean GeneChip	9027	Genes with altered expression regulated by alkaline stress	Ge et al., 2010
6	Flooding stress	HiCEP (29,388) high coverage expression profiling	97 genes and 34 proteins	Combined approach with proteomics	Komatsu et al., 2009

**Differentially expressed genes.*

COMBINING QTL MAPPING, GWAS, AND TRANSCRIPTOME PROFILING

QTL mapping and GWAS are very effective approaches to identify chromosomal region(s) associated with a particular phenotype. However, QTL spans large segments of chromosomes and it is also the same for GWAS where LD decay is slow as in case of soybean (Hyten et al., 2007). QTL or GWAS loci possess hundreds of genes that make the identification of candidate genes difficult (Sonah et al., 2012). This is similar in transcriptome profiling where thousands of genes have been found to be differentially expressed even with genetically similar isogenic lines (**Table 3**). Therefore combining QTL mapping or GWAS with transcriptome profiling will complement each other. For instance, candidate genes for grain number QTL in rice have been identified using microarray based transcriptome profiling of recombinant inbreed lines with contrasting phenotypes (Deshmukh et al., 2010; Sharma et al., 2011; Kadam et al., 2012). Similarly, a pair of soybean near-isogenic lines (NILs) differing in seed protein and an introgressed QTL segment (~8.4 Mb) have been used to study variation in transcript abundance in the developing seed (Bolon et al., 2010). The study identified 13 candidate genes in the QTL region using the Affymetrix Soy GeneChip and high-throughput Illumina whole transcriptome sequencing (Bolon et al., 2010). A combined approach of mapping and transcriptome profiling is based on an assumption that the quantitative trait is regulated by differential expression of candidate genes. This is not always true. Most of the time sequence variation present in candidate genes may cause defective proteins (Xu et al., 2013). Therefore, re-sequencing of QTL locus along with transcriptomics will also be a valuable approach to compliment mapping efforts.

PROTEOMICS IN SOYBEAN

Proteomics deals with structural and functional features of all the proteins in an organism. It is important to understand complex biological mechanisms including the plant responses to abiotic stress tolerance. Abiotic stress tolerance mechanisms involve stress perception, followed by signal transduction, which changes expression of stress-induced genes and proteins. Post-translational changes are also important in plant responses to abiotic stresses. A single gene can translate in several different proteins and a few genes can lead to a diverse proteome. Such inconsistency limits genomics and transcriptomic approaches more specifically, when post translational changes govern phenotype. Differential expression observed at the transcriptional (mRNA) level need not be translated into differential amounts of protein. To address this, several proteomic studies have been performed to understand abiotic stress tolerance mechanisms in soybean (Table S4).

Unexpected levels of changes in the soybean proteome can occur during stress response and these changes can lead to different defense mechanisms. Some common proteins involved in redox systems, carbon metabolism, photosynthesis, signaling, and amino acid metabolism have been found to be associated with various stress responses in soybean (Zhen et al., 2007; Aghaei et al., 2009; Yamaguchi et al., 2010; Qin et al., 2013). These candidate proteins can directly link to genetic regulation of stress response in soybean. Candidate protein information can be used for the functional annotation of genes present in QTL regions or found differentially expressed under stress conditions.

In the near future, various proteomics approaches will be routinely used in soybean research that will generate tremendous information regarding structural and functional attributes of proteins. A systematic cataloging of information in the form of a publically accessible database is very important. Recently, a proteome database has been developed that contains reference maps of the soybean proteome collected from several organs, tissues, and organelles (Mooney and Thelen, 2004; Brechenmacher

et al., 2009; Ohyanagi et al., 2012). Presently, these reference maps comprised information of about 3399 proteins from seven organs and 2019 proteins from four subcellular compartments that were identified using two-dimensional electrophoresis (http://proteome.dc.affrc.go.jp/soybean/). Volunteer deposition of proteomic information in such databases is necessary for effective utilization of available knowledge for the management of abiotic stress tolerance in soybean.

METABOLOMICS ADVANCES FOR ABIOTIC STRESS

Metabolomic studies in plants aim to identify and quantify the complete range of primary and secondary metabolites involved in biological processes. Therefore metabolomics provides a better understanding of biochemical pathways and molecular mechanisms. The knowledge of genes, transcripts and proteins involved cannot alone help to understand the biological process completely until knowledge of metabolites that are involved becomes available.

Several metabolomics studies have been performed to understand biochemical processes in soybean (Table S5). Development of new chromatographic and mass spectrometric platforms along with the enhancement of operational and analytical capabilities of existing platforms revolutionizes metabolomic investigations both in plant and animal sciences. The platforms such as gas chromatography mass spectrometry (GC-MS), fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), liquid chromatography mass spectrometry (LC-MS), capillary electrophoresis mass spectrometry (CE-MS), and nuclear magnetic resonance (NMR) are routinely used in plant sciences (Putri et al., 2013). Capability, limitations and specificity of these techniques has been recently reviewed in terms of effective utilization of these advanced resources (Putri et al., 2013). In-depth accurate analyses of metabolite information including the spectral data are the major challenge for the use of high-throughput techniques. Several statistical models and bioinformatics programs have been developed to analyze the metabolome in an interactive manner (Fernie et al., 2011; Putri et al., 2013).

IONOMICS IN SOYBEAN

Ionomics is the study of elemental composition of an organism that mostly deals with high-throughput identification and quantification. Ionomics is important to understand element composition and their role in biochemical, physiological functionality and nutritional requirements of plants. Phosphorus (P) and potassium (K) are the two key elements used as macronutrients in fertilizer to ensure better crop yield. However plants require many other elements and those are not uniformly distributed among different soil types. Plants have evolved with a diverse element uptake ability at different locations because of diverse soil types (Fujita et al., 2013). This justifies the need of integrating ionomics with genomics to explore existing genetic differences. An ionomic study has been performed to analyze concentrations of 17 different elements in diverse accessions and three RIL populations of Arabidopsis thaliana grown in several different environments (Buescher et al., 2010). Significant differences in elemental composition between the Arabidopsis accessions were detected and more than hundred QTL were identified for different elemental accumulation (Buescher et al., 2010). Most of the ionomics studies to date in soybean have been performed to analyze nutritive value of soybean products (Table S6).

The elemental composition of a plant is controlled by multiple factors including element availability, uptake capability of roots,

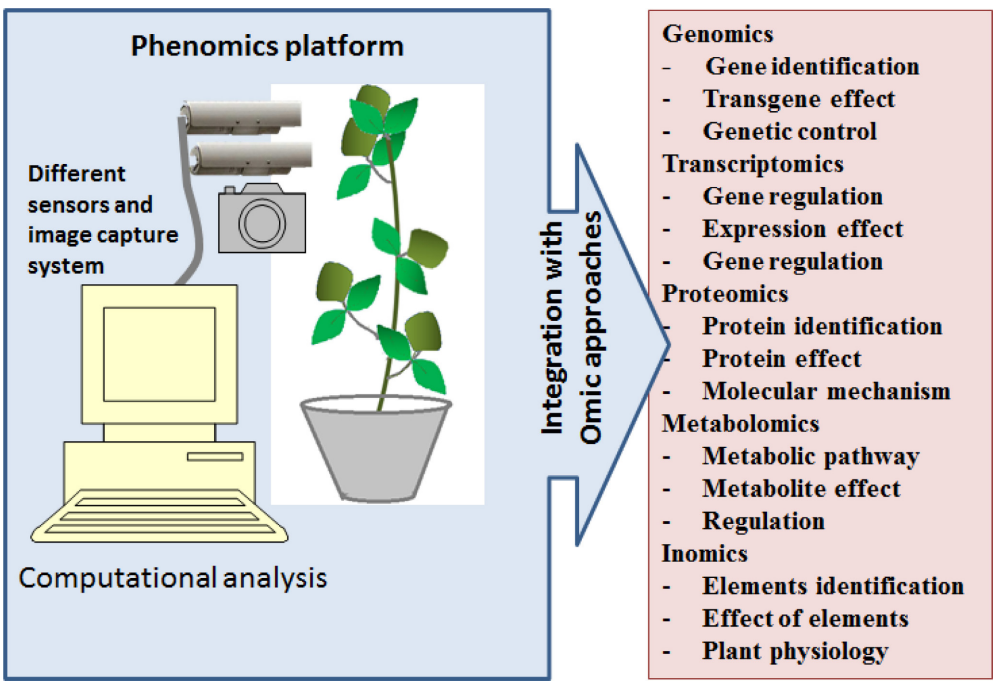

FIGURE 3 | Phenomics and its integration with other omics approaches.

transport, and external environment which regulate physiological processes such as evapotranspiration. Because of such factors, the plant ionome has become very sensitive and specific so that the element profile reflects different physiological states. Recently a study performed in barley has analyzed ionome of wild accessions and cultivar differing in salt tolerance, grown in presence of 150 and 300 mM NaCl (Wu et al., 2013) and observed decreased amounts of K, magnesium (Mg), P and manganese (Mn) in roots and K, calcium (Ca), Mg and Sulfur (S) in shoots at the seedling stage. In addition, significant negative correlation among the amount of accumulated Na and metabolites involved in glycolysis and tricarboxylic acid (TCA) cycle have been observed (Wu et al., 2013). This ionomic study suggests the possible rearrangement of elemental profiles and metabolic processes to modify the physiological mechanisms of salinity tolerance.

Improvement in abiotic stress tolerance with the application of several inorganic element has been observed (Liang et al., 2007; Pilon-Smits et al., 2009). For instance, silicon (Si) has shown beneficial effects against different abiotic stresses including high salinity, water stress, heavy metal stress, and UV-b (Liang et al., 2007). Previously, soybean has been considered as poor accumulator of silicon mostly because of the genetic differences existing in the germplasm and very few genotypes have been evaluated to draw this conclusion (Hodson et al., 2005). However, with the advancement in ionomics technologies, silicon transporter genes have been identified recently in soybean using the integrated omics approach (Deshmukh et al., 2013). This study has used computational genomics, transcriptomics, and ionomics information available in the model plant species such as Arabidopsis and rice. Besides this, high-throughput efforts for maximum number of elemental profiles in soybean in respective external environment are required. That will definitely improve the understanding of the soybean ionome and its subsequent utilization in the management of abiotic stress tolerance.

PHENOMICS PROSPECTIVE IN SOYBEAN

The phenotype is a physical and biochemical trait of an organism. Phenomics is a study involving high-throughput analysis of phenotype. Phenotype is the ultimate resultant from the complex interactions of genetic potential between an organism and environment. Precision phenotyping is important to understand any biological system. In plant as well as animal sciences, a particular phenotype (as symptoms) is used to understand biological status, such as disease, pest infestation or physiological disorders. With technological advances, genomic resources have been routinely used to predict phenotype based on the evaluation of genetic markers; it can be called "genetic symptoms." The success of genomics is based on how reliable connection is there between a genetic marker and the phenotype. In plant breeding, genetic improvement through omics approaches is being conducted to achieve ideal phenotype that will ensure higher and stable yield under diverse environmental conditions. Therefore phenomics integrated with other omics approaches has the most potential in the plant breeding (**Figure 3**).

Phenome has a broader meaning than what is being generally considered. It is not limited to the visible morphology of an organism but expectedly larger and complex. Unlike genomics, where the entire genome can be characterized by sequencing, the phenome cannot be characterized entirely. Therefore, the term phenomics being an analogy to genomics expected only study of particular set of phenotype at high-throughput level and not the entire set. In this regards, the technological development in image processing and the automation techniques have played important roles. Plant imaging with light sources from visible to near infrared spectrum provides an opportunity for non-destructive phenotyping. Therefore, real-time analysis of plant development became possible. Moreover, robotic technologies used in phenomic platforms have increased the precision and speed of phenotyping. This has allowed for incorporating additional aids such as precise irrigation and fertilization systems. For instance, "PHENOPSIS" an automated phenomic platform has been developed to study water stress in Arabidopsis and has a robotic arm loaded with a tube for irrigation and a camera (Granier et al., 2006). These types of advanced phenomic platforms have been developed and made available for wider range of crop plants (www.lemnatec.com). However, these platforms have not gained the expected popularity even though tremendous advancement in both imaging as well as robotic technology has been achieved.

In soybean, several phenomic efforts have been performed but most of these are pilot experiments (Table S7). Recently, a method has been developed to assess leaf growth in soybean under different environmental conditions (Mielewczik et al., 2013). This method can utilize different light sources that are available in a greenhouse as well as under field conditions. Marker tracking approaches (Martrack Leaf) have also been used to facilitate accurate analysis of two-dimensional leaf expansion with high temporal resolution (Mielewczik et al., 2013). Apart from this, phenomics has been used to facilitate efficient identification of soybean cultivars which is very important for germplasm resource management and utilization (Zhu et al., 2012). Zhu et al. (2012), used a laser light back-scattering imaging technology to analyze single seed. Images of laser light illuminated the soybean seed surface were captured by a charge-coupled device (CCD) camera. The characteristic pattern of laser luminance is analyzed by image processing technology to identify a particular cultivar. Such characteristic of laser light back-scattering can be used to assess quality and other seed characteristics as markers for selection in breeding programs.

Phenomics in soybean is lagging far behind genomics because hundreds of genomes and many genetic populations are re-sequenced. One best example is the 1000 genome re-sequencing project at the University of Missouri, MO, USA (http://soybeangenomics.missouri.edu/news2012.php). The 1000 genome project will generate a huge amount of genomic information which will require utilization of comparable phenomic data. This will be helpful to accelerate soybean research in many ways.

ROLE OF ONLINE DATABASES FOR EFFECTIVE INTEGRATION OF OMICS PLATFORMS

The recent advancement in the omic platforms has generated tremendous information which has been used to promote research activities in all possible dimensions. Utilization of available information has become possible because of computational resources that helps to catalog, store, and analyze available

Table 4 | Online databases exclusively developed to host soybean research data generated from different omics platforms.

Sr. No	Database	Features	Tools
1	SoyBase SoyBase and the Soybean Breeder's Toolbox, USDA and Iowa University, http://soybase.org/	Genetic and physical maps, QTL, Genome sequence, Transposable elements, Annotations, Graphical chromosome visualizer	BLAST search, ESTs search, SoyChip Annotation Search, Potential Haplotype (pHap) and Contig Search, Soybean Metabolic Pathways, Fast Neutron Mutants Search, RNA-Seq Atlas
2	SoyKB Soybean Knowledge Base, University of Missouri, Columbia, http://soykb.org/	Multi-omics datasets, Genes/proteins, miRNAs/sRNAs, Metabolite profiling, Molecular markers, information about plant introduction lines and traits, Graphical chromosome visualizer	Germplasm browser, QTL and Trait browser, Fast neutron mutant data, Differential expression analysis, Phosphorylation data, Phylogeny, Protein BioViewer, Heatmap and hierarchical clustering, PI and trait search, FTP/data download capabilities
3	SoyDB Soybean transcription factors database, Missouri University, http://casp.rnet.missouri.edu/soydb/	Protein sequences, Predicted tertiary structures, Putative DNA binding sites, Protein Data Bank (PDB), Protein family classifications	PSI-BLAST, Browse database, Family Prediction by HMM, FTP data retriever
4	SGMD The Soybean Genomics and Microarray Database, http://bioinformatics.towson.edu/SGMD/	Integrated view genomic, EST and microarray data	Analytical tools allowing correlation of soybean ESTs with their gene expression profiles
5	Deltasoy An Internet-Based Soybean Database for Official Variety Trials, http://msucares.com/deltasoy/testlocationmap.htm	Official variety trial (OVT) information in soybean, Mississippi OVT data, including yield, location, and disease information	Comparison tools for variety trail data, phenotypic data and disease related data
6	DaizuBase An integrated soybean genome database including BAC-based physical maps, http://daizu.dna.affrc.go.jp/	BAC-based physical map, Linkage map and DNA markers, BAC-end, BAC contigs, ESTs, full-length cDNAs	Gbrowse, Unified Map, Gene viewer, BLAST
7	SoyMetDB The soybean metabolome database, http://soymetdb.org	Soybean metabolomic data	Pathway Viewer
9	SoyProDB Soybean proteins database, http://bioinformatics.towson.edu	Several 2D Gel images showing isolated soybean seed proteins	Search tool for 2D spots, Navigation tools for protein data
10	SoyGD The Soybean GBrowse Database, Southern Illinois University, http://soybeangenome.siu.edu/	Physical map and genetic map, Bacterial artificial chromosome (BAC) fingerprint database, Associated genomic data	Sequence data retrieval tools, Navigation tool for sequence information of different builds
11	SoyTEdb Soybean transposable elements database, www.soybase.org/soytedb/	Williams 82 transposable element database	Browse for Repetitive elements, Transposable Element and Map position, Data retrieval tools
12	SoyXpress Soybean transcriptome database, http://soyxpress2.agrenv.mcgill.ca	Soybean ESTs, Metabolic pathways, Gene Ontology terms, Swiss-prot Identifiers and Affymetrix gene expression data	BLAST search, Microarray experiments, Pathway search etc

data and make it easily accessible through user friendly interfaces so called "databases." In this regard, several databases have been developed for soybean (**Table 4**). Among these, Soybean Knowledge Base (SKB, http://soykb.org) is a very useful database that provides a comprehensive web resource for omics data from several different platforms (Joshi et al., 2012). The SKB resources are helpful for bridging soybean translational genomics and molecular breeding research. It contains information of genes, proteins, microRNAs, sRNAs, metabolites, molecular markers, and phenomic information of soybean plant introductions (PI). It also provides interference to integrate multi-omics datasets and because of this, a galaxy of information becomes comparable and more useful. For instance, genes in the QTL region can be retrieved very easily along with the functional annotations, associated protein information in respect of structure and functional features, syntenic information with other model plants, sequence variation among different cultivars, gene expression data including tissue specific variations and many other types of information for soybean.

GENERAL CONCLUSION

Different omics tools have been employed to understand how soybean plants respond to abiotic stress conditions. We realize that the studies to integrate multiple omics approaches are limiting in soybean due to the increased cost and potential challenging integrated omic scale analysis. Recent developments in computational resources, statistical tools, and instrumentation have lowered the cost of omics in many folds but integrated analysis needs novel tools and technical wizards. The comprehensive nature of multi-omic studies provides an entirely new avenue and future research programs should plan to adapt accordingly. In soybean, genomics and transcriptomics have progressed as expected but the other major omic branches like proteomics, metabolomics, and phenomics are still lagging behind. These omic branches are equally important to get clear picture of the biological system. Notably, phenomic studies need to be extensively employed along with the other omics approaches. Desired phenotype is ultimate aim of crop sciences; therefore it needs to be understood intensely. Different omic tools and integrated approaches discussed in the present review will provide glimpses of current scenarios and future perspectives for the effective management of abiotic stress tolerance in soybean.

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SUPPLEMENTARY MATERIAL

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Virus-induced gene silencing is a versatile tool for unraveling the functional relevance of multiple abiotic-stress-responsive genes in crop plants

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Virus-induced gene silencing (VIGS) is an effective tool for gene function analysis in plants. Over the last decade, VIGS has been successfully used as both a forward and reverse genetics technique for gene function analysis in various model plants, as well as crop plants. With the increased identification of differentially expressed genes under various abiotic stresses through high-throughput transcript profiling, the application of VIGS is expected to be important in the future for functional characterization of a large number of genes. In the recent past, VIGS was proven to be an elegant tool for functional characterization of genes associated with abiotic stress responses. In this review, we provide an overview of how VIGS is used in different crop species to characterize genes associated with drought-, salt-, oxidative- and nutrient-deficiency-stresses. We describe the examples from studies where abiotic stress related genes are characterized using VIGS. In addition, we describe the major advantages of VIGS over other currently available functional genomics tools. We also summarize the recent improvements, limitations and future prospects of using VIGS as a tool for studying plant responses to abiotic stresses.

Keywords: abiotic stress, functional genomics of crop plants, plant viruses, post-transcriptional gene silencing, virus-induced gene silencing

INTRODUCTION

The recent advances in next-generation sequencing technology has enabled sequencing of stress-specific transcriptomes and genomes of stress tolerant and susceptible cultivars (Morozova and Marra, 2008). Furthermore, an inventory of genes showing altered expression under several abiotic stresses has been established for many crop species by expressed sequence tag (EST) analysis (Gorantla et al., 2007; Wani et al., 2010; Blair et al., 2011). In contrast to the enormous progress made in generating sequence information, functional analysis of genes is lagging behind. Although *in silico* approaches and comparative genomic strategies have provided initial clues about the identity and function of abiotic-stress-responsive genes in many crop species (Gorantla et al., 2007; Tran and Mochida, 2010; Soares-Cavalcanti et al., 2012), comprehensive functional characterization tools are necessary for understanding the precise role of these genes in combating abiotic stresses. Mutant plants generated by chemical mutagenesis (Saleki et al., 1993), T-DNA tagging (Koiwa et al., 2006), and transposon tagging (Zhu et al., 2007) have been used for understanding stress tolerance. However, the generation of large-scale mutant populations requires tedious and laborious efforts, and identification of mutated genes is a lengthy process. RNAi is another tool used for studying the functional relevance of various abiotic-stress-related genes (Guo et al., 2002; Senthil-Kumar and Udayakumar, 2010), but this requires time-consuming genetic transformation. Therefore, in order to quickly study the function of a large number of genes identified through abiotic-stress-specific transcriptome profiles in several crop species and their wild relatives, alternative high-throughput tools are needed. Virus-induced gene silencing (VIGS) has emerged as a successful gene knockdown technique in several crop species in part because it does not require transformation (Baulcombe, 1999; Burch-Smith et al., 2004; Senthil-Kumar and Mysore, 2011a) (Supplementary Table 1). Over the past several years, VIGS has been successfully used to understand the abiotic stress tolerance mechanisms in crop plants (Senthil-Kumar and Udayakumar, 2006; Senthil-Kumar et al., 2008; Manmathan et al., 2013). In this review, we discuss the utility of this powerful technique to study genes involved in abiotic stress tolerance. We also discuss the mechanism of VIGS and list the VIGS vectors available for a wide range of crops and novel ways for application of VIGS to carry out functional analysis of abiotic-stress-responsive genes. Further, the recent improvements in VIGS protocol, limitations and future prospects are discussed.

MECHANISM OF VIGS AND GENESIS OF VIGS VECTORS

VIGS is a post-transcriptional gene silencing (PTGS)-based technique (Baulcombe, 1999), and it exploits the natural defense mechanisms employed by plants to protect against invading viruses (Voinnet, 2001). Plants infected by viruses induce double

stranded RNA (dsRNA) mediated PTGS which degrades viral RNAs. For VIGS, the viral genomes are modified by removing genes which induce virus symptoms and cloning the cDNAs of viral genomes into binary vectors under CaMV35S promoter along with convenient multiple cloning sites to facilitate insertion of target gene fragments (Voinnet, 2001; Liu et al., 2002a,b). Viruses that do not have suppressors of gene silencing or have only weak suppressors are modified as VIGS vectors to induce PTGS-mediated degradation of target plant mRNAs (Li and Ding, 2001; Cao et al., 2005). VIGS vectors are constructed by cloning a fragment (usually 300–500-bp) of the plant target gene with efficient siRNA generation and no off-target genes into the modified viral genome (http://bioinfo2.noble.org/RNAiScan.htm) (Xu et al., 2006). The recombinant virus is then introduced into plant cells through *Agrobacterium tumefaciens*-mediated transient expression or *in vitro* transcribed RNA inoculation or direct DNA inoculation (Supplementary Table 2). After the recombinant virus is introduced into plant cells, the transgene is amplified along with the viral RNA by either an endogenous or a viral RNA-dependent RNA polymerase (RdRp) enzyme generating dsRNA molecules (Dalmay et al., 2000; Mourrain et al., 2000). These dsRNA intermediates are then recognized by DICER-like enzymes which cleave dsRNA into small interfering RNAs (siRNAs) of 21- to 25-nucleotides (Deleris et al., 2006). The double stranded siRNAs are then recognized by the RISC complex. The RISC complex uses the single stranded siRNAs and identifies complementary RNA sequences in the cell and degrades them (Fagard et al., 2000; Morel et al., 2002) (Supplementary Figure 1). VIGS has been shown to occur for a shorter period of approximately 3 weeks and the efficiency decreases after a month resulting in partial or complete recovery of plants from the silencing (Ratcliff et al., 2001; Hiriart et al., 2003; Ryu et al., 2004) (Supplementary Figure 2A). However, recent evidences suggest that some VIGS vectors can be used to maintain the gene silencing for several months by suitably modifying plant growth conditions that favor viral multiplication (Fu et al., 2006; Tuttle et al., 2008; Senthil-Kumar and Mysore, 2011b, 2014) (Supplementary Figure 2B) and can transmit to next generation (Senthil-Kumar and Mysore, 2011b) behaving like stable transgenic plants (Supplementary Figure 2C).

To date, about 35 DNA or RNA viruses have been modified as VIGS vectors (Senthil-Kumar and Mysore, 2011a). The VIGS vector resources available for crop plants are listed in Supplementary Table 1. Interestingly, the ability of certain viruses to infect a large number of host plants enabled the use of a single VIGS vector for gene silencing in several plant species (Robertson, 2004). For example, *Tobacco rattle virus* (TRV)-based VIGS vector is one of the most widely used VIGS vectors due to its ability to infect a wide range of host plants, systemic spread throughout the host plant including meristem, and lack of severe virus-associated symptoms in the infected plant (Valentine et al., 2004; Martín-Hernández and Baulcombe, 2008). TRV is a positive single stranded RNA virus with bipartite genome (RNA1 and RNA2). The RNA1 contains genes encoding RNA-dependent RNA polymerase, movement protein and 16K cysteine rich protein (Macfarlane, 1999). The RNA2 contains gene encoding coat protein and restriction sites for cloning the gene of interest (Liu et al., 2002b). Successful TRV-based VIGS requires infiltration of both RNA1 and RNA2 components. The TRV-based vector has been successfully demonstrated in functional analysis of abiotic-stress-responsive genes in model plants like *Nicotiana benthamiana* (Senthil-Kumar et al., 2007) and crop plants like tomato (*Solanum lycopersicum* and *S. pimpinellifolium*) (Senthil-Kumar and Udayakumar, 2006; Li et al., 2013; Virk et al., 2013), chili pepper (*Capsicum annuum*) (Lee et al., 2010; Choi and Hwang, 2012; Lim and Lee, 2014) and rose (*Rosa hybrid*) (Dai et al., 2012; Liu et al., 2013; Jiang et al., 2014).

Another source of VIGS vectors used for silencing of abiotic stress genes are the novel two-component system based on satellite-viruses along with helper viruses. In nature satellite-viruses are totally dependent on other viruses for replication (Tao and Zhou, 2004; Cai et al., 2007). An example of the DNA virus based two-component system is a satellite-virus-based vector, DNAβ, which was used along with *Tomato yellow leaf curl china virus* (TYLCCNV) as a helper virus to study the genes involved in abiotic stress responses in tomato (He et al., 2008; Guo et al., 2010). *DNAβ satellite virus* is devoid of the undesired effects of virus infection and instead functions to deliver the target gene fragment. RNA virus based VIGS systems with satellite and helper RNAs have also been developed. Here the satellite virus vector helps to deliver RNA into plants and the helper viruses supply replication and movement proteins. The advantage of two-component system is, it produces stronger silencing phenotypes compared to the satellite viruses alone (Gosselé et al., 2002).

In contrast to dicotyledonous plants, monocotyledonous plants have only a few VIGS vectors to date (Scofield and Nelson, 2009; Hema et al., 2013). Among these, the *Barley stripe mosaic virus* (BSMV)-based vector is the most widely used VIGS vector for functional analysis of abiotic stress genes in wheat (*Triticum aestivum*) (Kuzuoglu-Ozturk et al., 2012; Kang et al., 2013; Manmathan et al., 2013) and barley (*Hordeum vulgare*) (Liang et al., 2012). The availability of other vector resources and the potential of VIGS in monocotyledonous species have been comprehensively reviewed recently (Scofield and Nelson, 2009; Hema et al., 2013).

RECENT IMPROVEMENTS IN VIGS

Apart from a number of new VIGS vectors developed to suit a wide range of crop species, existing VIGS vectors and the technique have undergone several improvements in the recent past. For example, viral vectors have been modified to improve silencing efficiency. Recently, the RNA1 component of the bipartite TRV-vector was modified to serve as a VIGS vector which can infect plants systemically in the absence of RNA2 (Deng et al., 2013). This vector was developed by partially removing the 16K cysteine rich protein. The advantage of 16K protein removal is that it creates space for target gene cloning which otherwise cloned in RNA2 and also reduces the silencing suppression capacity of TRV. Furthermore, attempts have been made to identify gene-silenced tissues through a VIGS vector. For example, a *GREEN FLUORESCENT PROTEIN* (*GFP*) gene has been tagged to the coat protein gene of *TRV2* for easy identification of silenced tissue (Tian et al., 2014). This will help in tracing only green fluorescent tissues that have the virus, which are

expected to have silencing, and hence facilitate the use of these tissues for abiotic stress assays. Some VIGS vectors have also been used to induce transcriptional gene silencing (TGS). Cloning of endogenous target gene promoter into viral vector and delivery into plants results in the production of siRNAs homologous to the endogenous gene promoter. These siRNAs facilitate RNA-directed DNA methylation (RdDM) and histone modifications, resulting in RNA-mediated gene silencing (Kanazawa et al., 2011). This can help suppress the regulators of abiotic stress response. In addition to improvements in VIGS vectors, VIGS procedure has been modified to perform silencing in different tissues. Gene silencing has been demonstrated in detached plant parts like petals (Dai et al., 2012), leaves and fruits (Romero et al., 2011; Ramegowda et al., 2013). This will facilitate high-throughput silencing and multiple stress impositions. VIGS has also been used to silence genes during tissue culture and callus development (Anand et al., 2007) which can facilitate precise stress imposition and high-throughput screening.

VIGS FOR STUDYING ABIOTIC STRESS RESPONSES IN CROP SPECIES

VIGS has been used to investigate gene functions under abiotic stresses in model species. These studies involving model plants (Ahn et al., 2006; Moeder et al., 2007; Qian et al., 2007; Senthil-Kumar et al., 2007; Ahn and Pai, 2008; Cho et al., 2008; Hong et al., 2008; Sarowar et al., 2008; Govind et al., 2009; Ré et al., 2011) are not discussed in this review; instead, the main focus is given to studies involving crop plants. Recently, development of a wide range of VIGS vectors with high silencing efficiency has expanded the application of VIGS to several crop species for studying abiotic-stress-responsive genes (**Table 1**). The following sections enumerate the studies in which VIGS was used to characterize abiotic-stress-responsive genes in crop plants.

DROUGHT STRESS TOLERANCE

VIGS is a valuable tool for functional validation of drought-responsive genes identified from transcript profiling of plants exposed to drought stress. TRV-VIGS-mediated silencing of *lea4*, a gene encoding late embryogenesis abundant protein (LEA), resulted in increased susceptibility of tomato plants to drought stress. This gene was identified from a subtracted cDNA library for drought-stress-responsive genes (Gopalakrishna et al., 2001). At a given drought stress level, *lea4*-silenced plants wilted faster and recovered slower upon re-watering than the wild-type and vector control plants. *lea4*-silenced plants also exhibited reduced osmotic adjustment, reduced cell viability and higher superoxide radical levels (Senthil-Kumar and Udayakumar, 2006). In another study, a *GLUTAREDOXIN* gene, *SlGRX1*, was shown to regulate the drought stress response in tomato using a satellite-virus-based vector, DNAmβ (Guo et al., 2010). Under drought stress, silenced plants showed decreased chlorophyll content and decreased relative water content (RWC) compared to vector control plants (Guo et al., 2010). To study the role of mitogen-activated protein kinases (MAPKs) in drought tolerance of *S. pimpinellifolium*, a wild species of tomato, *SpMPK1*, *SpMPK2*, and *SpMPK3* genes were silenced individually or together using TRV-VIGS. Results suggested that co-silencing of *SpMPK1/SpMPK2* impaired ABA- and H_2O_2-induced stomatal closure and enhanced ABA-induced H_2O_2 production. But this response was not seen when *SpMPK1* and *SpMPK2* were silenced individually, suggesting these two genes are functionally redundant. This indicates that VIGS can be used to study functionally redundant genes. Reduced drought tolerance was also seen in *SpMPK3* alone and *SpMPK1/SpMPK2/SpMPK3* co-silenced plants (Li et al., 2013). Similarly, silencing of the *SlMPK4* gene in tomato resulted in early wilting and reduced tolerance of plants to drought stress (Virk et al., 2013). TRV-VIGS-mediated silencing of extracellular *PEROXIDASE 2* (*CaPO2*) in chili pepper resulted in increased susceptibility of silenced plants to mannitol-induced osmotic stress. Leaf disks from *CaPO2*-silenced leaves showed severe bleaching and higher chlorophyll loss than vector control plants (Choi and Hwang, 2012). Similarly, silencing of the *ABI3/VP1* transcription factor (*CaRAV1*) alone or together with *OXIDOREDUCTASE* (*CaOXR1*), using the TRV-VIGS vector, conferred reduced tolerance to mannitol-induced osmotic stress compared to vector control plants (Lee et al., 2010). This was accompanied by reduced expression of the known drought-stress-responsive genes *ANTIMICROBIAL PROTEIN* (*CaAMP1*) and *OSMOTIN* (*CaOSM1*) (Hong et al., 2004; Lee and Hwang, 2009). A recent study (Lim and Lee, 2014) implicated the involvement of *MILDEW RESISTANCE LOCUS O* (*CaMLO2*) in drought tolerance in chili pepper. Silencing of *CaMLO2* using the TRV-VIGS vector in chili pepper plants showed lower levels of transpirational water loss and lipid peroxidation in dehydrated leaves compared to wild-type plants. This study showed that *CaMLO2* acts as a negative regulator under drought stress conditions.

Another study demonstrated the usefulness of the TRV-based VIGS technique to study dehydration-responsive genes in rose flowers. Individual silencing of the *NAC TRANSCRIPTION FACTOR 2* (*RhNAC2*) and *A-TYPE EXPANSIN 4* (*RhEXPA4*) in rose petals and petal disks reduced the recovery of petals and petal disks during rehydration (Dai et al., 2012). Similarly, silencing of *NAC TRANSCRIPTION FACTOR 3* (*RhNAC3*) in rose petals has resulted in a decrease in cell expansion of the petals during rehydration along with concomitant down-regulation of several stress- and cell-expansion-related genes in the silenced petals compared to the vector control (Jiang et al., 2014). These genes are possible candidates for improving the shelf life of rose flowers through reduced water loss. Silencing of the *ACC SYNTHASE 1* (*RhACS1*) and *ACC SYNTHASE 2* (*RhACS2*) genes individually or co-silencing of both genes suppressed dehydration- and rehydration-induced ethylene in the sepals and gynoecia. Reduced ethylene production resulted in improved petal cell expansion during dehydration. On the contrary, silencing of an ethylene receptor, *RhETR3*, enhanced the inhibitory effect of dehydration on petal cell expansion (Liu et al., 2013). These results suggest that ethylene mediates dehydration-induced inhibition of cell expansion in rose petals.

VIGS has also been used to study drought stress response in monocotyledonous crop species. In a recent study (Manmathan et al., 2013), two drought-stress-responsive genes, *ENHANCED RESPONSE TO ABSCISIC ACID* (*Era1*) and *INOSITOL POLYPHOSPHATE 1-PHOSPHATASE* (*Sal1*), were individually silenced in wheat using the BSMV-VIGS vector. *Era1* gene

Table 1 | List of abiotic-stress-related genes silenced in crop plants using VIGS.

VIGS vector	Crop species	Silenced target gene	Abiotic stress	Changes in gene-silenced plants exposed to stress (compared to vector control plants)	References
BSMV	Wheat	*TaEra1* (*ENHANCED RESPONSE TO ABSCISIC ACID*), *TaSal1* (*INOSITOL POLYPHOSPHATE 1-PHOSPHATASE*)	Drought	Increased relative water content (RWC), increased water use efficiency (WUE), reduced stomatal conductance, reduced transpiration rate and higher plant vigor	Manmathan et al., 2013
		TaBTF3 (*BASIC TRANSCRIPTION FACTOR 3)*	Drought	Wilted and curled leaves under severe drought, higher water loss rate (WLR), decreased RWC and survival rate, lower free proline content, and increased membrane leakage	Kang et al., 2013
		TaPGR5 (PROTON GRADIENT REGULATION 5)	High light-induced photo-inhibition	Inhibition of photosynthesis, reduced non-photochemical quenching, increased membrane damage, anthocyanin and malondialdehyde (MDA) accumulation	Yuan-Ge et al., 2014
	Wild emmer wheat	*TdAtg8 (AUTOPHAGY-RELATED 8)*	Drought	Decreased chlorophyll content and increased MDA	Kuzuoglu-Ozturk et al., 2012
	Barley	*HvHVA1* (*H. VULGARIS ABUNDANT PROTEIN*)	Drought	Higher WLR in detached leaves, less survival, and retarded growth with reduced height and less total dry weight	Liang et al., 2012
		HvDhn6 (*DEHYDRIN*)	Drought	Less survival, retarded growth and reduced total dry weight	Liang et al., 2012
BPMV	Soybean	*GmRPA3* (*REPLICATION PROTEIN A*)	Iron deficiency	Reduced chlorosis, increased chlorophyll, stunting and shorter internode	Atwood et al., 2014
PEBV	Pea	*PsSym19* (*SYMBIOTIC*)	Arbuscular- mycorrhizal-symbiosis-associated Pi uptake	Less development of arbuscules and vesicles in the root cortex of silenced plants	Grønlund et al., 2010
		PsPT4 (*PUTATIVE PI TRANSPORTER*)	Arbuscular-mycorrhizal-symbiosis-associated Pi uptake	Reduced phosphate uptake in new roots	Grønlund et al., 2010
		TRX-F, *TRX-M* (*THIOREDOXIN*)	Oxidative stress	Pale-green phenotype, reduction in the following: Mg chelatase activity, 5-aminolevulinic acid synthesis, chlorophyll, carotenoid pigment, photosynthesis and expression of tetrapyrrole biosynthesis pathway genes and increased accumulation of ROS	Luo et al., 2012

(Continued)

Table 1 | Continued

VIGS vector	Crop species	Silenced target gene	Abiotic stress	Changes in gene-silenced plants exposed to stress (compared to vector control plants)	References
TRV	Tomato	*Sllea4* (*LATE EMBRYOGENESIS ABUNDANT PROTEIN 4*)	Drought or oxidative stress	Leaf wilting, reduced osmotic adjustment and cell viability, accumulation of higher superoxide radicals	Senthil-Kumar and Udayakumar, 2006
		SpMPK1 (*MITOGEN-ACTIVATED PROTEIN KINASE 1*), *SpMPK2 (MITOGEN-ACTIVATED PROTEIN KINASE 2*), *SpMPK3* (*MITOGEN-ACTIVATED PROTEIN KINASE 3*)	Drought or ABA or oxidative stress	Reduced survival, higher water loss in detached leaves, increased stomatal closure in response to ABA and increased H_2O_2 production in presence of ABA	Li et al., 2013
		SlMPK4 (*MITOGEN-ACTIVATED PROTEIN KINASE 4*)	Drought	Early leaf wilting	Virk et al., 2013
	Chili pepper	*CaPO2* (*PEROXIDASE 2*)	Salt or osmotic stress	Reduced chlorophyll content and increased lipid peroxidation	Choi and Hwang, 2012
		CaRAV1 (*RELATED TO ABI3/VP1*), *CaOXR1* (*OXIDOREDUCTASE 1*)	Salt or osmotic stress	Severe bleaching of leaf discs, loss of chlorophyll and increased lipid peroxidation	Lee et al., 2010
		CaMLO2 (*MILDEW RESISTANCE LOCUS O*)	Drought	Less water loss and lipid peroxidation	Lim and Lee, 2014
	Rose	*RhNAC2* (*NAC TRANSCRIPTION FACTOR 2*), *RhEXPA4* (*A-TYPE EXPANSIN 4*)	Dehydration	Reduced fresh weight, petal width and recovery from dehydration	Dai et al., 2012
		RhNAC3 (*NAC TRANSCRIPTION FACTOR 3*)	Dehydration	Reduced cell expansion during recovery	Jiang et al., 2014
		RhACS1 (*ACC SYNTHASE 1*), *RhACS2* (*ACC SYNTHASE 2*)	Dehydration	Reduced ethylene production and cell density decreased	Liu et al., 2013
		RhETR3 (*ETHYLENE RECEPTOR*)	Dehydration	Inhibition of petal expansion and cell expansion	Liu et al., 2013
TYLCCNV	Tomato	*SlGRX1* (*GLUTAREDOXIN 1*)	Oxidative or drought or salt stress	Reduced chlorophyll, leaf wilting, curled leaves and reduced RWC under drought; no further growth with wilted leaves and reduced chlorophyll under salt stress	Guo et al., 2010
		SlFRO1 (*FERRIC CHELATE REDUCTASE 1*)	Nutrient deficiency	Reduced ferric chelate reductase activity in roots	He et al., 2008

encodes the β-subunit of farnesyltransferase involved in ABA mediated stomatal closure by activating the guard cell S-type anion-channels and increasing the cytosolic Ca^{2+} concentration. The loss-of-function of *Era1* has been shown to enhance ABA sensitivity and hence reduced stomatal conductance and water loss (Cutler et al., 1996; Allen et al., 2002; Wang et al., 2005). Similarly, *Sal1* has been shown to act as a negative regulator of both ABA-independent and ABA-dependent stress response pathways. Its loss-of-function has shown to increase the sensitivity of plants to drought stress (Wilson et al., 2009). *Era1*- and *Sal1*-silenced plants subjected to drought stress showed increased RWC, improved water use efficiency (WUE) and better vigor compared to vector-inoculated plants. This suggests that down-regulation of *Era1* and *Sal1* genes enhances drought tolerance in wheat by decreasing sensitivity to ABA. In another study, *H. VULGARIS ABUNDANT PROTEIN* (*HvHVA1*) and *DEHYDRIN 6* (*HvDhn6*), genes encoding the LEA class of proteins, were individually silenced in wheat using the BSMV-based

VIGS vector (Liang et al., 2012). Under drought stress, both *HVA1*- and *Dhn6*-silenced plants showed lower survival rates than vector control plants. In addition, *HVA1*-silenced plants showed a higher rate of water loss under drought stress compared to vector control plants. However, the silenced plants also showed reduced vegetative growth and lower biomass even under well-watered conditions. This suggested the involvement of *HvHVA1* and *HvDhn6* in growth and development apart from drought tolerance (Liang et al., 2012). BSMV-VIGS-mediated silencing of the *BASIC TRANSCRIPTION FACTOR 3* (*TaBTF3*) gene in wheat resulted in a decreased plant survival rate, less free proline content, less RWC and increased membrane leakage compared to vector control plants under drought stress (Kang et al., 2013). Similarly, BSMV-VIGS-mediated silencing of *AUTOPHAGY-RELATED 8* (*TdAtg8*) from *Triticum dicoccoides* (wild emmer wheat) resulted in reduced chlorophyll content and an increase in malondialdehyde (MDA) content in silenced plants under drought stress (Kuzuoglu-Ozturk et al., 2012). The increased levels of MDA indicate membrane damage due to lipid peroxidation mainly by the effect of reactive oxygen species (ROS) (Zhang and Kirkham, 1994).

Taken together, these studies demonstrate the versatility of VIGS in deciphering the role of drought-stress-responsive genes in both dicotyledonous and monocotyledonous plants. In addition, the application of VIGS in silencing drought-stress-related genes in flowers (Dai et al., 2012) signifies its efficacy in studying the reproductive-tissue-associated genes which are important during terminal drought stress. Furthermore, VIGS has the potential to identify negative regulators of drought stress response during the reproductive stage.

SALT-STRESS TOLERANCE

The utility of VIGS in investigating salt stress tolerance in crop plants has also been demonstrated. *SlGRX1* gene silencing in tomato by a satellite DNAmβ-based VIGS vector resulted in yellowing of leaves under salinity stress compared to vector control plants due to a reduction in chlorophyll content, suggesting the role of *GRX1* in salt tolerance (Guo et al., 2010). Further, the role of *CaRAV1* and *CaOXR1* has been studied by TRV-VIGS in chili pepper (Lee et al., 2010). Leaf disks from *CaRAV1*-only silenced and *CaRAV1*/*CaOXR1* co-silenced plants exposed to different concentrations of NaCl showed severe bleaching due to loss of chlorophyll compared to vector control plants. Similarly, TRV-VIGS-mediated silencing of *CaPO2* resulted in a reduction in chlorophyll content and higher lipid peroxidation, leading to increased susceptibility of silenced chili pepper plants to salt stress compared to vector control plants (Choi and Hwang, 2012). Consistently, ectopic expression of *CaPO2* in Arabidopsis conferred enhanced tolerance to high salt stress, suggesting the role of *CaPO2* in salinity tolerance (Choi and Hwang, 2012). Taken together, these studies demonstrate the usefulness of VIGS in functional analysis of genes involved in salinity tolerance in crop plants.

OXIDATIVE STRESS TOLERANCE

ROS increases in plants challenged by drought, salinity, extreme temperatures, or high light stress (Pastori and Foyer, 2002); this in turn leads to oxidative stress (Apel and Hirt, 2004). VIGS has been used to study oxidative stress tolerance in the recent past. A few studies (Lee et al., 2010; Choi and Hwang, 2012) described earlier in this review that examined the role of chili pepper genes, like *CaRAV1*, *CaOXR1*, and *CaPO2*, have also described oxidative stress damage in the plants with these genes silenced. Silencing of *CaRAV1*, *CaOXR1*, or *CaPO2* individually, or co-silencing of *CaRAV1*/*CaOXR1* in chili pepper resulted in enhanced lipid peroxidation under stress (Lee et al., 2010; Choi and Hwang, 2012). Similarly, downregulation of *CaMLO2* expression in chili pepper using TRV-based VIGS resulted in lower MDA levels under drought stress compared to vector control plants (Lim and Lee, 2014). This indicated the plausible negative role of *CaMLO2* under drought as well as oxidative stress. In wheat, silencing of *TdAtg8* using BSMV-based VIGS resulted in higher MDA levels compared to vector control under drought stress, thus suggesting the possible involvement of *TdAtg8* under oxidative stress (Kuzuoglu-Ozturk et al., 2012). High light stress induces oxidative stress in chloroplast. A recent study (Yuan-Ge et al., 2014) used BSMV-based VIGS to silence the *PROTON GRADIENT REGULATION 5* (*TaPGR5*) gene in wheat to test its involvement in tolerance to photo-inhibition under high light treatment. High light inhibited the net photosynthesis and affected the maximal quantum yield of Photosystem II (Fv/Fm) in the silenced plants. Also, silenced plants showed increased membrane damage, anthocyanin accumulation and higher MDA, suggesting the role of *TaPGR5* in oxidative stress tolerance. In pea, PEBV-VIGS-mediated co-silencing of thioredoxin genes, *TRX-F*/*TRX-M*, resulted in a significant reduction in Mg-chelatase activity and 5-aminolevulinic acid synthesizing capacity. This was associated with reduced chlorophyll and carotenoid pigment contents, lowered photosynthetic capacity and reduced expression of tetrapyrrole biosynthesis pathway genes, leading to the accumulation of ROS (Luo et al., 2012). Altogether, these studies highlight the utility of VIGS in characterizing the genes that mitigate oxidative stress in crop plants.

VIGS FOR FUNCTIONAL ANALYSIS OF MINERAL NUTRITION-RELATED GENES IN CROP PLANTS

Differential expression of a large number of genes in response to nutrient deficiency or toxicity has been shown in plants (Wang et al., 2002; Becher et al., 2004; Hirai et al., 2004; Takehisa et al., 2013), but only a few of them have been functionally characterized. In a soybean (*Glycine max*) iron-inefficient line, Isoclark, a *Bean pod mottle virus* (BPMV)-based VIGS vector was used to silence a *REPLICATION PROTEIN A* (*GmRPA3*) gene. *GmRPA3*-silenced plants had smaller leaves, decreased internode length and higher chlorophyll content, and failed to respond to increased iron nutrition, suggesting a role of the *GmRPA3* gene in iron acquisition (Atwood et al., 2014). Using a satellite DNA (DNAmβ) virus system with TYLCCNV, the function of *FERRIC CHELATE REDUCTASE* gene (*FRO1*) was studied in tomato roots (He et al., 2008). Silencing of *FRO1* resulted in reduced ferric chelate reductase activity in roots. In pea (*Pisum sativum*), a *Pea early browning virus* (PEBV)-based vector was used to study arbuscular-mycorrhizal-fungi (AMF)-associated phosphate acquisition. Silencing of a symbiotic gene, *PsSym19,* reduced the

development of both arbuscules and vesicles at the root cortex. Similarly, silencing of a putative Pi transporter gene, *PsPT4*, using the PEBV-vector, reduced the phosphate uptake (Grønlund et al., 2010), suggesting the importance of these genes in phosphate assimilation in pea plants. Taken together, these studies suggest that VIGS can be effectively used to analyze gene function associated with nutrient deficiency in crop plants.

ADVANTAGES OF USING VIGS TO STUDY ABIOTIC STRESS TOLERANCE IN CROP PLANTS

VIGS has several advantages over most established functional genomics tools (Burch-Smith et al., 2004; Purkayastha and Dasgupta, 2009; Unver and Budak, 2009; Stratmann and Hind, 2011; Pflieger et al., 2013). (1) VIGS is faster and relatively easy to perform. VIGS can produce loss-of-function phenotype of a specific gene in a short period resulting in rapid functional characterization of genes (Dinesh-Kumar et al., 2003). (2) VIGS avoids plant transformation. Functional characterization of genes in difficult to transform species would be more easier once the VIGS system is established in that species (Burch-Smith et al., 2004). (3) VIGS allows functional analysis of genes whose loss-of-function produces lethal phenotype. It can be used to study genes related to embryonic development and seedling emergence and vigor (an important abiotic stress tolerance trait) (Ratcliff et al., 2001; Burch-Smith et al., 2004; Liu et al., 2004). (4) VIGS can overcome functional redundancy. Using the most conserved regions in VIGS, the multiple related genes or gene families can be silenced together (Ekengren et al., 2003; He et al., 2004). By silencing two or more members of the gene family with redundant functions the complex signaling components associated abiotic stresses such as drought can be deciphered. Though other functional genomics tools like antisense RNAs, artificial miRNAs, or RNAi can also be used for this purpose, but they are time consuming. (5) VIGS enables timely silencing of tissue-specific genes. For example, plants being infected only at the time of flowering or panicle development will predominantly have genes silenced in that organ. Besides, VIGS can be used to quickly silence genes in a particular gene mutant, stable RNAi or gene-overexpression plants. This will enable studying gene interactions under complex abiotic stresses in a large-scale and shorter time. In addition, VIGS is a feasible functional genomics tool over other PTGS-mediated gene silencing methods (Supplementary Table 3). VIGS is versatile, which allows rapid comparisons of gene function between species and works in different genetic backgrounds where genetic transformation is tedious and time consuming. VIGS also serves as a high-throughput forward as well as reverse genetics tool in plants. VIGS as a high-throughput reverse genetics tool can be performed by individually cloning fragments (usually 300–500 base pairs) from a large number of target genes into a suitable viral vector. The viral vector is delivered into plants using different methods. Abiotic stress can be applied 2–3 weeks after inoculation and the loss-of-function phenotype can be studied in the silenced plants to attribute function for the target gene under abiotic stress (Supplementary Figure 3). Similarly, VIGS as a forward genetics tool enables identification of critical players in stress tolerance. The stress specific cDNA pool can be cloned into binary vectors and transformed into *A. tumefaciens* in a high-throughput manner (Liu et al., 2002b). Each Agro-clone is inoculated into individual plants using a feasible inoculation method. The Agro-clones which produce interesting phenotype under abiotic stress can be quickly identified and sequenced to identify the inserted gene (Supplementary Figure 4). In addition to several general advantages, VIGS has some advantages pertinent to characterizing abiotic-stress-responsive genes.

LIMITATIONS OF VIGS IN STUDYING ABIOTIC STRESS TOLERANCE MECHANISMS AND SOLUTIONS TO OVERCOME THE LIMITATIONS

Though VIGS has been proved to be a robust tool for functional genomics studies, it has several limitations. These limitations and ways to overcome the same are listed below. (1) The virus vector may accumulate to high levels in the silenced plant if the silenced target gene is involved in the immunity of plants against the virus and such plants can become highly susceptible to subsequent abiotic stress. This will adversely influence studying the specific effect of gene silencing on abiotic stress tolerance. Quantification of viral load (Senthil-Kumar and Mysore, 2011b) in the silenced plants helps to decide whether the virus has accumulated higher than in the non-silenced control plant and this information can be used to choose different region of the target gene for silencing. (2) Virus infection by itself can interfere with abiotic stress response. For example, infection of *Brome mosaic virus* (BMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV) and TRV delayed the appearance of drought symptoms in various plant species (Xu et al., 2008). The VIGS vector along with abiotic stress can create a scenario like concurrent biotic and abiotic stress. The phenotype produced under this situation might be different from abiotic stress alone (Suzuki et al., 2014). This can be overcome by including appropriate non-silenced vector control plants and comparing the results with specific gene silenced plants. (3) Silencing can be affected by changes in environmental conditions during abiotic stress treatment. Temperature, relative humidity and light can influence silencing (Fu et al., 2005, 2006; Kotakis et al., 2010). VIGS efficiency is reduced under high temperatures due to reduced virus multiplication (Chellappan et al., 2005). This can be overcome by verifying the viral multiplication beforehand and maintaining the VIGS vector-inoculated plants under optimum environmental conditions until the silencing followed by abiotic stress imposition. Ways to overcome some of the limitations of VIGS to study abiotic-stress-associated genes are also described in our earlier review (Senthil-Kumar and Udayakumar, 2010).

CONCLUSION AND FUTURE PROSPECTS

VIGS, as both a forward and reverse genetics tool, offers opportunities for rapid functional analysis of abiotic-stress-related genes in both dicotyledonous and monocotyledonous crop species. Utilization of VIGS for understanding the mechanisms of abiotic stress tolerance and crop improvement is depicted in **Figure 1**. Currently, nearly 50 plant species have been shown to be amenable for VIGS (Lange et al., 2013), and VIGS is expected to be expanded to many other crop plants in future. Stress imposition protocols for VIGS plants have been optimized for several abiotic stresses, including drought, salinity and oxidative stress,

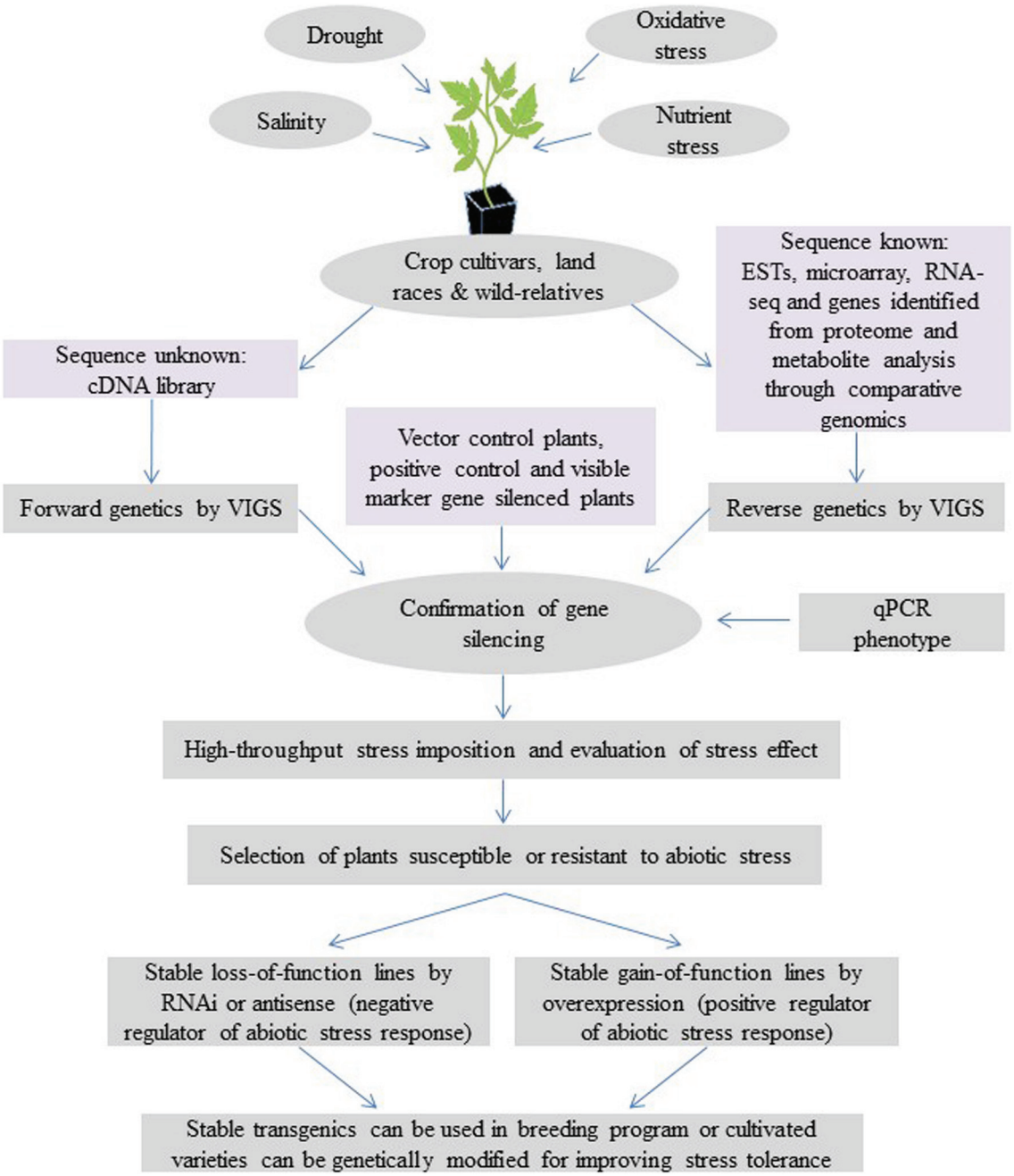

FIGURE 1 | Model showing the application of VIGS in understanding the mechanisms of abiotic stress tolerance and crop improvement. VIGS can be used as a powerful reverse genetic tool for functional analysis of abiotic-stress-responsive genes identified from cultivars, land races and their wild relatives though transcriptome analysis and comparative analysis of molecular marker, proteome and metabolite data. VIGS can also be used for a high-throughput forward genetics screening. This is achieved by cloning the cDNA libraries generated from abiotic-stressed plants directly into a VIGS vector, inoculating them on target plants and analyzing the knockdown plants under abiotic stress. Along with target-gene-silenced plants, vector control and visible marker gene (like *phytoene desaturase*, *PDS* or *magnesium protoporphyrin chelatase subunit H*, *ChlH*)-silenced plants showing a photo-bleaching/yellowing phenotype will aid in identifying the time of initiation and duration of gene silencing. Silencing of a gene known to be involved in the specific abiotic stress tolerance that leads to susceptibility under stress (positive controls) is useful for coinciding abiotic stress imposition at the time of target gene silencing. In addition, high-throughput stress imposition and stress effect quantification methods can be used to screen large numbers of gene-silenced plants (Ramegowda et al., 2013). Candidate genes identified from the screen can be further confirmed by generating stable RNAi or overexpression transgenic lines. The trait can then be transferred to elite cultivars through breeding or generating transgenics in amenable cultivars to develop stress-tolerant crop plants.

and extreme temperatures (Ramegowda et al., 2013). Recently, a modified virus vector has been developed to express artificial and endogenous miRNAs in plants (Tang et al., 2010). Virus-vector-mediated silencing using artificial miRNA will be useful for functional analysis of abiotic-stress-associated miRNAs in crop plants. This approach will combine the specificity of amiRNA and versatility of VIGS. VIGS could also assist plant breeding programs in validating quantitative trait loci (QTL) and genes associated with abiotic stress traits (Cheng et al., 2010). Most of the QTL identified by molecular marker technologies would have multiple candidate genes. VIGS could serve as an effective and robust functional genomics tool to validate each gene in the locus. For example, a combination of cDNA-amplified fragment length polymorphism (AFLP) and VIGS can be used to screen a large number of genes and identify genes associated with abiotic stress tolerance. In summary, VIGS can play a major role in understanding abiotic stress tolerance mechanisms. This will have a direct impact on developing crop varieties that are tolerant to abiotic stress.

AUTHOR CONTRIBUTIONS

Venkategowda Ramegowda and Muthappa Senthil-Kumar wrote the manuscript, and Kirankumar S. Mysore edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00323/abstract

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Comparative phylogenomics of the CBL-CIPK calcium-decoding network in the moss *Physcomitrella*, Arabidopsis, and other green lineages

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Land plants have evolved a host of anatomical and molecular adaptations for terrestrial growth. Many of these adaptations are believed to be elaborations of features that were present in their algal-like progenitors. In the model plant Arabidopsis, 10 Calcineurin B-Like proteins (CBLs) function as calcium sensors and modulate the activity of 26 CBL-Interacting Protein Kinases (CIPKs). The CBL-CIPK network coordinates environmental responses and helps maintain proper ion balances, especially during abiotic stress. We identified and analyzed CBL and CIPK homologs in green lineages, including CBLs and CIPKs from charophyte green algae, the closest living relatives of land plants. Phylogenomic evidence suggests that the network expanded from a small module, likely a single CBL-CIPK pair, present in the ancestor of modern plants and algae. Extreme conservation of the NAF motif, which mediates CBL-CIPK physical interactions, among all identified CIPKs supports the interpretation of CBL and CIPK homologs in green algae and early diverging land plants as functionally linked network components. We identified the full complement of CBL and CIPK loci in the genome of *Physcomitrella*, a model moss. These analyses demonstrate the strong effects of a recent moss whole genome duplication: CBL and CIPK loci appear in cognate pairs, some of which appear to be pseudogenes, with high sequence similarity. We cloned all full-length transcripts from these loci and performed yeast two-hybrid analyses to demonstrate CBL-CIPK interactions and identify specific connections within the network. Using phylogenomics, we have identified three ancient types of CBLs that are discernible by N-terminal localization motifs and a "green algal-type" clade of CIPKs with members from *Physcomitrella* and Arabidopsis.

Keywords: CBL-CIPK, calcium signaling, plant abiotic stress physiology, plant nutrition, evolution, molecular

INTRODUCTION

Of the events that have shaped our modern biosphere, the colonization of land by the predecessors of modern embryophytes stands out as an evolutionary advent that has profoundly affected our landscape and terrestrial ecology. Land plants arose roughly 450 million years ago from a lineage of multicellular freshwater green algae known as charophytes (Graham, 1996; Lewis and McCourt, 2004). Land plants have elaborated and expanded upon a molecular toolkit present in their charophyte ancestors and thereby developed novel anatomical and molecular adaptations to withstand life on land (Graham, 1996; Kenrick and Crane, 1997; Pittermann, 2010; Timme and Delwiche, 2010). The switch from aquatic to terrestrial growth imposed new and formidable abiotic stresses. Discontinuous access to water combined with labile, often unfavorable ion balances spurred the development of sophisticated mechanisms for the perception of water and ion availability, the communication of this information throughout the plant body, and the coordination of orchestrated responses to these stresses.

Calcium ions play a pivotal role in a host of signal transduction cascades in plants as well as in animals. Tightly localized spikes in cytosolic calcium concentration in response to particular environmental cues have been extensively documented in plant cells and are thought to act as early steps in plant signaling pathways (Gilroy et al., 1993; Evans et al., 2001). These bursts, known as calcium signals, are modulated by channels that allow calcium entry from both outside the cell and inside cellular stores (e.g., the vacuole, endoplasmic reticulum). Calcium signals are decoded by proteins that act as sensors; calcium sensors often contain helix-loop-helix motifs known as EF hands that bind calcium and induce conformational changes to modulate the activity of other proteins or domains (Hrabak et al., 2003; McCormack et al., 2005).

Calcineurin B-Like proteins or CBLs are a family of calcium sensors found in all studied land plants and some chlorophyte green algae (Weinl and Kudla, 2009; Batistic et al., 2011). CBLs are named based on their homology to the B regulatory subunit of the phosphatase calcineurin (Luan et al., 2002). CBLs

contain four calcium-binding EF hands and typically contain a subcellular localization signal at their N-terminus. The most thoroughly characterized CBLs to date contain a dual lipid modification motif (MGCXXS/T) at their N-terminus that is necessary and sufficient for targeting of fluorescent protein (FP)-fusions to the plasma membrane (Batistic et al., 2008, 2010). Other CBLs are reported to localize to the vacuole, and several of these CBLs contain a distinct N-terminal extension known as the Tonoplast Targeting Sequence (TTS) that targets FP-fusions to the tonoplast (Batistič, 2012; Tang et al., 2012). Uniquely, Arabidopsis CBL10 contains a putative N-terminal transmembrane helix that anchors it to the tonoplast (Kim et al., 2007; Batistic et al., 2010) or plasma membrane (Quan et al., 2007; Ren et al., 2013). Subcellular targeting has been shown to be critical for CBL functionality, and CBLs are responsible for the recruitment and localization of protein partners.

CBLs physically and functionally interact with CBL-Interacting Protein Kinases (CIPKs) and modulate their kinase activity (Shi et al., 1999; Batistic et al., 2011). Hence, the CBL-CIPK network serves to decode calcium signals and transmit these signals through reversible protein phosphorylation. CIPKs, also known as SnRK3 proteins, are serine/threonine protein kinases that consist of a N-terminal kinase domain similar to those found in other plant protein kinases and a unique C-terminal regulatory domain that acts as an autoinhibitory domain and mediates interactions with CBLs. CBLs bind to a short, conserved region within the C-terminal autoinhibitory domain of CIPKs known as the NAF or FISL motif (Shi et al., 1999; Albrecht et al., 2001; Guo et al., 2001). In addition to modulating the kinase activity of CIPKs, CBLs are thought to be the sole or primary determinants of CBL-CIPK complex localization, therefore they are thought to act as functional modules (Luan, 2009; Batistic et al., 2011). CBLs are believed to recruit CIPKs, which lack any sort of discernible targeting signals, to these surfaces, possibly in a calcium-dependent manner (Batistič and Kudla, 2009; Batistic et al., 2010).

Initial functional analysis of the CBL-CIPK network came from the genetic identification of the Salt Overly Sensitive (SOS) pathway. Together, CBL4/SOS3 and CIPK24/SOS2 modulate that activity of the plasma membrane Na^+/H^+ exchanger SOS1. Mutants lacking any component of the Salt Overly Sensitive (SOS) pathway display NaCl-hypersensitive phenotypes (Liu and Zhu, 1998; Liu et al., 2000; Shi et al., 2000). CBL4/SOS3 and CIPK24/SOS2 belong to large proteins families containing 10 CBLs and 26 CIPKs in Arabidopsis and similarly sized families in other angiosperms (Kudla et al., 1999; Kolukisaoglu et al., 2004; Weinl and Kudla, 2009). CBL-CIPK complexes have recently been implicated in sodium, potassium, nitrate, and proton transport (Li et al., 2006; Xu et al., 2006; Ho et al., 2009); therefore the CBL-CIPK network is currently thought to be a major regulator of ion homeostasis in angiosperms.

Though CBLs and CIPKs have been discovered among all studied land plants and certain green algal lineages, little is known about the functionality of the CBL-CIPK network outside of angiosperms. As an initial step toward functional analysis of the CBL-CIPK network in an early-diverging land plant, we analyzed the genomic content of CBLs and CIPKs in the model moss *Physcomitrella* and performed bioinformatic analyses of the CBL and CIPK families with an emphasis on relationships among *Physcomitrella* and Arabidopsis CBLs and CIPKs. We classified CBLs according to their phylogeny and N-terminal localization motifs and identified three ancient classes of CBLs. Using yeast two-hybrid analyses, we confirmed interactions among CBLs and CIPKs outside of angiosperms and characterized physical interactions among *Physcomitrella* CBLs and CIPKs. Through phylogenetic analyses, we identified a strongly supported clade that contains all CIPKs identified from green algae and two CIPKs from Arabidopsis and *Physcomitrella*. Using phylogenomic methods, we seek to characterize patterns of expansion of the CBL-CIPK network among land plant lineages to classify CBLs and CIPK in an evolutionarily and functionally meaningful manner to facilitate functional genetic work in early-diverging plants.

MATERIALS AND METHODS

HOMOLOG IDENTIFICATION, SEQUENCE ALIGNMENT, AND BIOINFORMATIC ANALYSES

CBL and CIPK homologs were identified using BLASTp and tBLASTn searches of the Uniprot and the NCBI protein and nucleotide databases, using previously identified CBLs and CIPKs from Arabidopsis as queries. Additional sequences were manually retrieved by annotation from UniProt using the keywords "calcineurin" and "CBL-interacting" (Jain et al., 2009). Genomic loci of CBL and CIPK homologs in *Physcomitrella patens* were identified in version 1.6 of the *Physcomitrella* genome, available at http://cosmoss.org (Zimmer et al., 2013). All charophyte CBL and CIPK sequences identified were predicted by assembly of homologous expressed sequence tags (ESTs) from transcriptome-level sequencing of diverse, representative charophyte genera (Timme and Delwiche, 2010; Timme et al., 2012). Other new CBL and CIPK protein sequences were predicted from EST sequences in the NCBI non-redundant (nr) nucleotide database identified by tBLASTn searches. Overlapping ESTs from the same taxa were assembled, and ESTs were translated using Geneious R6 (Biomatters), which was also used for all stages of phylogenetic analyses and figure preparation. Predicted CBL and CIPK homologs were verified by manual inspection of domain architecture and pBLAST searches of the NCBI non-redundant (NR) protein database; all protein sequences included in analyses showed expected domain architecture and yielded top BLASTp hits to previously identified CBLs and CIPKs. CBL and CIPK homologs identified in this study are listed in **Supplementary Tables S1**, **S2**, respectively. Protein sequences were aligned using MAFFT (algorithm G-INS-i) and edited and trimmed by eye to remove short, ambiguously aligned regions (see Supplementary Files S1,S2). Edited alignments were used to generate the phylogenetic trees shown Katoh et al. (2002). Phylogenetic trees were generated from the resulting multiple sequence alignments (MSAs) using PhyML with subtree pruning and regrafting (SPR) + nearest neighbor interchange (NNI) moves and X^2-like approximate likelihood ratio test (aLRT) clade support values, which serve as confidence scores much like bootstrap scores. Clades with aLRT scores > 0.95 were deemed to have strong phylogenetic support (Anisimova and Gascuel, 2006; Guindon et al., 2009). Specific model parameters are provided in the figure legend for each PhyML analysis

presented, however several additional MSAs and evolutionary models and parameters were tested for agreement with conclusions presented here (data not shown). Clades and evolutionary relationships mentioned in the text appeared consistently in independent phylogenetic analyses with different model parameters and MSAs.

CLONING AND SEQUENCING OF CBLs AND CIPK FROM THE MOSS *PHYSCOMITRELLA*

In order to verify expression and expected splice patterns of CBLs and CIPKs in an early-diverging land plant, we cloned CBLs and CIPKs identified from the model moss *Physcomitrella*. RNA was extracted from protonema and gametophores of *Physcomitrella patens* ssp *patens* ecotype Gransden 2004 using a CTAB/chloroform method similar to the one described by Chang et al. (1993). The RNA was reverse transcribed to produce cDNA using Superscript III Reverse Transcriptase (Invitrogen). Primers containing Invitrogen Gateway attB1 (forward primers) and attB2 (reverse primers) recombination sites were designed to amplify the coding sequences (CDSs) of each *Physcomitrella CBL* (*PpCBL*) and *CIPK* (*PpCIPK*) genes (see **Supplementary Table S3** for oligonucleotide sequences used in this study). CBL and CIPK transcripts were amplified using Phusion DNA Polymerase (Thermo-Fisher Scientific) following recommended manufacturer protocols on a MJ Research PTC-100 or PTC-200 model thermocycler. PCR products were visualized on a 0.8% agarose gel, and products of the expected sizes were extracted using a QIAquick gel extraction kit (Qiagen) and cloned into the pDONR™/Zeo vector (Invitrogen) by Gateway BP reaction, following manufacturer recommendations. Samples from three or more clones for each gene were submitted to Elim Biopharmaceuticals, Inc. (Hayward, CA) for DNA sequencing.

YEAST TWO-HYBRID ASSAYS

In order to verify physical interactions among CBLs and CIPKs in a non-angiosperm plant, we cloned the CDS of each full-length *CBL* and *CIPK* transcript identified in *Physcomitrella* and tested interactions among PpCBLs and PpCIPKs in yeast two-hybrid (Y2H) assays using the yeast strain AH109 (Clontech Inc.). This strain is auxotrophic for leucine, tryptophan, histidine, and adenine. The CDSs of *PpCBLs* and *PpCIPKs* were cloned by Gateway LR reaction into yeast two-hybrid gateway-compatible vectors (pGBT9-BS-GW and pGAD-GH-GW) derived from pGBT9-BS and pGAD-GH (Clontech). These vectors were transformed into yeast cells using the G-Biosciences FastYeast Transformation Kit and used to express CBL and CIPK fusions to the DNA-binding domain (BD) and activation domain (AD) of a split transcription factor. We screened CBL-BD fusions (pGBT9-BS-GW constructs) for interactions with CIPK-AD fusion proteins (pGAD-GH-GW constructs) and performed reciprocal screens among CIPK-BD and CBL-AD fusion proteins to verify that the interactions were not vector-dependent. As negative controls, we verified that CBL-BD or CIPK-BD fusion proteins did not interact with the pGAD-GH empty vector (EV).

To perform Y2H screens, co-transformed cells were cultured to mid-log phase in MP Biomedical drop out base (DOB) liquid media lacking leucine and tryptophan (-LT), to ensure retention of vectors containing bait and prey constructs. We then adjusted the cultures to $OD_{600} = 0.05$ and divided them into four 10-fold dilutions ($OD_{600} = 5 \times 10^{-2}$, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}). 6 μl droplets of each dilution were plated on agar DOB media (1) lacking leucine and tryptophan (-LT) to serve as a positive control for transformation and loading, (2) lacking leucine, tryptophan, and histidine (-LTH) to test for protein-protein interactions under low stringency, and (3) lacking leucine, tryptophan, histidine, and adenine (-LTHA) to test for interactions under stringent conditions. Cell growth was recorded at 48 h intervals over the course of 6 days.

RESULTS AND DISCUSSION

CBL-CIPK NETWORK COMPOSITION IN GREEN ALGAE, MOSS, AND OTHER LAND PLANTS

CBLs and CIPKs have been previously identified among various land plants and chlorophyte green algae, though other chlorophytes appear to lack CBL-CIPK homologs (Weinl and Kudla, 2009). Utilizing recently available transcriptome data, we identified CBL and CIPK homologs from several charophyte green algae species: *Coleochaete orbicularis*, *Klebsormidium flaccidum*, *Chaetospheridium globosum*, *Penium margaritaceum,* and *Chlorokybus atmophyticus*. Interestingly, we identified a single CBL and single CIPK in each of these lineages, with one exception. We could not confidently identify a CIPK homolog from *Chlorokybus*, though this may due to incomplete transcriptome coverage. Additional CBL or CIPK homologs may be present in these taxa but undetected due to incomplete sequencing coverage, or additional homologs may simply not be transcribed at sufficient levels under sampled growth conditions. In agreement with our current understanding of evolutionary relationships among these organisms, charophyte green algae sequences display greater sequence similarity to land plant CBLs and CIPKs than chlorophyte homologs. Although there is no currently available genome sequence for any charophyte, only a single CBL and single CIPK were identified in the complete genome sequence of the chlorophytes *Ostreococcus lucimarinus* and *Bathycoccus prasinos*, consistent with prior findings (Weinl and Kudla, 2009). Though it is difficult to make genomic inferences about any charophyte green alga without an available complete genome sequence, our analyses suggest that green algae commonly contain a single CBL-CIPK pair and that the CBL-CIPK network likely predates the split of chlorophyte and charophyte algae.

All CBLs and CIPKs analyzed in this study, including the most divergent homologs identified in algae, show strong conservation of domain architecture and important motifs. At approximately 200 amino acids (AAs) in length, CBLs contain one of a few variations of a localization at their N-termini, followed by 4 calcium-binding EF hand domains. The first EF-hand of CBLs is distinctive in that the calcium-binding loop is comprised of 14 rather than 12 AAs, however evidence suggests that it indeed binds calcium ions (Nagae et al., 2003). Identified full-length CIPKs are approximately 475 AAs in length and have a conserved domain architecture comprised of a N-terminal kinase domain and a C-terminal autoinhibitory region with a diagnostic NAF domain that mediates interactions with CBLs. One previously identified CIPK from the chlorophyte green alga *Chlorella*

(UniProt: C4P7Q5) differs, however, in that it possesses 2 NAF domains in its C-terminus, though the significance and accuracy of the published domain architecture is unknown. Our homology search results corroborate the assertion that CBLs and CIPKs are not found in certain chlorophyte green algae, including the models *Chlamydomonas* and *Volvox* (Weinl and Kudla, 2009; Batistic et al., 2011). This pattern parallels trends in calcium channel evolution. The *Chlamydomonas* genome encodes several voltage-dependent calcium channels (VDCCs) and transient receptor potential (TRP) channels, which play critical roles in environmental sensing in metazoans, whereas sequenced land plant genomes do not contain discernible homologs from either family (Wheeler and Brownlee, 2008; Verret et al., 2010). Like most metazoans, *Chlamydomonas* is motile and, in addition to performing photosynthesis, readily grows heterotrophically. *Chlamydomonas* cells contain an organelle unlike any found in plants, the eyespot, that is involved in the calcium-mediated process of phototaxis (Witman, 1993). Based on these observations, it appears that some components of the calcium signaling machinery of certain chlorophyte green algae, such as *Chlamydomonas*, more closely resemble animal signaling networks in some aspects than those of land plants.

Taking advantage of the published genome sequence of the moss *Physcomitrella patens*, we determined the genomic complement of *CBLs* and *CIPKs* in this early-diverging model plant. We identified a total 4 CBL and 7 CIPK predicted protein sequences in *Physcomitrella*, consistent with prior reports (Batistič and Kudla, 2009; Weinl and Kudla, 2009). One pair of *CBLs* (*PpCBL2+3*) and three pairs of *CIPKs* (*PpCIPK1+5*, *3+4*, and *6+7*) showed strikingly high sequence similarities at both the amino acid (73–93% pairwise identity) and genomic level (42–52% pairwise identity). Because of this observation and the inferred whole genome duplication (WGD) estimated to have occurred ∼45 million years ago in *Physcomitrella* (Rensing et al., 2007), we hypothesized that pairs of *CBLs* and *CIPKs* are products of the recent WGD and that the "unpaired" *CBLs* (*PpCBL1* and *PpCBL4*) and *CIPK* (*PpCIPK2*) may similarly possess cognate loci in the *Physcomitrella* genome. Consistent with this hypothesis, we identified paired loci for each gene and provisionally named these *PpCBL5*, *PpCBL6*, and *PpCIPK8* (**Figure 1**). Although these loci showed relatively low percentage identity to their cognate loci compared to previously detected *CBLs* and *CIPKs*, gene predictions using Augustus (Stanke et al., 2004) suggested these loci may encode partial or full-length proteins. Using RT-PCR, we amplified and cloned transcripts from *PpCBL5* and *PpCIPK8*, however we failed to amplify transcripts from the *PpCBL6* locus using several primer pairs validated on genomic DNA (data not shown), despite testing cDNA from different developmental stages (protonema, gametophores, sporophytes). Pairwise alignment of the *PpCBL4* and *PpCBL6* loci revealed a relatively low percentage identity, particularly in *PpCBL4* exonic regions, compared to other "sister" pairs of *CBLs* and *CIPKs*; these observations suggest that *PpCBL6* may be a pseudogene. Sequenced *PpCBL5* and *PpCIPK8* transcripts detected from both gametophyte and sporophyte cDNA were found to contain premature termination cassettes (PTCs) in their spliced forms (see **Figure 1**, Supplementary File S3), which suggests that these transcripts may not be translated, at least under conditions that we tested. *Physcomitrella CIPK8* contains a single nucleotide repeat (SNR), which are known to promote mutations and quickly change in length (Ellegren, 2004), spanning 31 bases in the retained PTC in cloned transcripts, further marking it as an unusual *CIPK*. Interestingly, *PpCBL5* and *PpCIPK8* show obviously stronger conservation in exonic regions (i.e., regions retained in spliced transcripts and that align to their sister gene's CDS) than intronic or regulatory regions (i.e., promoter and terminator). While these aberrant *CBL* and *CIPK* loci may simply be in early stages of pseudogenization, the unexpected finding that these loci are transcribed and spliced warrants further investigation into possible functions and may point toward a role for these transcripts as regulatory RNAs, as shown previously in animals (Korneev et al., 1999; Hirotsune et al., 2003; Tam et al., 2008). Like in *Physcomitrella*, expansion of the CBL-CIPK network in Arabidopsis, previously attributed to segmental duplications (Kolukisaoglu et al., 2004), can be traced to known WGD events in light of our current understanding of plant genome evolution (Cui et al., 2006). Independent expansion of other gene families in moss and angiosperms has been described, and this can obfuscate direct comparison and functional prediction of genes in widely divergent plants (Cui et al., 2006; Bowman et al., 2007; Rensing et al., 2008; Jiao et al., 2011).

PHYLOGENOMIC ANALYSIS OF CBL REVEALS CONSERVATION OF THREE UNIQUE N-TERMINAL MOTIFS

Phylogenomic methods extend the ability to determine relationships among distant homologs, facilitate functional prediction, and provide a framework for discovery of key features by identifying conserved regions of proteins (Eisen and Wu, 2002; Sjölander, 2004). Using maximum likelihood (ML) methods, we reconstructed the phylogeny of the CBL family in green lineages. Consistent with our hypothesis that land plant CBLs and CIPKs expanded from a simple module present in their common ancestor with algae, green algal CBLs cluster closely to one another with high confidence scores in phylogenetic analyses. Although algal CBLs do not consistently cluster with any particular clade of CBLs from land plants, they commonly show moderate phylogenetic affinity for a clade containing Arabidopsis CBL1 and CBL9 (**Figure 2**; see Supplementary Figure 1 for full tree), which play important roles in potassium nutrition through regulation of the AKT1 channel. Like Arabidopsis CBL1 and CBL9, green algal CBLs feature the dual-lipid modification motif MGCXXS/T or obvious relicts of this motif. Due to the retention of this motif among many embryophyte and green algal CBLs and the results of our phylogenetic analysis, we hypothesize that the dual-lipid modification motif is the ancestral localization mechanism of CBLs. This hypothesis is strengthened by the observation that distantly homologous neuronal calcium sensor (NCS) proteins feature a similar N-terminal motif (MGXXXS) that lacks the conserved cysteine residue but does trigger N-myristoylation of the conserved glycine residue (Li et al., 2011). We designate homologs with the dual lipid modification motif as Type I CBLs (**Figure 3** *top*). Consistent with the hypothesis that ancestral CBLs most closely resembled modern Type I homologs and gave rise to other types of CBLs, Type I CBLs are paraphyletic with respect to other CBLs. Arabidopsis CBLs containing the Type I dual lipid

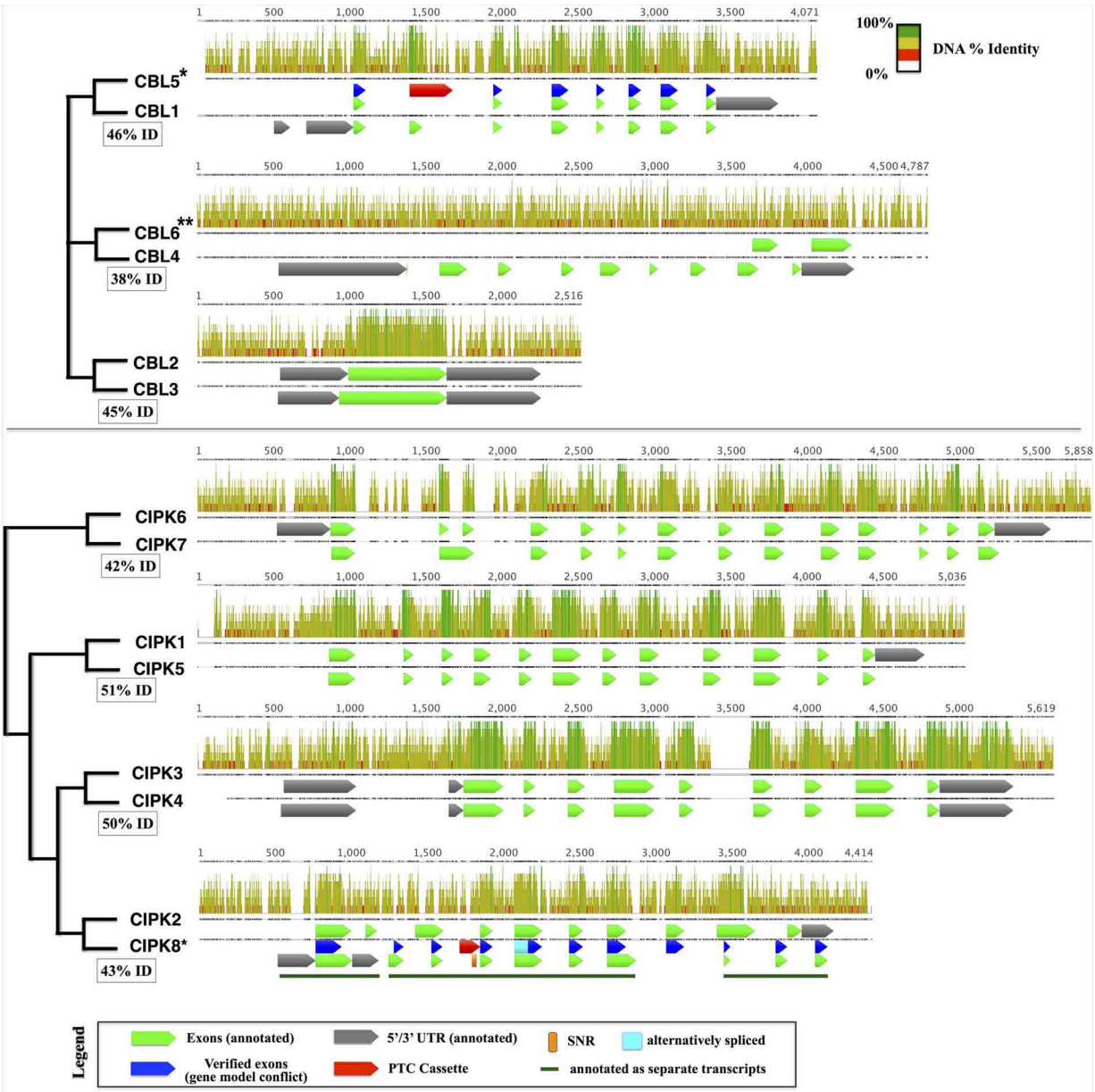

FIGURE 1 | Pairs of cognate *CBLs* (top) and *CIPKs* (bottom) in the *Physcomitrella* genome aligned using MAFFT. Displayed pairs of CBLs and CIPKs are genomic loci that are reciprocal best BLASTn hits within the genome, and inferred phylogenetic relationships are indicated by cladograms and described in the main text. Pairwise percentage nucleotide (nt) identity for pairs of genomic loci are displayed in boxes. Aligned nucleotides are displayed as bars shaded proportionally to percentage identity, and gapped regions in the alignment are represented by lines. Bar graphs indicate percentage identity (sliding window = 6 nt). Genes with cloned transcripts that do not encode full-length proteins under tested conditions are indicated with an asterisk (*) and genes lacking detectable transcripts are marked with two asterisks (**). In cases where our experimentally inferred gene model did not match the annotation, verified exons (blue), alternatively spliced regions (cyan), and premature termination cassettes (PTCs; red) are shown for comparison. *CIPK8*, which was annotated incorrectly as three separate transcripts, contains a long single nucleotide repeat (SNR) comprised of 31 thymidine (T) residues and described further in the main text. Sequences and associated annotations were extracted from the *Physcomitrella* genome v1.6 starting from 500 nucleotides (nt) upstream of the annotated 5′ UTR (750 nt upstream CDS for genes lacking 5′ UTR annotations) to 250 nt downstream of the annotated 5′ UTR (500 nt downstream the CDS) were extracted. Pairwise loci were aligned using MAFFT. Although *PpCBL6* has annotated exons, there is no experimental evidence that any part of this locus in transcribed.

modification motif have been shown to localize to the plasma membrane (D'Angelo et al., 2006; Cheong et al., 2007; Batistic et al., 2008). Mutational analyses using FP-fusions indicate that both N-myristoylation and S-acylation are required to target proteins to the plasma membrane, whereas either modification on its own results in endomembrane localization (Batistic et al., 2008). Although subcellular localization has not been investigated in early diverging plants or green algae, we speculate that the

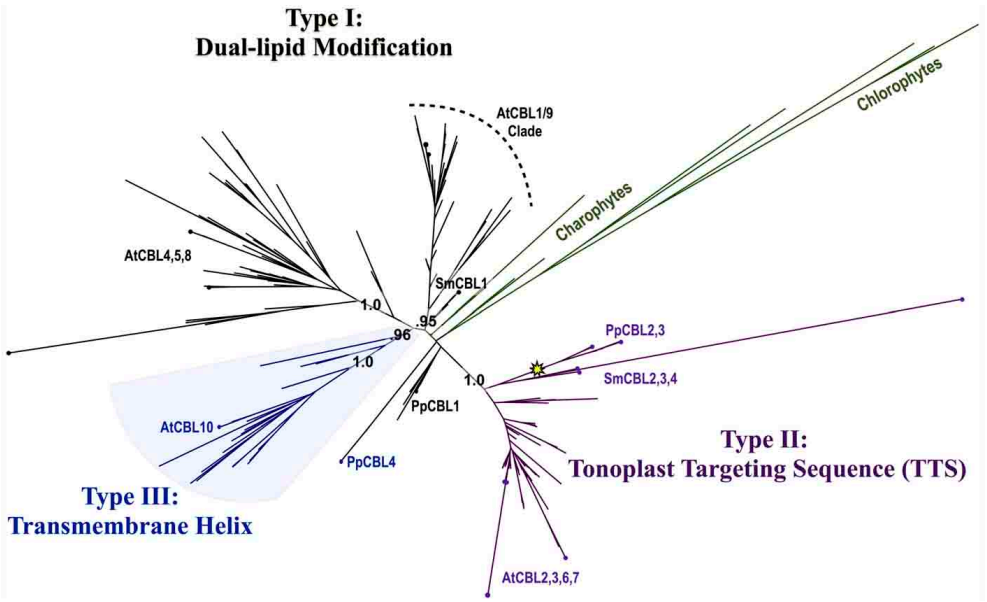

FIGURE 2 | Maximum Likelihood (ML) phylogenetic tree derived from multiple sequence alignment (MSA) of all CBL amino acid (AA) sequences analyzed in this study. Chlorophyte and charophyte green algal CBL and CIPKs are highlighted in green, and clades containing Type I–III CBLs identified in this study are annotated. The yellow star indicates an inferred intron loss event. See **Supplementary Table S1** for list of CBL sequences used in this study, Supplementary File S1 for MSA, and Supplementary File S2 for full phylogenetic tree.

ancestral CBL-CIPK module may have participated in the regulation of integral membrane proteins at the plasma membrane, given the observed evolutionary trends and our understanding of CBL-CIPK function and biochemistry in Arabidopsis.

Phylogenetic analyses revealed a strongly supported (aLRT = 1.0) clade that contains *Physcomitrella* CBL2 and CBL3 and Arabidopsis CBL2, CBL3, CBL6, and CBL7 (**Figure 4**). *Physcomitrella CBL2* and *CBL3* encode proteins that are 76% identical, and both genes lack introns, unlike other *CBLs* from Arabidopsis or *Physcomitrella*. The clade also contains homologs from other non-angiosperms, including three CBLs from the lycophyte *Selaginella moellendorffii*. Like Arabidopsis, *Selaginella* CBLs in this clade contain multiple introns and exhibit a conserved exon-intron structure (data not shown), leading us to infer there was a likely reverse transcription event in the *Physcomitrella* lineage not shared with the lineage leading to lycophytes and angiosperms. Experimental work is needed to determine functional consequences of intron loss in *Physcomitrella CBL2* and *CBL3*, however the effects and mechanisms of reverse transcription-mediated intron loss events and other means of intron loss are discussed elsewhere (Jeffares et al., 2006; Filichkin et al., 2010). Based on high sequence similarity, shared intron loss, and strong phylogenetic evidence, we infer that *PpCBL2* and *PpCBL3* are products of a lineage-specific gene duplication, likely the results of a recent WGD (Rensing et al., 2007, 2013). Both genes are orthologous to the four Arabidopsis *CBLs* contained in this clade. Arabidopsis *CBL2* and *CBL3* are also recent duplicates, as evidenced by their phylogenetic placement and very high sequence similarity (∼92% AA identity) throughout their entire lengths. *CBL3* and *CBL7* are tandem duplicates, although CBL7 is disparate from other Arabidopsis CBLs and contains a deletion in its N-terminus between a degenerate dual-lipid modification motif and its first EF hand (Batistič and Kudla, 2009). Arabidopsis CBL6, which features an unusual first EF hand relative to other CBLs, is more distantly related to the three other AtCBLs in this clade and forms a clade with orthologous CBLs from other eudicots.

Arabidopsis CBL2, CBL3, and CBL6 have been reported to localize to the tonoplast (Batistič and Kudla, 2009). In the case of CBL2 and CBL3, it has been rigorously shown that an N-terminal motif known as the tonoplast targeting sequence (TTS) mediates its subcellular localization (Tang et al., 2012). The TTSs of Arabidopsis CBL2 and CBL3, with the consensus motif MSQCXDGXKHXCXSXXXCF, span 19 AA; and the last three positions of the motif overlap with positions 2–4 in the dual lipid modification motif of Type I CBLs (i.e., MGCXXS/T), sharing a conserved cysteine residue found in all CBLs analyzed (see **Figure 3**, Supplementary File S1). This 19-AA fragment from either Arabidopsis CBL2 or CBL3 is necessary and sufficient for targeting of FP fusions to the tonoplast in Arabidopsis mesophyll cells (Tang et al., 2012), and strong sequence similarity suggests that CBL6 shares this targeting mechanism (**Figure 3** *middle*). CBL7 is reported to show a diffuse nuclear and cytosolic localization based on the analysis of fluorescent fusion proteins (Batistic et al., 2008), however we are unaware of any rigorous attempts to determine its subcellular localization. Therefore, it appears that tonoplast localization is a generally conserved feature among angiosperm CBLs in this clade. We identified a TTS-like motif in all three *Selaginella* CBLs in this clade and in PpCBL3. Unlike PpCBL3, PpCBL2 does not contain an extended N-terminus and

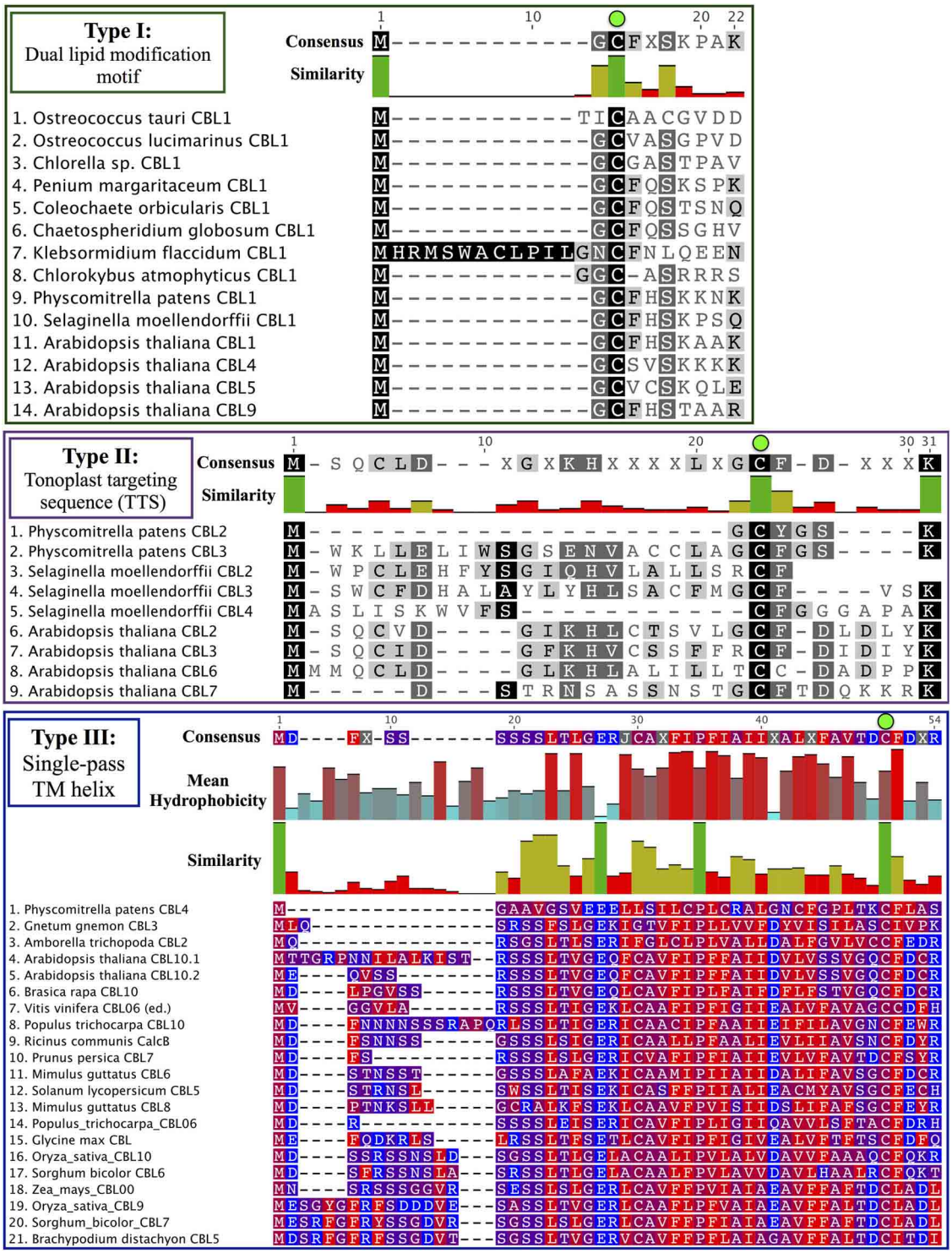

FIGURE 3 | CBL N-terminal localization motifs can be classified into three ancient types. Consensus sequences are provided above each MSA, and degree of conservation is indicated by bar graph and shading. Note the strictly conserved cysteine residues (green dots) in all three types of CBLs. (Top) Type I CBLs harbor a dual-lipid modification motif (MGCXXS/T) that triggers N-myristoylation of the glycine residue and S-acylation of the cysteine residue. Most green algal CBLs identified to date are Type I CBLs or appear to retain signatures of the dual-lipid modification motif. (Middle) Type II CBLs are characterized by a N-terminal extension called the TTS that is found in nearly all CBLs contained in the Type II clade. Phylogenetic evidence suggests that PpCBL2 has lost its TTS through a mechanism such as gene conversion. (Bottom) Type III CBLs feature a long N-terminal extension that is predicted to constitute a transmembrane helix. Residues are colored according to hydrophobicity (red) or hydrophilicity (blue), and mean hydrophobicity and similarity are indicated by bar graphs. Although PpCBL4 does not cluster with seed plant CBLs that share a similar N-terminal extension, we propose that it is targeted in a similar manner to other Type III CBLs based on sequence analysis of its N-terminal extension.

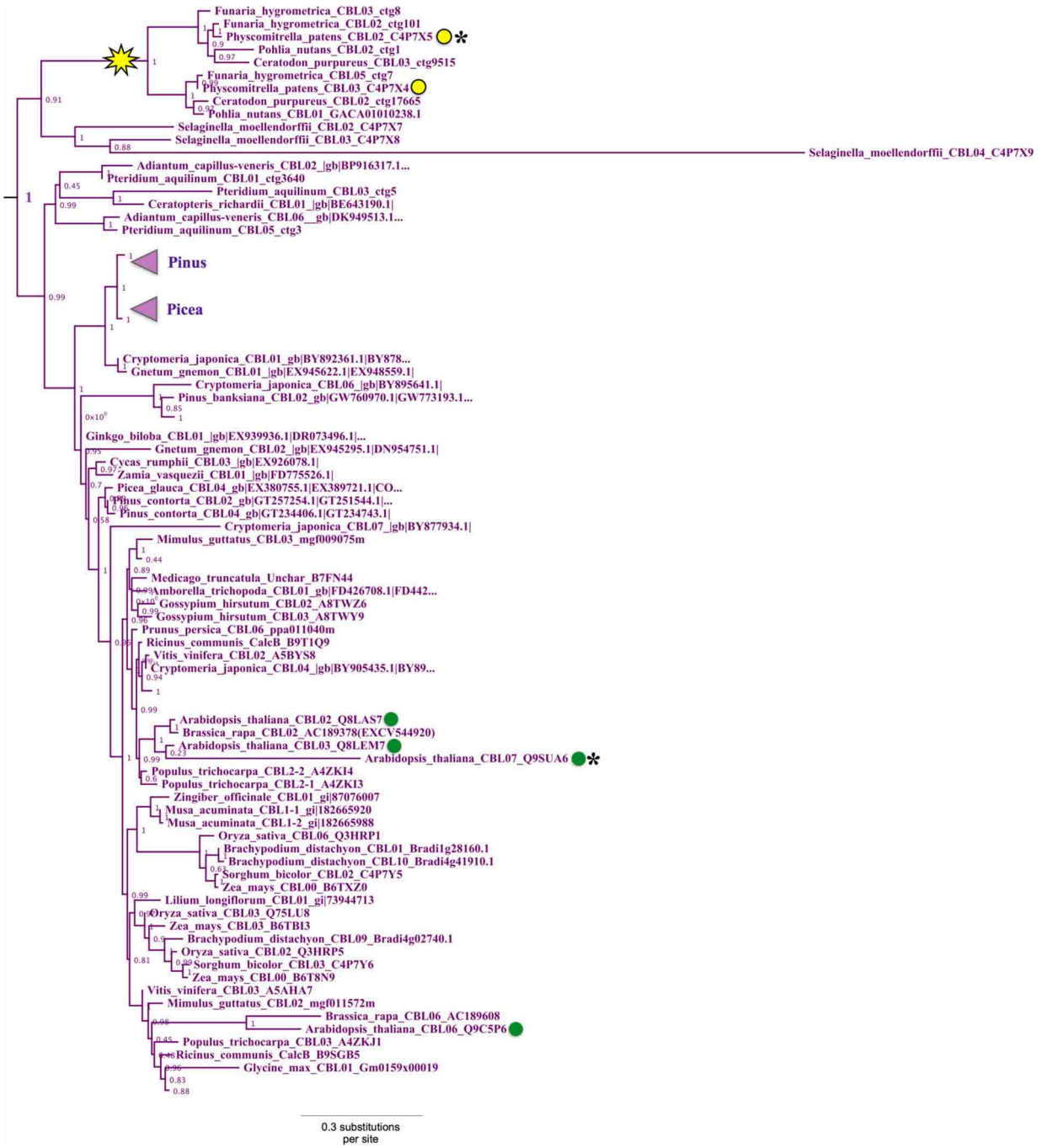

FIGURE 4 | Close-up of Type II CBL clade from ML tree shown in Figure 2. Confidence scores (aLRT) are shown for each clade, and the yellow star denotes an inferred intron loss event. Some clades are unlabeled or collapsed for clarity (see full tree in Supplementary File S2). Type II CBLs are distinguished by the presence of an N-terminal tonoplast targeting sequence (TTS), however certain members (asterisks) of the Type II clade have lost or degenerate TTSs. Arabidopsis CBLs in this clade (green dots) contain the TTS or a degenerate form of it; whereas the two *Physcomitrella* CBLs (yellow dots) in this clade sharply differ in that CBL2 has a Type I dual-lipid modification motif and CBL3 has a TTS. Presence of the TTS in *Selaginella* homologs within the clade (see **Figure 3**) suggests that CBL2 has lost its TTS, through a mechanism such as gene conversion, for example.

instead contains the Type I dual lipid modification motif. Given these trends, we posit that the TTS is a synapomorphy of this clade and that PpCBL2 lost its TTS via deletion or partial gene conversion, as described elsewhere (Jeffares et al., 2006). Based on strong phylogenetic support and TTS motif conservation, we designate homologs contained in this clade as Type II CBLs.

Phylogenetic analyses also identified a strongly supported clade that contains Arabidopsis CBL10, the only Arabidopsis CBL

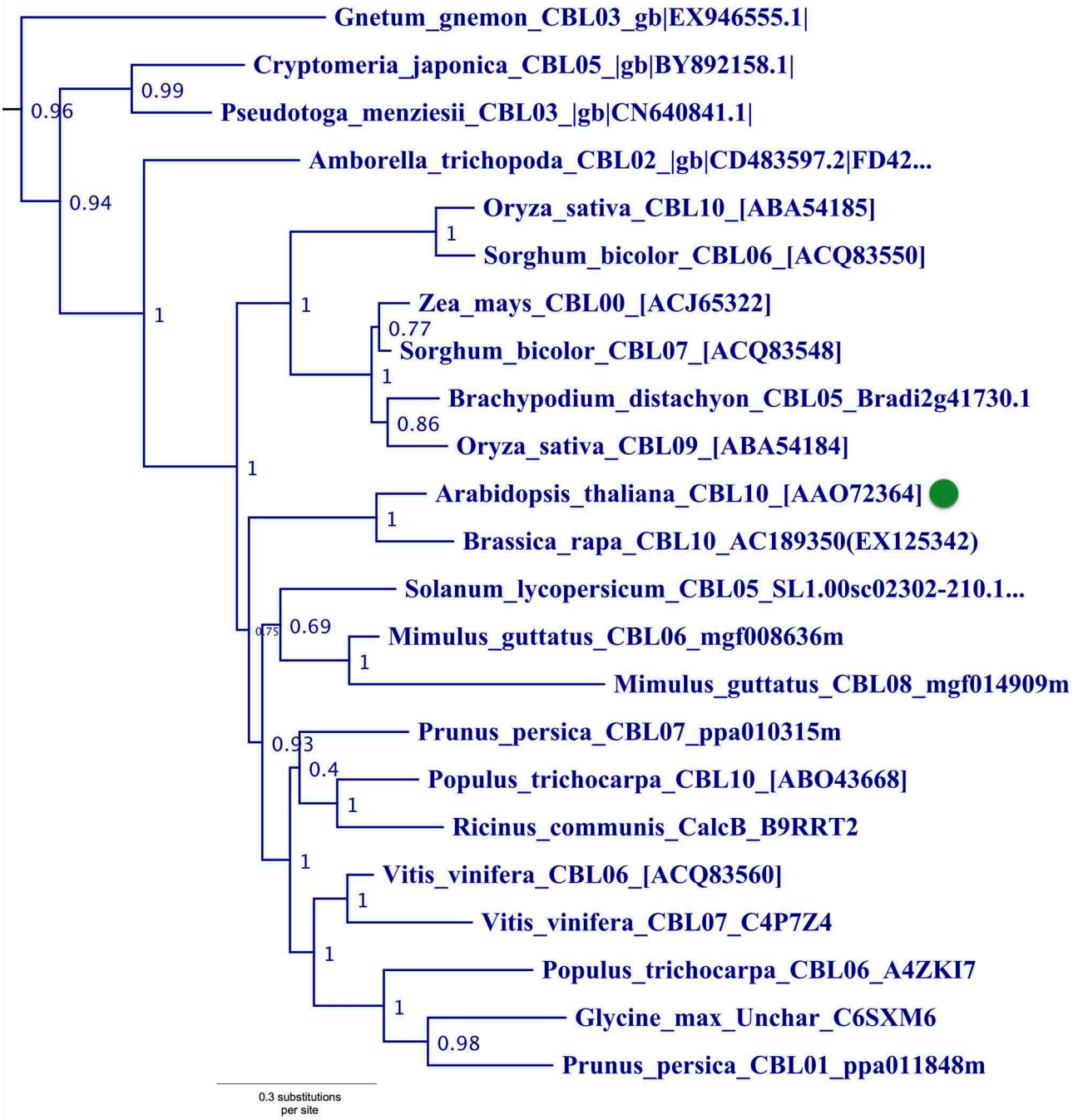

FIGURE 5 | Close-up of Type III CBL clade from ML tree shown in Figure 2. Confidence scores (aLRT) are shown for each clade. Arabidopsis CBL10 (green dot) and orthologs in angiosperms and gymnosperms that share the presence of a single N-terminal transmembrane helix (see **Figure 3**) form a well-supported clade (aLRT = 0.96). *Physcomitrella* CBL4, which also appears to contain a N-terminal transmembrane helix, does not phylogenetically cluster with this clade or other angiosperm homologs, possibly due to sparse taxon sampling among bryophytes.

predicted to contain a transmembrane (TM) helix for membrane association (**Figure 5**). This clade contains orthologs from all studied angiosperms and gymnosperms, indicating this clade is conserved among seed plants; and all members of this clade with full-length sequences exhibit a predicted N-terminal transmembrane helix. Like members of the AtCBL10 clade, *Physcomitrella* CBL4 contains an extended N-terminus, which we posited may form a transmembrane helix (**Figure 3** *bottom*). Various TM topology prediction methods disagree on whether AtCBL10 or PpCBL4 contain a predicted TM helix (data not shown), however visual inspection of hydrophobicity and patterns of conservation in MSAs suggests that both AtCBL10 and PpCBL4 contain

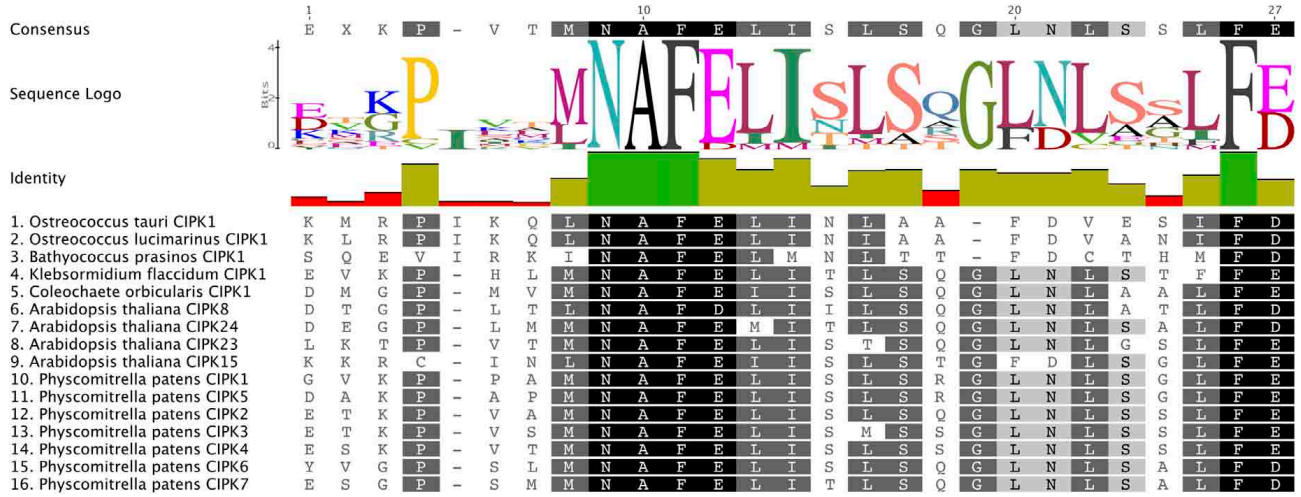

FIGURE 6 | The CBL-interacting or NAF motif is conserved in green algal and moss CIPKs. Consensus amino acid sequence of this motif and degree conservation, illustrated by bar graph, are provided above the MSA, which is shaded based on the degree of AA conservation. Strong conservation of this motif, responsible for CIPK interactions with CBLs, suggests that CBLs and CIPKs identified from green algae and early-diverging land plants constitute a functionally linked network.

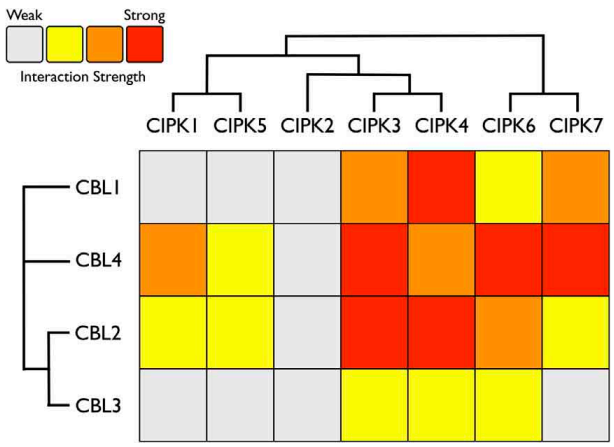

FIGURE 7 | Heat map summarizing yeast two-hybrid (Y2H) results for all *Physcomitrella* CBL and CIPK combinations. Each CBL and CIPK was fused to activation domain (AD) or DNA-binding domain (BD) of a split transcription factor and screened for interactions between CBL-AD/CIPK-BD fusion proteins and CBL-BD/CIPK-AD fusions. Interaction strength was inferred by serial growth dilutions on selective media lacking one or two auxotrophic markers and summarized qualitatively by heat map. Red boxes indicate vigorous growth on -LTHA plates (see Materials and Methods); orange boxes indicate weaker growth on -LTHA plates. Yellow boxes indicate robust growth on -LTH plates but no growth on -LTHA plates. Gray boxes indicate weak growth on -LTH plates, but each CBL-CIPK interaction conferred better growth than the empty vector (EV) control. Representative images of each assay are shown in Supplementary Figure S3. Inferred phylogenetic relationships of *Physcomitrella* CBLs and CIPKs are indicated by cladogram and described in the main text.

N-terminal TM helices. The presence of this TM helix raises the possibility that PpCBL4 may be an AtCBL10 ortholog, however our phylogenetic data neither favor nor disfavor this hypothesis. More thorough coverage of sequence data from early-diverging plants is likely required to test this possibility and determine whether Type III CBLs are monophyletic or not. The Arabidopsis *CBL10* transcript is reportedly processed into mRNAs that encode proteins with two distinct N-termini, though both share the same TM helical region. Alternative splicing is mediated by a unique 8th intron (other rice and Arabidopsis CBLs contain 6 or 7 introns) toward the 5′ pend of the transcript. Both *Physcomitrella CBL4* and Arabidopsis *CBL10* share a very similar exon-intron structure (data not shown), though we did not find evidence of alternative splicing in *PpCBL4*.

The typically short length and strong structural conservation of EF hand proteins like CBLs can complicate phylogenetic reconstruction, as relatively few substitutions can significantly influence results. Due to biophysical constraints, EF hand domains typically exhibit strong sequence conservation at positions that coordinate calcium ion binding. However, variation seen among EF hands of CBLs are predicted to have widely differing affinities for calcium ions, thereby facilitating functional diversity at the level of calcium binding. The 4th EF hand (EF4) of *Physcomitrella* CBL4 is unusual in that it contains non-polar residues at two of the positions that coordinate calcium ion binding, rather than negatively charged residues as seen in virtually all other EF hands. Therefore, it appears likely that it does not bind calcium. Indeed, studies of other calcium signaling pathways have underscored the plasticity of signaling components during evolution. The model yeast *Saccharomyces cerevisiae* contains a single-copy gene encoding a calmodulin (CaM), a widely studied type of calcium sensor in eukaryotes. This gene, *CMD1*, is indispensable for survival of the cell. Surprisingly, molecular genetic analysis suggests the CaM's ability to bind calcium ions is dispensable for its most vital functions, and its fourth EF hand is unable to bind calcium (Cyert, 2001). Plants contain a suite of typical CaMs and widely divergent CaM homologs, some of which either lack the ability to bind calcium ions or coordinate them in an unusual manner

(McCormack and Braam, 2003). Further work is needed to clarify the capacity and affinity of identified CBLs for calcium binding, particularly among non-angiosperm CBLs.

There has been some debate as to the localization of Arabidopsis CBL10; various reports indicate localization to the tonoplast (Kim et al., 2007; Batistic et al., 2010) or plasma membrane (Quan et al., 2007; Ren et al., 2013). Although the multiple isoforms of CBL10 may account for different localization patterns, Arabidopsis CBL10 is most strongly expressed in shoots and is suggested to participate in the regulation of a NHX-family, Na^+/H^+ exchanger believed to function in the sequestration of sodium ions within the vacuole. A model has emerged wherein CBL10 plays a regulatory role in the SOS pathway akin to that of CBL4/SOS3 (Kim et al., 2007; Tang et al., 2013). In root hair and cortical cells, the Type I Arabidopsis CBL4 forms a complex with CIPK24, and together they regulate the activity of the plasmalemma-localized Na^+/H^+ exchanger SOS1 (syn. NHX7) and facilitate the extrusion of sodium ions from the plant. In shoot mesophyll cells, CBL10 complexes with CIPK24, and together they putatively regulate the activity of an unidentified tonoplast-localized Na^+/H^+ exchanger and facilitate sequestration of sodium ions in the vacuole. A recent publication proposes a role for CBL10 in the regulation of the plasmalemma-localized potassium channel AKT1 (Ren et al., 2013), which has been rigorously shown to be subject to regulation by CBL1 and CBL9 acting in concert with CIPK23 (Li et al., 2006; Xu et al., 2006). Our phylogenetic results indicate that the single-pass N-terminal TM helix is a synapomorphy of the AtCBL10 clade. *Physcomitrella* CBL4 likewise contains a N-terminal TM helix and may be orthologous, therefore we designated these homologs Type III CBLs.

Different membranes of the eukaryotic cell have distinct phospholipid profiles, which can serve as a basis for subcellular targeting. Moreover, each particular membrane is commonly composed of distinct microenvironments with unique lipid and protein populations. Together, proteins and lipids are thought to form functional modules in cellular membranes, with membrane-targeted kinases recognized as common regulatory modules (Engelman, 2005). For these reasons, we expect that CBL-CIPK complexes are likely targeted not only to specific membranes but to precise sites within membranes where they interact and function with molecular partners (Bhatnagar and Gordon, 1997; Levental et al., 2010). Elevation of free calcium in the cytosol is localized and transient, partly due to effects of Ca^{++}-ATPases and Ca^{++}/H^+ antiporters and proteins that act as buffers. Because calcium signatures occur locally, calcium sensors must operate in close proximity to the channels responsible for calcium elevation (Fogelson and Zucker, 1985; Gilroy et al., 1993; Roberts, 1994; Clapham, 2007). In light of this, we interpret the conservation of CBL localization motifs among distantly related plants as a likely consequence of constraints on CBL-CIPK subcellular localization.

Although several studies have examined CBL localization, it remains unclear whether CBLs display a predominantly static or dynamic localization at protein maturity. Our analyses demonstrate that the cysteine residue occupying the third position in the Type I motif (MGCXXS/T) is perfectly conserved among CBLs from widely divergent organisms and paralogous clades. In Type I and Type II CBLs, this residue has been shown to be S-acylated, and the modification is required for known protein functions (Batistic et al., 2008; Batistič, 2012; Tang et al., 2012). Based on its striking conservation, we predict that S-acylation of this conserved residue is a shared among CBLs, at least under certain conditions. It is well established that S-acylation is a reversible post-translational modification and that it can strongly impact protein localization and can be critical for protein function (Bijlmakers and Marsh, 2003; Hemsley and Grierson, 2008). Prior research has pointed toward a role for S-acylation in fine-level targeting of proteins to specific membrane microenvironments (Bhatnagar and Gordon, 1997; Mumby, 1997; Dunphy and Linder, 1998; Levental et al., 2010). We predict that N-terminal S-acylation at this conserved residue functions, at least in part, as a mechanism for precise and dynamic localization of CBLs.

CONSERVATION OF THE NAF MOTIF AND CBL-CIPK INTERACTIONS IN *PHYSCOMITRELLA*

The CBL-CIPK network is mediated by a conserved CBL-interacting domain (also known as the NAF or FISL motif) in CIPKs. Our MSA of the CIPK family indicates that the NAF domain is strongly conserved, with many identical residues, among algal CIPKs and all CIPKs from Arabidopsis and *Physcomitrella* (**Figure 6**). This observation is consistent with our prediction that CBLs and CIPKs from green algae and early diverging embryophytes function together as a module. To confirm our presumption that *Physcomitrella* CBLs and CIPKs physically interact with each other and lend support to our interpretation of these protein families as functionally connected in early-diverging plants, we performed Y2H screening and characterized physical interactions between full-length PpCBLs and PpCIPKs in yeast cells.

Consistent with our expectations, CBLs and CIPKs from *Physcomitrella* showed physical interactions in yeast cells. All combinations of PpCBL and PpCIPK fusion proteins showed physical interactions in yeast (Supplementary Figure S2), but specific CBL-CIPK combinations showed very strong interactions with select partners, consistent with the hypothesis that particular CBLs show preferential interactions with cognizant CIPKs (**Figure 7**). We observed that "creeter" CIPKs displayed overlapping, though not identical, interaction profiles with their most closely related homolog. CIPK1 and CIPK5 interact moderately with CBL2 and CBL4 and weakly with CBL1 and CBL3. CIPK3 and CIPK4 interact weakly with CBL3 but moderately to strongly with CBL1, 2, and 4. CIPK6 and CIPK7 interact strongly with CBL4 and weakly to moderately with CBL1, 2, and 3. We observed only weak interactions between CIPK2, which lacks a "sister" CIPK, and any CBL, despite conservation of its NAF domain and phylogenetic proximity to the highly interactive CIPK3 and CIPK4.

Among the CBLs, CBL4 shows the highest number of strong connections to CIPKs, and it interacts very strongly with CIPK6 and CIPK7, members of the green algal clade of CIPKs. CBL1, a Type I CBL without clear phylogenetic affinities to angiosperm CBLs, most strongly interacts with CIPK4 and shows very weak interactions with CIPK1 and CIPK5. CBL3 shows clearly weaker interactions with CIPKs than its close paralog CBL2, although

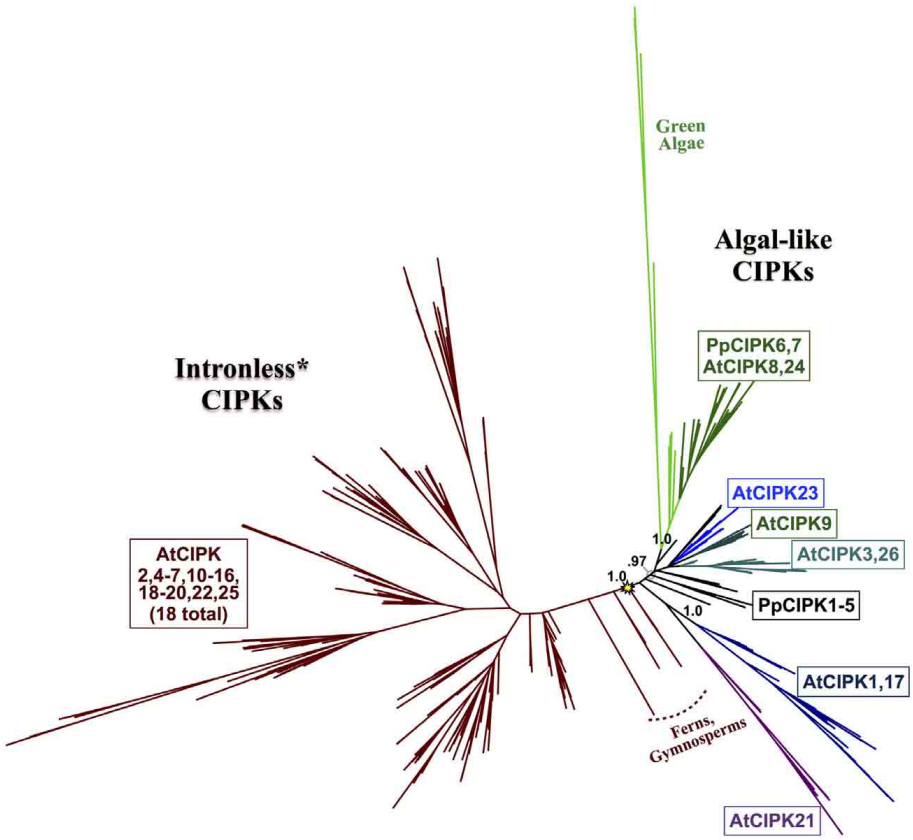

FIGURE 8 | ML phylogenetic tree derived from protein MSA of all CIPKs identified in this study. Confidence scores (aLRT) are shown for select clades. CIPKs from green algae phylogenetically cluster with land plant CIPKs, including *Physcomitrella* CIPK6 and CIPK7. Remaining *Physcomitrella* CIPKs cluster with Arabidopsis CIPK1, 17, and 21, which contain multiple introns, and a clade of "intronless" *CIPKs* (although *AtCIPK16* has gained one intron) derived from an inferred reverse transcription event (yellow star). See **Supplementary Table S3** for CIPKs in this study, Supplementary File S3 for MSA, and Supplementary File S4 for full phylogenetic tree with tip labels. (*Although AtCIPK16 has gained one intron.)

their interaction profiles are similar. CBL2 and CBL3 both interact most strongly with CIPK3, CIPK4, and CIPK6. These data support the model of a highly interconnected signaling network, however interaction patterns may differ significantly in moss cells due to differences in post-translational modifications, subcellular localization, expression, and other factors. Nonetheless, these results provide a guide for genetic analyses in moss and lend confidence to the interpretation that CBLs and CIPKs are functionally linked in early-diverging plants and constitute an ancient signaling network.

PHYLOGENOMIC IDENTIFICATION OF THE ANCESTRAL OR "GREEN ALGAL-TYPE" CLADE OF CIPKs

Phylogenomic analyses of the CIPK family were pursued, as described for CBLs, to decipher evolutionary patterns to facilitate identification of functionally meaningful groups, which would be expected to show conservation across diverse land plants. Our phylogenomic analyses of CIPKs (**Figure 8**; see Supplementary Figure S3 for full tree) indicated most Arabidopsis CIPKs (18 of 26) are contained within an "intronless" clade (although *CIPK16* contains a single intron that is inferred to be from an intron-gain event), consistent with prior analyses by Kolukisaoglu et al. (2004). We used conifer protein sequences from this clade as queries for tBLASTn searches of *Picea* chromosomal sequences (available at http://congenie.org) and did not identify introns in expected locations for intron-containing CIPKs (data not shown). Based on these observations, we posit that a reverse transcription event occurred before the split of gymnosperms and angiosperms and is a conserved feature of this clade. All *Physcomitrella CIPKs* contain multiple introns, and none cluster with the intronless clade. *Physcomitrella* CIPK1—CIPK5 share high sequence similarity (83–93% pairwise); and in our analyses, they were placed with strong confidence in a clade with homologs from other mosses, indicating they are paralogs in respect to their closest seed plant homologs. This clade of moss homologs is likely orthologous (aLRT = 0.97) to three clades of CIPKs conserved across seed plants: the aforementioned intronless clade, a clade containing AtCIPK21, and a clade containing AtCIPK1 and AtCIPK17.

Arabidopsis CIPK3+CIPK26, CIPK9, and CIPK23 each represent strongly supported (aLRT = 1.0; see Supplementary Figure S3) clades that cluster with one another and contain homologs in fully sequenced angiosperm genomes and, at least for the CIPK3 + CIPK26 and CIPK23 clades, in gymnosperms.

Whereas CIPK9 and CIPK23 regulate potassium transport and function in root and shoot tissues (Cheong et al., 2007; Pandey et al., 2007), CIPK3 has been implicated in abscisic acid (ABA)-dependent regulation of seed germination (Pandey et al., 2008), therefore homologs from seed plants as distantly related as gymnosperms might conceivably have a conserved regulatory role in seeds, given their strong conservation.

Physcomitrella CIPK6 and CIPK7 belong to a clade that contains Arabidopsis CIPK8 and CIPK24 and, importantly, contains all green algal CIPKs identified (**Figure 9**) with high confidence (aLRT = 1.0). Although *Physcomitrella* and Arabidopsis each contain two homologs in this clade, *Physcomitrella* CIPK6 and CIPK7 (72% AA pairwise identity) are the products of a gene duplication that occurred after the split between mosses and the lineage leading to vascular plants. In contrast, Arabidopsis CIPK8 and CIPK24 (60% pairwise identity) each represent a separate, strongly supported clade with orthologs in other angiosperms, implying that they derive from duplications that occurred during seed plant (most likely angiosperm) diversification. Based on our results, we posit that *Physcomitrella* CIPK6 and CIPK7 and

FIGURE 9 | Close-up of the "green algal-type" CIPK clade from ML tree shown in Figure 8. Confidence scores (aLRT) are shown for each clade. Phylogenetic evidence strongly supports the existence of a clade (aLRT = 1.0) containing all CIPK homologs identified from chlorophyte and charophyte green algae, as well as two CIPKs each from *Physcomitrella* (yellow dots) and Arabidopsis (green dots). *Physcomitrella* CIPK6 and CIPK7 are recent paralogs and sister to one another in our analyses. In contrast, Arabidopsis CIPK8 and CIPK24 each have clear orthologs in other sequenced angiosperms, and these clades appear to have arisen from a gene duplication that occurred around the time of divergence of angiosperms (arrow).

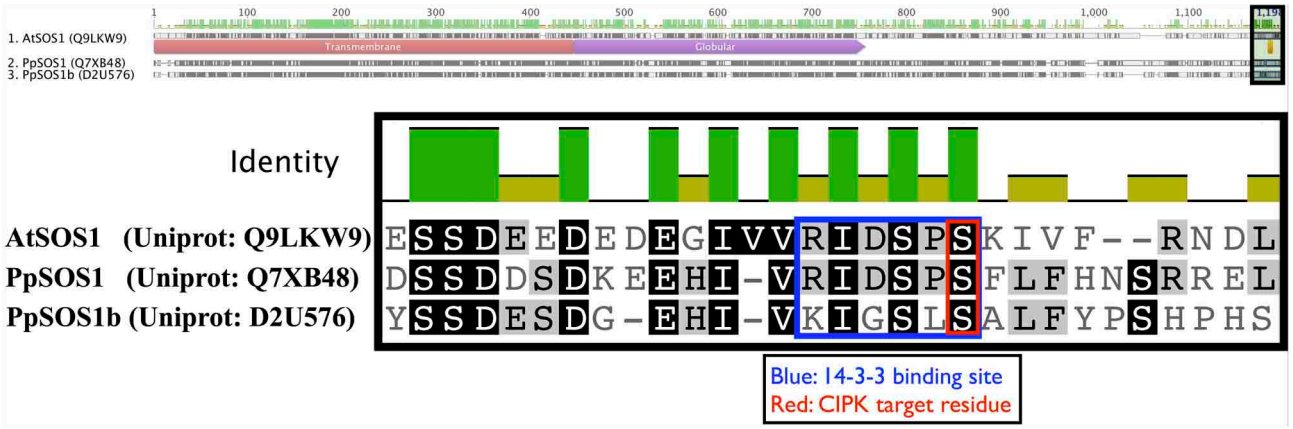

FIGURE 10 | Key regulatory residues at the C-terminus of Arabidopsis SOS1 (AtSOS1) are conserved in *Physcomitrella* homologs. The full-length protein sequence alignment is shown on top with the zoomed in region indicated by a shaded box. Arabidopsis CIPK24 (SOS2) phosphorylates the serine (S) residue marked in red in AtSOS1 and facilitates binding by a 14-3-3 protein. In Arabidopsis, these mechanisms are critical to plant sodium tolerance. Strong sequence conservation suggest similar mechanisms may be in place in *Physcomitrella*, though the cognate CBL-CIPK pair is currently unknown.

Arabidopsis CIPK8 and CIPK24 (SOS2) most closely resemble the ancestral or "green algal-type" CIPK and, due to their orthology, most likely to reflect ancestral function(s) of the CBL-CIPK network.

Arabidopsis CIPK8 is believed to be a positive regulator of the low-affinity phase of the primary nitrate response and has been implicated in glucose sensing, although mechanistic details are unknown at this time (Hu et al., 2009). Arabidopsis CIPK24, the first functionally characterized CIPK, plays a critical function in sodium tolerance through CBL4(SOS3)-modulated phosphorylation of the Na^+/H^+ exchanger SOS1. There is substantial evidence that orthologs of CBL4 and CIPK24 in other flowering plant lineages have similar functions (Martínez-Atienza et al., 2007; Tang et al., 2010). Given the phylogenetic proximity of Arabidopsis CIPK24 to green algal CIPKs, future work will test whether green algal CIPKs, and *Physcomitrella* CIPK6 and CIPK7, function in Na^+/K^+ homeostasis or possibly more broadly regulate ion transport. It has already been established that two orthologs of SOS1 in *Physcomitrella* (PpSOS1 and PpSOS1b) are required for proper K^+/Na^+ ratios and sodium tolerance (Quintero et al., 2011). Interestingly, a 6 AA C-terminal motif of AtSOS1 that is a phosphorylation substrate of CIPK24 and a 14-3-3 protein-binding site is 100% identical to PpSOS1 and 50% identical to PpSOS1b, and the target serine is conserved in both homologs (**Figure 10**). *Physcomitrella* SOS1 has further been shown to confer enhanced NaCl tolerance when heterologously expressed in yeast, and the effect is strengthened by coexpression with Arabidopsis CBL4 and CIPK24 (Fraile-Escanciano et al., 2010). Collectively, these observations suggest that the SOS pathway is conserved across land plants and may be conserved among some green algal lineages. Functional molecular analyses of CBLs and CIPKs in early-diverging plant and algal lineages could provide core insights and clarify the increasingly complex picture of calcium-regulated abiotic stress responses in Arabidopsis and agricultural species.

CONCLUSIONS

Prior publications (e.g., Batistič and Kudla, 2009; Weinl and Kudla, 2009) have mentioned the apparent expansion of the CBL-CIPK network in terms of the total numbers of CBLs and CIPKs found in algae and early diverging plants compared to their angiosperm counterparts. Here, we present phylogenetic evidence that the CBL-CIPK network has expanded independently in multiple plant lineages, including mosses and angiosperms. It appears that the common ancestor of mosses and vascular plants likely contained three CBLs distinguishable by N-terminal localization motifs, which likely are synapomorphies among ancient CBL subfamilies. We have identified a clade of CIPKs containing all green algal homologs and two representatives from *Physcomitrella* and Arabidopsis. Phylogenetic analysis demonstrates that the *Physcomitrella* and Arabidopsis members of this clade are the products of independent gene duplications and the earliest land plants likely contained a single homolog from this clade. The concurrent pairing of *CBLs* and *CIPKs* in available genomes and transcriptomes, the striking conservation of the NAF domain, and our Y2H results all point toward a physically and functionally connected CBL-CIPK network across plants and algae.

The function(s) of CBL-CIPK pairs found in green algae remains an open and intriguing question, and our identification of charophyte CBL-CIPK pairs expands the list of potential models for this inquiry. The conspicuous expansion of the network in several land plant lineages appears to have been driven largely by WGDs, and we hypothesize that duplicated members were adapted for novel signaling pathways and precise roles in particular cells and tissues. Research on molecular processes modulated by CBLs and CIPKs has intensified in recent years, and researchers are beginning to investigate CBL-CIPK functions in non-model angiosperm species. The field is prime for investigation of CBL-CIPK functions in earlier diverging land plants, and research in this area will enhance our understanding of the molecular evolutionary basis of the colonization of land by plants.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00187/abstract

Supplementary Table S1 | CBL sequences used in this study.

Supplementary Table S2 | CIPK sequences used in this study.

Supplementary Table S3 | Oligonucleotides used in this study.

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Allele diversity for abiotic stress responsive candidate genes in chickpea reference set using gene based SNP markers

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Chickpea is an important food legume crop for the semi-arid regions, however, its productivity is adversely affected by various biotic and abiotic stresses. Identification of candidate genes associated with abiotic stress response will help breeding efforts aiming to enhance its productivity. With this objective, 10 abiotic stress responsive candidate genes were selected on the basis of prior knowledge of this complex trait. These 10 genes were subjected to allele specific sequencing across a chickpea reference set comprising 300 genotypes including 211 genotypes of chickpea mini core collection. A total of 1.3 Mbp sequence data were generated. Multiple sequence alignment (MSA) revealed 79 SNPs and 41 indels in nine genes while the *CAP2* gene was found to be conserved across all the genotypes. Among 10 candidate genes, the maximum number of SNPs (34) was observed in abscisic acid stress and ripening (*ASR*) gene including 22 transitions, 11 transversions and one tri-allelic SNP. Nucleotide diversity varied from 0.0004 to 0.0029 while polymorphism information content (PIC) values ranged from 0.01 (*AKIN* gene) to 0.43 (*CAP2* promoter). Haplotype analysis revealed that alleles were represented by more than two haplotype blocks, except alleles of the *CAP2* and sucrose synthase (*SuSy*) gene, where only one haplotype was identified. These genes can be used for association analysis and if validated, may be useful for enhancing abiotic stress, including drought tolerance, through molecular breeding.

Keywords: chickpea, abiotic stress, single nucleotide polymorphism, genetic diversity, candidate genes

INTRODUCTION

Chickpea (*Cicer arietinum* L., $2n = 16$), a self-pollinated, diploid annual species which ranks second worldwide as a food legume crop, is primarily a crop of developing countries contributing to a larger part of human food and animal feed in these areas. Chickpea is a major source of nutrients to a vegetarian diet as it contain 20–30% protein, ∼40% carbohydrates and is also a good source of several minerals like calcium, magnesium, potassium, phosphorus, iron, zinc, and manganese. Global chickpea production is 11.6 million t from 12.3 million ha area with an average yield of less than one t/ha (FAO, 2012), much lower than its estimated potential of 6 t/ha under optimum growing conditions. Productivity of chickpea is adversely affected by several abiotic stresses of which drought, heat and cold are the major constraints affecting seed yield (Ruelland et al., 2002). Plant stress responses are generally controlled by a network of specialized genes through intricate regulation by specific transcription factors (Chen and Zhu, 2004). Application of available approaches to improve crop productivity under adverse environmental conditions requires a better understanding of the mechanisms involved during crop's response to abiotic stress. Genomic technologies and comparative genomics approaches that have emerged during the past decade can be exploited to identify some of the genes involved in drought tolerance mechanisms. Candidate genes for stress tolerance may be used in crop improvement programs directly (transgenic approach) or indirectly (through identification of linked SNPs) (Schena et al., 1995; Kudapa et al., 2013). The "chickpea mini core" comprising of 211 diverse genotypes (Upadhyaya and Ortiz, 2001) is a subset of the core collection (Upadhyaya et al., 2001) which represents the entire collection conserved in the ICRISAT Genebank. The reference set (Upadhyaya et al., 2008) includes four *C. reticulatum* genotypes and three *C. echinospermum* genotypes, but the majority (293 genotypes) is *C. arietinum* (Upadhyaya et al., 2006).

Although several genes have been found to be involved in abiotic stress tolerance in other crops, few studies have been carried out in chickpea. Candidate genes can be selected on the basis of prior knowledge from mutational analysis, biochemical pathways

or linkage analysis of the trait of interest (Zhu et al., 2008). The candidate genes we selected were; Snf-1 related kinase (*AKIN*), amino-aldehyde dehydrogenase (*AMADH*), abscisic acid stress and ripening (*ASR*) gene, a homolog of the *DREB2A* gene, known as the *CAP2* gene, dehydrin (*DHN*), drought responsive element binding protein (*DREB*), *ERECTA*, Myb transcription factor (*MYB*), sucrose phosphate synthase (*SPS*), and sucrose synthase (*SuSy*).

The *AKIN* (*SNF1 related protein kinase*) gene belongs to the CDPK–SnRK superfamily, which serves as important regulators modulating fundamental metabolic pathways in response to nutritional and environmental stresses in plants (Halford and Hey, 2009). An *AMADH* gene in sorghum was found to be related to osmotic stress tolerance, dehydration and salt stress tolerance (Wood et al., 1996) and the activity of *AMADH* in response to stress caused by mechanical damage in pea seedlings was evaluated by Petrivalský et al. (2007). *AMADH* is expected to play a role in physiological processes and metabolic pathways controlling response to abiotic stresses by detoxification of toxic aminoaldehydes (Stiti et al., 2011). *ASR* gene is a stress-inducible gene that has been reported exclusively in plants and belongs to a small gene family characterized by the presence of an ABA/WDS domain. Members of the *ASR* gene family are induced by abscisic acid (ABA), various abiotic stresses including water stress and during the process of fruit ripening (Carrari et al., 2004). *ASR* genes in various species respond to different abiotic stress factors including drought, salt, cold and limited light (Joo et al., 2013). Over-expression of *ASR* in transgenic *Arabidopsis* was shown to increase tolerance to drought and salt and decrease sensitivity to exogenous ABA (Yang et al., 2005). Characterization of the *ASR* gene family in rice identified the *ASR3* gene as a candidate for association studies related to drought tolerance (Philippe et al., 2010). The potential importance of the *ASR1* gene in drought tolerance in common bean was reported by Cortés et al. (2012a) who found low nucleotide diversity suggestive of strong purifying selection, in wild and cultivated accessions.

Dehydrins (*DHNs*) are among the most commonly observed proteins induced by environmental stress associated with dehydration or low temperature (Hanin et al., 2011). The *DHN* proteins have been estimated to comprise up to 4% of the total seed protein, and are thought to be involved in protecting the embryo and other seed tissues from osmotic stresses associated with the low water content of the mature seed (Wise and Tunnacliffe, 2004). A positive correlation between accumulation of *DHN* proteins and tolerance to freezing, drought, and salinity has been shown (Close, 1996; Allagulova et al., 2003). Transgenic plants overexpressing *DHN* showed better growth and tolerance to drought and freezing stress compared to controls (Puhakainen et al., 2004). *DREB* are transcription factors that induce a set of abiotic stress-related genes and impart stress endurance to plants. *DREB*s belong to the *ERF* (ethylene responsive element binding factors) clade of the *APETALA2* (AP2) family are distinctive to plants. Transcription factors *DREB1A*/CBF3 and *DREB2A* were identified as cold and drought stress–responsive genes expressed in *Arabidopsis thaliana* (Sakuma et al., 2006). Constitutively activated *DREB2A* resulted in significant drought stress tolerance in transgenic *Arabidopsis* plants and expression analysis revealed that *DREB2A* transcriptionally regulates many water stress-inducible genes (Sakuma et al., 2006). In rice, expression of *OsDREB2A* was induced by dehydration and high-salt stresses (Matsukura et al., 2010; Mallikarjuna et al., 2011). Based on physiological studies in several crop species, the *DREB2A* transcription factor is one of the most promising candidate genes for drought tolerance. Low sequence diversity of *DREB2A* was found in five crop species studied; chickpea, common bean, rice, sorghum, and barley (Nayak et al., 2009) as well as in studies of wild and cultivated common bean (Cortés et al., 2012b).

The *ERECTA* gene codes for a protein kinase receptor which mediates plants' responses to disease, predation and stress. *ERECTA* is involved in leaf organogenesis and reduces the density of stomata on the leaf under-surface, thereby reducing the evapotranspiration. In *Arabidopsis*, the *ERECTA* gene has been shown to control organ growth and flower development by promoting cell proliferation (Shpak et al., 2004). The contribution of *ERECTA* gene toward water use efficiency was confirmed using complementation assays on wilting mutant *Arabidopsis* plants (Masle et al., 2005). The *ZmERECTA* genes from maize are patented by Pioneer Hi-Bred International, Inc., which were involved in improving plant growth, transpiration efficiency and drought tolerance in crop plants (www.freepatentsonline.com/y2008/0078004.html). The *Myb* transcription factor family constitutes the largest and diverse class of DNA-binding transcription factors in plants (Riechmann et al., 2000). The roles of *Myb* genes in response to biotic and abiotic stress have been studied in a number of plant species (Romero et al., 1998; Du et al., 2012; Volpe et al., 2013). *SuSy*, a glycosyltransferase, and *SPS* are key enzymes involved in sugar metabolism. Sucrose-synthase transcript and protein levels have been shown to be modulated by dehydration and rehydration (Kleines et al., 1999) and the *Arabidopsis AtSUS3* gene in particular was shown to be strongly induced by drought and mannitol, thus behaving as a marker of dehydrating tissues (Baud et al., 2004).

Genetic diversity, representing the overall genetic makeup of a species, serves as a basis for a population to adapt to changing environments (Ross-Ibarra et al., 2007). Single nucleotide polymorphisms (SNPs) have gained much popularity in assessing the diversity because of automation and abundance. Though biallelic SNPs are generally less informative than multi-allelic simple sequence repeats (SSRs), their sheer abundance makes the development of high density SNP genetic maps possible, providing the foundation for subsequent population-based genetic analysis (Rafalski, 2002). In addition, a SNP is of great importance if it affects gene function and the function of the gene in stress response is known/understood and the SNP is associated with differences in plant performance. Assessing genetic diversity for stress responsive candidate gene sequences leads to the identification of a specific allele of the particular gene in that species associated with performance in response to a corresponding abiotic stress. Such information can therefore be further used in breeding programs to develop better varieties using modern molecular breeding approaches like marker assisted recurrent selection (MARS) or gene pyramiding. Allelic diversity (richness), one of the most important and commonly used estimators of genetic diversity in populations, mainly depends on the effective

population size and past evolutionary history (Petit et al., 1998). However, the number of alleles identified and their frequency distribution also depend on the genetic marker system used in these investigations. In the present study, the allelic diversity of candidate genes for abiotic stress tolerance was assessed in the chickpea reference set.

MATERIALS AND METHODS

PLANT MATERIAL AND DNA EXTRACTION

Young leaf tissues of each accession of the reference set from the greenhouse grown plants were harvested and immediately stored in 96-well plate and the total genomic DNA of all the genotypes was isolated using high-throughput mini- DNA extraction method (Cuc et al., 2008). The quality and quantity of extracted DNA was checked on 0.8% agarose gel. The DNA was normalized to 20 ng/μl concentration for further use.

IDENTIFICATION OF ABIOTIC STRESS RESPONSIVE GENES AND PRIMER DESIGNING

A set of 10 abiotic stress responsive genes conferring abiotic stress tolerance in model plants (*Arabidopsis* and Rice) and other crop species (*Glycine max* and *Medicago* spp.) were chosen based on available literature (**Table 1**). Different approaches were used for primer designing based on availability of gene sequence information in chickpea. In the first approach, heterologous primers were designed for *ASR*, *SuSy*, and *SPS* genes from corresponding *Medicago* sequences. The *ERECTA* gene in chickpea was isolated using consensus/degenerate primers designed at INRA, EPGV, France. In the second approach sequence-specific primers were designed, where in chickpea homologs of genes were isolated using chickpea ESTs developed for abiotic stress (Varshney et al., 2009) and available in NCBI EST database (DbEST- http://www.ncbi.nlm.nih.gov/dbEST/) (Roorkiwal and Sharma, 2012). The details of primers used in isolation of abiotic stress responsive candidate genes in chickpea are given in **Table 1**.

POLYMERASE CHAIN REACTION (PCR) AND SEQUENCING OF AMPLICONS

In order to amplify these candidate genes and confirm their presence, a pilot experiment was set to sequence amplicons from eight diverse genotypes of chickpea consisting of Annigeri, ICCV 2, ICC 4958, ICC 1882, ICC 283, ICC 8261, ICC 4411, and ICC 10029. PCR was set up with 20 μl reaction mixture comprising 5 ng of template DNA, 5 picomoles each of forward and reverse primers, 2 mM dNTP, 20 mM $MgCl_2$, 1X PCR buffer (AmpliTaq Gold) and 0.25 U of Taq polymerase (Ampli *Taq* Gold). PCR cycles comprising of denaturation of 94°C for 5 min, followed by 40 cycles of 94°C for 30 s annealing at temperature specific for each target gene for 40 s and 72°C for 1 min 30 s and a final extension was carried out at 72°C for 20 min. The amplified product (about 2 μl) was loaded on 1.2% agarose. The remaining PCR amplicons were purified using 1 unit of Exonuclease I and 1 unit of shrimp alkaline phosphatase (SAP) per 5 μl of PCR product. The Exo/SAP added PCR products were incubated for 45 min at 37°C followed by denaturing at 80°C for 15 min in the thermal cycler for deactivating unused exonuclease enzyme. The Exo/SAP treated amplicons were mixed with 1 μl of BigDye Terminator V3.1 (Applied Biosystems, California, USA), 2 μl of 5X sequencing dilution buffer and 3.2 μM of primer (forward and reverse, separately) and the volume was made to 10 μl by adding water. The sequencing PCR profile included an initial denaturation of 96°C for 30 s, followed by 60 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The PCR products were stored at 4°C until further use. Before sequencing, the PCR products were treated with 2.5 μl of 125 mM EDTA and 25 μl of absolute ethanol and incubated for 15 min at room temperature to precipitate the DNA. The plate containing the PCR product was centrifuged at 4000 rpm for 30 min at 4°C. The ethanol/ EDTA mix was poured off by inverting the plate, without losing the pellet. To each well, 60 μl of 70% ethanol was added and again spun at 4000 rpm for 20 min at 4°C. The ethanol was poured off as earlier. The plate was air-dried and 10 μl of HiDi formamide (Applied Biosystems, California, USA) was added and the products were denatured (94°C for 5 min, then immediately cooled to 4°C for 5 min) and sequenced using an ABI3700/ABI3130 automated sequencer (Applied Biosystems, California, USA).

ALLELE SEQUENCING AND SNP DETECTION

For allele sequencing, of candidate genes across the 300 genotypes of the reference set, PCR and purification were carried out as described above. Sequencing was carried out at MACROGEN, Korea using BigDye terminator cycle sequencing chemistry. Raw sequences were used to obtain contigs by assembling the forward and reverse sequences of each genotype using DNA Baser V 2.9 tool and gene identities were confirmed using BLAST (blastn and blastx). The sequences of each candidate gene were aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Multiple sequence alignment (MSA) files and fasta files were further used for identifying equence related parameters such as number of genotypes sequenced; length of sequences; number of indels; indel frequency; number of SNPs and their types (transition or transversion); SNP frequency; nucleotide and haplotype diversity and polymorphic information content (PIC) of SNPs and haplotypes using an in-house tool developed at ICRISAT called "DIVersity ESTimator" module (DIVEST) (Jayashree et al., 2009). Further, in order to identify if any of the haplotypes could be associated with the country of origin of the genotypes under study, NETWORK programme version 4.516 was used to determine haplotype networks for each candidate gene studied.

RESULTS

ISOLATION AND SEQUENCE ANALYSIS OF ABIOTIC STRESS RESPONSIVE CANDIDATE GENES

An *AKIN* homolog was amplified using the gene specific primer pair designed considering unigene sequence showing match with *Arabidopsis AKIN* (SNF-1 related protein kinase). The approximate amplicon size of *AKIN* was ∼800 bp. Amplification of an *AMADH* homolog yielded a product of ∼900 bp. The ABA stress and ripening (*ASR*) gene was isolated using the heterologous primers derived from *Medicago* sequence AC152054. A single amplicon of 700 bp was obtained for the chickpea genotypes used. A *DREB2A* homolog (also known as *CAP2* gene) and its promoter (*CAP2* promoter) were amplified using a

Table 1 | List of abiotic stress responsive genes and respective primers used for PCR amplification.

Gene	Putative function	Source sequence	GenBank/TC ID	Primer sequences (5′–3′)
SNF-1 related protein kinase (*AKIN*)	Response to nutritional and environmental stresses in plants	Chickpea ESTs	–	F: GTG GTT CAG GTG CAG ACT TG R: TCA GAA AGT GCC CAT CAC GC
Aminoaldehyde dehydrogenase (*AMADH*)	Osmotic stress, dehydration and salt stress tolerance	Chickpea ESTs	–	F: TTG GAA GAA GGT TGC AGG CTA G R: CCC ATT CTC CCA GTT CAC GG
Abscisic acid stress and ripening (*ASR*)	Tolerance to drought and salt stresses	*Medicago*	AC152054	F: GGG AAC TAA TCC TTT CCA AAC A R: CTG CAG CAC CTA ACT CAC CA
CAP2 gene (*DREB2A*)	Regulates expression of water stress-inducible genes	Chickpea	DQ321719	F: CGG CTT CCC TTC ATT CGA TCC A R: AGG CAC AAC ACA AGA ATC CA
CAP2 promoter	Induce a set of abiotic stress-related genes	Chickpea	–	F: TGT GCT TCA AGT TGC ACT CC R: CGG GGT CCT TAT ATA CTG CAG A
Dehydrin (*DHN*)	Induced by environmental stress, dehydration or low temperature	Chickpea ESTs	–	F: AAA GTG GTG TTG GGA TGA CC R: TCC TCT CTC CCG AAT TCT TG
Dehydration responsive element binding (*DREB1*)	Induced by dehydration and high-salt stresses	Chickpea ESTs	–	F: CTT CAT TCG ATC CAG ATT CGG R: AAC GCG AGT TTT CAG GCC CT
ERECTA (fragment 7F-5R)	Mediates plants' responses to disease and stress	Degenerate	–	F: GTG TAC AAA CCT TAA CAG CC R:CCA GTT AAT TCG TTG TTT TC
ERECTA (fragment 8F-8R)	Mediates plants' responses to disease and stress	Degenerate	–	F: GGT CAG CTA CAG AAC ATA GCA R: TCC ATT TTC CAT GTA GTC ATA A
Myb transcription factor	Response to biotic and abiotic stresses	Chickpea ESTs	–	F: ATG CTA CTG CTG CCT ACA AG R: ACC GCA GTA CAC TCC AAG AG
Sucrose synthase (*SuSy*)	Sugar metabolism pathway	*Medicago*	TC95820	F: GAT ACT GGC GGA CAG GTT GT R: CAT CCT TTG CTA GGG GAA CA
Sucrose phosphate synthase (*SPS*)	Induced by drought and mannitol	*Medicago*	BQ137986	F: TTT GGT CCA CGC GAT AAA TA R: TGA ATT GAT ATC CTC CCA AGA

primer pair as described by Nayak et al. (2009). The approximate amplicon size of the *CAP2* gene was 1000 bp while the *CAP2* promoter was ∼700 bp. A dehydrin homolog of chickpea was amplified using a primer pair designed for known dehydrin gene using chickpea unigene. The approximate amplicon size of dehydrin gene was ∼380 bp. A *DREB1* (Dehydration response element binding) homolog in chickpea was also amplified using a primer pair designed using unigene showing match against *DREB1* gene. The approximate amplicon size of the *DREB1* gene was ∼800 bp. About 4300 bp long ERECTA gene fragments were isolated from eight chickpea genotypes using consensus primers. An ∼350 bp long *MYB* gene was amplified using unigene sequence having match against *Glycine max* Myb transcription factor. For isolating the *SuSy* gene in chickpea, heterologous primers were designed from *Medicago* sequences TC95820 (homolog to *SUS2* Pea) and AJ131964 (*Medicago truncatula SUS1* gene). An ∼1500 bp amplicon was obtained for TC95820-derived sequences, while a 900 bp amplicon was obtained with AJ131964- derived sequences. Heterologous primers designed using *Medicago* sequence BQ137986 and CB893717 were used to isolate *SPS* in chickpea. Amplification across eight genotypes in chickpea yielded products of 400 bp in both cases (**Table 2**).

SEQUENCE DIVERSITY ANALYSIS OF CANDIDATE GENES

Forward and reverse sequences for all 10 abiotic stress responsive candidate genes and the *CAP2* gene promoter, were used for contig construction. The number of genotypes for which good quality sequences were obtained varied from 79 (*ERECTA* fragment obtained from 7f-5r primer pairs) to 236 genotypes (*SPS* gene), out of the 300 genotypes. Diversity analysis of the candidate genes using the DIVersity ESTimator (DIVEST) tool is presented in **Table 3**.

SNPs were manually inspected for possible sequencing errors and only those SNPs with clear peaks were considered further (**Figure 1A**). Sequences for each gene were aligned using CLUSTALW and positions of SNPs were identified (**Figure 1B**). The highest number of SNPs (34) was obtained for the *ASR* gene, amongst which 22 were transitions, 11 were transversions and one was tri-allelic. Apart from SNPs, two indels were also detected. The *CAP2* gene was found to be conserved across all 227 genotypes with no SNPs and indels. In the case of *CAP2* promoter, one SNP was found (which was the same observed when eight chickpea genotypes were sequenced as a pilot experiment). For the *ERECTA* gene, two fragments obtained from 7f-5r and 8f-8r primer pairs were sequenced. In total, 13 SNPs (9 transitions

Table 2 | Summary of abiotic stress responsive candidate genes showing match with previously reported accession/gene in other crop species.

Gene	Sequence length (bp)	Sequence similarity result	*e*-value
SNF-1 related protein kinase (*AKIN*)	772	SNF1-related protein kinase catalytic subunit alpha KIN10 [*Arabidopsis thaliana*] AKIN10	6.00E-41
Aminoaldehyde dehydrogenase (*AMADH*)	932	Betaine aldehyde dehydrogenase 1 [*Arabidopsis thaliana*]	2.00E-36
Abscisic acid stress and ripening (*ASR*)	680	(1) TC10668 similar to *ASR* protein homolog (2) *Medicago truncatula* clone (AC126014.6) (3) *Prunus armeniaca* (apricot) *ASR* (U93164.1)	2.80E-18 3.00E-29 0.003
CAP2 gene (*DREB2A*)	1000	DQ321719 (*CAP2* gene *Cicer arietinum*)	0.00
CAP2 promoter	700	–	–
Dehydrin (*DHN*)	381	Dehydrin 1 [*Cicer pinnatifidum*]	2.00E-04
Dehydration responsive element binding (*DREB1*)	776	Dehydration responsive element binding protein [*Cicer arietinum*]	2.00E-09
ERECTA	4300	LRR receptor-like serine/threonine-protein kinase ERECTA [*Medicago truncatula*]	
Myb transcription factor (*MYB*)	335	(1) MYB transcription factor MYB93 [*Glycine max*] (2) Myb-like transcription factor family protein [*Arabidopsis thaliana*]	2.00E-26 0.00
Sucrose phosphate synthase (*SPS*)	400	(1) *M. truncatula* (BQ137986) *SPS* like protein (2) TC103232 homolog to *Medicago sativa* SPS (Q9AXK3)	7.90E-60 9.60E-21
Sucrose synthase (*SuSy*)	900	(1) *M.truncatula SusS1* gene (AJ131964) (2) *Lotus japonicus* genomic DNA clone (AP009336.1) (3) *Vigna radiata* mRNA for *SUSY* (D10266.1)	2.00E-20 3.00E-18 3.00E-06

Table 3 | Estimation of sequence diversity in chickpea reference set/mini core collection using 10 abiotic stress responsive genes.

Candidate gene	***AKIN*** [#]	***AMADH*** [#]	***ASR***	***CAP2***	***CAP2* promoter**	***DHN*** [#]	***DREB1*** [#]	***ERECTA* _7f_5r**	***ERECTA* _8f_8r**	***Myb*** [#]	***SPS***	***SuSy***
Genotypes with successful sequences	208	209	193	227	137	198	191	79	147	200	236	230
Sequence length (bp)	772	932	621	367	629	381	776	921	1189	335	312	884
No. of Indels	2	3	2	0	0	7	23	1	0	2	1	0
Indel frequency	1/386.00	1/310.67	1/310.60	0	0	1/54.43	1/33.74	1/921.00	0	1/167.50	1/312.00	0
No. of SNPs	2	13	34*	0	1	7	14	13	20	6	3	1
Transition	2	6	22	0	0	5	8	9	10	1	2	1
Transversion	0	7	11	0	1	2	6	4	10	5	1	0
SNP frequency	1/386.00	1/71.69	1/18.26	0	1/629.00	1/54.43	1/55.43	1/70.86	1/69.46	1/55.83	1/104.00	1/884.00
Nucleotide diversity (Pi)	0.0004	0.002	0.0014	0	0	0.0022	0.0011	0.0029	0.0029	0.002	0.0011	0.0012
Average PIC of SNP	0.01	0.04	0.1	0	0.43	0.17	0.14	0.27	0.1	0.04	0.01	0.01
No. of haplotypes	3	9	4	1	2	6	33	4	3	6	4	1
Haplotype diversity	0.019	0.326	0.833	0	0.438	0.426	0.879	0.372	0.324	0.256	0.034	0.035
PIC of haplotypes	0.019	0.324	0.829	0	0.436	0.424	0.874	0.367	0.322	0.255	0.034	0.033

The sequence diversity was calculated using DIVEST tool (http://hpc.icrisat.cgiar.org/Pise/5.a/statistics_calculation/SNP_diversity_estimator.html) AKIN, SNF1 related protein kinase; AMADH, Aminoaldehyde dehydrogenase; ASR, Abscisic acid stress and ripening gene; DHN, Dehydrin; DREB1, Dehydration responsive element binding protein; Myb, Myb transcription factor; SPS, Sucrose synthase (SuSy) and sucrose phosphate synthase; [#]Gene was sequenced across 211 genotypes of chickpea mini core collection; [*]One SNP is tri-allelic.

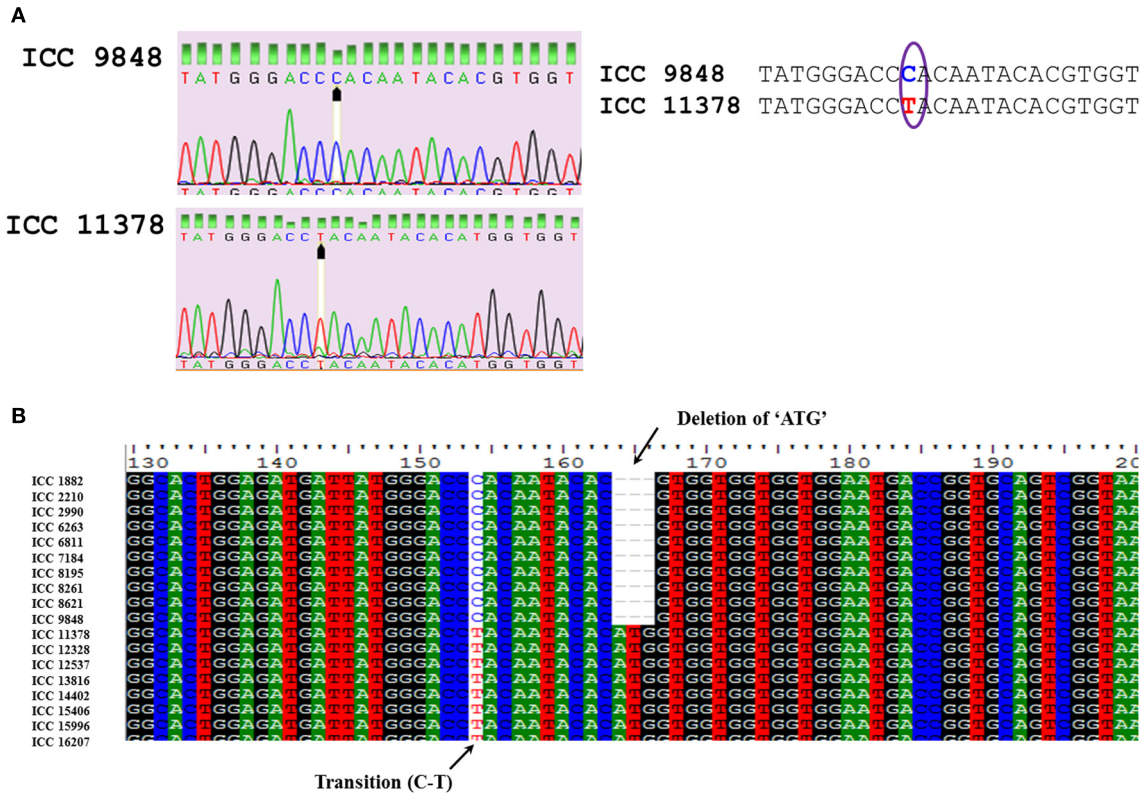

FIGURE 1 | (A) Comparison of sequence quality to confirm the true SNP using peak quality. The presence of SNP in *DHN* gene using sequence chromatogram is highlighted. **(B)**. Alignment of nucleotide sequences encoding *DHN* across various chickpea genotypes.

and 4 transversions) and one indel were obtained for *ERECTA* 7f-5r fragments while 20 SNPs (10 transitions and 10 transversions) were observed for *ERECTA* 8f-8r gene fragments. One indel and 3 SNPs were observed across *SPS* gene sequences. The *AKIN* gene showed the presence of two SNPs and two indels. A total of 13 SNPs (6 transitions and 7 transversions) and 3 indels were identified in the *AMADH* gene, while in the *DHN* gene 7 SNPs (five transitions and two transversions) were identified among 198 sequences analyzed. For the *MYB* gene only 6 SNPs (one transition and five transversions) and 2 indels were found in 200 *Myb* sequences under study. No nucleotide diversity was observed for the *CAP2* gene and promoter while in the case of *AKIN* it was 0.0004 and 0.0029 for both *ERECTA* fragments. The average polymorphic information content (PIC) value of SNPs ranged from 0 (*CAP2* gene) to 0.43 (*CAP2* promoter). Haplotype diversity ranged from 0.019 (*AKIN*) to 0.879 (*DREB1*). Average (PIC) of haplotypes values ranged from 0.019 (*AKIN*) to 0.874 (*DREB1*) (**Table 3**).

HAPLOTYPE NETWORKS FOR CANDIDATE GENES

Based on the sequence information, haplotype networks were drawn using the NETWORK program. The network figures show the number of haplotypes observed for each gene and the SNP position which separates one haplotype from the other. Network diagrams can be drawn only with the presence of more than two haplotype blocks. Haplotype frequency is depicted by circles, for example, the larger the haplotype circle, more genotypes are represented by that haplotype. The color code is given as per the country of origin of the genotypes (**Figures 2A–I**). *CAP2* and *SuSy* gene represented only one haplotype with all the genotypes sequenced while the *CAP2* promoter had only one SNP, forming two haplotype blocks. Hence haplotype network graphs could not be drawn for *CAP2* gene, its promoter and *SuSy* gene. The network analysis showed a linear relationship between haplotypes for most of the genes except for transcription factors *DREB1* and *Myb*, which showed network relationships between larger numbers of haplotypes.

In this study, although we could find more than two haplotype blocks in some of the candidate genes like *AKIN*, *AMADH*, *ASR*, *DHN*, *DREB, MYB, SPS, ERECTA* (7f-5r), and *ERECTA* (8f-8r), there was no clear distinction between the origin of the genotypes and the haplotype information. Haplotype network analysis for the *AKIN* gene reported three haplotypes, including one major (H2) and two minor haplotypes (H1 and H3) (**Figure 2A**). The *AMADH* gene showed the presence of nine haplotypes across the reference set of which, one major haplotype (H9) is connected to eight other haplotypes (**Figure 2B**). There were three minor haplotypes (H1, H2, and H4) derived from a major haplotype (H3) as observed in *ASR* haplotype networks with SNPs ranging from one to four (**Figure 2C**). *DHN* gene haplotype network indicated the presence of six haplotypes, of which one major haplotype (H2) was connected to three minor haplotypes (H1, H3, and H5) with

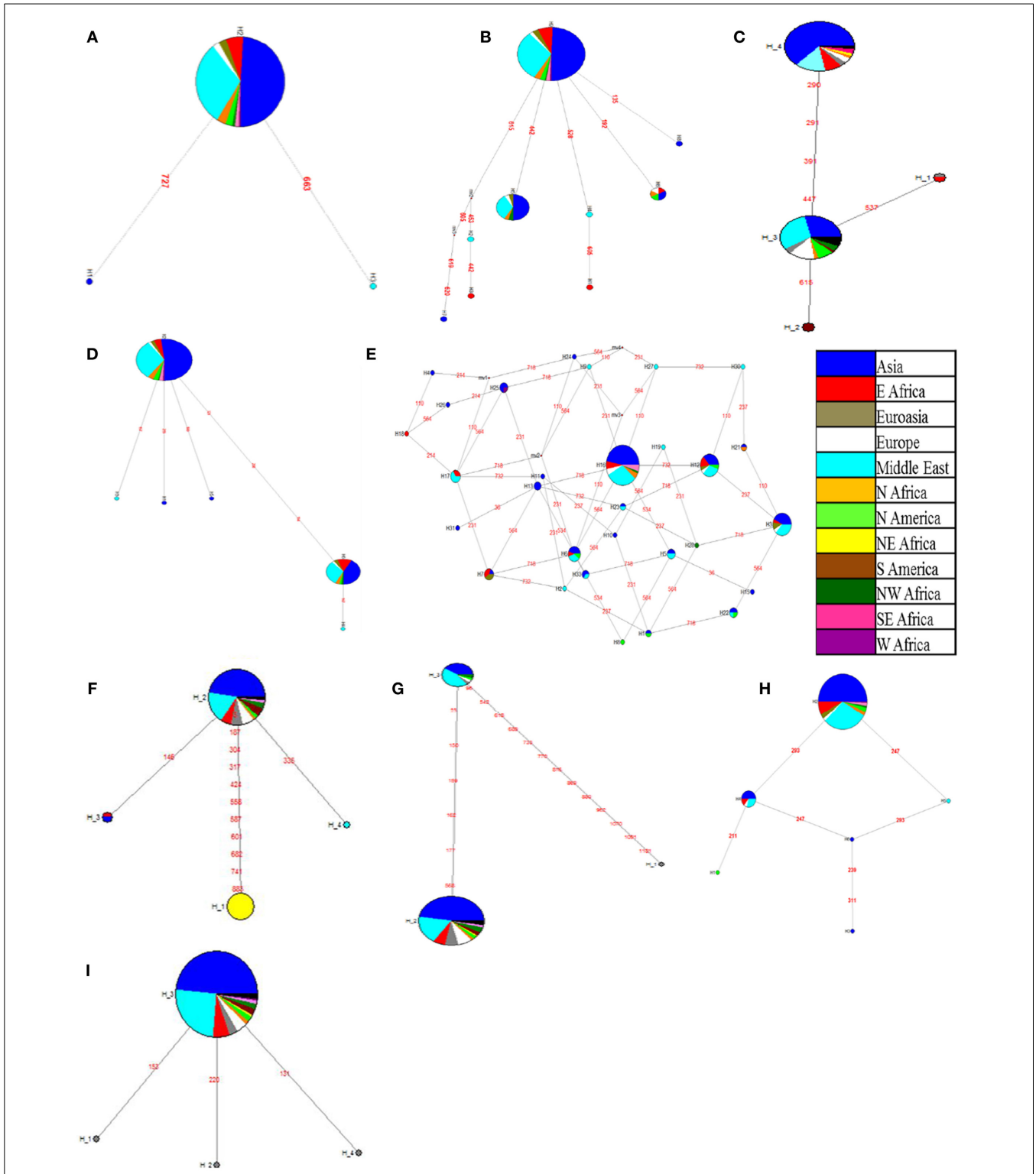

FIGURE 2 | Haplotype network of candidate genes developed based on country of origin of genotypes of the chickpea reference set. (A) *AKIN* gene; **(B)** *AMADH* gene; **(C)** *ASR* gene; **(D)** *DHN* gene; **(E)** *DREB1* gene; **(F)** *ERECTA* (*7f-5r*) gene; **(G)** *ERECTA* (*8f-8r*) gene; **(H)** *MYB* gene; **(I)** *SPS* gene; Each circle represents a haplotype and is labeled accordingly. Colors in the circles represent the countries of origin of chickpea genotypes. Circle size is in proportion to frequency (the larger the circle the more genotypes in the haplotype). Numbers in red represent the position of mutations separating the haplotypes.

one SNP and another haplotype (H6) with three SNPs which was further connected to one minor haplotype (H4) with one SNP (**Figure 2D**). The *DREB1* gene exhibits a complex haplotype network owing to the presence of 33 different haplotypes, which were connected to each other with 1–4 SNPs (**Figure 2E**). Three major haplotypes (H3, H12, and H16) covers 17, 19, and 62 individuals respectively (**Figure 2E**). Similarly, in *ERECTA*- 7f-5r gene fragment, one major haplotype (H1) defined by 10 SNPs and two minor haplotypes (H3 and H4) defined by single SNP were derived from major haplotype H2 (**Figure 2F**). In the case of the other *ERECTA* fragment (8f-8r) two haplotypes (H1 and H2) derived from H3 with 6 and 13 SNPs respectively (**Figure 2G**). Haplotype network of *Myb* gene showed the presence of six haplotypes, of which two major haplotypes (H2 and H4) are connected to four minor haplotypes with 1–2 SNPs (**Figure 2H**). SPS gene haplotype network showed presence of three minor haplotypes (H1, H2, and H4) derived from H3 with single nucleotide variation (**Figure 2I**). Accessions representing each haplotype were color coded according to their country of origin. In the present study, accessions in the major haplotypes were coming from Asia and Middle East in all the genes. The haplotype for ERECTA 7f-5r is unique to NE Africa. The network analysis showed linear relation between haplotypes in most of the genes except for *DREB1* and *Myb*, which are transcription factors. It is also interesting to note that these are the transcription factors which regulate many downstream genes in plant system.

DISCUSSION

The present study was initiated with the objective of the identification of favorable alleles in abiotic stress responsive genes in the chickpea reference set. These gene-based SNPs may be used to identify the suitable allele of a gene that enable the plant to survive in a stress environment. Due to lack of genome sequence information of the chickpea genome until recently (Varshney et al., 2013), identification of genes responsible for complex traits like drought tolerance was a daunting task at the time of initiation of this study. Identification of candidate genes responsible for drought tolerance was a part of an international collaborative project funded by the Generation Challenge Programme (GCP) entitled "Allelic Diversity at Orthologous Candidate genes (ADOC) in seven GCP crops"- one among them was chickpea. An extensive literature survey was carried out to identify possible candidate genes responsible for abiotic stress tolerance, which might have a consensus role in abiotic stress tolerance mechanism in model crops and other legume crops.

Most of the genes analyzed here, have not been previously studied in chickpea. Therefore, systematic efforts by using comparative genomics and bioinformatics approaches were made to determine the corresponding gene sequences in chickpea. For instance, a *DREB* homolog of chickpea was isolated by using sequence information available from chickpea. As *Medicago truncatula* is the known taxonomic ally of chickpea, the genomic information about *Medicago* was searched from different databases including NCBI, TIGR, and *Medicago* sequence repository (www.medicago.org). Putative candidate genes in chickpea namely *ASR*, *SuSy* and *SPS* were isolated using respective sequence information obtained from the *Medicago* candidate gene sequences. In addition, the remaining abiotic stress responsive genes (*AKIN, AMADH, DHN*, and *MYB*) were identified using a sequence similarity approach against the homolog genes present in model crops like *Arabidopsis* and *Medicago*. A large body of evidence demonstrated that the Snf1-related protein kinases (*AKIN*) serve as important regulators modulating fundamental metabolic pathways in response to nutritional and environmental stresses in yeast and mammalian cells (Hardie, 2007). To identify the *AKIN* homolog, chickpea ESTs were used for designing the primers for PCR amplification in eight chickpea genotypes based on sequence similarity with *Arabidopsis thaliana* (**Table 2**). Researchers have isolated the *AKIN* homolog in various plant species including *Arabidopsis,* wheat, rice, potato and tobacco and have established their role in abiotic stress response (Coello et al., 2012). The *AKIN* gene encodes two types of domains, catalytic kinase (highly conserved) domain and regulatory domain (highly divergent). In the present study, the *AKIN* gene was found to be mostly conserved except two unique alleles each reported in specific genotype, which indicates that in the present study we were able to amplify the conserved part of *AKIN* gene, i.e., catalytic kinase. Researchers can target the divergent regulatory domain to identify the SNPs actively involved in abiotic stress response. Similarly, a protective/curative role of the *AMADH* gene in response to stress events caused by mechanical injury was reported by Petrivalský et al. (2007) in pea seedlings. Since *AMADHs* works on degradation of reactive metabolites that show considerable toxicity, this enzyme was thought to serve as a detoxification enzyme. An *AMADH* homolog was amplified using primers designed from chickpea ESTs and BLASTN analysis confirmed its presence (**Table 3**). Over expression of the *AMADH* genes from *Arabidopsis* have been shown to affect stress responses (Missihoun et al., 2011). Based on various functional and characterization studies of the *AMADH* gene in rice, *Arabidopsis* and other crop species (Skibbe et al., 2002; Tsuji et al., 2003) makes this gene a suitable candidate for studying its similar role in chickpea. In our study, *AMADH* showed the second highest number of SNPs (13) across the chickpea mini core collection.

Expression of the *ASR* gene is regulated by water stress, salt stress and plant hormone ABA. Over-expression of the *ASR* gene in transgenic plants is known to induce water- and salt- stress tolerance (Kalifa et al., 2004). Although *ASR* gene function is not published in the case of *Medicago*, *ASR*-like sequences that were similar to some of the reported ASR sequences in other crops were used to design primers and amplified in chickpea. The sequence diversity across chickpea genotypes (193 sequences) showed 34 SNPs and two indels, highest among the candidate genes studied in the present study. The nucleotide diversity was found to be 0.0014 while haplotype diversity was 0.833. Cortés et al. (2012b) also analyzed the diversity of two *ASR* genes in a set of wild and cultivated beans and found two contrasting diversity patterns, most particularly for wild beans. A similar study in rice was carried out, where the polymorphism of four members of the *ASR* gene family was studied in a worldwide collection of 204 accessions of *Oryza sativa* and 14 accessions of wild relatives (*O. rufipogon* and *O. nivara*). This study provided a thorough description of the organization of the *ASR* family, and the nucleotide and

haplotype diversity of four *ASR* genes in *O. sativa* (Philippe et al., 2010).

The chickpea *CAP2* gene (a homolog of *DREB2A*) and its promoter, known to enhance tolerance to dehydration and salt stress, were isolated, characterized and expression studies were carried out in transgenic tobacco (Shukla et al., 2006). The sequence information was used to design nested primers in order to isolate the full-length *CAP2* gene during the present study. The study also showed extreme conservation of the *AP2* domain of the *DREB2* genes across five species studied (Nayak et al., 2009). *DREB* transcription factors bind to the dehydration responsive element (DRE) of the genes at the promoter region and regulate the expression of downstream genes. The DRE containing core sequence A/GCCGAC was identified as a cis-acting promoter element, which regulates gene expression in response to drought, high salinity and cold stresses in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994). The *CAP2* gene and its promoter were sequenced in 300 diverse chickpea genotypes. The occurrence of a SNP within a regulatory region, accounting for the loss of function of a seed shattering gene has been already shown in rice, which indicates that single sequence variants can cause major effects on the function of gene(s) (Konishi et al., 2006). Conservation of the *AP2* domain of the *DREB2A* gene was observed, not only within chickpea sequences, but also across other crop species; common bean, rice, sorghum and barley (Nayak et al., 2009). *DREB2A* diversity analysis in common bean (Cortés et al., 2012a) revealed a very high diversity level compared to *DREB2B* in these other species, indicative of adaptive selection and population expansion.

The *DHNs* are one of the several proteins that have been specifically associated with qualitative and quantitative changes in cold hardiness (Close, 1996). *Arabidopsis* plants engineered for *DHN* over-expression, showed improved survival when exposed to low temperature (Puhakainen et al., 2004). Similarly, transgenic tobaccos with increased level of expression of a citrus dehydrin protein have shown tolerance to low temperature (Hara et al., 2003) making *DHN* a suitable candidate gene for study in chickpea. Researchers have distinguished five different *DHN* genes *in silico*, which could be grouped into two types-*K2* and *SKn*. Three of the dehydrin genes reported several sequence variants which differ by multiple or single amino acid substitutions (Velasco-Conde et al., 2012). The role of *ERECTA* genes in drought tolerance pertains to their involvement in stomatal density and evapotranspiration (Shpak et al., 2004; Masle et al., 2005). Two fragments of *ERECTA* genes were isolated in the present study. In chickpea, a total of 33 SNPs (13 from fragment obtained from *ERECTA*-7f-5r and 20 from fragment obtained from *ERECTA*-8f-8r) making 7 haplotypes (4 in *ERECTA*-7f-5r and 3 in *ERECTA*-8f-8r) were observed. Nucleotide diversity was found to be 0.0029 which was high compared to all other candidate genes under study. The sequence diversity studies across the reference set of chickpea, provides the insights regarding existing haplotypes, which could be involved in drought tolerance mechanism. The role of plant Myb-proteins has been well characterized by using different genetic approaches. In most of the cases, the *Myb* domain binds to a specific DNA sequence (C/TAACG/TG) to facilitate transcriptional activation (Biedenkapp et al., 1988). A rice R2R3-type MYB transcription factor gene, *JAmyb*, whose overexpression causes tolerance to high salinity has been identified (Yokotani et al., 2013).

The *SuSy* and *SPS* genes encode for the enzymes involved in sugar metabolism and are known to be up-regulated in dehydration stress. The *SuSy* gene in chickpea is also associated with increased seed size (Kumar and Turner, 2009). A partial *SuSy* gene was isolated here, and sequencing discovered only 1 SNP across the chickpea reference set. The *SuSy* gene is a candidate gene for drought tolerance in many plant species (Gonzalez et al., 1995; Baud et al., 2004), and the *SPS* gene was found to be involved with drought tolerance in maize (Abdel-latif, 2007) and wheat (Fresneau et al., 2007). An *SPS* homolog was identified in chickpea in the present study. Diversity analysis of this gene on the reference set of chickpea showed the presence of three SNPs and one indel represented as four haplotypes across 235 chickpea genotypes. This observation indicates the conservation of this gene across chickpea genotypes. Studies on sequence diversity on the *SPS* gene are limited to date. Sequence diversity of an *SPS* gene was studied for two cultivars of sugarcane and 10 SNPs were identified in a 400 bp sequenced region. These SNPs were screened on a mapping population derived from the two cultivars. The SNP frequency did not vary in the two bulked DNA samples, suggesting that SNPs from this *SPS* gene family are not associated with variation in sucrose content. Estimation of genetic diversity serves many purposes concerning breeder's interest, like identification of distinct genetic groups for retention in germplasm, identification of genes responsible for phenotypic variation accrued during domestication (Ross-Ibarra et al., 2007) and inference of crop evolution. Allelic diversity studied through NETWORK indicated the distribution of different alleles across the globe based on the origin of the accessions. For some genes (ex: ERECTA 7F-5r), haplotypes identified were coming from particular geographic area (ex: H1 from NE Africa). Such haplotypes indicate a historical constraint as a result of selection, domestication or adaptation. In rice, a haplotype study of three genes revealed the difference in domestication pattern of cultivated and wild rice cultivars (Londo et al., 2006; Kovach et al., 2007). In the present study, linear haplotype networks were found in all genes except for transcription factors *DREB1* and *Myb*. Diversity of transcription factors at a sequence and functional level may affect downstream genes and their expression. Knowledge about genetic diversity and relationships within the diverse germplasm is also useful for breeders as it facilitates their decisions on the selection of the parents for hybridization when widening the genetic basis of breeding programs. Molecular variation in the germplasm can help in the selection of superior genotypes for the generation of new varieties for several agronomic traits. A total of 114 SNPs and 41 indels have been identified in these abiotic stress responsive genes across the chickpea reference set. These SNPs and indels were used for diversity estimation using DIVersity ESTimator (DIVEST). Among the 114 SNPs detected, 66 SNPs regions were transitions, whereas the other 49 were transversions, and one SNP was reported tri-allelic. The nucleotide diversity across the chickpea mini core collection ranged from 0.0004 to 0.0022 with overall mean diversity of 0.0015. The possibilities of association mapping can be explored

further by linking sequence diversity with the phenotype diversity in order to identify favorable alleles or haplotypes conferring drought tolerance in chickpea.

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