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PLANT RESPONSES TO FLOODING

Topic Editors
Pierdomenico Perata, Rens Voesenek,
Rashmi Sasidharan, and Chiara Pucciariello

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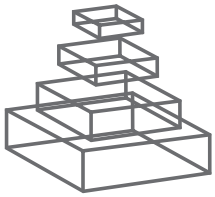

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PLANT RESPONSES TO FLOODING

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Flooded Rumex crispus. Photo credits: Ole Pedersen.

Global warming has dramatically increased the frequency and severity of flooding events worldwide. As a result, many man-made and natural ecosystems have become flood-prone. For plants, the main consequence of flooding is the drastic reduction of oxygen availability that restricts respiratory energy production and finally affects survival. Flooding can negatively influence crop production and wild plant distributions, since most plants are sensitive to excessively wet conditions. However, plants have evolved a broad spectrum of adaptive responses to oxygen deficiency that eventually leads to tolerance. Many of these morphological and physiological adaptations have been described in some crops and wild plant species and considerable progress has been made in understanding the molecular aspects governing tolerance traits. Moreover, the molecular mechanism of plant oxygen sensing has been recently elucidated. However, many other aspects concerning plant acclimation responses to flooding remain unanswered.

With this research topic we seek to build an online collection of articles addressing various aspects relating to "plant responses to flooding" which will reflect the exciting new developments and current state of the art in this vibrant and dynamic research field. All kinds of articles, including original research articles, short reviews, methods and opinions are welcome, in the attempt to broadly and freely disseminate research information, tools and protocols.

Table of Contents

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Plant responses to flooding

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Keywords: anoxia, flooding, hypoxia, low oxygen, submergence, waterlogging

Climate change models predict an increase in the frequency of flooding events globally, making flooding stress a major environmental threat for plants. Annually, crop damages due to unseasonal and severe flooding events amount to billions of dollars in yield losses. Despite the vulnerability of most crops to wet conditions, there is significant variation in the plant tolerance to flooding. Plant species adapted to wet areas have evolved specific strategies to deal with and even thrive under these conditions. In order to generate flood resilient, high yielding crops, it is of essence to not only understand the different elements that define flooding stress, but also to determine how plants sense and respond to these signals. In addition, the understanding of the genetic basis of tolerance variation and the underlying genes and processes will be key to discovering novel tolerance mechanisms and ultimately translating these to crops. In the last few years, the flooding research community has made leaps and bounds in understanding several of these aspects, unraveling more layers of the plant flooding response and paving the way for further research. This special issue entitled "Plant responses to flooding stress" brings together a collection of review and original research articles reflecting the broad scope and dynamics of the field of plant anaerobiosis.

The conditions during flooding can drastically affect survival. Large survival differences exist between plants submerged in complete darkness versus those submerged with some light, hinting at the importance of underwater photosynthesis. This importance is not only related to the production of carbohydrates but also, to the generation of molecular oxygen that accumulates in submerged plants and diffuses to tissues with less oxygen (e.g., roots). Pedersen et al. (2013) summarize recent advances and methods to quantify underwater photosynthesis in terrestrial plants in relation to leaf acclimations.

Among cereals, rice has the unique capacity to germinate and grow vigorously in flood-prone areas. Rice adaptive plasticity to different hydrological regimes has allowed the selection and characterization of some genotypes that have been subsequently used to breed high yielding and tolerant modern varieties. Miro and Ismail (2013) present an overview of the current understanding of the mechanisms associated with tolerant traits such as anaerobic germination and early vigor in rice that can help to develop tolerant varieties for direct-seeding systems. Narsai and Whelan (2013) employed a meta-analyses of microarray data to compare global low oxygen transcriptomic responses of tolerant rice with the relatively intolerant Arabidopsis. Based on their results they conclude that while Arabidopsis is simply responding to general stress conditions, rice displays transcriptome reconfigurations specific to low oxygen conditions.

Energy limitation during hypoxia, necessitates several resource limiting measures in plants. Amongst these is the use of pyrophosphate (PPi)-dependent enzymes that can take PPi as a substrate to catalyze reactions, thus sparing precious ATP reserves. Mustroph et al. (2013), execute a detailed expression analyses on the rice ATP and PPi-dependent phosphofructokinase gene family to establish their role during anoxic stress.

The study of flood adaptive strategies that evolved in nature and were selected by farmers will be crucial to develop stress-tolerant crop varieties. In this context, other environmental factors likely influence submergence tolerance, and stress combinations need to be explored toward the selection of crops tolerant to multiple stresses. Zeng et al. (2013) studied the combined response to waterlogging and salinity of barley varieties. They conclude that hypoxia is not the only factor determining the differential response of the genotypes to the stress combination and that the soil type strongly influence the presence of elemental toxicity.

The accumulation of toxic substances in flooded soils is a serious concern for plants. In some plant species, the formation of an apoplastic suberin barrier in the roots can help to prevent toxins entry and at the same time loss of oxygen. Watanabe et al. (2013) review the role of this barrier in waterlogging tolerance. In a similar context, Lamers et al. (2013) provide an overview of the effect of toxic sulfide accumulation in fresh and marine water. The accumulation of sulfide by microbial activity occurring in aquatic ecosystems is exacerbated by anthropogenic inputs. This makes sulfide-related pollution an urgent question to address.

Reactive oxygen species (ROS) are a major component of low oxygen stress. ROS production is expected to contribute to submergence adaptation. Owing to their transient nature ROS detection has been famously difficult to accurately quantitate. Steffens et al. (2013) outline the role of ROS during flooding and review the potential of techniques such as electron paramagnetic resonance to investigate ROS *in planta*.

Large trees encounter specific problems such as relatively deep floods (10–15 m) and prolonged durations of these floods (up to 7 months). Herrera (2013) reviews the biochemical responses of tropical trees to flooding in South America. The emphasis in this overview lies on leaf gas exchange, stomatal conductance, water status, and carbohydrate balance.

Beside plants, the unicellular green alga *Chlamydomonas reinhardtii* has received special attention for its response to low

oxygen due to its capacity to produce H_2, a source of renewable energy, under anoxia. Catalanotti et al. (2013) describe the fermentation pathway of *Chlamydomonas*, also discussing the fermentation process evolution.

This Frontiers special showcases the broad scope and importance of low oxygen stress studies in plants. We are grateful to all the authors for contributing to this collection. We would also like to acknowledge all the reviewers for taking the time to assess the work submitted here and helping to shape this focus issue.

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Responses to flooding of plant water relations and leaf gas exchange in tropical tolerant trees of a black-water wetland

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This review summarizes the research on physiological responses to flooding of trees in the seasonal black-water wetland of the Mapire River in Venezuela. Inter-annual variability was found during 8 years of sampling, in spite of which a general picture emerged of increased stomatal conductance (g_s) and photosynthetic rate (P_N) during the flooded period to values as high as or higher than in plants in drained wet soil. Models explaining the initial inhibitory responses and the acclimation to flooding are proposed. In the inhibitory phase of flooding, hypoxia generated by flooding causes a decrease in root water absorption and stomatal closure. An increase with flooding in xylem water potential (ψ) suggests that flooding does not cause water deficit. The P_N decreases due to changes in relative stomatal and non-stomatal limitations to photosynthesis; an increase in the latter is due to reduced chlorophyll and total soluble protein content. Total non-structural carbohydrates (TNC) accumulate in leaves but their content begins to decrease during the acclimatized phase at full flooding, coinciding with the resumption of high g_s and P_N. The reversal of the diminution in g_s is associated, in some but not all species, to the growth of adventitious roots. The occurrence of morpho-anatomical and biochemical adaptations which improve oxygen supply would cause the acclimation, including increased water absorption by the roots, increased rubisco and chlorophyll contents and ultimately increased P_N. Therefore, trees would perform as if flooding did not signify a stress to their physiology.

Keywords: flooding, photosynthesis, sap flux, stomatal conductance, tolerance

INTRODUCTION

Flooding brings about a number of changes to the soil environment, including among others reduction in oxygen concentration (Kozlowski, 1984), generation of reduced ions and, additionally, diminution of irradiance incident on leaves. Flood tolerance depends on the depth of the water column, the duration of the flooded period and specific plant traits (Colmer and Voesenek, 2009). Partial or total oxygen deficiency in the roots of higher plants causes morphological, anatomical and biochemical changes, and anoxia-tolerance seems to be strictly organ-specific (Drew, 1997); such changes allow plants of tolerant species to revert to normoxic condition as flooding progresses (Pezeshki, 1993).

A large number of articles have been published on physiological responses to waterlogging/flooding in temperate tolerant plants, both herbs and trees, as well as the tropical herb, rice, but less is known about the physiological, let alone biochemical or molecular, responses of tropical species, especially trees. I here review the existing literature on tropical tolerant trees, while comparing these with temperate tolerant and intolerant species.

Flood tolerance varies greatly with, among other factors, plant species and age, and time and duration of flooding (Kozlowski, 1997). A reduction in g_s and P_N are the earliest response to flooding observed in intolerant as well as tolerant species (Pezeshki, 1993; Batzli and Dawson, 1997; Fernández et al., 1999). In tropical flood-tolerant trees, reductions in P_N and g_s are reverted later on as flooding progresses (Fernández et al., 1999; Rengifo et al., 2005), as shown previously in temperate flood-tolerant trees (Pezeshki, 1993).

One of the main reasons why hypoxia due to flooding reduces g_s is decreased root water absorption (Kozlowski, 1984; Tournaire-Roux et al., 2003) through reductions in root hydraulic conductivity (Aroca et al., 2011). The decrease in P_N observed in flood-tolerant herbaceous and tree species is apparently governed not only by stomatal but also by non-stomatal (mesophyll) factors (Pezeshki, 1987, 1993).

In riparian Panamazonian forests, trees can suffer regular, long-lasting and deep flooding, when white-water rivers such as the Orinoco or black-water rivers such as the Rio Negro overflow, thus creating floodplain ecosystems called in the first case várzea and in the second, igapó (Prance, 1979). These ecosystems differ, among other traits, in quality of the waters; várzeas have nutrient-rich, turbid and higher pH waters, whereas in the igapós waters are very nutrient-poor, transparent and acidic. These characteristics are bound to affect plant physiology and growth differently. Amazonian wetlands cover over 1,000,000 km^2 (Melack et al., 2004), more than three times the area of the British Isles; therefore, knowing how tropical plants cope with flooding becomes of global importance.

Flood tolerance has been extensively examined in herbaceous or small floating and submerged angiosperms (revised by Colmer and Voesenek, 2009). Here, I will be dealing with large trees, which will surely make a difference because of resistances

encountered by long-distance transport of O_2 and the slow diffusion of this gas in water. In tropical wetlands, the water-column may be as high as 10–15 m and the flood period last up to 7 months (Vegas-Vilarrúbia and Herrera, 1993a; Parolin et al., 2006).

This review summarizes the results of research on physiological responses to flooding of trees in the seasonal igapó of the Mapire River. This igapó is formed when the black-water Mapire River, a northern tributary of the Orinoco, increases its flow due to rainfall and is additionally dammed by the Orinoco. Four broad phases can be defined in the flood cycle (**Figure 1A**): drainage (D, December–March), rising-waters (RW, April–May), full flood (FF, June–August), and falling-waters (FW, September–November). The igapó lake covers trees at various heights depending on tree size and position along a gradient from the savanna to the river channel (**Figure 1B**). The species that have been examined along this gradient are *Acosmium nitens* (Papilionaceae), *Campsiandra laurifolia* (Mimosaceae), *Eschweilera tenuifolia* (Lecythidaceae), *Pouteria orinocoensis* (Sapotaceae), *Symmeria paniculata* (Polygonaceae), and *Psidium ovatifolium* (Myrtaceae). The aspect of this ecosystem at drainage and full flood is shown in **Figure 2**. All species in the Mapire igapó are evergreen. Several, such as *C. laurifolia*, *S. paniculata*, and *P. ovatifolium*, retain leaves underwater.

Similar plant responses have been repeatedly observed during the various stages of the flood cycle. I will focus this review on results obtained in the field by Fernández et al. (1999), Rengifo et al. (2005), and Herrera et al. (2008a,b). In spite of inter-annual variability in gas exchange found during eight non-consecutive years of sampling, a general picture emerged of increased g_s and P_N during the flooded period to values as high as or higher than in plants in drained yet wet soil (**Figure 3**). The inter-annual variability may be due, among other factors, to differences in the exact timing of the flood phase, leaf developmental stage, rainfall, air temperature, and relative humidity. For example, measurements made in 1998 (Rengifo et al., 2005) were most certainly affected by the fact that this, as opposed to the other years of sampling, was a strong El Niño year. Based on these results and the literature, model are proposed (**Figure 4**) explaining physiological responses to the inhibitory and the acclimatized phases of flooding in species growing exclusively in this ecosystem.

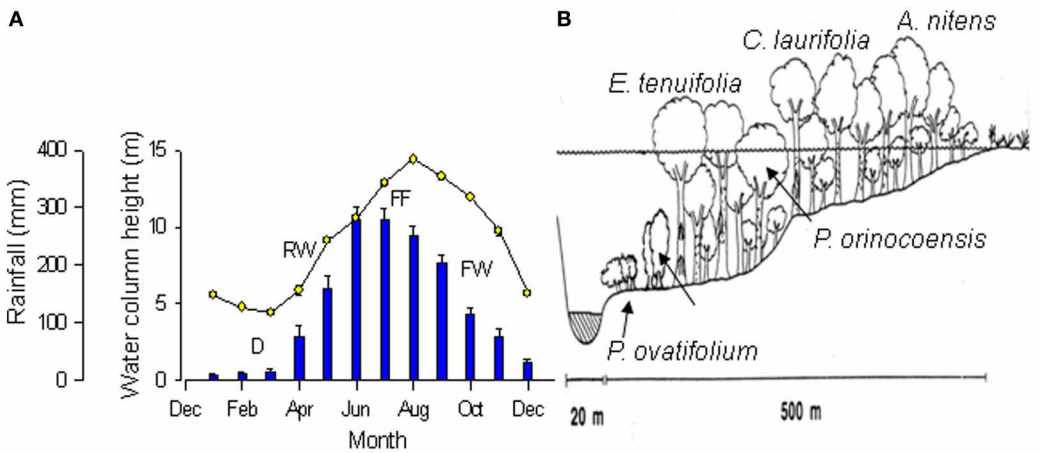

FIGURE 1 | (A) Changes over the year in rainfall (bars) and water column height (circles) of the Mapire River relative to the Orinoco. Values are mean ± *SE* ($n = 4$ years). Stages of the flooding cycle are indicated as drainage (D), rising-waters (RW), full flooding (FF), and falling-waters (FW). **(B)** Schematic view of the vegetation and water level along a gradient from the Mapire bed to the savanna. Approximate location of species indicated. Modified from Fernández et al. (1999) and Herrera et al. (2008b).

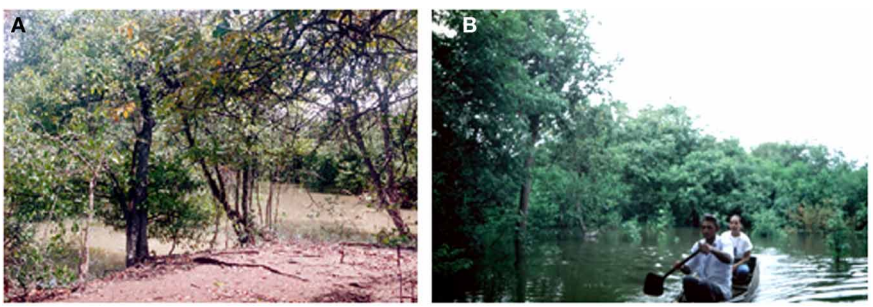

FIGURE 2 | The Mapire igapó forest during (A) drainage and (B) under full flood.

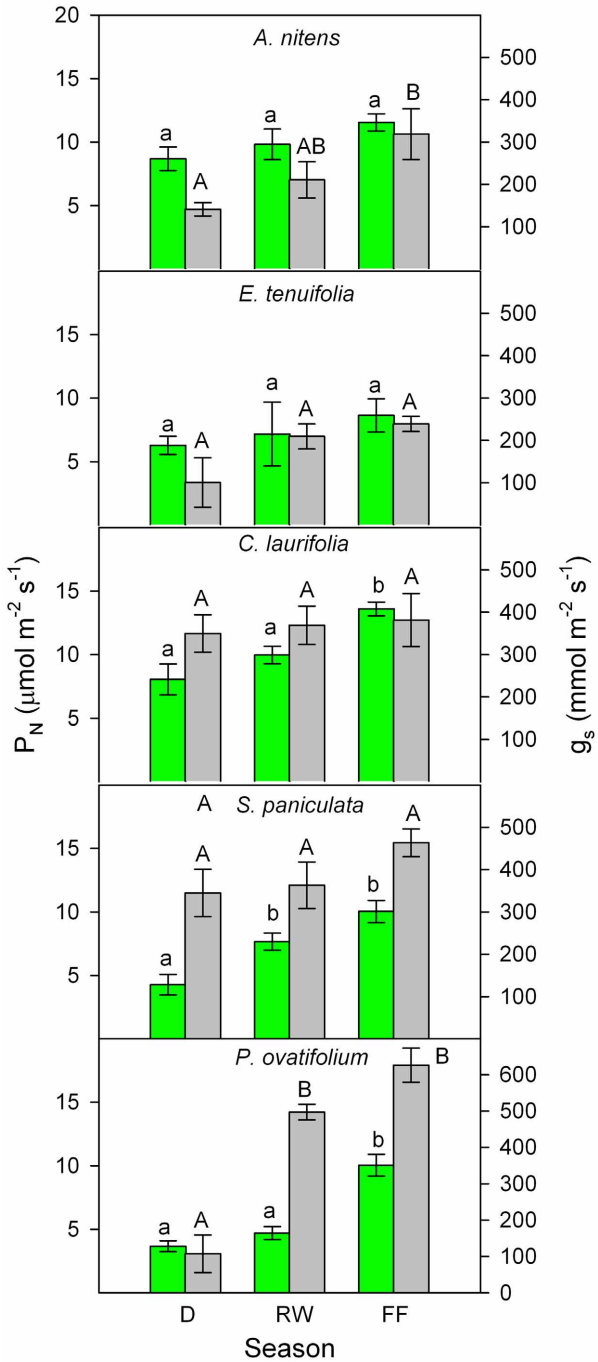

FIGURE 3 | Seasonal changes in photosynthetic rate (green) and stomatal conductance (gray) of the species indicated. Values are mean ± *SE* of measurements were made during 1995, 1996, 1998, 1999, 2000, 2003, 2004, and 2005. Different letters indicate significant differences at $p < 0.05$. Modified from Fernández et al. (1999), Rengifo et al. (2005), and Herrera et al. (2008a).

I will present evidence in favor or against the proposed models. Results on water status, leaf gas exchange and Total non-structural carbohydrates (TNC) are shown in **Table 1** and will be discussed where pertinent. The following issues will be examined: (1) Just how low does oxygen concentration become and how does it affect root water absorption and water status? (2) Are reductions in g_s and P_N under flooding reversible? (3) Does flooding cause photoinhibition? (4) Are changes in P_N under flooding caused by changes in stomatal and non-stomatal limitations? (5) How does leaf carbohydrate balance change during flooding?

The significance of under-water photosynthesis for whole-plant C balance and the possible changes under flooding in leaf anatomy will be examined, and some eco-physiological and ecological considerations made.

MODELS OF PHYSIOLOGICAL RESPONSES TO THE INHIBITORY AND THE ACCLIMATIZED PHASES OF FLOODING

Flooding at the inhibitory phase (RW) causes hypoxia, probably not anoxia. Hypoxia generates changes in hormone balance that cause stomatal closure and a decrease in absorption of water by roots, which may also promote stomatal closure. This brings about a decrease in intercellular CO_2 concentration, C_i and P_N. The decrease in g_s and P_N under high radiation and air evaporative demand could induce chronic photoinhibition. The decrease with flooding in soil N and absorption capacity of the roots creates a deficiency of N and possibly other nutrients, thus reducing the content and/or activity of rubisco, decreasing P_N, increasing relative non-stomatal limitation to photosynthesis (L_{NS}) and decreasing relative stomatal limitation (L_S) [for definitions see Herrera et al. (2008a)]. The L_{NS} could also increase due to down-regulation of PSII. Root TNC content decreases due to both an increase in root anaerobic respiration and a decrease in leaf TNC content due to both decreased P_N and increased root demand.

During the acclimatized phase, the appearance under full flood of morpho-anatomical adaptations which improve oxygen supply would cause the acclimation. These structures would result in a situation of normoxia for the tree and cause stomatal opening and increased N and water absorption by the roots because of synthesis of new aquaporins. The increase in N uptake would cause an increase in rubisco and chlorophyll contents, and P_N. Increased root water absorption would promote stomatal opening. This would also lead to increased P_N. In the process of acclimation of P_N to full flood, L_{NS} decreases and L_S increases. The role of hormones in this acclimation is presumed but no evidence for it is available. After acclimation, the tree would perform as if flooding did not signify a stress to its physiology, growth or reproduction.

OXYGEN CONCENTRATION UNDER FLOODING AND ITS EFFECTS ON ROOT WATER ABSORPTION AND WATER STATUS

Oxygen concentration in the water of the Mapire igapó decreases with flooding from 5 mg L^{-1} (300 μM) at the surface of the lake to 2 mg L^{-1} (120 μM) at a depth of 15 m (details in Herrera et al., 2008b). The K_m of cytochrome-c oxidase is 14 μM (Geigenberger, 2003); Armstrong et al. (2009) give a figure of 0.14 μM; whichever the values actually is, there is at least a ten times surplus of oxygen before tissues, especially in the roots, become anoxic. Therefore, even in the "worst" situation, plants do not seem to be exposed to severe oxygen stress. Nevertheless, without exact knowledge

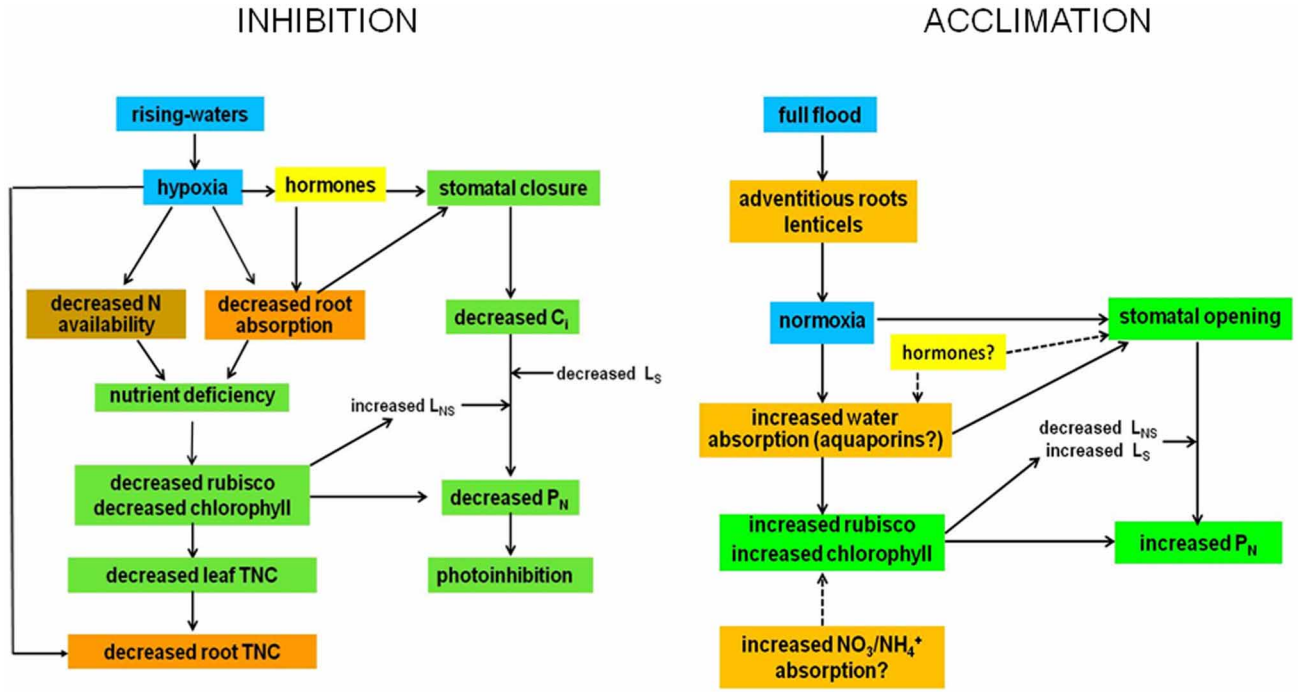

FIGURE 4 | Possible mechanisms of inhibition and acclimation of photosynthesis by flooding. Blue, water; brown, soil; orange, roots and stems; green, leaf; yellow, undetermined.

Table 1 | Changes from drainage (D) to full-flooding (FF) in morning leaf water (ψ) and osmotic (ψ_s) potential, maximum quantum yield of PSII (F_v/F_m), total chlorophyll content (Chl), photosynthetic rate (P_N), stomatal conductance (g_s), and total non-structural carbohydrate contents (TNC) as hexose equivalents of the species indicated.

Species	Season	ψ	ψ_s	F_v/F_m	*Chl*	P_N	g_s	*TNC*
		(MPa)		(μg cm^{-2})		(μmol m^{-2} s^{-1})	(mmol m^{-2} s^{-1})	(g hexose m^{-2})
A. nitens	D	−4.27	−4.13	0.79	28.9	8.1	207	9.4
	FF	−0.22	−3.50	0.79	22.6	12.3	399	14.4
E. tenuifolia	D	−1.53	−1.66	0.83	25.6	7.6	153	9.8
	FF	−0.18	−1.10	0.79	23.0	4.2	196	22.1
C. laurifolia	D	−1.07	−1.33	0.82	36.0	12.4	276	6.2
	FF	−0.47	−1.87	0.82	19.1	16.0	671	13.7
S. paniculata	D	−1.70	−2.02	0.81	34.0	15.3	1161	7.7
	FF	−0.28	−1.43	0.79	23.7	5.2	98	27.5
P. ovatifolium	D	−2.10	−2.53	0.84	24.0	13.3	269	15.4
	FF	−0.30	−1.50	0.80	22.2	5.7	109	26.6

Values are mean. Modified from Rengifo et al. (2005).

of internal root O_2 concentration, describing the situation as hypoxia or anoxia is simply speculative. The decrease in the ATP/ADP ratio at decreasing O_2 concentration is not paralleled by an increase in the NADPH/NAD$^+$ ratio until O_2 concentration reaches 1%, implying maintenance of aerobic respiration (Geigenberger, 2003).

Since O_2 diffuses very slowly in water, the question remains: how much of that 120 μM O_2 reaches root cells? The occurrence of anaerobic root respiration has been well documented in intolerant as well as tolerant species (Crawford, 1992). Anaerobic respiration has been reported in some tropical flood-tolerant trees (Joly and Crawford, 1982; Parolin and Wittmann, 2010). Indirect evidence for the occurrence of anaerobic metabolism in the Mapire igapó is a notable smell of alcohol near trees of *C. laurifolia* under FF which is not perceived at any other period of the flooding cycle. The sole direct evidence of alcoholic fermentation in a species from this ecosystem was provided by measurements of increased alcoholic dehydrogenase activity in seedlings of *A. nitens* grown under waterlogging (Izquierdo, 1988).

Changes in O_2 concentration from RW to FF should not totally compromise aerobic respiration; nevertheless, a 60% decrease in O_2 concentration could certainly be reflected in whole-plant and leaf performance, as seems to happen. This marked decrease in O_2 concentration would be alleviated after a short period under flooding by the production of new morpho-anatomical structures and the operation of physiological and biochemical processes, such as pressurized gas transport and aquaporin synthesis.

In an ingenuous approach to the issue of aquaporin influence on root water absorption, McElrone et al. (2007) accessed through caves the fine roots of trees growing at a depth of 18 m and determined that aquaporin activity contributed up to 45% of hydraulic conductivity. The collection of fine roots and measurement of aquaporin activity in flooded trees should help understanding the acclimation of aerial responses to flooding.

The improvement of oxygen transport to the roots by pressurized gas transport, experimentally demonstrated in some temperate tree species (Grosse et al., 1992) and tropical flood-tolerant herbs (Konnerup et al., 2011), is one of the known adaptations to flooding. In saplings of five Amazonian tree species, internal aeration of the roots was improved under conditions of pressurized gas transport as shown by measurements of oxygen exchange between root and rhizosphere. Using a tracer gas, Graffmann et al. (2005) showed gas permeability of transport pathways between the stem base and the roots of these saplings and concluded that pressurized gas transport significantly contributes to internal aeration of roots.

In intolerant species, the initial reduction under flooding in g_s is accompanied by decreased ψ and leaf water status (Crawford, 1982; Ruiz-Sánchez et al., 1996). In trees of the Mapire igapó, ψ was found to increase with flooding (**Table 1**), suggesting that flooding does not cause water deficit in these trees. Weak or no relationship between g_s and ψ was found, except for *A. nitens* ($r^2 = 0.43$), the one species growing along the gradient in the Mapire igapó that suffers from water deficit during the dry season. Since g_s diminished during the earlier stages of flooding, trees behaved as iso-hydric species. Similar increases with flooding in ψ without decreased P_N and, presumably, g_s were found in the evergreen várzea tree *P. glomerata* (Armbrüster et al., 2004). These results suggest a direct effect of flooding on gas exchange independent from water status.

The ABA content of leaves and xylem sap extracted under positive pressure increased from D to RW and FF in *C. laurifolia* and *P. orinocoensis*, together with the known decrease in g_s, suggesting a positive relationship between ABA and stomatal closure. In fact, the regression between ABA and g_s was linear with $r^2 = 0.98$ (Rengifo et al., 2006). A similar relationship was found in flooded tomato plants (Else et al., 1995). These results are subject to revision, since to obtain meaningful changes in sap ABA content, care must be taken when collecting sap, as overpressurizing stems may bring out ABA located in roots (Jackson, 1997), thus portraying an erroneous picture of ABA influence on g_s during the flooding cycle. No relationship was found in flooded tomato plants between stomatal closure and inhibition of gibberellin or cytokinin export (Else et al., 2009). Possible methodological errors in determination of data by Rengifo et al. (2006) may render their interpretation erroneous; nevertheless, they open interesting research avenues in tolerant trees.

Trees under flood in the igapó showed values of leaf osmotic potential (ψ_s) consistently lower than those of ψ (**Table 1**), suggesting the occurrence of osmotic adjustment (Rengifo et al., 2005); an osmotic adjustment of 0.26 MPa was shown to take place in flooded citrus seedlings (García-Sánchez et al., 2007). In four of the igapó species studied, soluble sugars contributed significantly to the decrease in ψ_s; in *A. nitens*, the contribution was low presumably because nitrogenous compounds supplied by nitrogen fixation in this legume made a significant contribution (Fernández et al., 1999). The accumulation in leaves of osmotically active metabolites, mostly sugars, may be a mechanism whereby these trees tolerate flooding by increasing turgor potential, which allows the resumption of high g_s and P_N.

When it is due to water transport from the roots to the atmosphere and not to replenishment of reservoirs, xylem sap flux may indicate the ability of roots to absorb water. In trees of *C. laurifolia*, sap flux changed seasonally; relative to the highest values measured during drainage in November, sap flux was 47% at the dry season, decreasing to 25% at rising-waters and resuming 49% under full flood [calculated from Herrera et al. (2008b)].

Since at RW dawn ψ remained high, it became apparent that the initial stages of flooding imposed a restriction to sap flux unrelated to water deficit. The decrease at RW in highest daytime sap flux was due to reduced leaf-specific hydraulic conductivity, whereas the recovery of daytime sap flux observed 1.5 months later was correlated to an increase in leaf-specific hydraulic conductivity, and attributed to acclimation.

The decrease with flooding in root hydraulic conductivity has been documented and attributed to cytoplasmic acidification due to increased CO_2 concentration and inhibition of aquaporin activity (Aroca et al., 2011). Evidence of this has been gathered in herbs and shrubs, mainly of agronomic interest and flood-intolerant, but no data are still available on flood-tolerant trees. Early flooding in the Mapire igapó apparently inhibited water absorption by roots and this inhibition was overcome later on at a higher water column through an acclimation process possibly involving the improvement of internal aeration by adventitious roots or by synthesis of new aquaporins.

A substantial night-time flux accompanied by nocturnal stomatal aperture was found in *C. laurifolia* under FF (Herrera et al., 2008b). Similar observations have been made in plants of other species and from other environments (e.g., arid) and a role for night-time transpiration in N absorption has been suggested (Snyder et al., 2008). Why would flood-tolerant trees implement a mechanism for nutrient acquisition during the night remains to be elucidated. One possibility is that the development of positive nocturnal root pressure generated by active transport of ions into the xylem, together with open stomata, results in increased sap flux and nutrient supply to the shoot.

STOMATAL CLOSURE AND REDUCTION IN P_N UNDER FLOODING ARE REVERSIBLE

In three out of four tropical tree species that grow on drained soils in drylands, experimentally subjected to waterlogging (Lopez and Kursar, 1999), g_s diminished 30% on average, one of the species,

Prioria copaifera, showing no change. In this species P_N was significantly reduced after 45 days of experimental flooding but, in contrast to the other three species, resumed drained values after 90 days of flooding, the authors deeming this a flood-tolerant species.

In **Table 2**, values of maximum P_N of evergreen trees in a Brazilian várzea and the Mapire igapó are compared during the drained and flooded periods. None of the várzea species showed changes with flooding, whereas in the igapó P_N increased in all the species. The observation that although igapó waters are poorer and more acidic than várzea waters, average maximum P_N at FF was similar between wetlands merits further comparative research.

The reversal of diminished g_s to values under drainage was associated in *C. laurifolia* and *S. paniculata* to the growth of adventitious roots (**Figure 5**). The occurrence with flooding of adventitious roots and hypertrophied lenticels has been reported in trees of several species in the central Amazonian floodplains (Parolin, 2001). Amelioration of leaf gas exchange through improvement in aeration by adventitious roots and hypertrophied lenticels has been reported in temperate tolerant species (Kozlowski, 1984) such as *L. laricina*, in which such roots increase root hydraulic conductivity apart from improving oxygenation (Islam and Macdonald, 2004).

FLOODING AND PHOTOINHIBITION

Despite decreased g_s and P_N and high radiation and air temperature, emerged leaves of tolerant trees do not become photoinhibited during RW, when decreased P_N is often observed. Values of maximum quantum yield of PSII, F_v/F_m (**Table 1**), were always higher than 0.70, the threshold proposed by Bolhàr-Nordenkampf and Öquist (1993) for determining that photoinhibition occurs. The F_v/F_m was also higher than 0.70 in plants of *S. paniculata* growing in the Solimões River várzea that had been submerged at 1 m in the dark for 6 months, and leaves submerged deeper did become photoinhibited ($F_v/F_m = 0.20$) but regained high values several weeks later upon re-emergence (Waldhoff et al., 2002).

In the Mapire igapó, most leaves of plants of *S. paniculata* and *P. ovatifolium* under FF for approximately 6 months may become photoinhibited because the entire tree is covered with a 15-m-high water column. This, together with a reduction of 50% in radiation at a depth of 60 cm in June (Fernández et al., 1999), could mean that deeply submerged leaves do reduce P_N to zero. Leaves of *S. paniculata* that developed at D and remained submerged for approximately 4 months were fully functional when they emerged at FW (Fernández et al., 1999), indicating that they were not negatively affected by submergence.

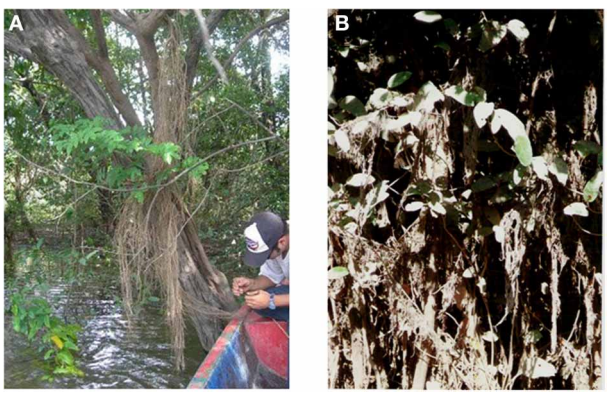

FIGURE 5 | Adventitious roots observed in October at falling-waters in trees of: (A) *Campsiandra laurifolia* and (B) *Symmeria paniculata*. Note in **(B)** the mud deposits on leaves from previous flood.

Table 2 | Maximum values of photosynthetic rate in evergreen trees growing in a várzea and an igapó during drainage (D) and flooding (FF).

Species	Wetland	P_N (μmol m^{-2} s^{-1})		References
		D	FF	
Pouteria glomerata	várzea	10.0 ± 1.3	10.4 ± 2.0	Parolin et al. (2006)
Pouteria glomerata	várzea	12.0	12.2	Armbrüster et al. (2004)
Cecropia latiloba	várzea	16.8 ± 1.5	15.0 ± 4.6	Parolin (2000)
Senna reticulata	várzea	20.0 ± 4.0	18.4 ± 4.3	
Nectandra amazonum	várzea	9.3 ± 2.4	7.6 ± 3.1	
All		**13.6**	**12.7**	
Acosmium nitens	igapó	8.7 ± 0.9	11.6 ± 0.7	
Eschweilera tenuifolia	igapó	6.3 ± 0.7	8.6 ± 1.3	
Campsiandra laurifolia	igapó	8.0 ± 1.2	13.6 ± 0.5	
Symmeria paniculata	igapó	4.3 ± 0.8	10.0 ± 0.9	
Psidium ovatifolium	igapó	3.7 ± 0.4	10.0 ± 0.9	
All		**6.2**	**10.8**	

Values are mean ± SE for species and mean for each wetland (in bold). Data for the igapó are means of value reported by Fernández et al. (1999), Rengifo et al. (2005), and Herrera et al. (2008a,b).

REVERSIBLE REDUCTION IN P_N UNDER FLOODING IS ASSOCIATED TO CHANGES IN STOMATAL AND NON-STOMATAL LIMITATIONS

It has not been systematically examined whether in flood-tolerant trees P_N decreases under flood due to decreased g_s only. In flooded plants of the tropical species *Genipa americana*, photosynthesis was co-limited by stomatal and non-stomatal factors (Mielke et al., 2003). In seedlings of the non-tolerant temperate species *Nothofagus menziesii* and *N. solandri*, P_N, g_s, and non-stomatal conductance, i.e., mesophyll conductance plus carboxylation efficiency, decreased markedly in response to waterlogging (Sun et al., 1995). In the igapó species *P. orinocoensis*, carboxylation efficiency in experimentally submerged seedlings decreased 70% and CO_2-saturated P_N 61% relative to drained seedlings (Fernández, 2006), indicating co-limitation of photosynthesis by stomatal and non-stomatal factors.

In order to determine whether in species of the Mapire igapó P_N is reduced due to changes in L_S and L_{NS}, response curves of P_N to C_i were done (**Figure 6**). Photosynthetic capacity was affected by flooding, as indicated by variations in CO_2-saturated P_N (**Figures 6**, **7**). The L_S (the difference in CO_2-saturated P_N and P_N at ambient CO_2 concentration divided by the former) decreased, whereas L_{NS} (CO_2-saturated P_N at a given time divided by the maximum CO_2-saturated P_N) increased relative to drainage (**Figure 7**). The increase in L_{NS} was related to a decrease in total soluble protein (TSP), an indirect measure of rubisco content, and chlorophyll content (**Figure 7**). Nevertheless, it was previously reported that rubisco content was similar in emerged and submerged leaves of *P. ovatifolium* and higher in submerged than emerged leaves of *S. paniculata* (Fernández et al., 1999), indicating no loss of rubisco due to submergence or even flooding. It remains to determine whether this maintenance of rubisco content is a general response in the rest of the species in the igapó. A 54% change in P_N was due to changes in g_s, confirming that the limitation imposed by flooding to P_N was not only stomatal (Herrera et al., 2008a).

Soil NO_3^- content in the Mapire igapó decreases markedly during the flooded period, whereas NH_4^+ content progressively increases until drainage (Barrios and Herrera, 1994). The decrease in NO_3^- content could be relevant for metabolism in non-leguminous but not in leguminous trees. Approximately 30% of the species in the Mapire igapó, including *A. nitens* and *C. laurifolia*, are legumes (Rosales, 1988). Setting aside differences in habit and habitat, the positive effect in the pasture legume *Lotus corniculatus* of low O_2 concentration on nodulation, N_2 fixation and growth reported by James and Crawford (1998) suggests that a similar response of N_2 fixation to low O_2 could be expected in flood-tolerant legume trees.

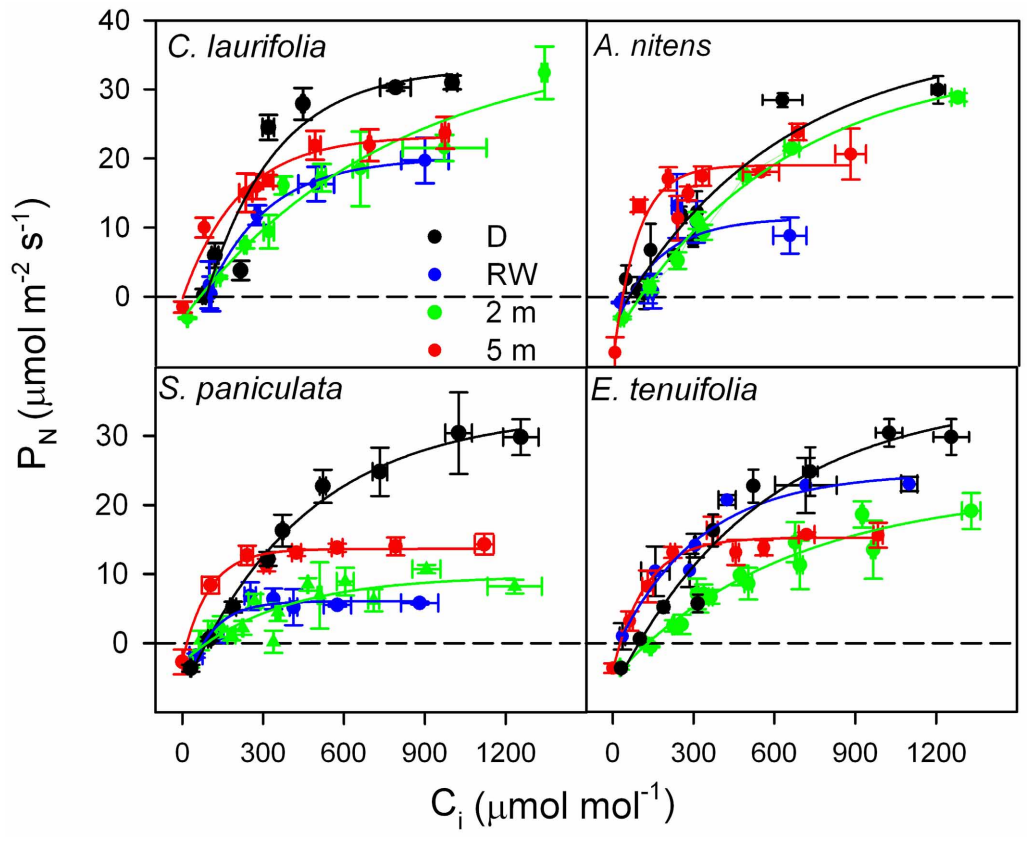

FIGURE 6 | Response curves of photosynthetic rate to intercellular [CO_2] in the species indicated during drainage (black), rising-waters (blue), flooding by 2-m water column (green), and full flood by 5-m water-column (red). Modified from Herrera et al. (2008a).

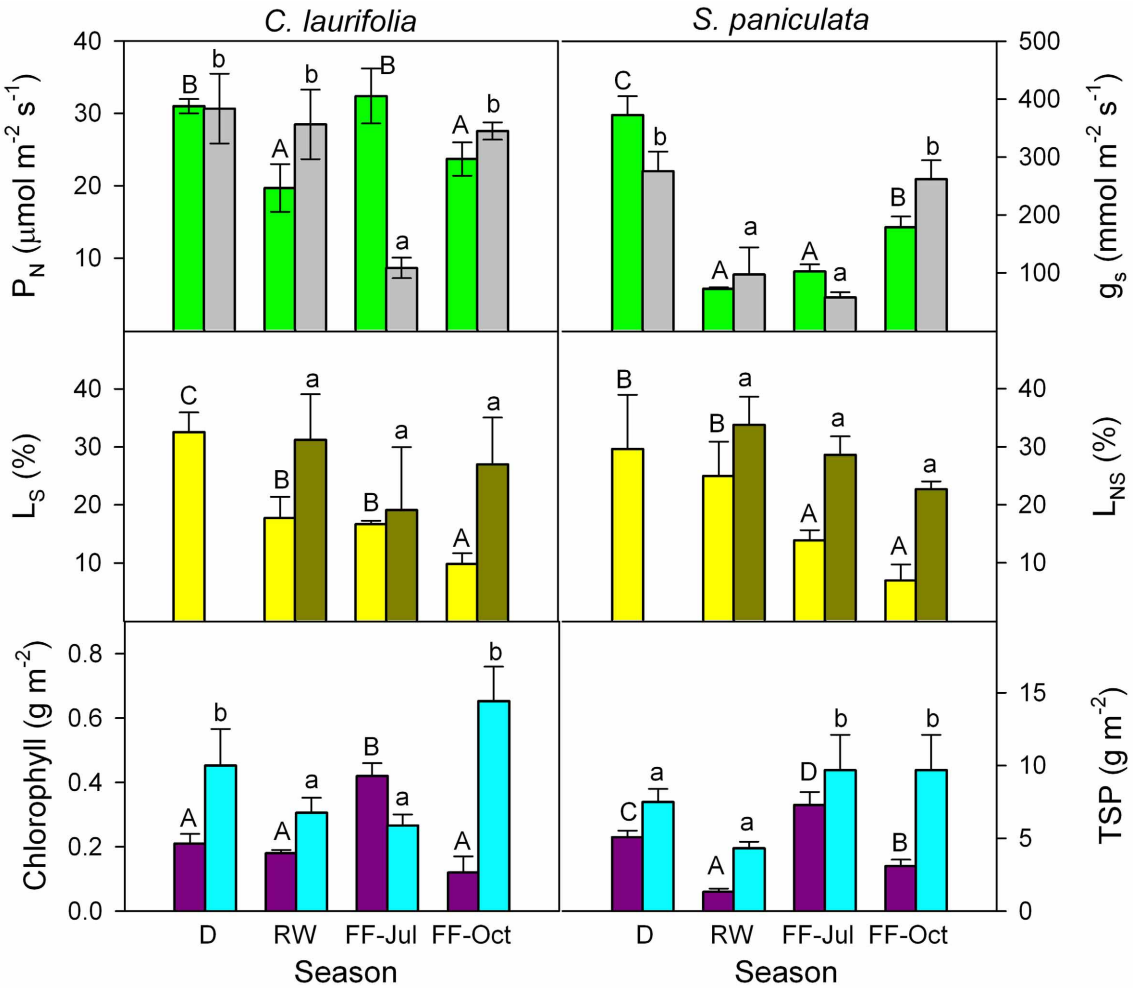

FIGURE 7 | Seasonal changes in CO_2-saturated photosynthetic rate (green), stomatal conductance at ambient CO_2 concentration (gray), relative stomatal (yellow) and non-stomatal (dark green) limitations of photosynthesis, chlorophyll (purple), and total soluble protein (cyan) content of the species indicated. D, drainage; RW, rising-waters; FF-Jul, full flood in July; and FF-Oct, full flood in October. Modified from Herrera et al. (2008a).

An increase with flood in L_{NS} in legumes would not necessarily involve a decrease in rubisco content due to low N supply, since legumes do not depend on NO_3^- availability for protein synthesis. Instead, the increase in L_{NS} and decrease in TSP of legumes of the Mapire igapó could be due to low availability/absorption of other nutrients, extremely low O_2 concentration in the nodules or toxicity caused by reduced ions. Uptake of NO_3^- and NH_4^+ by saplings of *Fagus sylvatica* (flood-intolerant) was severely inhibited by waterlogging, whereas in *Populus tremula* × *P. alba* (flood-tolerant) absorption of both N forms was unaffected (Kreuzwieser et al., 2002). We have no information on which form of nitrogenous compounds trees, whether legumes or non-legumes, absorb at D or FF.

Leaf N content of species in the Mapire igapó (Rosales, 1988) was similar to that in the Rio Negro igapó (Parolin et al., 2002), with an average of 2% (by dry mass). No data are available for seasonal variations in leaf N content in the Mapire igapó; in the Rio Negro igapó, content varied between the drained and the flooded phases, with no consistent trend, since in some species content decreased, whereas in others it increased with flooding. Changes in content were related to leaf development, content being higher in new leaves which flushed mostly during the flooded period. Therefore, the reduction in soil N availability is not apparently reflected in leaf N content.

LEAF CARBOHYDRATE BALANCE CHANGES DURING FLOODING

Information on changes in TNC content of flood-tolerant species is scant. In seedlings of the várzea species *Himatanthus sucuuba* experimentally subjected to submergence in the dark, root starch content diminished while alcoholic dehydrogenase activity remained relatively constant after an initial increase (Ferreira et al., 2009). It has been proposed that flood tolerance is proportional to the capacity of roots for TNC storage (Crawford, 1992). In the Mapire igapó, difficulty in accessing roots has impeded the

determination of root TNC content; therefore, leaf TNC balance has been used as a surrogate measure.

Leaves of all the trees studied in the Mapire igapó accumulated TNC under flooding (**Table 1**) but this accumulation was not always related to a higher P_N. Similarly, flooded seedlings of the temperate tolerant species *L. laricina* showed a marked increase in shoot starch after 27 days of flooding, as opposed to seedlings of the intolerant species *Picea mariana* (Islam and Macdonald, 2004), which suggests that starch accumulation in leaves is a tolerance mechanism. In our investigation, TNC accumulation began at RW, when P_N of all the species was lower than the maximum (**Table 1**). This suggests that photosynthates could not be translocated to other sinks and the mechanisms responsible for alleviating hypoxia had not begun operating. In all the species TNC content began to decrease at FF or FW, coinciding with the resumption of high P_N (Rengifo et al., 2005). Enhanced starch and sugar accumulation was observed in woody species under flooding, suggesting that this is a consequence of reduced phloem translocation from shoot to root (Kreuzwieser et al., 2004).

SIGNIFICANCE OF UNDER-WATER PHOTOSYNTHESIS FOR WHOLE-PLANT C BALANCE

In the Mapire igapó, some of the tree species maintain live leaves submerged for as long as 6 months. Submerged leaves brought to the air showed values of P_N similar to emerged leaves (**Figure 8**), indicating that leaves may be photosynthetically active under water.

The significance for whole-tree physiology of underwater photosynthesis has not been examined yet; the energy-costly process of maintaining live leaves under water may be compensated by continued O_2 supply to the roots produced by the photosynthetic activity of these submerged leaves. Significant daily oscillations in TNC content of submerged leaves of *P. ovatifolium* and *C. laurifolia* that were 53 and 131% of those in aerial leaves, respectively (Fernández et al., 1999), support the hypothesis that these leaves are in effect photosynthesizing underwater. Additionally, the balance could resided in having leaves that are fully functional when waters fall; in this case, the payback would lie in a higher whole-tree leaf area, hence higher productivity, as opposed to trees which do not maintain live leaves under water.

The occurrence of radial oxygen loss in submerged plants has been interpreted as evidence of photosynthetic O_2 production and transport to roots (Armstrong et al., 2006), demonstrating a positive contribution of underwater photosynthesis to whole-plant survival.

Indirect evidence of the occurrence of underwater photosynthesis is contradicted by the finding in seedlings of *P. orinocoensis* that O_2 consumption by submerged leaves measured with an O_2 electrode became almost zero after 12 days and remained so for 45 days, while emerged leaves, although continually reducing P_N and g_s during 45 days of treatment, had values of P_N after 12 days of 70% the value at the beginning of flooding (Fernández, 2006). The author argues that such responses would be expected from seedlings in the field because to them, in contrast to adult trees, flooding would be abrupt and stressful.

The case of *S. paniculata* and *P. ovatifolium* is particularly interesting, because plants of these species are covered by water earlier and remain submerged longer than other species in the igapó forest. Most of the foliage of these plants remains under water at 25% of incident radiation, yet is fully functional as soon was waters fall (Fernández et al., 1999; Rengifo et al., 2005). An important issue related to the possible underwater photosynthetic activity is that of whether enough CO_2 is available. The CO_2 concentration calculated at the pH and alkalinity of the water from data of Vegas-Vilarrúbia and Herrera (1993b) was at any season one order of magnitude higher than the $K_m(CO_2)$ of rubisco (Fernández et al., 1999); therefore, CO_2 availability would not be a limiting factor as long as the gas diffused into the leaf.

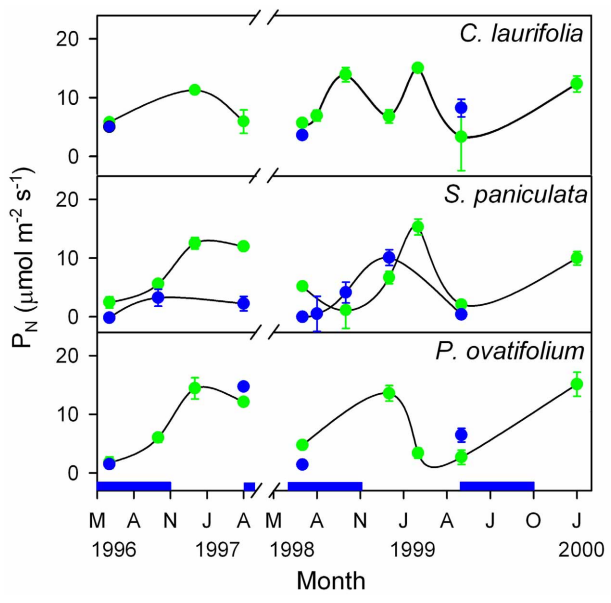

FIGURE 8 | Seasonal changes in photosynthetic rate of emerged and submerged leaves of the species indicated. Green circles, emerged leaves; blue circles, submerged leaves. The blue indicates the duration of the flood period. Modified from Herrera et al. (2001).

LEAF ANATOMY REMAINS UNCHANGED UNDER FLOODING

In many tolerant was well as intolerant species, flooding leads to a programmed destruction of cells in the leaves as well as stems that ends in the formation of aerenchyma, in a process designed to improve aeration of organs. In the Mapire igapó, as observed in tolerant trees of the Solimões River igapó (Waldhoff and Furch, 2002; Waldhoff, 2009), leaf anatomy remained practically unchanged regardless of phase of the flood cycle (Herrera et al., 2009; **Figure 9**). Emerged leaves under FF are apparently new and, using lack of sediment deposition as an indicator, produced by branches not covered by water. No signs of branch or petiole elongation under flooding, a phenomenon observed in flooded herbs (Bailey-Serres and Voesenek, 2008), have been observed but their occurrence may not be ruled out without detailed time-series on labeled individuals.

A xeromorphic leaf anatomy, such as described for drought-tolerant trees (Roth and Lindorf, 1991), was observed, with

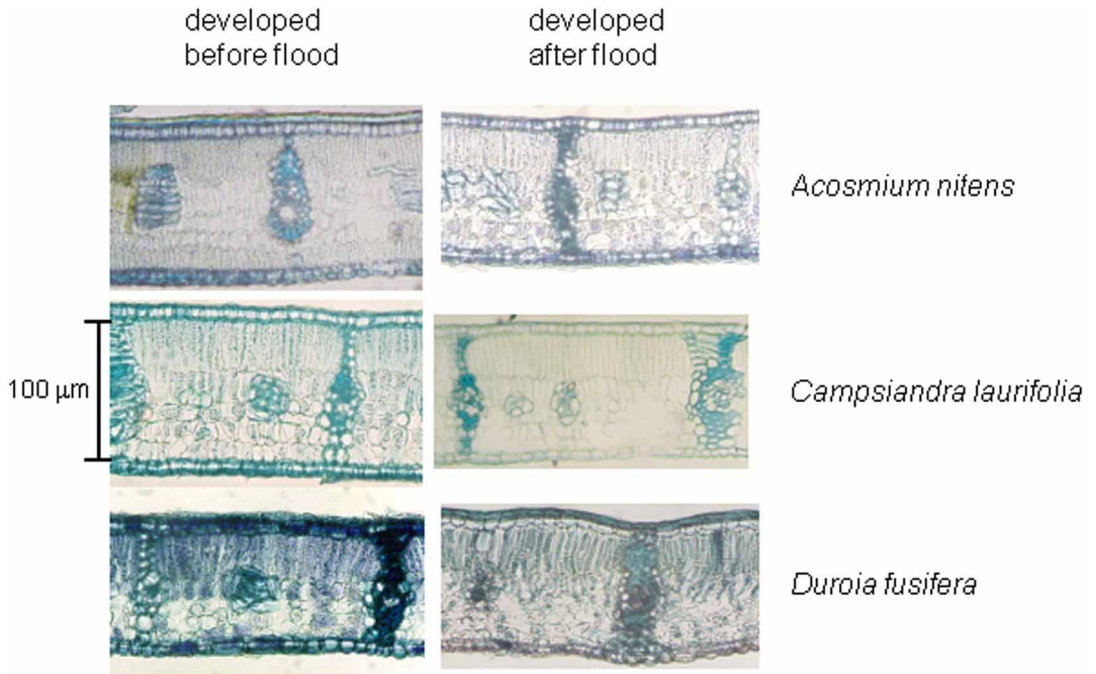

FIGURE 9 | Cross-sections of emerged leaves of the species indicated produced during drainage and after full flooding. The sections were stained with toluidine blue. Modified from Herrera et al. (2009).

abundant sclerenchyma separating mesophyll sections into areoles, and no aerenchyma formation (Herrera et al., 2009). The significance of the occurrence of heterobaric leaves in this environment is unclear and merits further investigation.

Leaves collected in the Mapire igapó at FW from trees on drained soil and trees under flooding had few differences in proportional tissue thickness, the sole significant ones being a reduction in whole-leaf thickness of *E. tenuifolia*, *P. orinocoensis*, and *S. paniculata* and a change in relative thickness of parenchymata in *E. tenuifolia* (Herrera et al., 2009). These results contrast with observations done on the tropical species *Alchornea triplinervia* (Roças and Scarano, 2001), where palisade and spongy parenchymata of flooded plants growing in the field were 1.4 times as thin as those in unflooded plants.

Specific leaf area (SLA = area/mass) decreased with flooding, being higher in young than mature leaves and similar in mature emerged and submerged leaves (Herrera et al., 2009). In *Rumex palustris*, a wetland herbaceous plant, leaf thickness and SLA were 20 and 58% lower in submerged than emerged leaves (Mommer et al., 2005). This was attributed the function of facilitating O_2 diffusion through the liquid phase into the mesophyll. In trees of the Mapire igapó the acclimation to flooding of leaf gas exchange may involve an increase in mesophyll conductance to CO_2 in spite of augmented dry mass per area, which may have increased due to increased total soluble protein (Herrera et al., 2008a).

ECO-PHYSIOLOGICAL AND ECOLOGICAL CONSIDERATIONS

The occurrence in all the species of the Mapire igapó examined of values of g_s and P_N as high under D as under FF suggests that even though these species are indeed tolerant to flooding, they thrive under drainage as long as the soil is wet. This is supported by the finding that in *C. laurifolia*, *A. nitens*, *P. orinocoensis*, and *P. ovatifolium*, a strong relationship between the formation of new wood rings and the fluctuation of the river level during the non-flooded months suggested that an increase in the river water level during drainage promoted growth probably because of better access to ground water (Dezzeo et al., 2003). This could be interpreted as meaning that these species chose to occupy wetlands because of the competitive advantages tolerance gives them, not because of a strict requirement of flooding to grow. Trees of *A. nitens* and *C. laurifolia* grow vigorously at the savanna-end of the gradient, where they experience flooding of low height and short duration, and trees of *Andira inermis*, among others, grow to very large size in areas of the savanna far removed from the igapó. The apparent lack of need for flooding in these species contrasts with the requirement of salt in other flood-tolerant trees, mangroves, in many of which P_N and growth increase in response to salinity up to an optimum (Wang et al., 2011).

Flooding is frequently considered a generalized stress (e.g., Jackson and Colmer, 2005; Bailey-Serres and Voesenek, 2008; Perata et al., 2011), even when dealing with flood-tolerant plants (Parolin and Wittmann, 2010). In agreement with Otte (2001), I argue here that flooding would be stressful if it were not normally encountered by plants and that waterlogging and flooding are not stressful to wetland plants, but only to non-adapted dry land plants. The aim in this argument is not semantic; rather, I have endeavored to demonstrate that initial "negative" responses to flooding of tolerant trees are part of the adaptation.

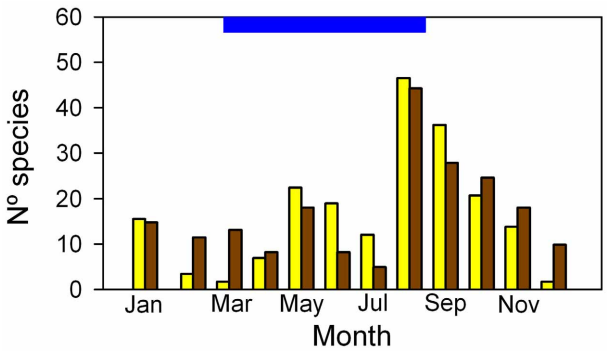

FIGURE 10 | Frequency distribution of flower (yellow) and fruit (brown) production in trees of the Mapire igapó. The blue bar on the ordinate indicates the length of the flood period. Modified from Rosales (1988).

Additionally, the mere observation that ψ increases when waters rise supports the idea of not describing flooding as a stress to tolerant plants.

Of the 85 species identified in the Mapire igapó, 85% flowered and 73% set fruit during the flooded period, mostly during August-September (**Figure 10**). Detailed time-series of leaf production and reproduction in conspecific trees growing under flood and in dry lands would be welcome when dealing with flood tolerance. Together with high P_N values and maintenance of leaf area, the reproductive phenological behavior seems to indicate that flooding increases fitness through its two components, survival and fecundity. This supports the idea that flooding, far from constituting a stress to these wetland plants, is a natural and necessary part of the suite of environmental variables that make their presence in this ecosystem possible.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Thus far, a wealth of information is available on the responses to flood of leaf gas exchange and leaf and whole-plant water status but we know very little about mechanisms underlying such responses. The following is important to solve this issue:

1. In order to gain knowledge on plant tolerance to hypoxia/anoxia in tropical tolerant trees, the morpho-anatomical, biochemical and molecular characteristics of roots must be literally unearthed. *De novo* synthesis of root aquaporins and the occurrence of pressurized O_2 transport could help explain acclimation to flooding. Difficulty in accessing the root system of large trees has hindered progress in this area and data from more feasible experiments with seedlings may not necessarily be extrapolated to adult trees.
2. Examination of the hormonal balance of these trees under flooding seems imperative; preliminary data on leaf and xylem ABA contents point in the direction of promising research.
3. Long-term phenological observations under continually drained conditions should help elucidate whether these species are dependent on flooding for the completion of their life cycle.

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Underwater photosynthesis of submerged plants – recent advances and methods

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We describe the general background and the recent advances in research on underwater photosynthesis of leaf segments, whole communities, and plant dominated aquatic ecosystems and present contemporary methods tailor made to quantify photosynthesis and carbon fixation under water. The majority of studies of aquatic photosynthesis have been carried out with detached leaves or thalli and this selectiveness influences the perception of the regulation of aquatic photosynthesis. We thus recommend assessing the influence of inorganic carbon and temperature on natural aquatic communities of variable density in addition to studying detached leaves in the scenarios of rising CO_2 and temperature. Moreover, a growing number of researchers are interested in tolerance of terrestrial plants during flooding as torrential rains sometimes result in overland floods that inundate terrestrial plants. We propose to undertake studies to elucidate the importance of leaf acclimation of terrestrial plants to facilitate gas exchange and light utilization under water as these acclimations influence underwater photosynthesis as well as internal aeration of plant tissues during submergence.

Keywords: flooding tolerance, light extinction, carbon dioxide, wetland plants, photorespiration

Knowledge of plant and environmental factors determining photosynthesis by submerged plants is essential for understanding aquatic plant ecophysiology and ecosystem productivity, as well as submergence tolerance of terrestrial plants. Following the pioneering studies by Arens (1933) and Steemann Nielsen (1946) on the use of dissolved inorganic carbon (DIC) for photosynthesis of aquatic plants, numerous studies on the regulatory role of light and DIC for underwater photosynthesis of aquatic plants have been conducted. Particularly, the use of DIC by aquatic plants has fascinated researchers and been reviewed several times (e.g., Madsen and Sand-Jensen, 1991; Maberly and Madsen, 2002; Raven and Hurd, 2012) because this process is important for growth and survival and the uptake mechanisms are very different from those of terrestrial, amphibious, and floating leaved plants exposed to atmospheric air (definitions of these life forms and examples of species are in Sculthorpe (1967)). Since the physical conditions differ markedly between water and air, we have often been approached by researchers asking for practical advice, unavailable in the literature, before engaging in work with underwater photosynthesis. Thus, this review serves to offer the background and a practical guide for measurements of carbon fixation by plants when under water.

Moreover, a growing number of researchers are interested in tolerance of terrestrial plants during flooding (**Figure 1A**). Torrential rains sometimes result in overland floods that inundate terrestrial plants (Vervuren et al., 2003) and with the current projection on climate change, the frequency of such flooding events are expected to increase (Parry et al., 2007). We therefore predict that research on underwater photosynthesis will extend greatly beyond its current focus on aquatic plants as natural wetlands and many crops will become submerged in future flooding events. Researchers engaging in underwater photosynthesis should be aware that physical restrictions on light availability and gas exchange are much more profound under water than on land (Sand-Jensen and Krause-Jensen, 1997). Moreover, the aquatic sources and mechanisms of inorganic carbon use are complex, difficult to study, and often challenging to fully understand (Madsen and Sand-Jensen, 1991; Raven and Hurd, 2012).

Photosynthesis provides sugars and O_2. The importance of underwater photosynthesis to internal O_2 status (**Figure 1B**), including via internal long-distance transport into roots growing in anoxic sediments, has been demonstrated for aquatic species (e.g., Borum et al., 2005; Sand-Jensen et al., 2005; Holmer et al., 2009; Pedersen et al., 2011) and terrestrial wetland plants when completely submerged (Pedersen et al., 2006; Winkel et al., 2011, 2013). By contrast, during the night submerged plants rely on O_2 uptake from the surrounding water to sustain their respiration and belowground organs can suffer from O_2 deficiency.

The majority of studies on photosynthesis by submerged aquatic plants have been carried out on detached leaves and algal thalli. These may experience very different environmental conditions than entire communities of submerged plants or plant dominated ecosystems, which have rarely been examined (e.g., Sand-Jensen et al., 2007; Christensen et al., 2013). We thus recommend undertaking studies on communities and ecosystems because they may reveal very different principles of regulation of greater relevance for the ecology and natural performance of

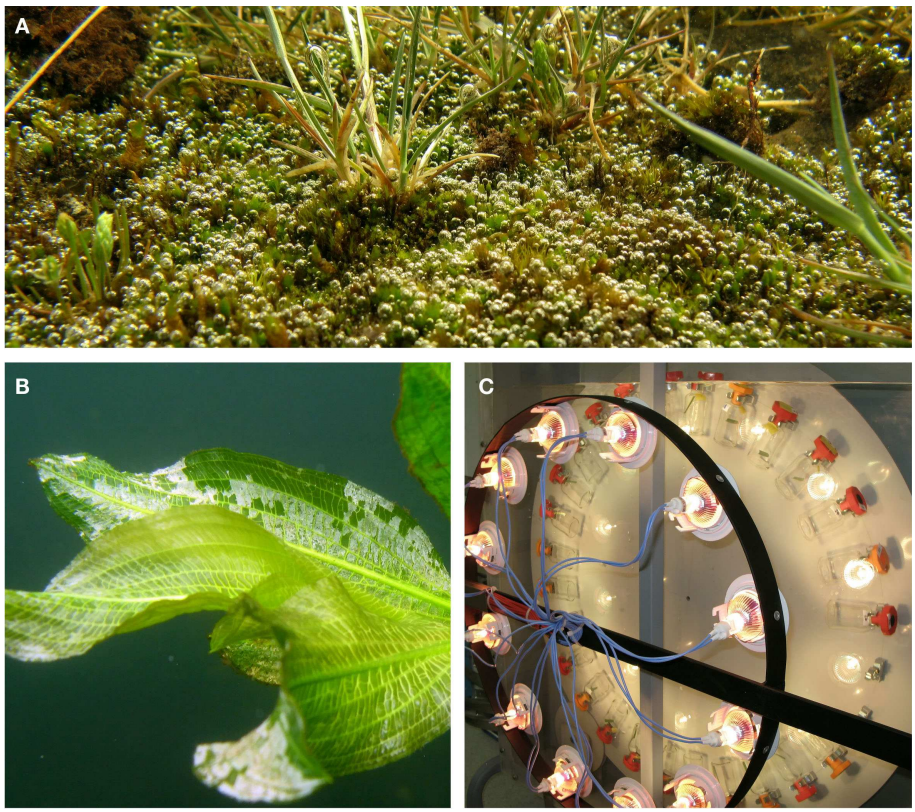

FIGURE 1 | Completely submerged terrestrial vegetation (A), white flakes of $CaCO_3$ on leaves of a pondweed (*Potamogeton lucens*) (B) and an incubator with a vertically rotating wheel holding vials with leaf segments for measurements of underwater net photosynthesis (C) or dark respiration when in complete darkness. The bubbles on the submerged mosses **(A)** are obvious signs that underwater photosynthesis takes place with O_2 evolution causing the bubble formation. Moreover, the submerged grasses possess superhydrophobic self cleansing leaf surfaces that retain a thin gas film when under water, evident as a silvery reflecting surface. In **(B)**, high pH on the adaxial leaf surfaces following extensive underwater photosynthesis has resulted in precipitation of $CaCO_3$ (See "Challenges Under Water – Reduced Gas Diffusion and Light Penetration"). Photos: a shallow puddle on Öland, Sweden **(A)**, the bicarbonate rich (1.8 mmol DIC L^{-1}) Lake Slåen, Denmark **(B)** and the custom built wheel incubator by Ray Scott at the University of Western Australia **(C)**; photos by Ole Pedersen.

submerged aquatic plants, as well as survival of terrestrial species during overland floods.

With the present review, we describe the general background and the recent advances in underwater photosynthesis of phytoelements (shoots, excised thalli, or leaf portions), communities, and plant dominated aquatic ecosystems and present contemporary methods tailor made to quantify photosynthesis and carbon fixation under water.

UNDERWATER PHOTOSYNTHESIS

CHALLENGES UNDER WATER – REDUCED GAS DIFFUSION AND LIGHT PENETRATION

The 10^4-fold lower diffusion coefficient of gases in water, compared with in air, presents a major challenge to submerged plants (Armstrong, 1979; Maberly and Madsen, 2002). Diffusive boundary layers (DBL) develop on all surfaces and their thickness adjacent to leaves in water is of the same order of magnitude as those for leaves in air (Vogel, 1994; Raven and Hurd, 2012). Although the transport distance for gases across the DBL is similar, the much lower diffusion coefficient in water results in a 10^4-fold lower flux for the same concentration gradient and thus the DBL constitutes a much larger proportion of the total resistance to gas exchange for leaves under water than in air (Maberly and Madsen, 2002). The "bottleneck effect" of the DBL on underwater gas exchange was demonstrated in a study of four submerged aquatic species, where the DBL accounted for 90% of the total resistance to carbon fixation (Black et al., 1981). Hence, inorganic carbon limitation of photosynthesis is a much more common and prominent feature for aquatic than terrestrial leaves (Stirling et al., 1997). On average, the underwater photosynthesis increased threefold in a study of 14 submerged freshwater species at saturating relative to ambient supply of DIC supply (Nielsen and Sand-Jensen, 1989). The immediate acclimation of photosynthesis of five fast growing annual terrestrial species by doubling of atmospheric CO_2 was 1.6- to 2.1-fold while the average increase of relative growth rate over 56 day was 1.25-fold and only significant for one of the five species (Stirling et al., 1998).

The restricted gas exchange under water has resulted in evolution of a suite of adaptive features in aquatic leaves and macroalgal thalli to reduce the influence of DBL on the exchange of O_2 and

CO_2, including the supplementary use of HCO_3^- (bicarbonate) (Sculthorpe, 1967; Maberly, 1990; Colmer et al., 2011). In seawater and in many freshwaters, the pool of HCO_3^- is several fold higher than of CO_2 and thus presents an attractive alternative to CO_2. Use of HCO_3^- has evolved many times among unicellular algae, macroalgae, and angiosperms in freshwater and marine environments (Raven and Hurd, 2012) and can involve direct uptake into the cells or external conversion to CO_2 in the DBL catalyzed by surface bound carbonic anhydrase and/or extrusion of protons in acids bands (charophytes; Lucas and Smith, 1973) or lower leaf surfaces in e.g., species of *Potamogeton* and *Elodea* (Prins et al., 1980) often with precipitation of $CaCO_3$ on the alkaline upper leaf surfaces (**Figure 1B**). While the DBL reduces the direct HCO_3^- flux to the leaf surface, the "stagnant" layer is required to forming high CO_2 concentrations by acid titration of HCO_3^- (Helder, 1985). Use of HCO_3^- is prominent for marine macroalgae and seagrasses, freshwater charophytes, and in 50% of 80 tested species of freshwater angiosperms (Sand-Jensen and Gordon, 1984; Maberly and Madsen, 2002), but the ability is absent among mosses and pteridophytes. Also 12 amphibious species alternating between emergent and submerged forms in Danish lowland streams relied solely on CO_2 use although high HCO_3^- concentrations may still benefit photosynthesis by stabilizing pH and permitting rapid uncatalyzed replenishment of the CO_2 consumed (Maberly, 1990). The proportion of HCO_3^- users among angiosperm species in lakes increases significantly with alkalinity and, thus, availability of HCO_3^- (Maberly and Madsen, 2002) in accordance with the increasing advantage of HCO_3^- use for photosynthesis and growth. Assuming for simplicity a 10-fold higher apparent preference for CO_2 than HCO_3^- by leaves in alkaline water containing 0.015 mmol L^{-1} CO_2 and 1.5 mmol L^{-1} HCO_3^-, the supply rate of HCO_3^- would be 10-fold higher than that of CO_2. In softwater containing only 0.15 mmol L^{-1} HCO_3^- the supply rate of the two carbon species would be the same. Terrestrial plant species lack these adaptive features for aquatic life, and when underwater their leaves show dramatically reduced net photosynthesis (Sand-Jensen et al., 1992; Nielsen, 1993) and dark respiration (Colmer and Pedersen, 2008; Pedersen et al., 2009). Thus, 13 terrestrial species submerged in CO_2 rich stream water were unable to use HCO_3^- and median rates of underwater net photosynthesis were sevenfold lower than of 10 permanently submerged stream plants and the terrestrial species were unable to support substantial growth (Sand-Jensen et al., 1992; **Figure 2**).

The extraction capacity of the DIC pool is only some 1–4% for obligate CO_2 users while it is typically 40–70% among HCO_3^- users; 16 of 19 species (Madsen and Sand-Jensen, 1991). This is because of the ability of HCO_3^- users to continue photosynthesizing despite very high external pH and low DIC in the water. In vegetation rich water bodies of high pH, HCO_3^- users can eventually out compete all obligate CO_2 users (Sand-Jensen et al., 2010). Submerged aquatic plants unable to use HCO_3^- typically have final pH's in the external medium of the order of 8.6–9.8 in alkaline solutions and final CO_2 concentrations equivalent to CO_2 compensation points of 2–10 μmol L^{-1}, while active HCO_3^- users typically have final pH's of 9.8–11.0 and final CO_2 concentrations mostly below 0.3 μmol L^{-1} (Sand-Jensen et al., 1992).

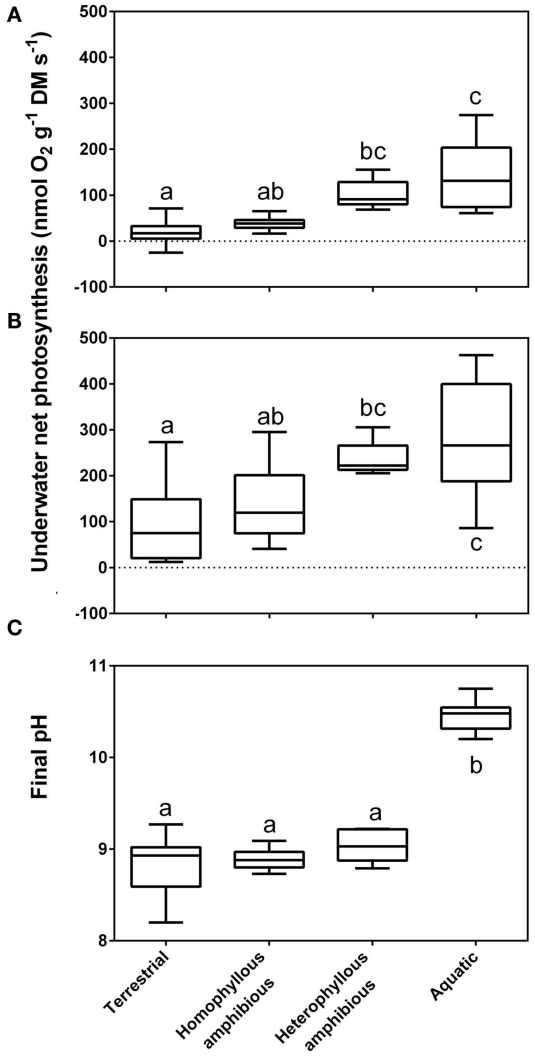

FIGURE 2 | Rates of underwater net photosynthesis (nmol O_2 g^{-1} DM s^{-1}) in ambient [(A), 90–400 μmol CO_2 L^{-1}] and CO_2 enriched water [(B), 800 μmol CO_2 L^{-1}] and final pH from a pH drift experiment; Section "pH Drift Approach to Establish CO_2 Compensation Points" (C) in four groups of life forms defined in Sculthorpe (1967). The box whisker plot indicates the range of the observations (bars), 50% of the observations (boxes) and the median (horizontal line). The amount of free CO_2 present at median final pH of the four groups was 6.20, 6.00, 4.80, and 0.04 μmol L^{-1}, respectively. Leaves of aquatic plants species ($n = 10$), heterophyllous amphibious plants ($n = 5$), homophyllous amphibious plants ($n = 7$) were all produced under water while leaves of terrestrial plant species were formed in air ($n = 12$). Letters indicate significant differences ($P < 0.05$); Tukey test. Data from Sand-Jensen et al. (1992), with measurements taken at 15°C.

For a large collection of stream plants, median final CO_2 values among the supposedly obligate CO_2 users were 6.0 μmol L^{-1} for homophyllous and 4.8 μmol L^{-1} for heterophyllous amphibious plants, within the typical range of CO_2 compensation points, while the median final CO_2 concentration for the putative HCO_3^- users was only 0.04 μmol L^{-1} reflecting the supplementary use of HCO_3^- (**Figure 2**). Heterophyllous amphibious species form morphologically and anatomically distinct leaf types under water

as compared to in air (Sculthorpe, 1967). The underwater leaf forms are an acclimation that enhances underwater gas exchange (Sand-Jensen et al., 1992; Colmer et al., 2011).

Photosynthesis of submerged aquatic plants and flooded terrestrial plants can also be severely limited by light (Kirk, 1994). In water, light is exponentially attenuated with depth following the equation: $I_Z = I_0(1 - f)\ e^{-Z\varepsilon}$; where I_z is the available irradiance at a given depth (z), I_0 is the irradiance at the surface, and ε is the attenuation coefficient. The proportional reflection and back scattering at the water surface (f) is variable but typically about 0.1 such that the proportion of down welling irradiance is 0.9 (Kirk, 1994). The attenuation coefficient of pure water averaged across the photosynthetic spectrum is about 0.03 m^{-1}, so in ultra clear water such as oligotrophic oceanic water, rooted plants could grow as deep as 70 m with 10% of surface irradiance still being available, which happens to be the approximate lower depth limit of seagrasses (Duarte, 1991). In most cases, however, colored dissolved organic matter (CDOM), pigments in planktonic algae and suspended particles, together reduce light penetration much more profoundly (Staehr et al., 2012b). Because freshwaters compared with marine waters are typically richer in nutrients, phytoplankton, CDOM exported from land and particles suspended from shallow sediments, attenuation coefficients typically range from 0.3 to 10 m^{-1} and thus have lower depth limits of rooted plants from 7 m to only 0.2 m (Middelboe and Markager, 1997). Flooding after heavy rain is commonly associated with erosion, high particle loads and high release of CDOM from inundated terrestrial soils. Flooded terrestrial plants can, therefore, experience extreme shading corresponding to attenuation coefficients between 1 and 8 m^{-1} (Parolin, 2009) making light limitation also of terrestrial plants a prominent feature during flooding events (Colmer et al., 2011).

UNDERWATER PHOTOSYNTHESIS IN SUBMERGED AQUATIC PLANTS AND RECENT ADVANCES

The net process of photosynthesis (Eq. 1) is often described simply as the fixation of CO_2 (or HCO_3^- in water; Eq. 2) catalyzed by several enzymes, including Rubisco, driven by light and resulting in production of organic matter, O_2 and OH^-:

$$CO_2 + H_2O \rightarrow CH_2O + O_2 \quad (1)$$

$$HCO_3^- + H_2O \rightarrow CH_2O + O_2 + OH^- \quad (2)$$

The rate of the process can be determined by the production of O_2 and new organic matter (e.g., by isotopic tracing with ^{13}C and ^{14}C) and the consumption of CO_2, HCO_3^-, or more generally the pool of DIC: CO_2, HCO_3^- and CO_3^{2-}. Photosynthesis is an alkalinization process as reflected by the release of OH^- in Eq. 2 and the equivalent consumption of CO_2 and protons in Eq. 1 (i.e., $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$) such that photosynthesis can be determined by the pH increase when converted to DIC consumption accounting for the buffer capacity [mainly due to carbonate alkalinity (CA), See, The CO_2 Equilibria in Water].

In charophytic macroalgae, use of HCO_3^- in photosynthesis can be closely coupled stochiometrically to carbonate precipitation (McConnaughey, 1991):

$$Ca^{2+} + 2HCO_3^- + H_2O \rightarrow CH_2O + O_2 + CaCO_3 \quad (3)$$

This process is pH neutral because conversion of HCO_3^- to CO_3^{2-} generates the necessary proton for conversion of HCO_3^- to CO_2 for assimilation. Thus, HCO_3^- is equally divided between production of organic matter and $CaCO_3$ and the photosynthetic quotient (PQ: mol O_2 evolved mol^{-1} DIC consumed) is only about 0.5 compared with the typical value of 1.0 in Eqs 1 and 2. The active processes are apparently active antiport of H^+ and Ca^{2+} in the acid bands and Ca^{2+} extrusion in the alkaline bands resulting in gradual accumulation of $CaCO_3$ from inside the carbonate layer (McConnaughey, 1991). Carbonate precipitation closely coupled to photosynthesis is also found in coralline red algae, several green algae, and numerous freshwater angiosperms developing polar leaves with high pH and carbonate precipitation being confined to the upper leaf surfaces (Raven and Hurd, 2012). However, it remains to be tested whether active Ca^{2+} extrusion is involved in angiosperm use of HCO_3^- as in charophytes (Prins et al., 1980). Even though photosynthesis and carbonate precipitation may not be closely coupled, the alkalinization process in Eqs 1 and 2 may still result in carbonate precipitation on leaf surfaces (**Figure 1B**) or in the surrounding water because of increase of pH and CO_3^{2-}, though with a variable ratio to the fixation of CO_2 in organic matter. Consequently, O_2 evolution is a more reliable measure of underwater photosynthesis, while DIC use and production of organic matter and carbonate are essential parameters in the analysis of plant growth and carbon dynamics in ecosystems and on regional and global scales (McConnaughey et al., 1994; Cole et al., 2007). While coupled calcification photosynthesis leads to extensive drawdown of DIC and sediment accumulation of organic carbon and carbonates, carbonate formation *per se* generates H^+ tending to reduce pH and increase CO_2. During geological periods of intense formation of coral reefs, CO_2 concentrations are suggested to have increased in the ocean and the atmosphere (Opdyke and Walker, 1992). The coupled photosynthesis calcification process has three major ecological or physiological implications: (i) in coral and coralline algae carbonates are directly used to build up the structural skeleton, (ii) in all phototrophs, surface precipitates will protect them against grazing animals, and (iii) calcification will prevent alkalinization during intensive photosynthesis which could otherwise have led to such high pH levels that tissues are damaged and photosynthesis is severely inhibited.

The photosynthetic capacity under optimum light, temperature, and DIC conditions varies among species and within species depending on their investment in active transport processes and catalytic machinery. On dry mass basis, maximum rates of photosynthesis of detached leaves of submerged aquatic plants from lakes typically vary 25-fold and dark respiration only fourfold between slow-growing, oligotrophic isoetid species and fast growing, eutrophic elodeid species (Nielsen and Sand-Jensen, 1989). Photosynthetic rates per unit dry mass increase significantly with reduced leaf thickness, higher relative surface area, and higher concentrations of pigments and nitrogen in structural and catalytic proteins, including Rubisco (Madsen et al., 1993). Because metabolism on a dry mass basis increases with declining leaf thickness, photosynthesis expressed per surface area only varies eightfold among species (Nielsen and Sand-Jensen, 1989). A comparison

with terrestrial leaves characterizes the aquatic leaves by their lower chlorophyll and Rubisco concentrations and lower photosynthetic rates per surface area mainly due to the thin leaves of most aquatic species. This finding has been interpreted by Maberly and Madsen (2002) as a result of selection of submerged plants to match the low rates of carbon influx predominantly because of high transport resistance. Thin submerged leaves with chloroplasts in epidermal layers will also increase the cost effectiveness of light use and can also be regarded as a particular advantage in a low light aquatic environment with no risk of desiccation damage to the epidermal layers.

Realized rates of underwater photosynthesis for a given plant tissue varies from zero at compensation levels for light and DIC to maximum rates at saturating light and DIC. Light and DIC levels required to saturate photosynthesis increase with temperature and are highly dependent on the extent of self shading and, therefore, the scale of analysis of either detached leaves, individuals or populations (See Underwater Photosynthesis – Approaches and Methods). On a daily level, light limitation takes place early in the morning (low light but plenty of CO_2, **Figure 3**) and co-limitation of both light and CO_2 takes place late in the afternoon where also CO_2 is low (**Figure 3**). On a seasonal level, light limitation is present from late autumn to early spring outside the tropical regions. Populations in deep or turbid waters and dense populations with high self shading face permanent light limitation. Photosynthetic rates at saturating light and DIC increase with temperature due to stimulation of enzyme activity up to an optimum level depending on the adaptation and acclimation of the species but are usually located between 25 and 32°C for temperate submerged aquatic plants (Santamaría and van Vierssen, 1997). The temperature exponent gradually declines and reaches zero as temperature approaches the optimum temperature and it turns negative above the optimum due to increasing influence of photorespiration (Long, 1991) and risk of damage of macromolecules, membranes, and structural organization of membrane proteins (Johnson et al., 1974; Lambers et al., 2008). Respiration continues to increase up to higher temperatures than photosynthesis resulting in proportionally greater losses of organic matter and optima for growth being located at lower temperature than optima for photosynthesis (Olesen and Madsen, 2000; Pilon and Santamaría, 2001).

Ninety nine percent of all studies of aquatic photosynthesis have been carried out with detached leaves or thalli and this selectiveness influences the perception of the regulation of aquatic photosynthesis (Sand-Jensen and Krause-Jensen, 1997). The influence of light, DIC, and temperature on underwater photosynthesis show mutual interdependencies and are, moreover, strongly dependent on the spatial scale. From detached phytoelements to closed communities, light compensation points typically increase three- to eightfold and light saturation levels increase from 200 to 400 μmol m^{-2} s^{-1} to more than the maximum irradiances at noon of about 1500 μmol m^{-2} s^{-1} (**Table 1**; Sand-Jensen et al., 2007). The stimulation of photosynthesis in alkaline water by rising CO_2 concentrations from 20 (close to air equilibrium) to 250 μmol L^{-1} (more than 10-fold above air equilibrium) is about ninefold for detached leaves and only 1.9- to 2.5-fold for dense communities of freshwater CO_2 users while for efficient HCO_3^- users the

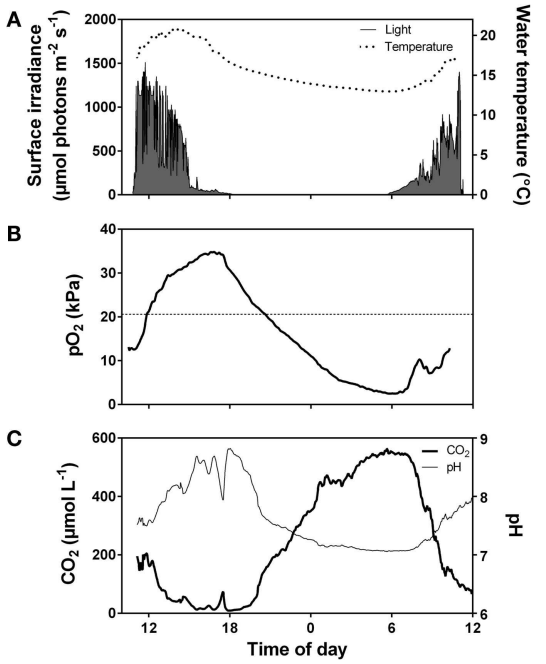

FIGURE 3 | Diurnal fluctuations in surface irradiance and water temperature (A), O_2 partial pressure (B), and CO_2 concentration and pH (C) in the water of a vegetation rich pond in Western Australia. The pond was densely vegetated by *Meionectes brownii* which in late afternoon had extracted CO_2 from the water down to below air equilibrium (<15 μmol L^{-1}). During the night, total system respiration consumed O_2 (pO_2 dropped to 2.5 kPa early in the morning) and CO_2 rose to 550 μmol L^{-1}. Data extracted from Rich et al. (2013).

CO_2 stimulation is only about twofold for individual leaves and insignificant for dense communities (**Table 2**; Sand-Jensen et al., 2007). Open communities of less self shading take an immediate position between detached individual leaves and dense communities. Profound self shading and light limitation of photosynthesis in dense aquatic communities imply that the influence of temperature and inorganic carbon supply is smaller than observed for well illuminated phytoelements. The full scale influence of temperature and CO_2 on community photosynthesis is confined to tissues in the upper part of the canopy receiving irradiances above light saturation.

Up scaling of metabolic analyses from communities of submerged aquatic plants to entire ecosystems dominated by rooted plants have only been done in a few instances. Kelly et al. (1983) studied a shallow, densely vegetated stream (Gryde Stream, Denmark) by open water O_2 measurements and confirmed that incoming irradiance was the main determinant of daily and seasonal variations of underwater photosynthesis which was only light saturated for a few hours at noon on clear summer days. The high CO_2 concentrations (typically 10-fold air equilibrium) in lowland streams is a prerequisite for the high photosaturated rates and strong light dependency of submerged plants in general and CO_2 users in particular (Sand-Jensen and Frost-Christensen, 1998). With natural CO_2 concentrations close to air equilibrium, as observed in most lakes and ponds and in streams in the

Table 1 | Photosynthetic parameters for thallus segments and communities of *Fucus serratus* of varying leaf area index (LAI).

	Thallus (mean)	Community LAI (m^2 m^{-2})			
		3.0	**6.3**	**8.9**	**13.8**
GP_{max} (μmol O_2 m^{-2} s^{-1})	7.95	11.5	17.4	22.0	23.7
A (μmol O_2 mol^{-1} photon)	0.064	0.031	0.049	0.069	0.072
E_C (μmol photon m^{-2} s^{-1})	22	67	99	94	175
E_K (μmol photon m^{-2} s^{-1})	Approx. 300	Not sat.	Not sat.	Not sat.	Not sat.

GP_{max}: light saturated photosynthesis, α: the initial linear slope of photosynthesis versus irradiance, E_c: the light compensation points when net photosynthesis is zero, E_k: the irradiance for onset of light saturated photosynthesis. From Binzer and Sand-Jensen (2002b).

Table 2 | Increase (x-fold) of maximum gross production of O_2 at high (250 μmol L^{-1}) relative to low (20 μmol L^{-1}) CO_2 concentration in alkaline water (5000 μmol L^{-1} DIC) of leaves and freshwater plant communities at two densities (LAI; 2 or 10 m^2 m^{-2}).

	Temperature (°C)	Leaves	Community (LAI 2 m^2 m^{-2})	Community (LAI 10 m^2 m^{-2})
HCO_3^- USERS				
Potamogeton crispus	15	2.1	1.45	1.16
Potamogeton pectinatus	15	2.0	1.33	0.96
CO_2 USERS				
Callitriche cophocarpa	15	9.0	3.32	2.46
	25		2.14	1.91
Sparganium simplex	15	9.3	4.24	1.91
	25		4.16	1.54

The ratio GP_{max} (high CO_2):GP_{max} (low CO_2) is shown. Leaf values are from Sand-Jensen (1983) and community values from Sand-Jensen et al. (2007).

afternoon after several hours of planktonic photosynthesis (Sand-Jensen and Frost-Christensen, 1998; Christensen et al., 2013), CO_2 plays a stronger regulatory role for photosynthesis particularly in open plant stands of low self shading (Sand-Jensen and Frost-Christensen, 1998). Moreover, the species rich group of terrestrial plants in lowland streams would be unable to survive if the water had not been greatly supersaturated as their CO_2 compensation points resemble or exceed the CO_2 concentrations at air equilibrium (Sand-Jensen and Frost-Christensen, 1999). Recent use of open water measurements of O_2 and pH in shallow, alkaline ponds dominated entirely by charophytes documents that high biomass densities in late summer are attained by sustained slow growth over the preceding 3 months at very low nutrients concentrations in the water, and that daily photosynthesis is mostly limited by light (**Figure 4**) and only briefly by DIC at high pH (>9.5), and with virtually no CO_2 available in the afternoon (Christensen et al., 2013). Only submerged aquatic plants capable of using HCO_3^- and concentrating CO_2 internally at the site of Rubisco can thrive in this environment (Sand-Jensen et al., 2010). Plant species forming dense communities in shallow ponds must also be able to tolerate substantial diurnal variations in temperature (e.g., 18–32°C) and O_2 (hypoxic to twice air equilibrium) (Christensen et al., 2013). Daily photosynthesis and respiration were high in the pond and closely interrelated showing that newly produced organic matter was mostly rapidly respired by plants and bacteria.

Overall, the analyses of individual phytoelements, communities, and ecosystems confirm that the relative roles of light and DIC for determining photosynthesis are closely interrelated and highly dependent on plant density and species affinities for CO_2 and HCO_3^-. Maximum photosynthetic rates under light and inorganic carbon saturation are quite variable both between and within species depending on selected strategies and the investment in catalytic machinery coupled to supply of resources (e.g., nutrients). While photosaturated photosynthetic rates are strongly dependent on species, acclimation, and temperature, light limited rates are rather temperature independent and relatively similar among species (Frost-Christensen and Sand-Jensen, 1992). As the importance of light limitation for community photosynthesis

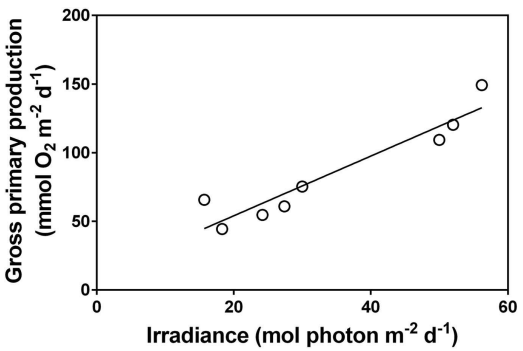

FIGURE 4 | Relationship between daily surface irradiance and community gross primary production in a shallow pond in Öland, Sweden over a daily time course of 8 days with naturally variable light conditions (each data point represents 1 day). The dominant submerged aquatic plant in the pond was, *Chara virgata* (data extracted from Christensen et al., 2013). Pearson correlation = 0.96, $P < 0.001$.

increases in dense plant stands, the influence of species, temperature, and DIC supply decline and enable us to predict community photosynthesis primarily from the overall distribution and absorptance of light in the canopy (Binzer and Sand-Jensen, 2002a; b; Binzer et al., 2006).

RECENT ADVANCES IN UNDERWATER PHOTOSYNTHESIS IN TERRESTRIAL WETLAND PLANTS

Terrestrial wetland plants grow in waterlogged soils and/or sediments with shallow standing water, so that a large proportion of the shoot is in contact with air. So, aerial photosynthesis predominates but these plants can experience episodes of complete submergence during floods. Although much more tolerant of submergence than non-wetland terrestrial species, submergence is regarded as a serious abiotic stress for terrestrial wetland plants, but species (and genotypes within a species) differ markedly in submergence tolerance (Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009). The impeded gas exchange under water restricts respiration and photosynthesis (See Challenges Under Water – Reduced Gas Diffusion and Light Penetration); photosynthesis can also be limited by low light when submerged (See Challenges Under Water – Reduced Gas Diffusion and Light Penetration and Underwater Photosynthesis in Submerged Aquatic plants and Recent Advances). Thus, submergence disrupts energy metabolism of terrestrial plant species as a result of a reduced O_2 supply (at least during the night, in some tissues) and/or diminished carbohydrate status because of the restricted photosynthesis when under water.

Terrestrial wetland species lack most of the adaptive leaf features for inorganic carbon acquisition for photosynthesis as described in Section "Underwater Photosynthesis in Submerged Aquatic Plants and Recent Advances" for aquatic and acclimated amphibious plants. Thus, when compared with leaves of aquatic plants, those of terrestrial plants generally have larger overall apparent resistance to diffusion of CO_2 from the bulk medium to chloroplasts, so that slow CO_2 uptake restricts underwater photosynthesis. Underwater photosynthesis by leaves of terrestrial wetland species is lower than that achieved by aquatic species, when compared per unit of leaf dry mass (Sand-Jensen et al., 1992; Colmer et al., 2011).

The few studies available show, however, that the low photosynthesis when under water enhances survival of submerged terrestrial plants (Vervuren et al., 1999; Mommer et al., 2006b; Vashisht et al., 2011). Both the sugars and O_2 produced would likely contribute to enhanced survival when submerged (Mommer and Visser, 2005), and in the case of sugars especially when submergence lasts more than a few days and internal carbohydrates become depleted. Depletion of carbohydrates during submergence is considered a major factor influencing survival of submerged rice (Setter and Laureles, 1996) and determining recovery following desubmergence and ultimately grain yield in flood-prone areas (Bailey-Serres et al., 2010; Mackill et al., 2012). The O_2 produced in photosynthesis can travel from leaves to roots via aerenchyma, and so this endogenously produced O_2 improves the internal aeration of submerged plants (e.g., rice; Pedersen et al., 2009; Winkel et al., 2013).

A recent review (Colmer et al., 2011) highlighted there are few studies of underwater photosynthesis by terrestrial wetland plants, and few of these compared rates underwater with those achieved by leaves in air. Similarly, a quantitative understanding of the potential role of underwater photosynthesis to whole plant carbon budgets during submergence seems to be lacking for terrestrial wetland species, whereas carbon budgets for several aquatic species (e.g., van der Bijl et al., 1989) and systems (e.g., Christensen et al., 2013) have been evaluated. For some terrestrial wetland species, only a few crude leaf level estimates of carbon budgets have been considered (e.g., in Colmer and Pedersen, 2008), but the potential contribution of underwater photosynthesis to carbon gain was demonstrated in growth studies of completely submerged rice, albeit under controlled conditions (e.g., Pedersen et al., 2009). Studies of whole plant carbon budgets in field conditions are generally lacking, even understanding of this aspect for the important wetland crop rice submerged in various field scenarios appears to be incomplete.

Detailed studies of underwater photosynthesis of terrestrial wetland species have focused on production and performance of submergence acclimated leaves. New leaves produced when under water by some terrestrial wetland species are better acclimated for underwater photosynthesis than the aerial leaves (Mommer et al., 2007). The acclimated leaves have a thin cuticle and overall are also thinner and of less breath (Mommer and Visser, 2005). These morphological and anatomical differences as compared with the usual leaves produced in air, reduce the resistance to CO_2 (and O_2) diffusion between the bulk medium and chloroplasts in submerged leaves, owing to narrower DBLs (suggested by Colmer et al., 2011), lower cuticle resistance (Mommer et al., 2006b), and shorter internal diffusion path lengths (Mommer et al., 2006a). However, although a reduced cuticle that enhanced underwater gas exchange occurs in several terrestrial wetland species (few species have been evaluated to date), the magnitude of the reduction in apparent resistance to gas exchange with the medium was not correlated with submergence tolerance for the species tested (Mommer et al., 2007), highlighting the need for further experimental investigations.

Some recent work on underwater photosynthesis by submerged terrestrial wetland plants has evaluated the contribution of gas films on superhydrophobic leaf surfaces to gas exchange with floodwaters. Leaf surface hydrophobicity (i.e. water repellence) is a feature that sheds off water in wet aerial environments (Smith and McClean, 1989; Brewer and Smith, 1997) and promotes "self cleansing," enhancing leaf performance and reputably lowering susceptibility to pathogens (Neinhuis and Barthlott, 1997). Some terrestrial wetland species have super hydrophobic leaves that when submerged retain a gas film, e.g., rice (Raskin and Kende, 1983) and *Phragmites australis* and others (Colmer and Pedersen, 2008). Gas films enhance CO_2 uptake for underwater photosynthesis (Raskin and Kende, 1983; Colmer and Pedersen, 2008; Pedersen et al., 2009) and O_2 entry for respiration in darkness (Colmer and Pedersen, 2008; Pedersen and Colmer, 2012). The enhancement by leaf gas films of CO_2 uptake (in light) and of O_2 (in darkness) was demonstrated by the marked declines in underwater photosynthesis and respiration when the films were experimentally removed (Colmer and Pedersen, 2008; Pedersen et al., 2009; Pedersen and Colmer, 2012). In addition, leaves produced in air by terrestrial wetland species that did not form gas films

when submerged (i.e. leaves of these species were not sufficiently hydrophobic), had lower rates of underwater photosynthesis than those that did form gas films (Colmer and Pedersen, 2008; Colmer et al., 2011). As one example, leaf segments of rice with dissolved CO_2 set as in a field pond and with underwater photosynthesis measured as described in Section "The Rotating Wheel Incubator," showed rates four- to fivefold higher for leaf segments with intact gas films compared to those with the films experimentally removed (Winkel et al., 2013). Moreover, a field study using *in situ* monitoring of O_2 in rhizomes of *Spartina anglica* demonstrated the benefit of having leaf gas films to internal aeration during complete submergence, both during day and night tides (Winkel et al., 2011). Summing up, leaf gas films enhance underwater photosynthesis and internal aeration of some terrestrial wetland plants when submerged, with benefits also demonstrated to growth when submerged in controlled experiments (e.g., rice; Pedersen et al., 2009).

UNDERWATER PHOTOSYNTHESIS – APPROACHES AND METHODS

Conventional infrared gas analyzer (IRGA) systems following CO_2 exchange in air do not work under water, so dedicated measuring systems are required to quantify underwater net photosynthesis and dark respiration. DIC can be measured by injection of small aliquots of water into concentrated acid in a bubble chamber purged with gaseous N_2 carrying the released CO_2 into an IRGA (Vermaat and Sand-Jensen, 1987). However, photosynthesis measurements based on DIC determinations are thus based on discrete measurements at selected times and can be complicated because of the large and variable combined pool of DIC in water (See Underwater Photosynthesis in Submerged Aquatic plants and Recent Advances and The CO_2 Equilibria in Water). Indirect methods to track DIC changes can be based on continuous measurements of pH in solution (Maberly, 1996). The DIC technique to measure photosynthess has potential errors if: (i) DIC is removed by external carbonate precipitation, (ii) internal DIC accumulates in tissues or colony gels, (iii) DIC dissolution of solid carbonates occurs, or (iv) DIC is released from internal pools (McConnaughey et al., 1994; Sand-Jensen et al., 2009). External measurements of pH to estimate DIC changes have the same potential errors as above and, moreover, also due to direct exchange of protons from tissues not always being closely coupled to DIC exchange. Therefore, most methods for studies of underwater net photosynthesis are based upon O_2 detection.

In contrast to gas exchange measurements of photosynthesis by leaves in air using open systems and CO_2 detection, underwater measurements commonly use closed systems and detection of O_2. In addition to the rationale for O_2 detection described in the preceding paragraph, O_2 detection also enables measurements in waters of substantially different DIC concentrations (e.g., soft-water lakes up to 100 μmol L^{-1}, ocean approximately 2000 μmol L^{-1} and hardwater lakes up to 10000 μmol L^{-1}). The drawback of closed systems is that these are non-steady-state (i.e. DIC declining and O_2 increasing with time). Use of open systems with O_2 detection is constrained by reliable continuous detection of differences in O_2 concentrations between incoming and outgoing solutions from an appropriate chamber.

Changes in O_2 concentration over time are straightforward to measure with Clark type amperometric electrodes or more recently by use of O_2 sensitive optodes. Oxygen partial pressure (pO_2) or dissolved O_2 can be continuously monitored in water with an accuracy of 0.01 kPa or 0.2 μmol L^{-1} (Strickland and Parsons, 1972). Photosynthesis determined from changes in O_2 and DIC pools dissolved in the surrounding water requires that those are much greater than changes in such pools within the plant tissue (Sand-Jensen and Prahl, 1982). This is best achieved by having large incubation volumes relative to plant volumes. Alternatively, changes in tissue pools can be measured (Sand-Jensen et al., 2005) or be deduced by establishment of true steady state where tissue concentrations remain constant or quasi steady state where tissue concentrations changes proportionally to external concentrations (Sand-Jensen and Prahl, 1982). Measurements of underwater photosynthesis based upon O_2 evolution can include great error when plants with highly porous tissues (perhaps variable in volume and having much higher "solubility" of O_2 than water; See Medium and Tissue) are incubated in small chambers (Hartman and Brown, 1967; Richardson et al., 1984). On the other hand, measurements of underwater photosynthesis based upon changes in DIC can include extreme error when plant tissues (or colony matrices in the case of algae and cyanobacteria) hold very large pools of DIC that do not change in concert with those in the surrounding water. For example, DIC in the colony gel of *Nostoc zetterstedtii* continues to support photosynthesis after water pools have been exhausted, and in darkness respiratory CO_2 replenishes this internal pool before being released to the water (Sand-Jensen et al., 2009).

Measurements of radioactive labeling of the DIC pool with ^{14}C and the use of pulse amplitude modulated (PAM) fluorometry are also methods to measure photosynthetic performance under water; these technique are beyond the focus of the present paper so readers are referred to e.g., Adams et al. (1978) or Kemp et al. (1986) for methods on ^{14}C and to Silva et al. (2009) or Suggett et al. (2011) and chapters therein for PAM approaches.

THE CO_2 EQUILIBRIA IN WATER

Understanding the chemistry of dissolved DIC and the proportional changes in its three constituents (CO_2, HCO_3^- and CO_3^{2-}) depending on ionic strength, temperature, and primarily pH (Mackereth et al., 1978) is essential because it determines the availability of the preferred CO_2 source and the supplementary HCO_3^- source for underwater net photosynthesis. When CO_2 dissolves in water, the following equilibrium is established:

$$CO_{2(aq)} + H_2O \leftrightarrow (H_2CO_3) \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (4)$$

CO_2's reaction with water (H_2O) forming carbonic acid (H_2CO_3) is a time dependent process which in some organisms is catalyzed by the enzyme carbonic anhydrase. H_2CO_3 can dissociate immediately into a proton (H^+) and bicarbonate (HCO_3^-) so the dissolution of CO_2 into water causes pH to drop. At high pH, HCO_3^- can further dissociate into a second H^+ and carbonate (CO_3^{2-}). The relative distribution of the three main inorganic carbon species with pH is shown (**Figure 5**). The pKa_1 is 6.532 and

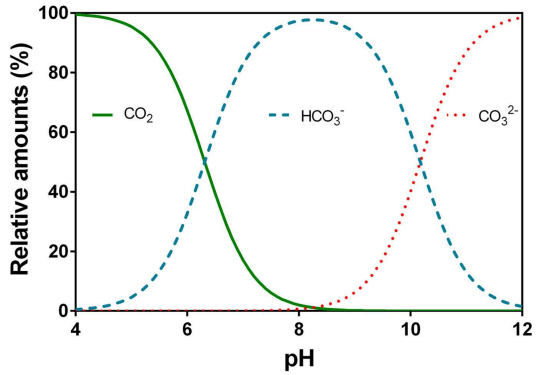

FIGURE 5 | Relative speciation (%) of carbon dioxide (CO_2), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) in water as a function of pH. The example given is at 20°C and electrical conductivity of 250 μS cm^{-1}. Data were calculated using Gutz (2012) with the apparent $pK_1 = 6.532$ and $pK_2 = 10.329$ (Schwarzenbach and Meier, 1958).

is referred to as the apparent pKa_1 as only little CO_2 is converted into carbonic acid (hence the brackets in Eq. 4) while the majority remains in solution as CO_2(aq) also referred to as free CO_2; pKa_2 is 10.329 (Schwarzenbach and Meier, 1958; Stumm and Morgan, 1996; Gutz, 2012). Below pH 6, most of the DIC is present as CO_2, which is usually more readily used for underwater photosynthesis than HCO_3^-. Between pH 7 and 10, HCO_3^- dominates, a carbon species that can be used as an additional carbon source among species in most taxonomic groups of aquatic plants except for pteridopytes and mosses (Raven and Hurd, 2012). Only at pH higher than 10, a significant proportion of the DIC is in the form of CO_3^{2-} which apparently is not taken up by any phototrophs in ionic form but can perhaps be made available in acid zones on plant surfaces by back titration with released protons (conversion toward the left in Eq. 4).

In freshwaters and seawater, the alkalinity (sum of alkaline ions buffering added H^+; units in mequiv. L^{-1} or mmol L^{-1} for monovalent HCO_3^- in water which is in air equilibrium of negligible OH^- and CO_3^{2-}) is almost entirely controlled by the carbonate systems with insignificant contribution from silicate and phosphate, and with some contribution by borate in seawater. At pH above 9, OH^- has a significant contribution to alkalinity being 0.074 mmol L^{-1} at pH 10 and 0.74 mmol L^{-1} at pH 11 at an alkalinity of 2 mmol equivalents L^{-1} (**Table 3**). It is thus convenient to distinguish between the total alkalinity (TA) and the CA (Dickson, 1981; Stumm and Morgan, 1996). The chemical species contributions to the two alkalinities are:

$$CA = (HCO_3)^- + 2(CO_3)^{2-} \quad (5)$$

$$TA = CA + (H_2BO_3)^- + 2(HBO_3)^{2-} + (OH)^- - (H)^+ \quad (6)$$

Purging an aqueous solution with pure CO_2 alters the CA through the addition of ionic carbon species and also through pH related shifts in the partitioning of carbon species already present in the solution (Eqs 4 and 5). However, the TA is not affected by bubbling with CO_2 as every negatively charged ion is balanced by a proton (Eq. 6). For example, water fresh from the tap often contains CO_2 above air equilibrium and so bringing it to equilibrium by purging with atmospheric air would thus lower pCO_2 until a new equilibrium has been reached. According to Eq. 5, CA would decrease slightly as both CO_3^{2-} and HCO_3^- decrease equivalent to the rise of OH^- and pH.

For experimental purposes, an aqueous photosynthesis solution is usually prepared with a certain amount of DIC and then pH is adjusted with acid or base to that required to achieve the desired concentration of "free" (i.e. dissolved) CO_2 and HCO_3^-. **Table 3** lists the relationship between pH and amounts of CO_2, HCO_3^-, CO_3^{2-}, and OH^- at 20°C and a fixed TA, calculated from Gutz (2012). The examples provided in the sections below demonstrate how to apply all the above information in practice.

In the next sections (See "The Rotating Wheel Incubator" to "The Open Natural System") we describe methods in use for measurements of underwater photosynthesis. The methods scale from phytoelements to communities. The approaches involve laboratory and field techniques and so have different levels of control of key environmental variables influencing photosynthesis.

THE ROTATING WHEEL INCUBATOR

Principle: Leaf samples or algal thalli are incubated in glass vials of a known concentration of CO_2 in an aqueous medium, and the sealed vials of known volume are rotated on an incubator under well defined light and temperature conditions. O_2 produced during incubation is measured by an electrode/optode and underwater net photosynthesis can be calculated based on e.g., leaf area, fresh mass, dry mass, and/or chlorophyll. Alternatively, consumption of DIC can be used as a photosynthetic measure. Incubation in darkness provides data on dark respiration.

Medium and tissue

The choice of medium is basically between an artificial medium with a well defined ion and gas composition or ambient water with the ion and gas composition of natural habitats (essential chemical parameters such as pH, DIC, and alkalinity should be characterized). An example of an artificial medium is the Smart and Barko (1985) general purpose culture medium. This medium contains (mmol L^{-1}) 0.62 Ca^{2+}, 0.28 Mg^{2+}, 0.28 SO_4^{2-}, and 1.24 Cl^- and $KHCO_3$ (sometimes mixed with $NaHCO_3$) is used to generate the required DIC. HCl, NaOH (or KOH), atmospheric air or gas mixtures of known pCO_2 can be used to adjust pH to a required value based on the desired amount of free CO_2. Since all incubations are short term, there are no micro nutrients or vitamins in this medium. Some studies have also used submergence solutions or "ambient" water from streams or lakes in order to establish a rate of photosynthesis under specific conditions (Sand-Jensen et al., 1992; Nielsen, 1993; Sand-Jensen and Frost-Christensen, 1998) and these can also be adjusted to predefined pH, CO_2 and/or O_2 levels. Any production of O_2 by microalgae or consumption by microbial organisms in ambient water is accounted for in the blanks; micro-filtration of water is commonly used to remove background microflora.

Photorespiration, as previously demonstrated for rice (Setter et al., 1989) and the aquatic pteridophyte, *Isoetes australis*, (Pedersen et al., 2011), during incubation is a potential issue as the

Table 3 | Distribution of DIC, CO_2, HCO_3^-, CO_3^{2-}, and OH^- as a function of pH at constant total alkalinity of 2 mmol H^+ equivalents L^{-1} at 20°C.

pH	DIC (mmol L^{-1})	CO_2 (mmol L^{-1})	HCO_3^- (mmol L^{-1})	CO_3^{2-} (mmol L^{-1})	OH^- (mmol L^{-1})
6.00	6.5073	4.5074	1.9998	0.0001	0.0000
6.05	6.0170	4.0172	1.9997	0.0001	0.0000
6.10	5.5801	3.5803	1.9997	0.0001	0.0000
6.15	5.1907	3.1909	1.9997	0.0002	0.0000
6.20	4.8436	2.8438	1.9996	0.0002	0.0000
6.25	4.5343	2.5345	1.9996	0.0002	0.0000
6.30	4.2586	2.2588	1.9995	0.0002	0.0000
6.35	4.0128	2.0131	1.9995	0.0002	0.0000
6.40	3.7938	1.7941	1.9994	0.0003	0.0000
6.45	3.5986	1.5990	1.9994	0.0003	0.0000
6.50	3.4246	1.4250	1.9993	0.0004	0.0000
6.55	3.2696	1.2700	1.9992	0.0004	0.0000
6.60	3.1313	1.1318	1.9991	0.0004	0.0000
6.65	3.0082	1.0087	1.9990	0.0005	0.0000
6.70	2.8983	0.8989	1.9988	0.0006	0.0000
6.75	2.8004	0.8011	1.9987	0.0006	0.0000
6.80	2.7132	0.7139	1.9985	0.0007	0.0000
6.85	2.6354	0.6362	1.9984	0.0008	0.0001
6.90	2.5661	0.5670	1.9982	0.0009	0.0001
6.95	2.5042	0.5053	1.9979	0.0010	0.0001
7.00	2.4491	0.4503	1.9977	0.0011	0.0001
7.05	2.3999	0.4013	1.9974	0.0012	0.0001
7.10	2.3561	0.3576	1.9971	0.0014	0.0001
7.15	2.3169	0.3186	1.9968	0.0016	0.0001
7.20	2.2820	0.2839	1.9964	0.0018	0.0001
7.25	2.2509	0.2530	1.9959	0.0020	0.0001
7.30	2.2230	0.2254	1.9954	0.0022	0.0001
7.35	2.1982	0.2008	1.9949	0.0025	0.0002
7.40	2.1760	0.1789	1.9942	0.0028	0.0002
7.45	2.1561	0.1594	1.9935	0.0031	0.0002
7.50	2.1383	0.1420	1.9927	0.0035	0.0002
7.55	2.1223	0.1265	1.9919	0.0039	0.0003
7.60	2.1080	0.1127	1.9909	0.0044	0.0003
7.65	2.0951	0.1004	1.9898	0.0050	0.0003
7.70	2.0835	0.0894	1.9885	0.0056	0.0004
7.75	2.0730	0.0796	1.9871	0.0062	0.0004
7.80	2.0635	0.0709	1.9856	0.0070	0.0005
7.85	2.0548	0.0632	1.9838	0.0078	0.0005
7.90	2.0469	0.0562	1.9819	0.0088	0.0006
7.95	2.0396	0.0501	1.9797	0.0098	0.0007
8.00	2.0328	0.0446	1.9772	0.0110	0.0007
8.05	2.0265	0.0397	1.9745	0.0123	0.0008
8.10	2.0205	0.0353	1.9714	0.0138	0.0009
8.15	2.0149	0.0314	1.9680	0.0155	0.0010
8.20	2.0094	0.0279	1.9641	0.0173	0.0012
8.25	2.0041	0.0248	1.9598	0.0194	0.0013
8.30	1.9989	0.0221	1.9550	0.0217	0.0015
8.35	1.9937	0.0196	1.9497	0.0243	0.0017
8.40	1.9884	0.0174	1.9437	0.0272	0.0019
8.45	1.9830	0.0155	1.9371	0.0304	0.0021
8.50	1.9774	0.0138	1.9297	0.0340	0.0023

(Continued)

Table 3 | Continued

pH	DIC (mmol L^{-1})	CO_2 (mmol L^{-1})	HCO_3^- (mmol L^{-1})	CO_3^{2-} (mmol L^{-1})	OH^- (mmol L^{-1})
8.55	1.9716	0.0122	1.9214	0.0380	0.0026
8.60	1.9655	0.0108	1.9122	0.0424	0.0029
8.65	1.9590	0.0096	1.9020	0.0474	0.0033
8.70	1.9520	0.0085	1.8907	0.0528	0.0037
8.75	1.9445	0.0075	1.8781	0.0589	0.0041
8.80	1.9364	0.0067	1.8642	0.0656	0.0047
8.85	1.9277	0.0059	1.8489	0.0729	0.0052
8.90	1.9182	0.0052	1.8319	0.0811	0.0059
8.95	1.9079	0.0046	1.8133	0.0901	0.0066
9.00	1.8967	0.0040	1.7928	0.0999	0.0074
9.05	1.8846	0.0036	1.7703	0.1107	0.0083
9.10	1.8714	0.0031	1.7457	0.1225	0.0093
9.15	1.8570	0.0027	1.7189	0.1353	0.0104
9.20	1.8415	0.0024	1.6898	0.1493	0.0117
9.25	1.8246	0.0021	1.6582	0.1643	0.0131
9.30	1.8065	0.0018	1.6241	0.1806	0.0147
9.35	1.7870	0.0016	1.5874	0.1981	0.0165
9.40	1.7661	0.0014	1.5480	0.2167	0.0185
9.45	1.7438	0.0012	1.5061	0.2366	0.0208
9.50	1.7201	0.0010	1.4615	0.2576	0.0233
9.55	1.6950	0.0009	1.4144	0.2797	0.0262
9.60	1.6686	0.0008	1.3649	0.3028	0.0294
9.65	1.6408	0.0007	1.3132	0.3269	0.0329
9.70	1.6118	0.0006	1.2594	0.3518	0.0370
9.75	1.5817	0.0005	1.2039	0.3773	0.0415
9.80	1.5506	0.0004	1.1469	0.4033	0.0465
9.85	1.5186	0.0003	1.0887	0.4295	0.0522
9.90	1.4858	0.0003	1.0297	0.4559	0.0586
9.95	1.4525	0.0002	0.9703	0.4820	0.0657
10.00	1.4188	0.0002	0.9109	0.5077	0.0738
10.05	1.3847	0.0002	0.8519	0.5327	0.0828
10.10	1.3505	0.0001	0.7936	0.5568	0.0929
10.15	1.3162	0.0001	0.7364	0.5797	0.1042
10.20	1.2820	0.0001	0.6807	0.6012	0.1169
10.25	1.2478	0.0001	0.6267	0.6211	0.1312
10.30	1.2138	0.0001	0.5747	0.6391	0.1472
10.35	1.1800	0.0001	0.5249	0.6550	0.1651
10.40	1.1462	0.0000	0.4776	0.6686	0.1853
10.45	1.1124	0.0000	0.4327	0.6797	0.2079
10.50	1.0786	0.0000	0.3905	0.6881	0.2333
10.55	1.0446	0.0000	0.3508	0.6937	0.2617
10.60	1.0101	0.0000	0.3138	0.6963	0.2937
10.65	0.9750	0.0000	0.2794	0.6956	0.3295
10.70	0.9389	0.0000	0.2475	0.6914	0.3697
10.75	0.9017	0.0000	0.2181	0.6835	0.4148
10.80	0.8628	0.0000	0.1910	0.6718	0.4654
10.85	0.8220	0.0000	0.1662	0.6558	0.5222
10.90	0.7788	0.0000	0.1435	0.6353	0.5859
10.95	0.7327	0.0000	0.1228	0.6099	0.6574
11.00	0.6831	0.0000	0.1039	0.5792	0.7377

Calculated from Mackereth et al. (1978) and Gutz (2012). See ***Figure 5*** *for the relative distribution between CO_2, HCO_3^- and CO_3^{2-} versus pH. The yellow highlight refers to Example 1, Section "Medium and Tissue."*

evolved O_2 is trapped in solution of the closed glass vial. The risk of photorespiration is increased during experiments at high temperature (Long, 1991) and with very low DIC and CO_2 concentrations leading to low ratios of CO_2 to O_2 at the site of Rubisco (Maberly and Spence, 1989; Sand-Jensen and Frost-Christensen, 1999). Therefore, the starting partial pressure of O_2 (pO_2) should be brought down to approximately 50% of air equilibrium, i.e., 10 kPa. This is sufficient to address the issue of photorespiration (provided that incubation do not last long periods so that O_2 produced increases above air equilibrium) and at the same time there is still enough O_2 in the medium to prevent tissue anoxia before photosynthesis starts producing O_2 (Colmer and Pedersen, 2008). In practice, equal volumes of medium (including all ions) are bubbled with either air or N_2. After mixing the two solutions, the pO_2 will be approximately 10 kPa and HCO_3^- can be added to the medium and pH adjusted accordingly to achieve the desired amount of free CO_2 (see example below).

In some situations, an organic buffer may be used to maintain a constant pH in the medium during incubation. In practice, however, HCO_3^- is a natural and often sufficient buffer in itself and we do not recommend using buffers if the CA is above 1 mmol L^{-1} as HCO_3^- would be sufficient to buffer against large pH fluctuations during incubation (Sand-Jensen et al., 1992; Colmer and Pedersen, 2008). Moreover, organic buffers can also modify membrane porters and pH at plant surfaces modifying HCO_3^- use and influx/efflux of CO_2 (Price and Badger, 1985; Larsson and Axelsson, 1999; Moulin et al., 2011). pH of the medium should be measured in a sample taken of the initial solution and then also in vials after incubations. With the ongoing advancement of optodes, pH may even be measured without opening the vials if applying pH sensitive microdots (See "O_2 Measurements" for description of O_2 sensitive microdots). If additional buffering is required, i.e. pH measurements after incubation show unacceptable drift in pH, then MES or TES buffers may be used, e.g., at a concentration of 5 mmol L^{-1} (Pedersen et al., 2009, 2010), though the possible influence of these buffers on HCO_3^- use must be kept in mind.

The vials (10–100 mL glass vials with ground glass stoppers) are filled with medium using a siphon. By siphoning the medium into the bottom of each vial, exchange of O_2 and particularly CO_2 with the atmosphere is minimized; prepare sufficient medium to flush the vials at least twice the volume, and fill the vials completely. An air bubble can hold 36-fold more O_2 as the same volume of deionized (DI) water at 25°C, so bubbles in the vials introduce significant error to the net photosynthesis measurements. A set of vials without tissue serves as blanks and is incubated along with the vials containing tissue samples in the rotating incubator. The blanks serve to provide the starting pO_2 in the vials and also to correct for any O_2 production or consumption (e.g., by algae, bacteria, or chemical processes) if ambient water is used as medium. Glass beads ($\varnothing = 3$–5 mm; two in each 25 mL vial) are added to each vial to provide mixing as the vials are rotating in the incubator.

The amount of tissue added to each vial depends on the activity of the tissue, the amount of DIC and free CO_2, the light level (PAR), and the temperature. At saturating light and CO_2 levels and at 25°C, 0.5 mg fresh mass mL^{-1} medium is often a good choice as this will result in a rise of pO_2 by approximately 2–5 kPa within an hour of incubation providing reproducible and accurate determination of O_2 regardless of the technique employed (see below). However, both microelectrodes and optodes have a resolution of approximately 0.01 kPa so a change in 1 kPa could also be sufficient. At lower CO_2 and/or light levels, more tissue may be required or alternatively, longer incubation times are needed. However, small tissue samples are preferred to prevent self shading and to promote good mixing in the vials so that tissues are well exposed to light and chemicals during incubation.

Example 1: Preparation of artificial floodwater with CA of 2.0 mmol L^{-1} and 200 μmol free CO_2 L^{-1}. Prepare a solution of DI water containing Ca^{2+}, Mg^{2+}, SO_4^{2-}, and Cl^- at the concentrations described above. Divide the solution into two containers and bubble one half of the solution with air and the other half with N_2 for 20 min and then mix the two solutions. Add the required amount of DIC (**Table 3**, highlighted in yellow for this example) which is 2.2 mmol L^{-1}. Add the DIC in the form of $KHCO_3$, $NaHCO_3$ or a mixture, and acidify the solution to pH 7.35 using HCl. This results in a solution with a CA of 2 mmol L^{-1} (in mmol L^{-1}: 1.995 $HCO_3^- + 0.002$ CO_3^{2-}) and 200 μmol L^{-1} CO_2 (**Table 3**).

Incubator with light and temperature control

The incubator provides constant temperature and mixing throughout the incubation. It consists of a vertically rotating wheel where glass bottles or vials can be clipped on facing the light source. The wheel rotates at about 10 rpm in a tank with temperature controlled water and a transparent glass or Perspex wall for illumination at various irradiances (**Figure 1C**).

The rotating wheel incubator was originally invented for photosynthesis measurements in phytoplankton (Steemann Nielsen, 1952) and the typical light source in commercially available models consists of a rack of fluorescent tubes. However, it is hard to achieve PAR levels much above 500 μmol photons m^{-2} s^{-1} with fluorescent light so high pressure metal halide lamps (mercury or sodium) or light emitting plasma lamps are required to provide the levels of light needed to light saturate net photosynthesis by leaves of many terrestrial species and some macroalgae with thick thalli.

Photosynthesis *versus* light curves (i.e. light response curves) are obtained by: (i) regulating light intensities by varying the distance of the light source to the incubator, (ii) placing neutral shading filters in front of the light source, (iii) placing a box with neutral shading filters of variable transmission in front of individual vials, or (iv) by wrapping the vials in layers of neutral shading mesh, or by a combination of these various approaches.

O_2 measurements

The O_2 produced or consumed during incubation can be measured directly in the glass vials using O_2 electrodes or optodes. In the absence of good electrodes or optodes, the Winkler titration can also be applied; see Strickland and Parsons (1972) for details.

Contemporary methods for O_2 measurements in water involve either Clark type electrodes or optodes. A Clark type O_2 electrode measures pO_2 as molecular O_2 transverses a membrane before the electrochemical reaction on the cathode results in a current which is linearly proportional to the pO_2 of the medium. Since the electrode consumes O_2, a conventional large O_2 electrode is quite

stirring sensitive and it is thus much more convenient to use an O_2 microelectrode which consumes little O_2 to address the stirring issue during measurements; O_2 microelectrodes can have a stirring sensitivity of less than 1% (Revsbech and Jørgensen, 1986; Revsbech, 1987). Oxygen microelectrodes typically have a temperature coefficient of approximately 1–3%°C^{-1} (Revsbech, 1987; Gundersen et al., 1998) so temperature control during measurements is essential. The temperature effect on electrodes (and optodes, see below) is primarily caused by changes in diffusion and electrochemistry. In addition, temperature also influences solubility of gases, and metabolic rate of the tissues.

The measuring principle of optodes is quite different from that of a Clark type electrode. In the optode, light excites a fluorophore coated onto the tip of fiber optics and the excited light is subsequently transmitted back and measured by a spectroradiometer (Klimant et al., 1997). Alternatively, the fluorophore can be coated onto tiny plastic patches which (microdots) can be mounted directly in the medium where O_2 is to be measured; the microdot with the fluorophore is then illuminated from the outside through the transparent wall of the container. Molecular O_2 quenches the florescence so that the transmitted signal can be calibrated toward O_2 in the medium; the relationships between quenching and pO_2 is non-linear. Optodes do not consume O_2 and are thus completely insensitive to stirring. However, O_2 optodes can have higher temperature coefficients than Clark type microelectrodes and require even better temperature control during measurements (Kragh et al., 2008). On the other hand, optodes can be built into the individual glass vials (microdots glued onto the glass wall inside the vial) and the O_2 concentration can be measured in a non-destructive manner (Kragh et al., 2008). The great advance of this approach is that vials can remain sealed and be returned to the rotating wheel if a preliminary reading shows that longer incubation is required in order to obtain the necessary accuracy, or O_2 evolution can be followed over time to ensure *quasi* steady state measurements or to elucidate possible temporal patterns.

Supporting measurements and calculations

After measuring O_2 of each vial, the tissue must be processed according to standard procedures to establish the area, the fresh mass or dry mass, the chlorophyll concentration, or all of the above. The underwater net photosynthesis is calculated as the net O_2 evolution rate per unit tissue per unit time. In practice, the change in O_2 content in each vial (change in O_2 concentration multiplied by the volume of the vial; individual volumes of vials (i.e. minus the volume of the glass beads, etc.) must be established) divided by the incubation time and divided by the amount of tissue (i.e., mass, area or any other of the above mentioned parameters used to scale photosynthesis per sample unit). An example of a CO_2 response curve established with the technique described here in Section "The Rotating Wheel Incubator" is shown in **Figure 6**.

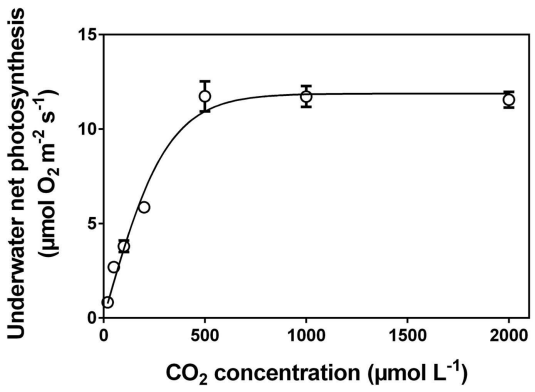

FIGURE 6 | Underwater net photosynthesis *versus* CO_2 concentration in the medium for excised leaf segments of *Hordeum marinum*. Leaf segments (30 mm) were incubated in 35 mL glass vials with various well defined CO_2 concentrations on a rotating wheel with PAR of 350 µmol photons m^{-2} s^{-1} at 20°C (see **Figure 1C**). O_2 evolution was measured with a Clark type O_2 microelectrode and underwater net photosynthesis was calculated as O_2 evolution per projected area per unit time (See "The Rotating Wheel Incubator"). Data (mean ± SE, $n = 5$) from (Pedersen et al., 2010). Note: leaves of *H. marinum* are superhydrophobic and so possess a gas film when underwater.

THE CLOSED CHAMBER WITH INJECTION PORTS

Principle: a leaf or algal thalli sample is incubated in a closed chamber with internal mixing and possessing injection ports and fitted with an electrode/optode that follows O_2 concentration. The amount of free CO_2 can be manipulated by injection of acid or base while a fitted pH electrode allows calculation of the exact CO_2 level. The approach enables production of a complete light or CO_2 response curve based on the same sample, and underwater net photosynthesis can be calculated based on e.g., leaf area, fresh mass, dry mass, and/or chlorophyll concentration. Incubation in darkness can provide data on dark respiration.

Chamber with light and temperature control

The chamber for measurements of underwater net photosynthesis enables measurements with light, temperature, and CO_2 manipulations in water, with monitoring of O_2 with time. Chambers are commercially available for underwater photosynthesis measurements on macro algae, phytoplankton, or isolated chloroplasts and these are made from glass, acrylic glass, or acetal. These chambers can also be custom built to match specific electrodes, light sources, and fitted with extra ports for temperature and PAR sensors and injection of acid/bases or inhibitors. The chamber must be made from a material the can be sterilized and also have a least one transparent side to enable illumination of the sample. The light source can be diode based (650 nm red diode) or "full spectrum" halogen light to simulate white sunlight. Pay attention to the fact that some lighting devices are unable to produce sufficient light to saturate the photosynthesis of some terrestrial leaves or thick macroalgae thalli. Illumination (even by means of fiber optics) produces heat, so cooling of the chamber by a water jacket is crucial.

A light sensor small enough to measure inside the chamber is also essential. The spherical PAR sensor US-SQS/L (Walz, Effeltrich, Germany) is of a size ($\varnothing = 3.7$ mm) that enables permanent installation in most chambers.

Finally, the issue of mixing must be addressed. The simplest solution is to use a glass coated stir bar (avoid Teflon coated stir bars as these can hold O_2) which is isolated from the sample with a coarse mesh to prevent contact with the tissue. It may be necessary

to fix the tissue in the swirling current; if the tissue rotates with the water current in the chamber, the DBL will be larger than if the tissue is fixed. The thicker DBL increases the apparent resistance to CO_2 uptake or O_2 escape.

O_2 and pH measurements

O_2 measurements in the closed chamber are similar to O_2 measurements in the vials described in Section "O_2 Measurements." An O_2 sensor (Clark type electrode or optode) is fixed in the chamber in one of the ports, or if an optode is used, a patch with fluorophore can be glued onto the interior wall. A pH electrode is fitted in a second port and the signals from both sensors are logged onto a computer with data acquisition software. Calibration of both O_2 and pH sensors should be performed in the chamber to avoid stirring related artifacts to the calibrations. Remember to pay extra attention to temperature if using O_2 optodes. It may take a while for the temperature of the solution inside the chamber to equilibrate with that of the cooling jacket, and working in a constant temperature room or keeping solutions in a thermostated water bath will significantly reduce the time it takes before a temperature steady state is obtained; always measure temperature directly in the chambers. Temperature influences electrode or optode performance, solubility of gases, and metabolic rate of the tissues (see Section "O_2 Measurements"). After insertion of tissue and filling of the chamber with medium (see below), pH can be manipulated by injection of small amounts of acid or base through one of the injection ports. Fit a 27G needle in one of the injection ports and let it function as "over pressure valve" to prevent pressurization during injection of acid or base (or inhibitors); the needle may be left in the stopper during the experiment as diffusion of gases in water is too slow to result in experimental artifacts.

As described in Section "The Rotating Wheel Incubator" for incubations of tissues in closed vials on the wheel, substantial photorespiration can occur if O_2 is allowed to build up in the medium. Therefore, the susceptibility to photorespiration should initially be established for each tissue type. The linearity of O_2 production with increasing external pO_2 is easily tested the following way: a medium with total DIC of 5.0 mmol L^{-1} is prepared from $KHCO_3$ in a 5.0 mmol L^{-1} TES buffer adjusted to pH 8.00 and with a pO_2 of 10 kPa. The tissue is then allowed to photosynthesize up to a pO_2 of 30 kPa. Here, approximately 500 μmol O_2 has been produced from 500 μmol CO_2 and because of the TES buffer the pH has remained at 8.0. Although the DIC pool has declined to 4.5 mmol L^{-1}, free CO_2 has changed by only 10% from 110 to 100 μmol L^{-1}. If the O_2 evolution occurs linearly in this range, it means that the approximately threefold lower CO_2:O_2 in the medium, with likely even greater changes in internal CO_2:O_2, has not increased photorespiration. If the curve exhibits a saturation tendency (i.e. declining rate of net O_2 production with increasing pO_2), photorespiration has probably increased with increasing pO_2 in the chamber.

Medium and tissue may be prepared as described in Section "Medium and Tissue." However, as a CO_2 response curve in the closed photosynthesis chamber often involves conversion of HCO_3^- into free CO_2 (dissolved), e.g., by manipulation of pH, enough HCO_3^- must initially be present in the medium to produce the required levels of free CO_2. Following injection of small amounts of acid or base to manipulate free CO_2, the rate of net photosynthesis changes accordingly so that a new rate of net O_2 production (slope of dissolved O_2 with time) is established at each dissolved CO_2. However, pH may also change slightly in the time interval because CO_2 is extracted from the system as it is fixed via photosynthesis (Eqs 1 and 4). Hence, for every rate of underwater net photosynthesis determined in a time interval, the mean CO_2 concentration must be calculated in order to present the CO_2 response curve of the tissue.

Example 2: average free CO_2 concentration in the pH range from 7.25 to 7.30 in a medium with total DIC of 2.0 mmol L^{-1}. According to Gutz (2012), CA of such a solution at pH 7.25 would be 1.77 mmol L^{-1} having 223 μmol CO_2 L^{-1}; at pH 7.30 CA would be 1.80 mmol L^{-1} and have 203 μmol CO_2 L^{-1}. Consequently, the average free CO_2 concentration in the pH range was 213 μmol CO_2 L^{-1}.

After each experiment, the incubated tissue must be characterized to enable calculation of underwater net photosynthesis rates; the supporting measurements are as those described in section "Supporting Measurements and Calculations."

PH DRIFT APPROACH TO ESTABLISH CO_2 COMPENSATION POINTS

Principle: Leaf or algal thalli samples are incubated in glass vials for 16–18 h where after pH and CA or DIC are measured. CO_2 compensation points and carbon extraction capacity of tissues can be calculated. The method is also used as a diagnostic test for bicarbonate (HCO_3^-) use in underwater photosynthesis.

These long term incubations are used to test how far net photosynthesis of a given plant sample at saturating light can extract DIC, i.e. to deplete CO_2 and HCO_3^- and drive up pH. Because the objective is to determine the ultimate DIC extraction capacity and maximum upper pH in a standardized way, all incubation vials are prepared to have an equal standard DIC concentration (usually 1–2 mmol L^{-1} for alkaline waters and 0.1–0.3 mmol L^{-1} for softwaters) and a pH, CO_2, and O_2 concentration corresponding to air equilibrium (Sand-Jensen et al., 1992, 2009). Artificial media and natural waters can be applied. However, to minimize O_2 build up and the risk of photorespiration during extended incubation the initial O_2 can be reduced to 20–50% of air equilibrium. To ensure the maximum possible DIC depletion, the amount of plant material is typically three times larger than in the incubations described in Sections "The rotating Wheel Incubator" and "The Closed Chamber with Injection Ports" though it must still be able to move freely in the vials to ensure adequate mixing.

The initial and final DIC and pH must be determined in order to calculate the DIC extraction capacity during incubation and the CO_2 compensation point after incubation. Provided no internal pools of DIC and protons interfere with conditions in the water/medium and no precipitation or dissolution of carbonates takes place, DIC can be determined in the medium from CA, pH, temperature, and ionic strength; CA in turn can be determined by acidimetric titration (Stumm and Morgan, 1996). The risk of carbonate precipitation is small in artificial media of $KHCO_3$ and much larger in natural waters and artificial media where $Ca(HCO_3)_2$ dominates, the reason being that K_2CO_3 is highly soluble and $CaCO_3$ is poorly soluble. Calcium carbonate precipitation is likely to take place in pH drift experiments where final

pH exceeds 10. Therefore, it is always recommended to directly measure DIC. This can be done by injecting of small water samples into concentrated acid in a bubble chamber purged with N_2 gas carrying the released CO_2 into an IRGA (Vermaat and Sand-Jensen, 1987). Water samples may need to be filtered (with no atmospheric contact) if minute $CaCO_3$ crystals have been formed in the external water of high pH. It is generally recommendable to determine (or check) CO_2 compensation points by depletion experiments in media of low initial DIC ($<50\,\mu mol\,L^{-1}$) and low pH (<6.5) where the interference by HCO_3^- is low and $CaCO_3$ is not formed.

The pH drift technique has also been used to determine DIC consumption at intervals during the ongoing drift of pH upwards (Maberly and Spence, 1983; Spence and Maberly, 1985). DIC, pH, the proportion of carbon species and O_2 change during the time of incubation. Because all parameters may influence photosynthesis, and exchange with internal DIC and proton pools in the incubated tissue may interfere with calculations, we cannot recommend the procedure for determining rates of net photosynthesis considering the much more accurate and straightforward methods being available today (as described in this review).

COMMUNITY PHOTOSYNTHESIS IN LARGE CHAMBERS

Principle: Community photosynthesis is measured in large closed chambers with linear dimensions of 0.5–0.6 m, or larger, to minimize edge effects and make certain that natural changes of plant density, tissue capacity and irradiance through the canopy are maintained. Photosynthetic rates are measured by O_2 and DIC, as for phytoelements in small chambers (See The Closed Chamber with Injection Ports), but photosynthetic parameters and their dependence on DIC and temperature are markedly different for communities than phytoelements.

Submerged aquatic plants grow in communities of variable density where the spatial structure and self shading are prominent features (Sand-Jensen, 1989). Light limitation is substantial and the efficiency of photosynthesis at low light is therefore important (Binzer and Sand-Jensen, 2002a,b). The photosynthetic chamber needs to be large enough to include tall communities (Binzer et al., 2006; Middelboe et al., 2006). It is made of glass or transparent acrylic glass and viewed from above, the shape of photosynthetic chambers can be cylindrical, rectangular, or quadratic. The cylindrical shape can be advantageous because the surface area of side walls relative to chamber volume is smaller than in rectangular or quadratic chambers, and these two latter types may also have "dead corners" with stagnant waters. The light sources are high pressure metal halide lamps (mercury or sodium) or light emitting plasma lamps because only those provide a sufficiently high irradiance ($>1000\,\mu mol\ photon\ m^{-2}\ s^{-1}$). The light sources must be placed more than 0.5 m above the photosynthetic chamber and the light path both above the chamber and around the chamber walls are surrounded by totally reflecting material to reduce the influence of distance with depth in the chamber both when plants are absent or present. Irradiance is measured with depth in the water and through canopies of different densities using a small spherical PAR sensor. To ensure statistically reliable determinations of vertical attenuation a series (e.g., 10) of measurements are performed at different positions (Middelboe et al., 2006). Temperature, O_2, DIC, and pH are set and measured as described in Section "The Closed Chamber with Injection Ports," while mixing is provided by large submersible pumps ensuring current velocities above 2 cm s^{-1}. Temperature control is attained by direct cooling and warming of the water in the incubation chamber or by placing it in a larger temperature controlled holding tank. In the latter case some temperature variations (1–3°C) is difficult to avoid between light and darkness.

Algal communities for measurements can be collected attached to stones or established over a period of one or several years on artificial tiles of desired size set out in the field and later brought to the laboratory for measurements in the photosynthetic chamber (Binzer et al., 2006; Middelboe et al., 2006). Rooted submerged plants can be harvested from natural stands with the 3D structure kept intact when roots and rhizomes are interwoven. In other cases, individual plants are placed in a homogeneous pattern on the chamber bottom in small plastic bags surrounding the root system. Alternatively the individuals are tied to a frame on the chamber bottom. Plant density is determined as fresh mass, dry mass, or plant surface area normalized to bottom area. Leaf area indices (LAI) ranging from 1 to 12 are useful for comparisons among species. Vertical distribution of plant biomass and surface can be determined by cutting the plants sequentially in well defined strata starting at the top of the canopy.

The setup is suited to evaluate the influence on community photosynthesis by variable irradiance, temperature, DIC (including variable CO_2 and HCO_3^-), canopy density, and spatial structure (Sand-Jensen et al., 2007).

Community photosynthesis can also be determined over longer periods of time by employing the large chambers in an open mode. This allows for exchange of O_2 and CO_2 with the atmosphere to prevent that the chambers undergo too extensive accumulation and depletion in the water during several days of alternating light or dark periods. For calculation of photosynthesis and respiration, exchange rates between air and water must be determined. The flux (F_{exch}, $mol\,m^{-2}\,s^{-1}$) between water and air for O_2 is given by the equation:

$$F_{exch} = K\left(C_{act} - C_{equ}\right) \quad (7)$$

where K is the exchange coefficient (piston velocity, ms^{-1}), C_{act} is the actual and C_{equ} is the equilibrium concentration of O_2 ($mol\,m^{-3}$) in water at the actual temperature (Staehr et al., 2012b). Piston velocity is controlled by surface turbulence and can, therefore, be considered a constant for a given mixing regime determined by the strength and location of the pumps and the dampening influence of the plant community. Thus, K must be directly measured for a given plant density and mixing regime. This is best done in the dark, where only dark respiration ($mol\,m^{-2}\,s^{-1}$) takes place, by modifying C_{act} to for example 10 or 30 kPa and measuring the total O_2 flux (F) over time as a result of respiration and exchange with the atmosphere above from time changes in O_2 concentrations in the water:

$$F = R + K(C_{act} - C_{aqu}) \quad (8)$$

From 30 kPa the actual pO_2 will first rapidly decline as a combined result of respiration and loss to the atmosphere and gradually decline less rapidly as pO_2 approaches equilibrium with the atmosphere and respiration alone drives pO_2 further downwards. Calculations of pO_2 changes over time in relation to differences in the pO_2 gradient between water and air produces a straight line (Eq. 8) permitting calculation of R and K assuming that they remain constant for a given mixing intensity and plant density.

Community measurements operated in an open mode have the main advantage for future application that fluxes of O_2, DIC, Ca^{2+}, H^+, and nutrient ions (NH_4^+, NO_3^-, and PO_4^{3-}) can be determined during repeated diel light dark cycles for several weeks while the submerged plants may also grow. Combined field measurements have been operated in open chambers and mesocosms under a strict mixing regime under natural temperature and light conditions both for phytoplankton (e.g., Markager and Sand-Jensen, 1989), submerged aquatic plants (e.g., Liboriussen et al., 2005), and flooded terrestrial plants (e.g., Setter et al., 1988).

THE OPEN NATURAL SYSTEM

Principle: Natural ecosystems dominated by submerged aquatic plants have free undisturbed gas exchange with the atmosphere and input/output of water. Determination of ecosystem metabolism by open water measurements requires accurate calculations of atmospheric exchange of O_2 and CO_2. The main advantages of the ecosystem approach is that environmental conditions and processes are natural and temporal patterns can be followed over months or years, while allowing plant density and acclimation to gradients in light, DIC, and other environmental variables to develop.

Photosynthesis of submerged aquatic plants derived from analysis of ecosystems can only be determined when rooted plants or macroalgae are the main phototrophs responsible of more than 90% of ecosystem photosynthesis. Only then can the patterns obtained be referred to the metabolism of macrophytes accepting that a minor error (<10%) due to photosynthesis of microalgae may be present. The dominance of submerged aquatic plants can be realized in shallow plant rich ponds, lakes, streams, and coastal lagoons. Open water measurements are used to follow changes in O_2, DIC, pH, temperature, and irradiance, and enable calculation of ecosystem net production, plant gross production, and community respiration assuming fully mixed conditions (Odum, 1956; Staehr et al., 2012a). Meteorological observations of wind direction, wind velocity, atmospheric pressure, etc., in standing waters and current velocity, water depth, slope, and bed roughness in flowing waters, can be used to estimate physical exchange coefficients of gases (i.e. piston velocities) and thus calculate fluxes between water and atmosphere using empirical models (Sand-Jensen and Staehr, 2011). Flow chambers, floating chambers, inert gases, and coverage of water surfaces by impermeable floating plastic can be used for direct determination of exchange coefficients which are critical in all determinations of ecosystem metabolism (Staehr et al., 2012a,b). Rooted plants with gas filled lacunae formation and release of gas bubbles can introduce error. Oxygen storage may delay establishment of steady state exchange of O_2 following dark light switches by some 10–20 min for most rooted plants (Westlake, 1978) and loss of bubbles is negligible in swift flowing waters, while bubble release may account for 10% of net O_2 release in slow flowing waters (Kragh et al., unpublished data).

The strength of these measurements is that they provide natural rates under fully realistic and undisturbed environmental conditions. They can reveal the coupling between O_2 and carbon metabolism, the natural precipitation and dissolution of carbonates and the direct involvement of accumulation and release of acids in the photosynthetic process. Measurements have shown fast exchange rates of protons between macrophytes and water following diurnal light dark switches partly uncoupled from exchanges of DIC during photosynthesis and respiration; a phenomenon that is not unraveled in short term laboratory measurements with detached phytoelements (Kragh et al., unpublished data). Ecosystem measurements can also reveal how early summer growth in biomass and late summer senescence influence plant metabolism and how ongoing desiccation of ponds may suddenly stop photosynthesis and accelerate decomposition, while refilling may restart photosynthesis and growth (Christensen et al., 2013). Modeling approaches, as successfully used for canopy level understanding of terrestrial systems systems (Ainsworth and Long, 2005), should also be applied more widely in studies of aquatic systems (e.g., Binzer and Sand-Jensen, 2002a,b). All techniques for measuring and calculating ecosystem process are basically available (Staehr et al., 2012a) and awaits broad scale application.

OUTLOOK

Studies of photosynthesis by aquatic and submerged wetland plants are few compared with research on photosynthesis in air, but underwater systems are attracting more attention. Light and CO_2 availability under water are often low to submerged plants. Low CO_2 together with impeded escape of O_2 can result in high photorespiration as a component determining net photosynthesis. Focus studies of contrasting species and systems are required to develop our understanding of "models" since the environment under water is more complex than in air and there is a diversity of photosynthetic mechanisms (i.e. C_3, C_4, CAM, and bicarbonate use) in aquatic species.

The physical and chemical environments of overland floods are only poorly known and few data exist on light extinction and CO_2 and O_2 concentrations in floodwaters. Such data are crucial to design relevant laboratory experiments on submergence tolerance of terrestrial plants and to establish, for example, carbon budgets during submergence on leaf lamina as well as for whole plants. Also, studies on leaf acclimation of terrestrial plants to facilitate gas exchange and light utilization under water are also only in their infancy; these acclimations influence underwater photosynthesis as well as internal aeration of plant tissues during submergence.

Finally, a challenge also exists to assess the influence of light, inorganic carbon, and temperature on natural aquatic communities of variable density instead of only studying detached leaves in the scenarios of rising CO_2 and temperature. Use of mathematical modeling, both at the leaf and community levels, will provide valuable additional understanding of underwater photosynthesis.

Improved knowledge of plant and environmental factors determining rate of underwater net photosynthesis at various scales (leaf-to-community) is essential for understanding aquatic plant ecophysiology, submergence tolerance of terrestrial plants, and productivity of the many aquatic and flood-prone ecosystems worldwide.

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Fermentation metabolism and its evolution in algae

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Fermentation or anoxic metabolism allows unicellular organisms to colonize environments that become anoxic. Free-living unicellular algae capable of a photoautotrophic lifestyle can also use a range of metabolic circuitry associated with different branches of fermentation metabolism. While algae that perform mixed-acid fermentation are widespread, the use of anaerobic respiration is more typical of eukaryotic heterotrophs. The occurrence of a core set of fermentation pathways among the algae provides insights into the evolutionary origins of these pathways, which were likely derived from a common ancestral eukaryote. Based on genomic, transcriptomic, and biochemical studies, anaerobic energy metabolism has been examined in more detail in *Chlamydomonas reinhardtii* (*Chlamydomonas*) than in any other photosynthetic protist. This green alga is metabolically flexible and can sustain energy generation and maintain cellular redox balance under a variety of different environmental conditions. Fermentation metabolism in *Chlamydomonas* appears to be highly controlled, and the flexible use of the different branches of fermentation metabolism has been demonstrated in studies of various metabolic mutants. Additionally, when *Chlamydomonas* ferments polysaccharides, it has the ability to eliminate part of the reductant (to sustain glycolysis) through the production of H_2, a molecule that can be developed as a source of renewable energy. To date, little is known about the specific role(s) of the different branches of fermentation metabolism, how photosynthetic eukaryotes sense changes in environmental O_2 levels, and the mechanisms involved in controlling these responses, at both the transcriptional and post-transcriptional levels. In this review, we focus on fermentation metabolism in *Chlamydomonas* and other protists, with only a brief discussion of plant fermentation when relevant, since it is thoroughly discussed in other articles in this volume.

Keywords: anoxic, anaerobiosis, hypoxic, fermentation, pyruvate metabolism

INTRODUCTION

Chlamydomonas AS A MODEL ORGANISM

Chlamydomonas reinhardtii (*Chlamydomonas* throughout) is a soil-dwelling, unicellular green alga that is considered a model organism for studying photosynthetic energy metabolism, and the production of molecular hydrogen (H_2) under anoxic conditions (Melis and Happe, 2001, 2004; Ghirardi et al., 2007). This alga has several metabolic features in common with those of vascular plants, although it also has structures and activities (e.g., flagella and eyespot) that were lost during vascular plant evolution. *Chlamydomonas* represents a robust system for probing biological processes with sophisticated molecular tools. The sequencing of all three *Chlamydomonas* genomes (nuclear, chloroplast, and mitochondrion; Lilly et al., 2002; Maul et al., 2002; Merchant et al., 2007) has facilitated the capture of information about gene and genome structure and potential regulatory sequences, including promoter regions, 3′- and 5′-UTRs and intron–exon junctions. Forward and reverse genetic screens have been developed to generate mutant strains with specific phenotypes, or that are disrupted for specific genes (Dent et al., 2005; Pootakham et al., 2010; Gonzalez-Ballester et al., 2011). Most information discussed in this manuscript on responses of algae to hypoxia/anoxia was derived from studies of *Chlamydomonas*, although information for other algae has been used to strengthen generalizations. Furthermore, we briefly discuss the evolution of the fermentation processes in prokaryotes and non-photosynthetic eukaryotes, but do not discuss plants since other contributions in this volume detail the responses of plants to hypoxic conditions.

BASIC ENERGY-GENERATING PROCESSES

Whether in aerobic or anaerobic environments, the challenge for organisms to maintain viability can only be met if they can stay far from equilibrium. To achieve this situation, they must use energy to satisfy their metabolic demands, which includes continuous synthesis of the cellular energy currency (mostly ATP) along with maintenance of redox and ionic balances. Aerobic metabolism is used by several eukaryotic and prokaryotic organisms to efficiently synthesize ATP through oxidative phosphorylation; O_2 serves as the terminal electron acceptor of the respiratory electron transport chain (Bailey-Serres and Chang, 2005). Nevertheless, life in low O_2 (hypoxia) environments, or even in environments totally devoid of O_2 (anoxia), is common on our planet. Diminished levels of O_2 in various biotopes can result from geochemical or physical conditions, including flooding, excess rainfall, and winter ice encasement, but may also be a consequence of high metabolic activity of bacteria in habitats that are not well aerated. While

anoxia is often transient, it can also be protracted, extending from diurnal periods, to months or years, and even to millennia or more (Grieshaber et al., 1994; Burnett, 1997; Danovaro et al., 2010). Furthermore, even though an organism may live in an oxic habitat, it may still perform anoxic metabolism under certain circumstances. For example, in the presence of sufficient levels of a fermentable substrate, many yeast strains will forego using O_2 as a terminal electron acceptor and maintain vigorous fermentation of available substrates (van Dijken and Scheffers, 1986; Pronk et al., 1996).

REDOX BALANCE THROUGH FERMENTATION

For cells to sustain viability during hypoxia/anoxia they must produce ATP and recycle the NAD(P)H and $FADH_2$ generated by catabolic pathways, usually glycolysis. These compounds must be re-oxidized in a process involving the transfer of electrons to suitable terminal acceptor molecules, which are then typically secreted. Among eukaryotes, there are only two processes for maintaining redox balance and conserving energy when organisms experience anoxic conditions: (i) fermentation, which usually entails substrate-level phosphorylation (SLP), and (ii) anaerobic respiration which involves terminal electron acceptors like NO_3^- and SO_4^{2-} instead of O_2 (Atteia et al., 2013). Anaerobic metabolism provides cells with low levels of chemical bond energy, generating ~2–3 ATP molecules per molecule of glucose metabolized; this compares to the over 30 ATP molecules generated by the oxidative metabolism of glucose.

METABOLIC ENERGY GENERATION

INTRODUCTORY REMARKS

Glycolysis oxidizes glucose to two molecules of pyruvate while generating two ATP molecules. During the oxidation of glucose there is also the production of two NADH molecules (four reducing equivalents). To maintain glycolytic flux and energy production, the cells must re-oxidize the NADH. In the absence of a functional TCA cycle under anaerobic conditions, *Chlamydomonas* places reducing equivalents into partially oxidized metabolic intermediates. The following section reviews the main anaerobic pathways activated in many organisms, including prokaryotic bacteria, eukaryotic fungi, and animals, when they are exposed to hypoxic/anoxic conditions.

IN BACTERIA (Figure 1)

In the absence of O_2 and under conditions that favor catabolite repression (e.g., excess glucose), *Escherichia coli* does not utilize a complete TCA cycle. However, it can use enzymes of this cycle to synthesize succinyl-CoA and 2-oxoglutarate; these metabolites represent the reductive and oxidative branches of the TCA cycle, respectively (Wolfe, 2005). This branched form of the TCA cycle does not generate energy but instead provides the precursor metabolites needed for cell viability. Therefore, ATP must come from glycolysis and SLP is associated with the phosphotransacetylase-acetate kinase pathway (Brown et al., 1977).

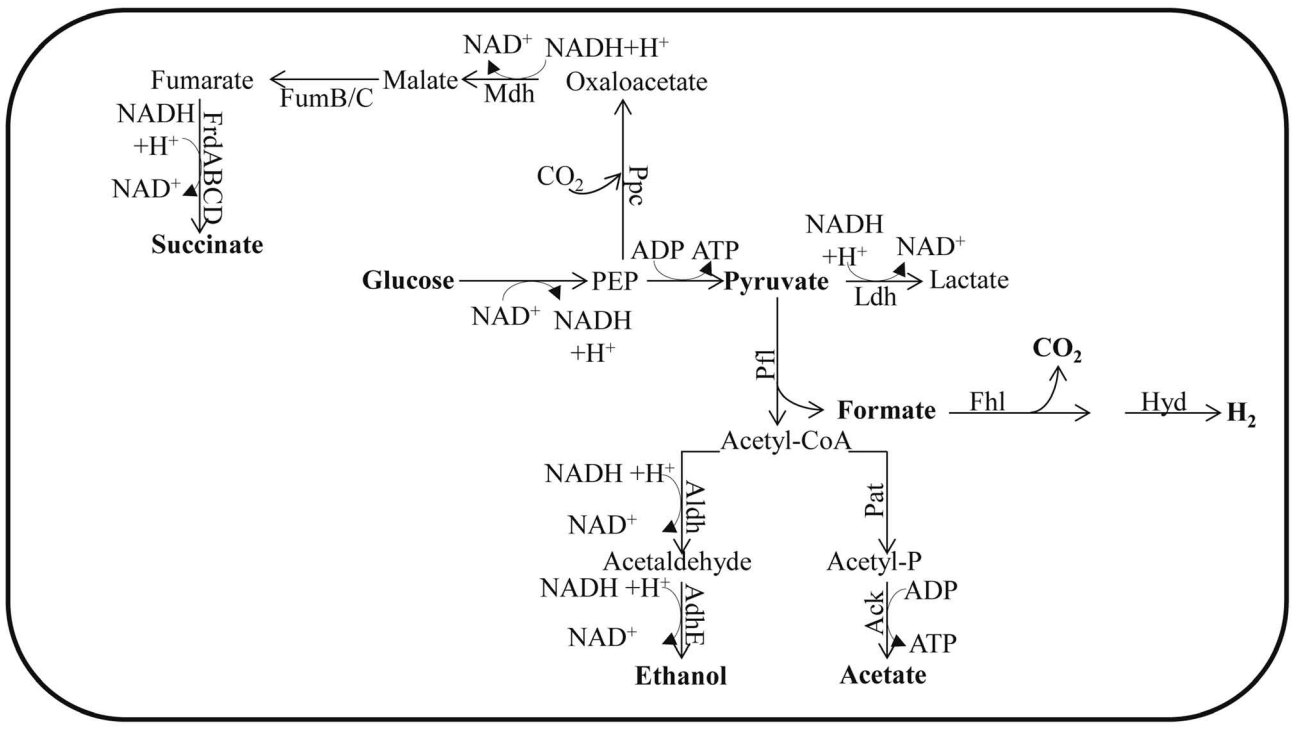

FIGURE 1 | Fermentation pathways of *E. coli*. The enzyme designations are: Ack for acetate kinase; AdhE for alcohol dehydrogenase; Aldh for aldehyde dehydrogenase; Fhl for formate hydrogen lyase; FrdABCD for fumarate reductase; FumB for fumarase B (anaerobic); FumC for fumarase C; Ldh for lactate dehydrogenase; Mdh for malate dehydrogenase; Pfl for pyruvate formate lyase; Ppc for phosphoenolpyruvate carboxylase and Pat for phosphotransacetylase. In *Chlamydomonas*, Aldh and AdhE activities are fused in the enzyme ADH1.

To sustain the flow of glycolytic metabolites when O_2 availability severely limits aerobic respiration, the cells must re-oxidize NADH. In many bacteria the sugars are fermented to a mixture of ethanol and organic acids. This is achieved by reducing partially oxidized metabolic intermediates and forming, predominantly, the metabolites D-lactate, succinate, and ethanol, which are excreted into the environment along with formate and acetate (Wolfe, 2005; **Figure 1**). During anaerobiosis, pyruvate is the major metabolite synthesized as a consequence of glycolysis. The pyruvate can be converted to formate and acetyl-coenzyme A (acetyl-CoA) by pyruvate formate lyase (Pfl; Wolfe, 2005; **Figure 1**). This conversion is a non-oxidative reaction, which contrasts with oxidative decarboxylation that is mediated by the pyruvate dehydrogenase complex (Pdh, also sometimes designated Pdhc), which functions during respiratory metabolism. Pfl and its activating enzyme are widespread in facultative and obligate anaerobic eubacteria, as well as in archaea (Sawers and Watson, 1998). Mutants of *E. coli* devoid of Pfl do not grow anaerobically on glucose, but can grow if the medium is supplemented with acetate (Varenne et al., 1975). Under such conditions, *pfl* mutants maintain glycolytic ATP synthesis by reducing pyruvate to lactate. The generation of an *ldh* mutant in the *pfl* strain eliminates the remaining fermentation pathway for sustaining glycolysis. The formate derived from the Pfl reaction may be further metabolized to H_2 and CO_2 through the activity of formate hydrogen lyase (Fhl; Gottschalk, 1985) while the acetyl-CoA generated in this reaction can be converted to acetate or reduced to ethanol. Full conversion of acetyl-CoA to ethanol would not allow for redox balance since a single NADH is generated for each pyruvate that is synthesized from sugars, and two NADH molecules are required to convert pyruvate to ethanol. In order to achieve redox balance, *E. coli* must also synthesize additional products from the pyruvate, such as acetate and/or succinate (Dien et al., 2003).

The type and amount of fermentation end products excreted by bacteria, and the level of NADH generated for recycling, are highly dependent upon the substrate being metabolized by the bacterium. For example, bacteria using sorbitol, a highly reduced carbon compound, produce three NADH molecules per molecule of substrate, while a highly oxidized carbon compound such as glucuronic acid generates no NADH. To regenerate NAD^+ from the NADH formed during the oxidation of sorbitol, bacteria synthesize and excrete ethanol (Wolfe, 2005). In contrast, cells growing on glucuronic acid are redox balanced and therefore no ethanol will be synthesized; instead, most pyruvate will be converted to acetate (Alam and Clark, 1989). The composition of excreted fermentation products also depends on the oxidation state of the cells and the pH of the medium. At neutral or higher pH, the main end products are acetate, ethanol, and formate, with moderate levels of succinate (Belaich and Belaich, 1976). However, as the pH becomes more acidic, cells produce lactate instead of acetate and formate (Bunch et al., 1997).

The conversion of acetyl-CoA to acetate is catalyzed by the phosphoacetyltransferase–acetate kinase (Pat–Ack also known as Pta-AckA) pathways. The Pat–Ack pathway generates one ATP per molecule of pyruvate metabolized, but consumes no NADH (**Figure 1**). In contrast, reduction of acetyl-CoA to ethanol is catalyzed by the bifunctional acetaldehyde/alcohol dehydrogenase (AdhE). While this reaction consumes reducing equivalents, it does not result in the generation of ATP (Wolfe, 2005). By coordinating the amount of ethanol and acetate (and other organic acids) synthesized and excreted into the medium, bacteria can efficiently balance their energy requirement with the need to recycle redox carriers (as reviewed by Wolfe, 2005).

There are two major acetate-producing pathways in *E. coli*; these are pyruvate oxidase (PoxB) and Pat–Ack (mentioned above). While PoxB decarboxylates pyruvate to acetate aerobically, the Pat–Ack complex is active under both aerobic and anaerobic conditions, converting acetyl-CoA to acetate (Hahm et al., 1994; Yang et al., 1999). The Pat–Ack reactions are sequential, reversible, and considered important for balancing the cellular carbon flux during exponential, aerobic and anaerobic growth (Chang et al., 1999; Avison et al., 2001). Pat converts acetyl-CoA and inorganic phosphate to acetyl phosphate (acetyl-P) and CoA, while Ack catalyzes the formation of ATP and acetate from acetyl-P and ADP (Rose et al., 1954). In *E. coli*, the *pat* and *ack* genes are organized in an operon (Kakuda et al., 1994). Mutants defective for Pat can neither synthesize acetate nor grow anaerobically (Gupta and Clark, 1989).

Under conditions in which anaerobically maintained *E. coli* cells are accumulating high levels of pyruvate or growing in a low pH medium, they can convert pyruvate to lactate through the activity of lactate dehydrogenase (Ldh; Clark, 1989; **Figure 1**). Alternatively, pyruvate or phosphoenolpyruvate (PEP) can be converted to a C4 intermediate of the TCA cycle by the catalytic addition of CO_2 (Clark, 1989; **Figure 1**). In some cases, malic enzymes can carboxylate pyruvate forming malate, while phosphoenolpyruvate carboxylase (Ppc) can catalyze the formation of oxaloacetate (OAA) from PEP and CO_2 (Clark, 1989; **Figure 1**). Both OAA and malate are then further reduced to succinate (Clark, 1989; **Figure 1**). This conversion is catalyzed by the sequential action of malate dehydrogenase (Mdh), fumarase (FumB and FumC), and fumarate reductase (FrdABCD; Clark, 1989; **Figure 1**); the gene encoding fumarase B is induced under anaerobic conditions (Woods et al., 1988). Since the amount of NADH generated varies with the nature of the substrate and the composition of the fermentation products generated, the redox balance and recycling of the NADH can be achieved by modulating the activities of the various fermentation pathways, which would result in a mix of end products, including ethanol, formate, acetate, and lactate (when necessary). Hence, *E. coli* mutants of *ldh* show no growth defects under anaerobic conditions because of compensatory pathways (Mat-Jan et al., 1989). Tarmy and Kaplan (1968) reported that fermentative Ldh is allosterically regulated and that its activity increases as the cellular pyruvate concentration increases; when pyruvate concentrations are low, the enzyme has very low activity. In contrast, *E. coli adhE* mutants do not synthesize alcohol dehydrogenase and cannot grow anaerobically on sorbitol, glucose, or gluconate since they cannot maintain redox balance, but they are able to ferment glucuronate (as reviewed by Clark, 1989).

IN ALGAE (Figure 2)

Fermentation of stored organic compounds by phototrophic microorganisms can represent a significant part of their overall

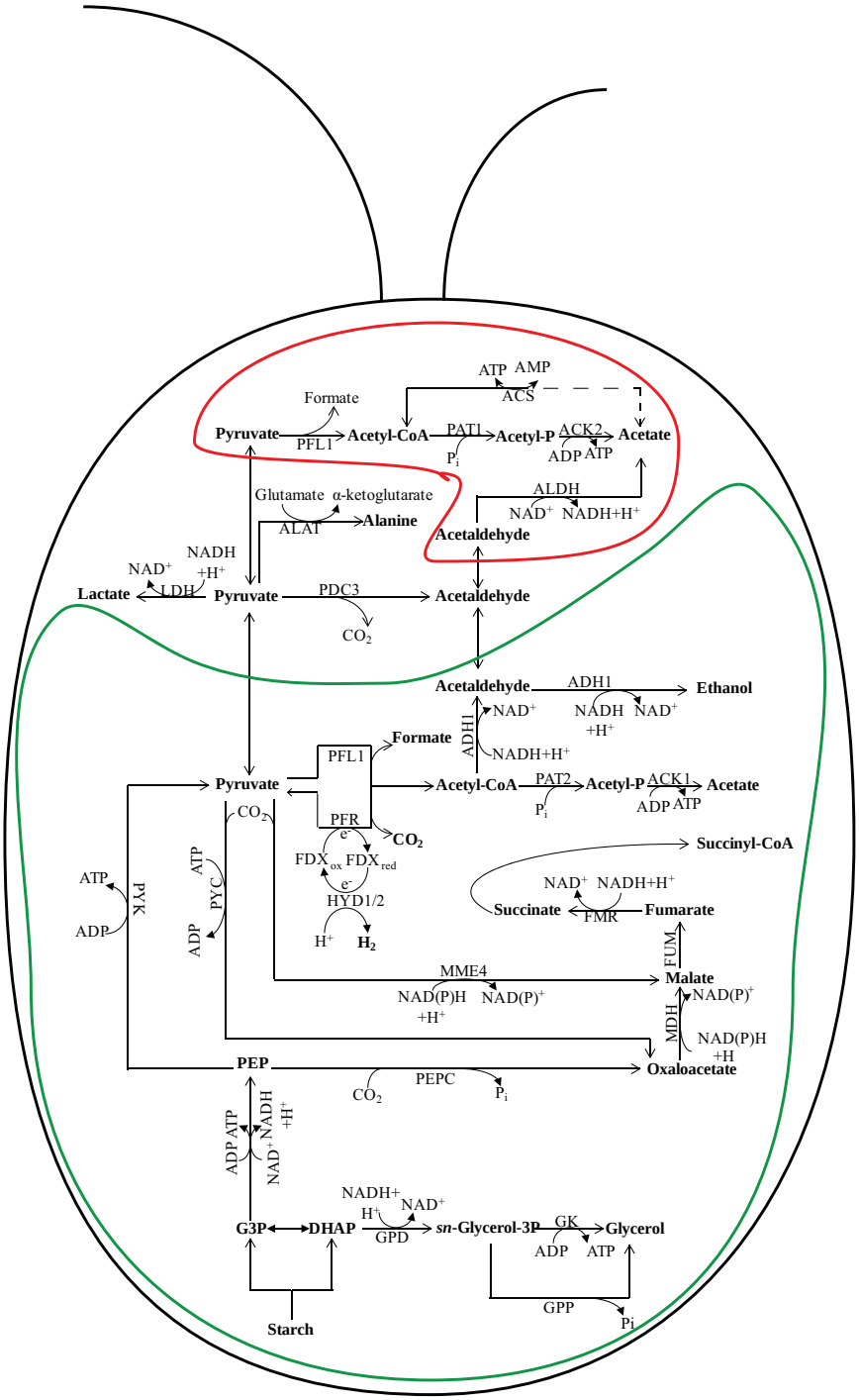

FIGURE 2 | Fermentation pathways in *Chlamydomonas*. In wild-type (WT) *Chlamydomonas* cells, the major fermentative products are formate, acetate, and ethanol, with CO_2 and H_2 generated as minor products. The pathway leading to fermentative succinate generation is not readily detected in WT cells grown under laboratory conditions, but becomes prominent in the *hydEF-1* mutant (Dubini et al., 2009). An increase in lactate production, which is almost undetectable in fermenting WT cells, is observed in the *pfl1* mutants (Philipps et al., 2011; Burgess et al., 2012; Catalanotti et al., 2012). Glycerol accumulation occurs in the *adh1* single and the *pfl1–1adh1* double mutants (Catalanotti et al., 2012; Magneschi et al., 2012). The protein designations in this figure are: FMR for fumarate reductase; MME4 for malic enzyme 4; PDC3 for pyruvate decarboxylase 3; PEPC for phosphoenolpyruvate carboxylase; PYC for pyruvate carboxylase; PYK for pyruvate kinase; PFR for pyruvate ferredoxin oxidoreductase; ACS for acetyl-CoA synthase; FDX for ferredoxin; HYDA1 and HYDA2 for the hydrogenases; GK for glycerol kinase; GPD for sn-glycerol-3 phosphate dehydrogenase and GPP for glycerol 3-phosphate phosphatase. See **Figure 1** for ACK1; ACK2; ADH1; ALDH; FUM; LDH; MDH; PAT1; and PAT2 designations. The enclosure delineated by a green line represents the chloroplast while the enclosure delineated by a red line represents the mitochondrion. The placement of some of the enzymes into specific subcellular compartments is speculative.

energy budget as many of these ecologically important organisms spend much of their lifecycle under light-limited, hypoxic/anoxic conditions. Several species of water-oxidizing, photosynthetic algae can metabolize endogenous polysaccharides or secondary metabolites when the environment becomes anoxic, enabling them to generate the ATP necessary to drive metabolic and energy-requiring processes (Gfeller and Gibbs, 1984, 1985; Kreuzberg, 1984; Gibbs et al., 1986; Ohta et al., 1987). During dark fermentation, cellular polysaccharide reserves are catabolized, generating the needed ATP, while the co-produced NADH must be re-oxidized. The primary fermentation pathways used during anoxia vary among different algal species (Ohta et al., 1987; Atteia et al., 2013). Green algae such as *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Chlorogonium elongatum*, and *Chlorella fusca* ferment starch to a variety of end products including acetate, ethanol, formate, glycerol, lactate, H_2, and CO_2 (Gaffron and Rubin, 1942; Ben-Amotz, 1975; Klein and Betz, 1978; Grossman et al., 2007; Mus et al., 2007). The heterofermentation patterns vary among green algal species (and sometimes among strains) and can also significantly vary with changes in environmental conditions, including the medium composition and carbon source. For *Chlamydomonas*, dark fermentation leads to the production of formate, acetate, and ethanol in a 2:1:1 ratio (Mus et al., 2007; **Figure 2**). In contrast, *Chlamydomonas moewusii* cells do not excrete formate during exposure to dark anoxic conditions; the major end products synthesized by this organism are acetate, glycerol, and ethanol (Klein and Betz, 1978; Meuser et al., 2009).

Some algae do not excrete fermentation products, but instead store them (reviewed by Müller et al., 2012; Atteia et al., 2013). *Euglena gracilis* synthesizes ATP when maintained under anoxic conditions with the concomitant accumulation of up to 60% fatty acids by dry weight (Inui et al., 1982). When the cells are returned to oxic conditions, the stored fatty acids can be converted back to acetyl-CoA, which can then be oxidized to CO_2 or used to form paramylon reserves (Inui et al., 1982).

Diatoms and dinoflagellates are present in anoxic marine sediments (Jewson et al., 2006). The diatoms that inhabit these sediments accumulate high concentrations of nitrate (Lomstein et al., 1990), which is used as an electron acceptor in respiratory metabolism (e.g., generating ammonium) allowing these organisms to survive under dark anoxic condition (Kamp et al., 2011).

Enzymes of fermentation in Chlamydomonas (Figure 2)

Currently, most information on fermentation metabolism in algae comes from studies of *Chlamydomonas* (Gfeller and Gibbs, 1984, 1985; Kreuzberg, 1984; Gibbs et al., 1986; Ohta et al., 1987; Hemschemeier and Happe, 2005; Grossman et al., 2007, 2011; Mus et al., 2007; Hemschemeier et al., 2008; Dubini et al., 2009; Philipps et al., 2011; Burgess et al., 2012; Catalanotti et al., 2012; Magneschi et al., 2012). Genes encoding proteins associated with a diverse set of fermentative pathways have been identified on the *Chlamydomonas* genome, while a number of biochemical studies have revealed various fermentation circuits. The flexibility among the different pathways for catabolism of stored carbon under dark, anoxic conditions has been demonstrated through analyses of various mutants perturbed for these pathways (Mus et al., 2007; Dubini et al., 2009; Philipps et al., 2011; Burgess et al., 2012; Catalanotti et al., 2012; Magneschi et al., 2012). This flexibility allows *Chlamydomonas* to satisfy its energy requirements as O_2 from the surrounding environment is depleted.

Over the course of the day there is a natural cycle for storage and utilization of fixed carbon. In phototrophic organisms, polysaccharides (sometimes lipids) accumulate in cells during daylight hours when photosynthetic CO_2 fixation is a dominant metabolic process. During the evening, much of the starch reserve can be hydrolyzed to sugars by amylase activity (Ball, 1998; Dauvillee et al., 2001a,b; Zabawinski et al., 2001) and then, through the activity of glycolysis, be converted to pyruvate (**Figure 2**). As in bacteria, pyruvate fuels fermentation processes, serving as substrate for pathways that generate various organic acids, acetyl-CoA, alcohols, CO_2, and H_2. *Chlamydomonas* has multiple pathways for converting pyruvate to acetyl-CoA (Hemschemeier and Happe, 2005; Atteia et al., 2006; Grossman et al., 2007; see **Figure 2** for details). Three enzymes involved in these pathways are pyruvate formate lyase (PFL1), pyruvate ferredoxin oxidoreductase (PFR, often referred to as PFOR), and the pyruvate dehydrogenase (PDH) complex. As PDH generates NADH, a product that must be re-oxidized to sustain fermentation metabolism, it is presumed that PFL1 and PFR are the favored pathways for pyruvate catabolism in hypoxic/anoxic cells (**Figure 2**). While PFL1 catalyzes the conversion of pyruvate to acetyl-CoA and formate, in the PFR reaction pyruvate is oxidized to acetyl-CoA and CO_2 with the concomitant generation of reduced ferredoxin (FDX). FDX can then pass reducing equivalents to hydrogenases to generate H_2 (Happe and Naber, 1993; Ghirardi et al., 1997, 2000, 2007; Melis et al., 2000; Melis and Happe, 2001; Müller, 2003). However, the reduced FDX can also serve as a substrate for nitrite and sulfate/sulfite reductases (Ghirardi et al., 2008).

The acetyl-CoA produced by PFL1 and PFR reactions is either reduced to ethanol by alcohol/aldehyde dehydrogenase 1 (ADH1; Hemschemeier and Happe, 2005; Atteia et al., 2006; Dubini et al., 2009), or metabolized to acetate by the PAT–ACK (Atteia et al., 2006). An alternative pathway for ethanol production may be direct decarboxylation of pyruvate to CO_2 and acetaldehyde through the activity of pyruvate decarboxylase (PDC3). The acetaldehyde generated in this reaction can be reduced to ethanol by ADH (either the same enzyme that catalyzes acetyl-CoA reduction or a distinct enzyme, e.g., ADH2). While the conversion of acetyl-CoA to ethanol by ADH1 oxidizes two NADH molecules, only a single NADH is oxidized in the PDC pathway.

Mutants in specific branches of fermentative metabolism have proven extremely valuable for elucidating the various routes of fermentation metabolism in *Chlamydomonas*, which are shown in **Figure 2**.

Formate production

Formate was demonstrated to be the dominant, secreted organic acid synthesized by *Chlamydomonas* maintained in anoxic conditions at near neutral pH in dark (Kreuzberg, 1984; Gibbs et al., 1986). The synthesis of formate by PFL1 uses a free-radical mechanism to catalyze the homolytic cleavage of pyruvate into

formate and acetyl-CoA. This reaction depends upon a radical *S*-adenosyl methionine-dependent activating enzyme, designated PFL-AE (Atteia et al., 2006; Hemschemeier et al., 2008), which is usually present as an inactive form in aerobic cells, and is allosterically activated by pyruvate. In *Chlamydomonas*, PFL1 appears to be located in both mitochondria and chloroplasts (Kreuzberg et al., 1987; Atteia et al., 2006).

Algal strains deficient for PFL1 activity were isolated by independent groups (Philipps et al., 2011; Catalanotti et al., 2012) using different strategies (Burgess et al., 2012). The elimination of PFL1 activity in *Chlamydomonas* led to a marked accumulation of extracellular lactate, elevated pyruvate decarboxylation, and extracellular ethanol accumulation (**Figure 2**). The accumulation of lactate in the medium of *pfl1* mutants allows for recycling of NADH as a consequence of pyruvate reduction by LDH. Catalanotti et al. (2012) also demonstrated that the *pfl1* mutant accumulates elevated intracellular levels of lactate and alanine. Additionally increased intracellular levels of succinate, malate, and fumarate were observed, suggesting operation of the left branch of the reverse TCA reactions to recycle NADH.

Ethanol production

Acetyl-CoA produced by PFR/PFL1 activities can be metabolized to generate ATP by conversion to acetate or to help maintain redox balance by conversion to ethanol (Mus et al., 2007). *Chlamydomonas* possesses three distinct enzymes potentially important for ethanol production when the cells become anoxic: ADH1 (putative dual-function alcohol/acetaldehyde dehydrogenase; Mus et al., 2007; Hemschemeier et al., 2008; Magneschi et al., 2012), and two other putative alcohol dehydrogenases that were identified based on protein homology, designated ADH2 (Augustus version 5.0 protein identifier 516421) and ADH3 (Augustus version 5.0 protein identifier 516422). ADH1 has been localized to chloroplasts (Terashima et al., 2010).

A *Chlamydomonas* mutant devoid of ADH1 was unable to synthesis either ethanol or CO_2 when the cells were transferred to anoxic conditions (Magneschi et al., 2012). The inability of the *adh1* mutant to accumulate ethanol and CO_2, while synthesizing low levels of formate, suggests that the acetaldehyde synthesized by PDC3 and the acetyl-CoA synthesized by PFL1 and PFR cannot be rapidly reduced in the mutant. These findings also indicate that ADH1 is the only acetaldehyde-alcohol dehydrogenase in *Chlamydomonas* capable of reducing acetyl-CoA or acetaldehyde to ethanol under the conditions used in this study. Interestingly, the *adh1* strain was able to compensate for its inability to reduce acetyl-CoA or acetaldehyde to ethanol by reducing a significant amount of pyruvate to lactate. This elevated lactate accumulation was not as high as the level measured in *pfl1* (Philipps et al., 2011; Burgess et al., 2012; Catalanotti et al., 2012). However, the *adh1* mutants also accumulated high extracellular and intracellular levels of glycerol relative to anoxic wild-type (WT) cells. This acclimation response removes a significant amount of the C3 metabolites at the dihydroxyacetone phosphate (DHAP) step of the glycolytic pathway, which is prior to the reduction of NAD^+ to NADH; the DHAP is then used as a substrate to re-oxidize NADH in the synthesis of glycerol (**Figure 2**).

Acetate production

The acetyl-CoA that is produced by PFL1 or PFR activities can be converted to acetate by PAT and ACK (Mus et al., 2007). Two parallel pathways have been identified in *Chlamydomonas*; PAT1–ACK2 appear to be mitochondrial while PAT2–ACK1 are in the chloroplast (Atteia et al., 2006, 2009; Terashima et al., 2011; **Figure 2**). Interestingly, the *PAT2* and *ACK1* genes are contiguous on the genome while *PAT1* and *ACK2* are far apart on the same chromosome (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html).

While PAT–ACK activities comprise the predominant pathways for acetate formation under dark anaerobiosis, other enzymes are present on the *Chlamydomonas* genome that may play a role in acetate synthesis. Four genes encoding homologs of acetyl-CoA synthase (ACS) and eight genes encoding homologs of aldehyde dehydrogenase (ALDH) have been identified on the *Chlamydomonas* genome (Kirch et al., 2004, 2005; Brocker et al., 2012). The ACSs catalyze the putatively reversible conversion of acetate to acetyl-CoA (dash line in **Figure 2**). The ALDH reaction produces NAD(P)H during the conversion of acetaldehyde to acetate, therefore it is unlikely that these enzymes are active in fermentative metabolism when the cells require regeneration of reducing power (Kirch et al., 2004, 2005; Brocker et al., 2012). To date, there is no biochemical evidence to demonstrate that these alternative pathways for acetate generation are active in *Chlamydomonas*. In bacteria, the two pathways active under aerobic conditions that generate acetate are the Pat–Ack pathway, which is active in exponentially growing cells, and the PoxB pathway, which dominates during late exponential and stationary phase (Dittrich et al., 2005). It is uncertain whether or not similar regulatory features occur in *Chlamydomonas*.

The presence and/or production of acetate as *Chlamydomonas* cells become anoxic was found to be critical for maintenance of anoxic conditions in the light since acetate assimilation promotes O_2 utilization (Kosourov et al., 2007; Morsy, 2011). The level of acetate accumulation during fermentative metabolism has proven to be difficult to predict, probably because it can also be used for the biosynthesis of key metabolites in anoxic cells, provided sufficient ATP and NAD(P)H is available. The *adh1* mutant exhibits a higher ratio of acetate production under anoxic conditions compared to WT cells, which reflects the elimination of ethanol production from the acetyl-CoA that is generated by PFL1 and PFR activities in the mutant strain; glycerol and lactate production serve as the primary NADH re-oxidation mechanisms in this mutant (Magneschi et al., 2012). In contrast, the *pfl1* mutant strains exhibit strongly reduced acetate accumulation (Burgess et al., 2012; Catalanotti et al., 2012); this decrease is likely due to a diminished intracellular acetyl-CoA pool.

H_2 production

The mitochondria of cells maintained in aerobic conditions use PDH to convert pyruvate to acetyl-CoA; the acetyl-CoA generated can be metabolized to CO_2 by the TCA cycle. In some animals PDH can function under anaerobic conditions (reviewed by Tielens and van Hellemond, 1998; Tielens et al., 2002; Hoffmeister et al., 2005; Tucci et al., 2010; Atteia et al., 2013). However, in many prokaryotes and eukaryotes, pyruvate oxidation in the absence of

O_2 is typically mediated by PFR. PFR belongs to a large family of thiamine pyrophosphate (TPP)-dependent enzymes. It catalyzes the oxidative cleavage of the carbon–carbon bond of the carboxyl group of pyruvate to liberate CO_2 and reducing equivalents, with the attachment of the resulting acetyl group to CoA. However, unlike PDH, PFR can also function in the reverse direction catalyzing the production of pyruvate from CO_2 and acetyl-CoA (Evans et al., 1966; see below), with FDX or flavodoxin serving as electron donors (Charon et al., 1999; Ragsdale, 2003; **Figure 2**). In *Chlamydomonas*, the reduced FDX generated from pyruvate oxidation by PFR activity can be re-oxidized by hydrogenases, generating H_2 (Müller, 2003), or by reactions that enzymatically reduce nitrite and sulfate/sulfite. Hydrogenases are widespread among prokaryotes, whereas they are not as common among eukaryotes, and are restricted to a subset of unicellular eukaryotes, including photosynthetic algae (Meuser et al., 2011; Müller et al., 2012). *Chlamydomonas* hydrogenases belong to the class of [FeFe]-hydrogenases in which a [4Fe4S] cluster is linked through a cysteine residue to a 2Fe− cluster (Peters et al., 1998; Mulder et al., 2011).

Hydrogen production in algae is likely to have significant impacts on redox poising, photoprotection, and fermentative energy metabolism. Hydrogen production is coupled to cellular metabolism in a variety of ways, all of which are associated with O_2 limitation: (i) direct biophotolysis, (ii) indirect biophotolysis, and (iii) dark fermentative metabolism (**Figure 3**). Direct biophotolysis involves light-dependent oxidation of water by photosystem II (PSII), the transfer of electrons from PSII to photosystem I (PSI), light-dependent excitation of PSI with the concomitant reduction of FDX and the subsequent transfer of electrons from FDX to hydrogenase (Benemann et al., 1973; Greenbaum, 1982; Happe and Naber, 1993; Miura, 1995; Ghirardi et al., 2007). During direct biophotolysis, the O_2 generated by PSII must be reduced in order to prevent the accumulation of O_2 to levels that would inhibit the hydrogenase. Indirect biophotolysis involves non-photochemical reduction of the PQ pool by NAD(P)H generated as a consequence of catabolic metabolism, followed by light-dependent FDX reduction by PSI and the subsequent transfer of electrons from FDX to hydrogenase (Cournac et al., 2000; Kosourov et al., 2003; Mus et al., 2005; Chochois et al., 2009). In the third H_2-production pathway, starch catabolism provides electrons to the hydrogenases under dark fermentative conditions (Gfeller and Gibbs, 1984; Kreuzberg, 1984; Ohta et al., 1987; Happe et al., 1994; Ghirardi et al., 1997; Melis and Happe, 2001; Posewitz et al., 2004; Mus et al., 2007; Dubini et al., 2009).

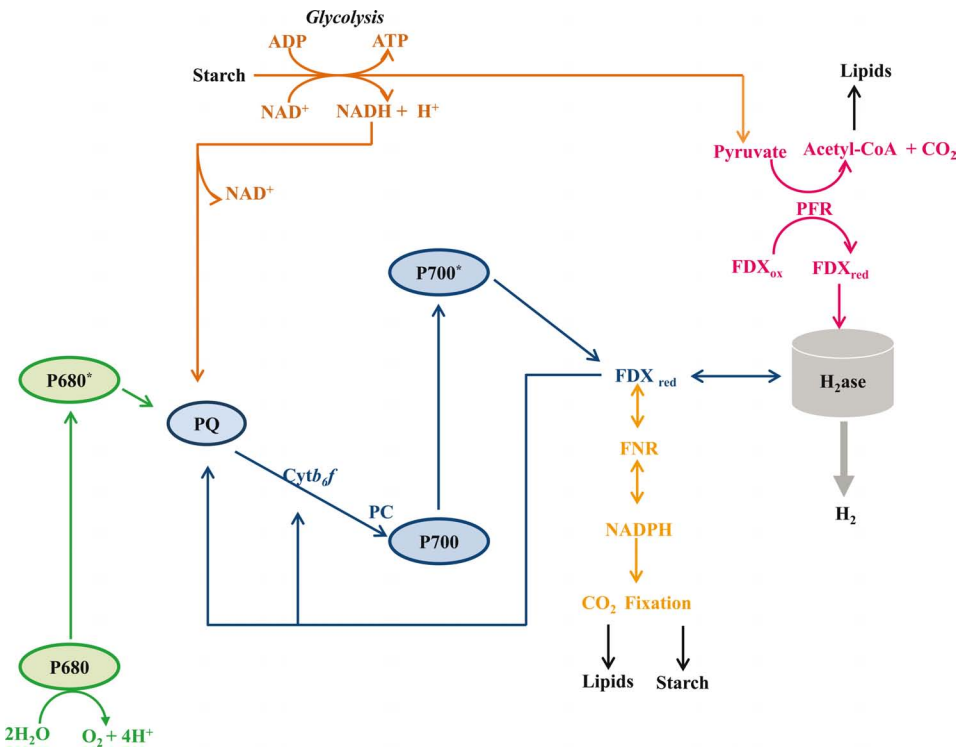

FIGURE 3 | Metabolic pathways associated with hydrogenase activity. Five distinct metabolisms are depicted: (1) H_2 production dependent on the complete photosynthetic electron transport system (PSII, PSI, FDX, H_2ase; green and blue lines); (2) H_2 production requiring starch catabolism and PSI activity (starch, glycolysis, PQ, PSI, FDX, H_2ase; brown and blue lines); (3) H_2 production in the dark from pyruvate oxidation (starch, glycolysis, pyruvate, FDX, H_2ase; brown and magenta lines); (4) H_2 oxidation coupled to CO_2 reduction, with respiratory O_2 uptake used to generate ATP (H_2, H_2ase, FDX, FNR, NAD(P)H, CO_2 fixation; orange line); (5) H_2 oxidation coupled to PSI-driven cyclic electron flow and ATP production (H_2, H_2ase, FDX, PSI, FDX and continued cycling; blue line). Abbreviations are: Cytb_6f, cytochrome b_6f complex; FDX, ferredoxin; FNR, ferredoxin NAD(P)$^+$ reductase; H_2ase, hydrogenase enzyme; PC, plastocyanin; PFR, pyruvate ferredoxin oxidoreductase; PQ, plastoquinone pool; P700, reaction center of photosystem I; P680, reaction center of photosystem II. For simplicity we have not tried to scale the energy potential of the electron carriers downstream of FDX.

Hydrogenases also function in H_2 uptake, with two distinct uptake pathways described in *Chlamydomonas* (Gaffron, 1944; Kessler, 1974; Maione and Gibbs, 1986a,b; Chen and Gibbs, 1992a; **Figure 3**). In the first pathway, H_2 oxidation and cyclic PSI activity in the light are linked to RuBisCO-mediated anaerobic CO_2 fixation. Electrons from H_2 are used to reduce FDX, which then reduces FDX-NAD(P) oxidoreductase (FNR), leading to the generation of NAD(P)H which, along with the ATP generated by cyclic electron flow, can be used to fix CO_2. This pathway requires the absence of O_2 evolution from PSII. In the second pathway, termed the oxyhydrogen reaction, H_2 oxidation occurs concomitantly with the uptake of low levels of O_2 in a process that can be coupled to CO_2 fixation (Gaffron and Rubin, 1942; Gaffron, 1944; Russell and Gibbs, 1968; Kessler, 1974; Chen and Gibbs, 1992a). Although not well characterized, it is posited that H_2 oxidation provides the reducing equivalents for CO_2 fixation and that the low levels of O_2 present are respired to provide ATP (Gaffron and Rubin, 1942; Gaffron, 1944; Maione and Gibbs, 1986a; Chen and Gibbs, 1992a).

Recently, mutants were obtained in each of the two *HYDA* genes of *Chlamydomonas*, *HYDA1* and *HYDA2* (Meuser et al., 2012). The phenotypes of the single (*hydA1* and *hydA2*) and double (*hydA1–hydA2*) mutants were analyzed under both light and dark anoxic conditions. Both single mutants could catalyze H_2 production from reductant generated from either fermentative or photosynthetic metabolism. However, the contribution of the HYDA2 enzyme to H_2 photoproduction under the conditions tested was approximately 25% of that of HYDA1 (Godman et al., 2010; Meuser et al., 2012).

The impact of the *hydEF-1* lesion on fermentation is more interesting since it demonstrates the flexibility of *Chlamydomonas* anaerobic metabolism (see below). This mutant is unable to assemble the inorganic constituents of the hydrogenase active site, and consequently cannot catalyze H_2 synthesis (Posewitz et al., 2004).

Succinate production

Anoxic cultures of the *Chlamydomonas hydEF-1* mutant exhibit lower CO_2 evolution and reduced extracellular formate, acetate, and ethanol accumulation. Interestingly, the mutant synthesizes elevated levels of extracellular succinate (Dubini et al., 2009; **Figure 2**), indicating activation of a fermentative pathway that is not operating at significant levels in WT cells. Microarray data and metabolite analyses suggest that carboxylation of pyruvate in the *hydEF-1* mutant leads to the synthesis of either malate or OAA (or both), which is subsequently converted to succinate via reverse reactions of the TCA cycle. Activation of the reductive TCA branch as a means of recycling NADH was previously observed in anaerobic bacteria (Gray and Gest, 1965; Schauder et al., 1987; Buchanan and Arnon, 1990; Beh et al., 1993; Yoon et al., 1999), in the green alga *Selenastrum minutum* (Vanlerberghe et al., 1989, 1990) and in vascular plants (Sweetlove et al., 2010), but was not known to occur in *Chlamydomonas*.

The alternative pathway suggested by Dubini et al. (2009) not only explains succinate accumulation under anaerobic conditions, but also raises the possibility that *Chlamydomonas* could potentially operate a complete reverse TCA cycle. This would require that PFR functions in the direction of pyruvate synthesis under the appropriate metabolic conditions. Various researchers have suggested that PFR could function in the synthesis of pyruvate in *Chlamydomonas* (Chen and Gibbs, 1992b; Melis et al., 2007; Terashima et al., 2011; **Figure 2**). Chen and Gibbs (1992b) detected ATP-citrate lyase, as well as PFR and α-ketoglutarate synthase activities in *Chlamydomonas* cell extracts, speculating that the existence of these three key enzyme activities indicated that the reverse TCA cycle could operate in *Chlamydomonas*. These authors showed that a *Chlamydomonas* mutant with a compromised Calvin–Benson cycle takes up CO_2 in the dark under minimal aerobic conditions (1% O_2), and that the CO_2 uptake is coupled to H_2 oxidation (Chen and Gibbs, 1992a), suggesting that the reverse TCA cycle could be a significant pathway for CO_2 assimilation when the Calvin–Benson cycle is compromised (Chen and Gibbs, 1992b). Hence, under these conditions H_2 oxidation would provide the reducing equivalents to drive the reverse TCA cycle and to allow PFR to synthesize pyruvate, leading to the accumulation of an array of biosynthetic precursors. The possibility of PFR-dependent synthesis of pyruvate has also been observed in many hydrogenosome-containing eukaryotic organisms experiencing anaerobic conditions (Lindmark and Müller, 1973). Furthermore, in the unicellular microaerophilic eukaryote *Trichomonas vaginalis*, MME and PFR are central to carbohydrate metabolism in the hydrogenosomes (Müller, 1993). In addition, PFR and MME activities have been linked to malate production in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, also suggesting reductive carboxylic acid cycle activity (Fukuda et al., 2005). The association of PFR and MME with pyruvate metabolism in hydrogenosome-containing anaerobic eukaryotes, the findings that a similar set of anoxic-induced proteins are associated with *Chlamydomonas* chloroplasts, and the metabolite data obtained with various *Chlamydomonas* strains exposed to anoxic, reductant-rich conditions, all suggest that the TCA cycle may operate in the reverse direction in *Chlamydomonas* chloroplasts in anoxic cells that have sufficient reducing equivalents and ATP.

Lactate and glycerol production

Glycerol and lactate are usually minor end products of green algal fermentation (Gfeller and Gibbs, 1984; Kreuzberg, 1984). Glycerol is synthesized from DHAP, and its synthesis results in recycling of one NADH. The reaction precedes the formation of pyruvate and the C3 oxidation (NADH formation) step in glycolysis. Hence, glycerol and lactate production in the *adh1* mutant would allow for efficient recycling of NADH, maintenance of redox balance and sustained glycolytic production of ATP even though the cells are unable to reduce acetaldehyde or acetyl-CoA to ethanol (Magneschi et al., 2012; **Figure 2**). The *pfl1–1adh1* double mutant cannot synthesize either formate or ethanol (Catalanotti et al., 2012; **Figure 2**). This strain, like *pfl1*, secretes significant levels of lactate, however, like the *adh1* mutant, it also synthesizes and secretes high levels of glycerol and acetate. Hence, this mutant exhibits a complete rerouting of glycolytic carbon to lactate and glycerol, transforming *Chlamydomonas* cells from a formate/acetate/ethanol to a glycerol/lactate fermenter (Catalanotti et al., 2012; **Figure 2**).

IN OTHER EUKARYOTES

Eukaryotes specialized to thrive under aerobic conditions generally possess simple cytosolic fermentation pathways that enable them to tolerate short-term anoxia; these pathways facilitate accumulation of end products such as lactate, ethanol, and glycerol (reviewed by Müller et al., 2012). Some eukaryotes, including many algae, experience frequent exposure to anoxic conditions, where they are unable to use O_2 as a terminal electron acceptor. These organisms have evolved a modest set of energy-generating pathways, which are reviewed below.

Ethanol, lactate, and glycerol fermentation (Figures 4 and 5)

When O_2 in the environment is depleted, plants can use PDC to convert pyruvate to acetaldehyde, which is metabolized to ethanol by ADH (Gibbs and Greenway, 2003; Bailey-Serres and Voesenek, 2008). The ethanol generated in plant roots can rapidly diffuse into the rhizosphere, which limits its toxicity. Plants can also synthesize lactate under conditions of low O_2. The transition from lactic to ethanolic fermentation appears to be controlled by the pH of the cytoplasm of the cell. A ∼0.6 unit decrease in cytosolic pH favors PDC activity, which promotes ethanol production and limits lactate synthesis (reviewed by Bailey-Serres and Voesenek, 2008). This lactic to ethanolic switch is critical for maintaining cytosolic pH (Roberts et al., 1989). In addition to eliciting metabolic changes, low O_2 can trigger alterations in plant morphology which include petiole or internode elongation, altered anatomy and cell ultrastructure in leaves and roots, development of lateral or adventitious roots and the formation of aerenchyma cells (Bailey-Serres et al., 2012).

Ethanol, lactate, and glycerol are common end products of fermentative metabolism in many organisms. The synthesis and excretion of ethanol allows carp to survive anaerobiosis for up to ∼5 months (van Warde et al., 1993) and goldfish to withstand anoxia for several weeks (van den Thillart et al., 1983). It is notable

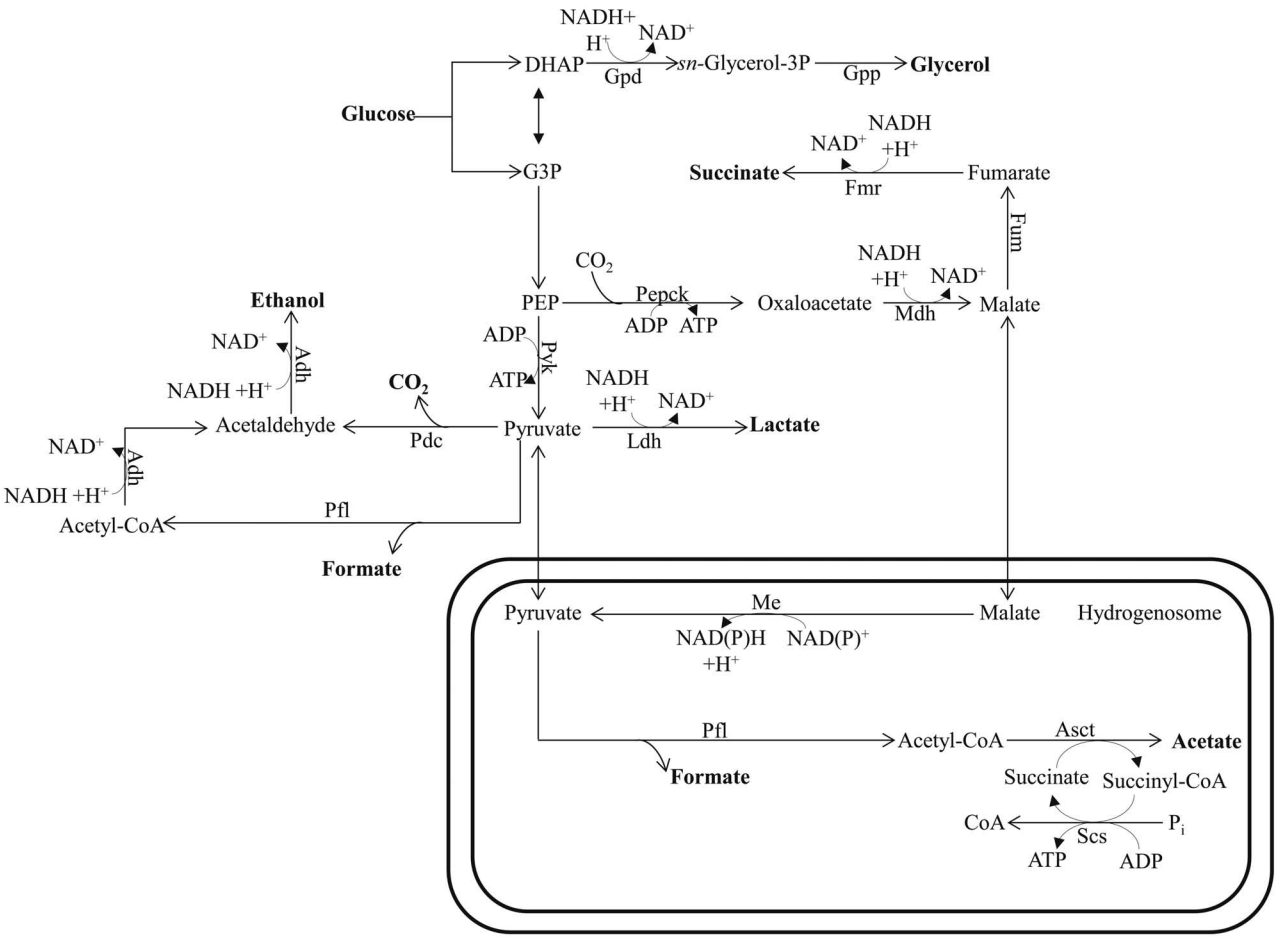

FIGURE 4 | Mixed-acid fermentative metabolism of the hydrogenosome-bearing anaerobic chytridiomycete fungus *Piromyces*. The circuitry is drawn based on data reported by Wang et al. (2001) and Boxma et al. (2004). This fungus uses pyruvate formate lyase for pyruvate catabolism in their hydrogenosomes. Glucose can also be metabolized in the cytosol to the end products succinate, lactate, formate, and ethanol. Bifunctional alcohol dehydrogenase (Adh), having both alcohol dehydrogenase and acetaldehyde dehydrogenase activities, mediates the cytosolic formation of ethanol. The protein designations in this figure are: Asct for acetate succinyl-CoA-transferase; Me for malic enzyme; Pepck for phosphoenolpyruvate carboxykinase; Scs for succinyl-CoA synthase; See **Figure 1** for Adh; Fum; Ldh; Mdh; and Pfl designations and **Figure 2** for Fmr; Gpd; Gpp; and Pdc designations. DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; PEP, phosphoenolpyruvate.

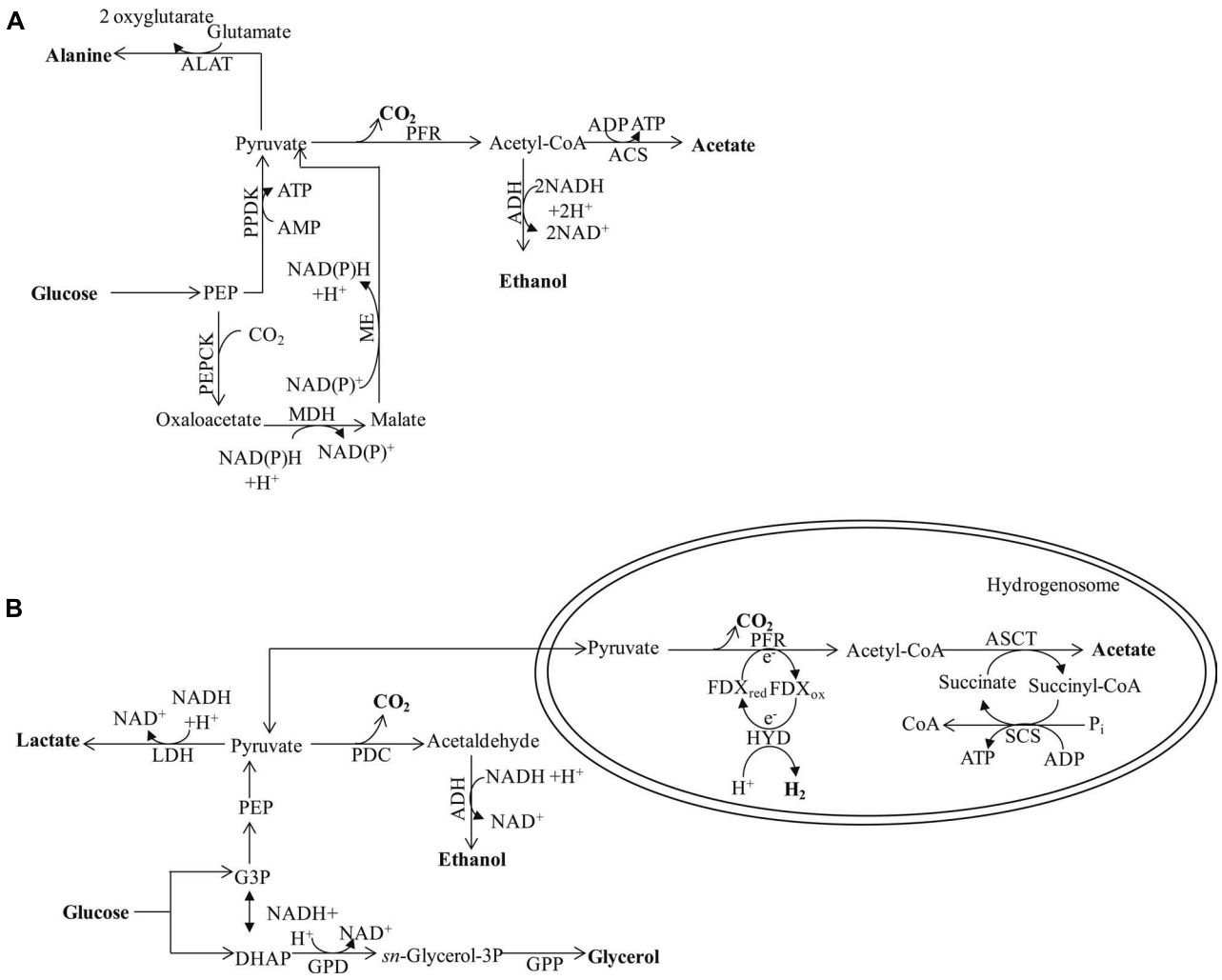

FIGURE 5 | (A) Major metabolic pathways in the anaerobic intestinal parasite *Entamoeba histolytica*. The map is adapted from Müller et al. (2012). The energy metabolic pathways are localized in the cytosol. Pyruvate ferredoxin oxidoreductase is used for pyruvate decarboxylation/oxidation, ATP is synthesized through SLP via acetyl-CoA synthase (ADP forming). **(B)** Major pathways of anaerobic, molecular H_2-producing, fermentative metabolism in *Trichomonas vaginalis*. Hydrogenosomal pyruvate breakdown involves PFR and functional [FeFe]-hydrogenase (HYD) in *Trichomonas*. Additional major end products of cytosolic fermentation in *Tvaginalis vaginalis* include alanine, lactate, ethanol, and glycerol. The protein designations in this figure are: ALAT for alanine aminotransferase and PPDK for pyruvate:orthophosphate dikinase. See **Figures 1–3** for the other protein designations.

that the fermentation pathways used for these reactions appear to have their origins in a typical yeast-type PDC and ADH (van Warde et al., 1993; **Figure 4**). The fungi, a highly diverse group, can also ferment carbohydrates to lactate, glycerol, and ethanol. Glycerol acts as a redox valve under anaerobic conditions since it enables re-oxidation of NADH that is generated during the conversion of sugars into biomass. While fungi may also excrete organic acids, the levels are generally low; these acids include formate, acetate, lactate, and succinate (**Figure 4**). Formate production is not uncommon in fungi as a result of the activity of a cytosolic (and hydrogenosomal) PFL, which provides the acetyl-CoA for ethanol production (Boxma et al., 2004; **Figure 4**).

Finally, pathogenic amoebozoa such as *Entamoeba histolytica* often experience anaerobic conditions; their main end products of anaerobic energy metabolism are alanine, CO_2, ethanol, and acetate (Müller et al., 2012). The enzymes responsible for generating these products are exclusively in the cytosol (Müller, 2003). The initial reactions of the pathway involve conversion of PEP to pyruvate by pyruvate orthophosphate dikinase (PPDK; Reeves, 1968; **Figure 5A**), which also generates ATP. The pyruvate is then oxidized via PFR to CO_2 and acetyl-CoA, with the latter converted into a mixture of acetate and ethanol (**Figure 5A**). Alternatively the PEP can be carboxylated to OAA by PEP carboxytransferase, reduced to malate by malate dehydrogenase (MDH) and the malate then converted to pyruvate by the malic enzyme (ME; **Figure 5A**; as reviewed by Müller et al., 2012). *Entamoeba* possesses a bifunctional aldehyde/alcohol dehydrogenase (ADH), which represents a fusion protein that contains an N-terminal aldehyde dehydrogenase domain and a C-terminal alcohol dehydrogenase domain. This enzyme regenerates 2 NAD^+, is present

in many eukaryotes, and has been found to be highly expressed in *Chlamydomonas* (Mus et al., 2007; Catalanotti et al., 2012; Magneschi et al., 2012), other green algae and protozoan parasites such as *Giardia intestinalis*, *Trichomonas*, and euglenids.

Trichomonas vaginalis synthesizes ethanol from pyruvate in the cytosol via PDC and ADH. However, the main end products of *T. vaginalis* fermentative metabolism are distributed between the cytosol (glycerol, lactate, and ethanol) and the hydrogenosome (CO_2, H_2, and acetate; **Figure 5B**). Similarly, *Chlamydomonas* can synthesize glycerol from DHAP, which is catalyzed by glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, and LDH can catalyze lactate accumulation. However, while both reactions in *T. vaginalis* occur in the cytosol, their locations in *Chlamydomonas* are not clear.

Malate dismutation and acetate and propionate production (Figure 6)

Fermentation in animals often involves malate dismutation. It is not uncommon for parasitic worms to switch to complete anaerobic metabolism once they are established in the host tissue. In parasitic mode they convert the PEP generated by glycolysis to OAA, which is then reduced to malate via a cytosolic malate dehydrogenase (**Figure 6**). This reaction results in re-oxidation of one molecule of NADH. The malate is then imported into the mitochondrion where dismutation occurs; a portion of the malate is oxidized to acetate (via pyruvate), and another is reduced to succinate. In the latter reaction, malate is converted to fumarate by FUM and the fumarate is reduced to succinate (**Figure 6**); this pathway is similar to the alternative fermentation pathway activated in the *Chlamydomonas hydEF-1* mutant. Many organisms excrete succinate produced by malate dismutation rather than decarboxylating the succinate to generate propionate plus an extra molecule of ATP (Pietrzak and Saz, 1981; Müller et al., 2012; **Figure 6**). Interestingly, in the parasite system, fumarate reduction is performed by a membrane-associated, anaerobiosis-specific enzyme (FRD) that is coupled to an electron transport chain that functions specifically under anaerobic conditions. Electrons are transferred from NADH to fumarate via rhodoquinone (RQ; **Figure 6**) instead of ubiquinone (UQ, which is normally used under oxic conditions); the lower redox potential of RQ (relative to UQ) allows for the thermodynamically favorable use of electrons in the synthesis of succinate by FRD (as reviewed by Müller et al., 2012).

Intracellular metabolite accumulation

O_2 deficiency is often associated with a wide range of excreted metabolites, but may also trigger more complicated responses involving sequestration of specific end products. Plants experiencing low O_2 accumulate alanine and γ-aminobutyric acid (GABA; reviewed by Bailey-Serres and Voesenek, 2008). Upon re-oxygenation, alanine can be recycled back to pyruvate, and GABA can be converted to succinate. This set of amino acid oxidation reactions may minimize the decline in cytosolic pH and reduce the loss of fixed carbon as ethanol or lactate. Alanine also accumulates in *T. vaginalis* and in many animals belonging to the Excavata taxa (Edwards et al., 1989) as a minor end product.

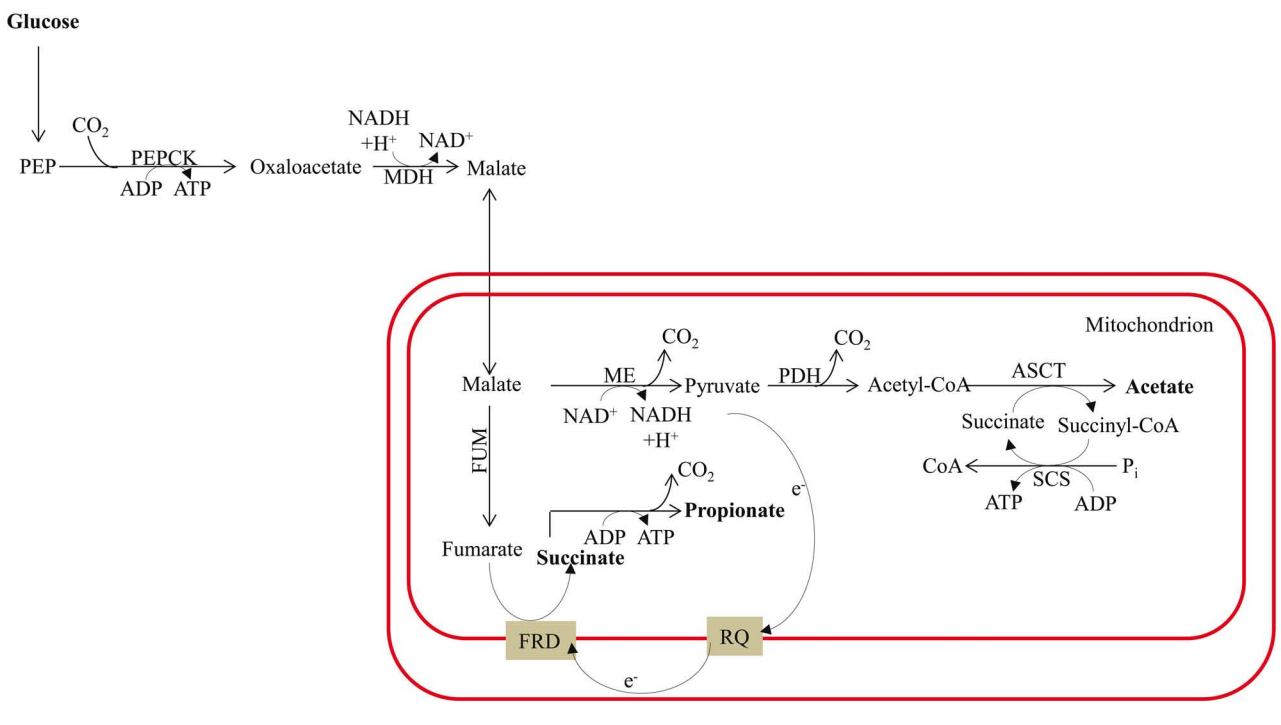

FIGURE 6 | Malate dismutation and energy metabolism in anaerobic mitochondria. The map is redrawn based on the review of Müller et al. (2012). The main end products are acetate and propionate, with minor amounts of succinate. The designations in this figure are: FRD for fumarate reductase; PDH for pyruvate dehydrogenase complex; and RQ for rhodoquinone. See previous figure for other designations.

A less common fermentation process, but largely used in marine environments, involves opine formation. This pathway is localized to the cytosol and involves pyruvate condensation with an amino acid in a redox reaction that regenerates NAD^+. A possible advantage of this alternative pathway for balancing cellular redox is that opine is less acidic than lactate. Moreover the process maintains an osmotic equilibrium since one amino acid is consumed per opine synthesized (Ballantyne, 2004).

Denitrification (Figure 7)

The capacity for nitrate respiration is widespread among bacteria, fungi, and other eukaryotic organisms (Morozkina and Kurakov, 2007). Details of the denitrification pathway have been studied in fungi and bacteria (see previous paragraphs). On the other hand, the enzymes required for nitrogen metabolism in foraminifera and diatoms are not well characterized, although the occurrence of the pathway was noted (Risgaard-Petersen et al., 2006; Kamp et al., 2011).

Numerous reports have demonstrated the presence of two main pathways for denitrification; one is localized in the mitochondrion and usually occurs under low O_2 conditions, while the other, often referred to as ammonia fermentation, is localized in the cytosol (Zhou et al., 2001; Takasaki et al., 2004; Morozkina and Kurakov, 2007; **Figure 7**). The latter pathway appears to be activated under strict anoxic conditions and involves reduction of nitrate to ammonia using reductant generated by the catabolic oxidation of ethanol (the donor of electrons) to acetate, which is coupled to SLP. As shown in **Figure 7**, the ethanol is oxidized to acetaldehyde by an alcohol dehydrogenase (designated Ald), which is converted to acetyl-CoA by acetaldehyde dehydrogenase (AddA). The acetyl-CoA is then converted to acetate and CoA, with the concomitant production of ATP by Ack (Zhou et al., 2001). Under hypoxic conditions the ethanol is oxidized to acetate and the electrons generated in the reaction are used to reduce nitrite to N_2O, which is excreted from cells (Zhou et al., 2001; **Figure 7**). Nitrate and nitrite reductases catalyze the reduction of nitrogen oxides to ammonia using NADH as the electron donor, and are assimilatory enzymes.

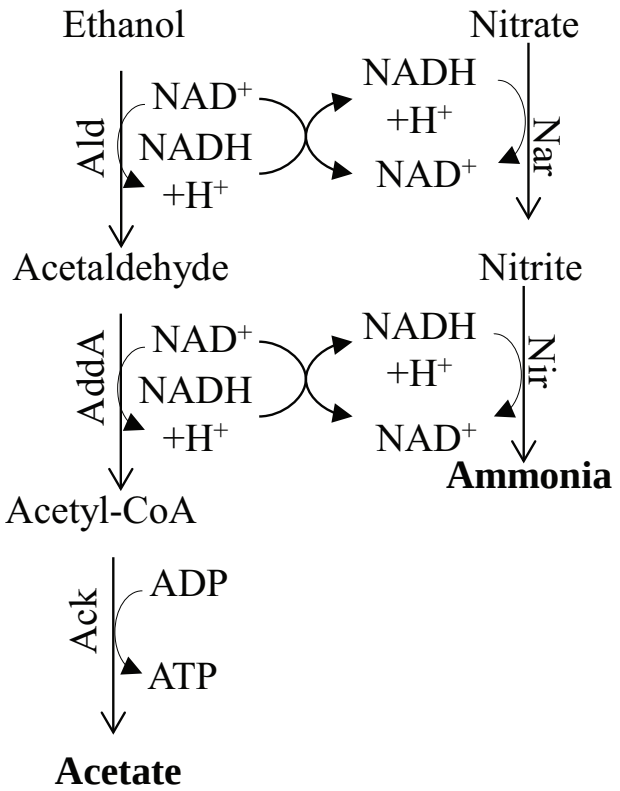

FIGURE 7 | Metabolic pathway for ammonia fermentation coupled to acetogenic oxidation of ethanol and substrate-level phosphorylation. The protein designations in this figure are: Ald, alcohol dehydrogenase; Adda, acetaldehyde dehydrogenase; Nar, nitrate reductase; and Nir, nitrite reductase.

H_2 and CO_2 production

H_2 and CO_2 are generated in an ancestral anaerobic pathway that is present in many green algae. The generation of H_2 in algae often serves as a redox valve. This pathway can be in chloroplasts, as in *Chlamydomonas* and other algae, in mitochondria-like organelles, as in the Stramenopiles, or in the hydrogenosomes of the amoebozoa, some opisthokonta and Excavata. H_2 production is often associated with PFR activity, which oxidizes pyruvate to acetyl-CoA and CO_2. Reduced ferredoxin transfers electrons to a hydrogenase that can convert protons and electrons into H_2 (**Figure 5B**).

METABOLITE PARTITIONING, ORGANELLE COMMUNICATION AND ITS EVOLUTION

CARBON PARTITIONING BETWEEN ORGANELLES

Glycolysis is the backbone of eukaryote carbon and energy metabolism, leading to the production of pyruvate, ATP, and NADH. Further metabolism of the pyruvate can occur in the cytosol, mitochondrion, or plastid. For some eukaryotic organisms fermentation occurs entirely in the cytosol; the organisms included in this group are the protistan parasites such as *Giardia* and *Entamoeba* (Müller, 1996). Fermentations can also occur partly in hydrogenosomes, as is the case for *Trichomonas* (Müller, 1993). Among animals, fermentation often entails malate dismutation, involving segments of the mitochondrial electron transport chain, as in the case of the anaerobic mitochondria of many marine invertebrates and parasitic worms (Tielens et al., 2002; Tielens and van Hellemond, 2009).

A number of metabolic reactions can occur in more than one compartment in the cell and some enzymes may be routed to more than one cellular location; one example of this is PFL, which appears to occur in both chloroplasts and mitochondria, but dual localizations of proteins is not uncommon in eukaryotes (Atteia et al., 2006; Martin, 2010; Müller et al., 2012). Examining the network of activities in *Chlamydomonas* exposed to anoxic conditions raises some fundamental questions; one very important question is "How can an entire metabolic pathway be transferred to a new compartment?" This issue is still far from being resolved and more detailed biochemical and evolutionary analyses are necessary. However, it is becoming evident that over evolutionary time, enzymes and pathway can readily undergo re-compartmentation

among subcellular locations in the cell including the mitochondrion, cytosol, hydrogenosome, and chloroplast. Small changes in targeting sequences might result in mistargeting, which could explain how individual activities, as well as entire pathways are found in more than one cellular compartment (Martin, 2010).

EVOLUTIONARY INSIGHTS

This review presents information indicating that overall, the different groups of eukaryotic organisms share the same core pathways for hypoxic/anoxic energy metabolism. Although distinct mechanisms are used by obligate and facultative anaerobes, there is a certain set of enzymes consistently associated with fermentation metabolism among a variety of organisms ranging from the bacteria to algae, fungi, and metazoans. The heterofermentation that is associated with the algae differs from lactate or ethanol homofermentation that occurs in yeast and various multicellular organisms including plants and animals; fermentation patterns in *Chlamydomonas* show some similarities to mixed-acid fermentation, which is common in the enteric bacteria (Neidhardt et al., 1990). The *Chlamydomonas* genome appears to contain a complete (or near complete) spectrum of genes involved in anaerobic energy metabolism across all eukaryotes (Müller et al., 2012; Atteia et al., 2013). However, the ancestry of these genes, whether from single or multiple origins, remains to be established. Müller et al. (2012) in a recent review favor the hypothesis that many of the enzymes associated with anaerobic energy metabolism in eukaryotes share a common ancestor, which is supported by the finding that different eukaryotic lineages possess different subsets of the same ancestral collection of genes. Furthermore, if the various genes for anaerobic metabolism in protists were derived from multiple ancestral genes, then evidence for the lateral transfer of genes from multiple sources should be apparent. The fact that no eukaryotes perform sulfate reduction, ammonium oxidation, or methane oxidation suggests that the independent lateral transfer of anoxic pathway genes to eukaryotes is not a common occurrence. Instead, different lineages of eukaryotic anaerobes use distinct enzyme combinations selected from a limited core inventory of fermentative pathways. Although a common origin of these pathways is speculative at this point, Müller et al. (2012) observes that there is no pattern of lineage specific acquisition, and it remains unclear why alternative anoxic strategies are not widely observed in eukaryotes.

CONCLUSION

Chlamydomonas is a metabolically versatile organism that can perform photosynthetic CO_2 fixation, aerobic respiration, and anaerobic fermentation. This alga has served as a model system to examine many aspects of photosynthetic metabolism and recently has been used in studies of anaerobic metabolism; these latter studies have shown that *Chlamydomonas* contains a large and complex repertoire of anaerobic enzymes that are distributed among the different compartments of the cell. Initial characterizations have demonstrated that *Chlamydomonas* has flexible, mixed-acid fermentation, with features common to bacterial-, plant-, and yeast-type fermentation. Many pathways and enzymes associated with fermentation metabolisms in this alga are just being defined, and there is almost nothing known about the mechanisms by which these pathways are regulated and the trafficking of fermentation products among the different compartments in the cell. In general, photosynthetic algae appear to have a broad inventory of fermentative enzymes and, based on evidence discussed in this review, it appears that anaerobic respiration among eukaryotic algae is comparatively rare while anaerobic fermentation is widespread. Most enzymes for fermentative metabolism in the algae, often inferred from genomic and metabolic studies, have not been characterized biochemically. Expression patterns of genes encoding these enzymes and the biochemical properties of these enzymes and pathways need further characterization in a broader spectrum of algal systems. In addition, the diversity of end products that the various algae can synthesize during anaerobic fermentation is still mostly unknown. This information will be critical for developing a clear understanding of the metabolic diversity both within and among the different algal groups and the ways in which fermentation pathways have evolved and are shaped by environmental conditions. Finally, fermentation metabolism in the algae appears to represent a significant ecological component of carbon flux in soils (and sediments) that has a strong impact on its content of organic acids, alcohols, and H_2; more focus on fermentation in the future is likely to unmask a relatively unexplored aspect of carbon cycling in the environment.

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Sulfide as a soil phytotoxin—a review

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In wetland soils and underwater sediments of marine, brackish and freshwater systems, the strong phytotoxin sulfide may accumulate as a result of microbial reduction of sulfate during anaerobiosis, its level depending on prevailing edaphic conditions. In this review, we compare an extensive body of literature on phytotoxic effects of this reduced sulfur compound in different ecosystem types, and review the effects of sulfide at multiple ecosystem levels: the ecophysiological functioning of individual plants, plant-microbe associations, and community effects including competition and facilitation interactions. Recent publications on multi-species interactions in the rhizosphere show even more complex mechanisms explaining sulfide resistance. It is concluded that sulfide is a potent phytotoxin, profoundly affecting plant fitness and ecosystem functioning in the full range of wetland types including coastal systems, and at several levels. Traditional toxicity testing including hydroponic approaches generally neglect rhizospheric effects, which makes it difficult to extrapolate results to real ecosystem processes. To explain the differential effects of sulfide at the different organizational levels, profound knowledge about the biogeochemical, plant physiological and ecological rhizosphere processes is vital. This information is even more important, as anthropogenic inputs of sulfur into freshwater ecosystems and organic loads into freshwater and marine systems are still much higher than natural levels, and are steeply increasing in Asia. In addition, higher temperatures as a result of global climate change may lead to higher sulfide production rates in shallow waters.

Keywords: global change, iron, microorganism, oxygen, plant, roots, sulfur, symbiosis

INTRODUCTION: ANAEROBIOSIS AND SOIL SULFUR TRANSFORMATIONS

Although sulfur (S) is one of the six macronutrients for plant growth and low availability of S may therefore limit primary production (Marschner, 1995; Leustek and Saito, 1999), the accumulation of reduced sulfur in sediments of aquatic systems and permanent or riparian wetlands (including estuarine and marine) generally causes physiological toxicity stress for the community involved, including its plants, animals and micro-organisms (Bagarinao, 1992). Unlike the first billion years of life on earth, when sulfide oxidation was an integral part of life generating energy, sulfide accumulation has become much less common as a result of biogenic oxygen production, and sulfide has become toxic to many organisms inhabiting the top layer of soils, including plants (Olsen, 2012). Atmospheric oxygen levels started to increase 2.5 billion years before present (BYBP), and reached levels above 15% since 0.6 BYBP. Oscillations in oxygen and reciprocal oscillations in sulfide levels may even have contributed to mass extinctions (Olsen, 2012).

During flooding and waterlogging of wetland soils, hydrogen sulfide (H_2S) is produced as a metabolic end product by prokaryotes that oxidize organic compounds using sulfate as a terminal electron acceptor. This group of dissimilatory sulfate reducers includes both Bacteria (e.g., *Desulfovibrio*, *Desulfobacter*) and Archaea (e.g., *Archaeoglobus*). If the sulfide produced cannot be sufficiently sequestered in the soil by metals such as iron, free (dissolved) sulfide will accumulate. Sulfide concentrations in sediment porewaters show a large range up to 15 mmol L^{-1} in marine sediments (Bagarinao, 1992). The reduced sulfur compound acts as a potent phytotoxin (equally toxic as cyanide), by inhibiting the activity of cytochrome *c* oxidase in mitochondria, leading to a subsequent blocking of energy production, and by negatively affecting a range of other metal containing enzymes (Koch et al., 1990; Bagarinao, 1992; Raven and Scrimgeour, 1997). The chemical speciation of sulfide (H_2S, HS^- and S^{2-}) depends on soil pH (pK1 = 7.2; pK2 = 13.7 for freshwater). Although all forms seem to be equally toxic (Armstrong and Armstrong, 2005), the gaseous H_2S will normally prevail over both ionic forms in

freshwater systems as the pH of most anaerobic soils is buffered around 6–7 as a result of the HCO_3^- - CO_2 buffering mechanism, resulting in relative H_2S abundances of 95–60%. In marine systems, however, pH is often around 7.5, leading to a relative abundance of only 30% for H_2S, and 70% for HS^-. As a result of the release of acidic compounds and oxygen from roots, pH in the rhizosphere may, however, be lower than in the bulk soil, and the proportion of H_2S consequently be higher.

In marine and brackish ecosystems, sulfate concentrations are 10 to 1000 times higher compared to freshwater systems (Marschner, 1995), stimulating sulfate reducers that play an imminent role in decomposition (Jørgensen, 1982) and concomitant sulfide production. Hence, the role of sulfide as a potential natural toxin in saline sediments has been well-established (Carlson and Forrest, 1982; Ingold and Havill, 1984; Webb and Mendelssohn, 1996; Raven and Scrimgeour, 1997; Koch and Erskine, 2001; Pedersen et al., 2004). Sulfide toxicity may also occur when levels and inputs of sulfur remain unchanged, but increased loading with organic matter boosts sulfate reduction rates by providing electron donors from its decomposition (Jørgensen, 1982; Armstrong and Armstrong, 2001; Ruiz-Halpern et al., 2008; Van der Heide et al., 2012). In many coastal systems worldwide, organic loading has strongly increased as a result of land use change in the catchment of rivers (Ver et al., 1999). In addition, increased inorganic nutrient loading (from rivers, run-off, urbanization, atmospheric deposition) fuels local organic matter production (e.g., Van Beusekom and De Jonge, 2002). This makes sulfide-related questions here even more urgent than in more pristine areas.

As a result of anthropogenic forcing, plants in freshwater wetlands and aquatic systems are facing much higher concentrations of sulfur at a global scale nowadays (Lamers et al., 1998). The emission of sulfur to the atmosphere and airborne inputs of anthropogenically-derived sulfur into freshwater wetlands have increased considerably over the last decades as a result of extensive mining for fossil fuels and associated combustion (Gorham, 1976; Schindler et al., 1980; Benkovitz et al., 1996; Schlesinger, 1997). Although S deposition has decreased in Europe and North America during the last decade as a result of effective legislation, rates are still much higher than natural background levels, and in Asia, South America and South Africa, S emission and deposition rates are still strongly increasing (Shah et al., 2000; Vallack et al., 2001). Moreover, sulfate loading of groundwater has increased due to aerobic oxidation of deposited sulfide minerals as a result of water table lowering for agriculture (Schuurkes et al., 1988; Heathwaite, 1990; Lamers et al., 1998), and from anaerobic oxidation of reduced sulfur compounds by chemolithoautotrophic coupling of sulfide oxidation and nitrate reduction in nitrate-loaded catchments and wetlands (Haaijer et al., 2006; Burgin and Hamilton, 2008; Smolders et al., 2010). As a result of the discharge of this groundwater and run-off from pastures and shores suffering from drought, surface waters have become richer in sulfate too. The S in terrestrial soil and subsoil originates in part from increased anthropogenic airborne inputs (S legacy), but also from natural marine and estuarine deposits in the Quaternary or in earlier periods. In addition, recent hydrological changes such as increased inputs of riverine water to compensate for water shortage in both agricultural areas and nature reserves (Roelofs, 1991; Smolders and Roelofs, 1993; Lamers et al., 1998), as well as the intrusion of seawater (salinization; Fogli et al., 2002; Chambers and Pederson, 2006) have contributed to increased S inputs into freshwater wetlands.

DIFFERENTIAL SENSITIVITY THRESHOLDS FOR SULFIDE

Research on sulfide toxicity and physiological stress originally focused on rice (*Oryza sativa*) as a crop plant in relation to acid sulfate soils that accumulate high concentrations of sulfide during anaerobiosis (Okajima and Takagi, 1955; Vámos, 1959; Hollis et al., 1972). Seedlings appeared to be particularly sensitive to sulfide (Joshi et al., 1975). Since then, sulfide toxicity has also been reported for many other wetland species in both freshwater and saline systems, with a wide range of threshold levels for different species.

In **Table 1**, a literature overview is given for sulfide toxicity of different plant species, grouped by ecosystem type, showing the differential threshold levels and ecophysiological responses to sulfide. As high levels of free sulfide are only present in wetland soils (including aquatic systems), dryland species are not represented in this table. In addition, no data are available on phytotoxic effects for macroalgae and phytoplankton. As phytoplankton only occurs in the photic zone of the water column that contains oxygen, sulfide toxicity is very unlikely to play an important role. Sulfide toxicity will be much less common for macroalgae than for vascular plants, because they only possess rhizoids and do not protrude into the anoxic sediment, but are often attached to substrates such as rock and coral. At low tide, however, sulfide may well accumulate under dense mats of macroalgae. As an example, anoxic conditions and high ammonium levels were measured in *Cladophora* mats, hampering seagrass growth (Hauxwell et al., 2001). Although sulfide was not measured in this study, it can be expected to have led to sulfide accumulation as well, as shown for *Ulva* mats in coastal lakes (Viaroli et al., 1996). Direct effects of sulfide on macroalgae have, as far as we know, not been tested yet. Algal cover can, however, lead to increased sulfide toxicity to seagrasses (Holmer et al., 2011; Thomsen et al., 2012). Sulfide may only accumulate to high concentrations in the surface water if the water is anoxic and its oxidation is prevented, for instance by the cover of floating-leaved vascular plants such as *Eicchornia crassipes*, *Pistia stratiotes*, *Lemna* spp., and floating ferns such as *Salvinia* spp. and *Azolla* spp. Dense layers of these plants effectively block oxygen intrusion from the atmosphere (e.g., Van Kempen et al., 2012). Phytoplankton is lacking in this dark layer, due to photon deficiency.

As can be expected in sulfate-rich environments (particularly when they are permanently submerged), seagrass species are relatively tolerant to sulfide (thresholds generally 2000–6000 μmol L^{-1}), although negative effects on growth rates have also been reported at levels of 200–500 μmol L^{-1}, especially for small species (**Table 1**). The saltmarsh species *Spartina alterniflora* is also known to survive high concentrations of sulfide up to 8000 μmol L^{-1} (Lee, 1999; Van der Heide, unpubl. results), but lower concentrations may already impair its growth (King et al., 1982). For mangroves, *Rhizophora* seedlings appeared to be more tolerant than those of *Avicennia*, but adult trees of the latter

Table 1 | Overview of sulfide toxicity effects reported in marine, brackish and freshwater plants.

Ecotype *Species*	Concentration (μmol L^{-1})	Observation	Method	References
SEAGRASS MEADOWS				
Halodule wrightii	2000	AD	Glucose add. to increase SO_4 red.	Koch et al., 2007
Posidonia oceanica	>1800	AD	Glucose add. to increase SO_4 red.	Frederiksen et al., 2008
Halophila ovalis	>150	AP, RP,	Raised T (25–30°C) in aq. exp.	Holmer et al., 2011
Posidonia oceanica	1500	AP, AD	Field Fe addition to lower HS^-	Marbà et al., 2007
Thalassia testudinum	>500	AD	Field observation	Borum et al., 2005
Thalassia testudinum	5500	AD	Glucose add. to increase SO_4 red.	Koch et al., 2007
Thalassia testudinum	6000	AD (only high T and Sal.)	H_2S in hydroponic culture	Koch and Erskine, 2001
Thalassia testudinum	5000	AD	org. matter to increase SO_4 red.	Ruiz-Halpern et al., 2008
Zostera marina	600/1000	NP (low/high light)	H_2S inject. microcosm sediment	Goodman et al., 1995
Zostera marina	>1800	No indication of AD	Glucose add. to increase SO_4 red.	Frederiksen et al., 2008
Zostera noltii	>200	AP	Omission of *Loripes* bivalves	Van der Heide et al., 2012
Zostera noltii	>500	LE (from patches)	org. matter to increase SO_4 red.	Govers et al. pers. observ.
Zostera marina	600	AP, AD	Raised T (18°C) in aq. exp.	Hoffle et al., 2011
SALT MARSHES				
Agrostis stolonifera	500	AP, NU	H_2S in hydroponic culture	Van Diggelen et al., 1987
Halimione portulacoides	500	AP, NU	H_2S in hydroponic culture	Van Diggelen et al., 1987
Salicomia dolichostachya	>500	AP	H_2S in hydroponic culture	Van Diggelen et al., 1987
Salicomia brachystachya	>500	AP	H_2S in hydroponic culture	Van Diggelen et al., 1987
Spartina alterniflora	1000	AP	Field observation	King et al., 1982
Spartina alterniflora	1130	AP, RD	H_2S in hydroponic culture	Koch and Mendelssohn, 1989
Spartina alterniflora	2000–3000	AP, RA, NU	H_2S in hydroponic culture	Koch et al., 1990
Spartina alterniflora	8000	AP	Field observation	Lee, 1999
Spartina anglica	500	AP	H_2S in hydroponic culture	Van Diggelen et al., 1987
MANGROVES				
Avicennia marina (sl)	500–1000	AP, RP	H_2S inject. microcosm sediment	McKee, 1993
Avicennia marina	>4000	AP	Field observation	McKee, 1993
Rhizophora mangle (sl)	>1000	AP	H_2S inject. microcosm sediment	McKee, 1993
Rhizophora mangle	>1000	AP	Field observation	McKee, 1993
FRESHWATER AQUATIC SYSTEMS				
Ceratophyllum demersum	>500	AP	SO_4 addition mesocosms	Geurts et al., 2009
Elodea nutallii	100	AP	SO_4 addition enclosures	Van der Welle et al., 2007a
Elodea nutallii	150–500	AP	SO_4 addition mesocosms	Geurts et al., 2009
Hydrilla verticulata	100	NP	H_2S in root hydroponic culture	Wu et al., 2009
Nitella flexilis	50	AP	H_2S injection aquarium sediment	Van der Welle et al., 2006
Potamogeton compressus	150–500	AP	SO_4 addition mesocosms	Geurts et al., 2009
Statiotes aloides	10–100	RD	H_2S in root hydroponic culture	Smolders and Roelofs, 1996
Stratiotes aloides	100–600	AP	SO_4 addition enclosures	Van der Welle et al., 2007a
Stratiotes aloides	500	AP	SO_4 addition mesocosms	Geurts et al., 2009
FRESH WATER WETLANDS				
Calamagrostis epigejos (sl)	30–50	AP	Natural production in microcosm	Grootjans et al., 1997
Calla palustris	150	AP	SO_4 addition mesocosms	Geurts et al., 2009
Caltha palustris	170	AP, Y	H_2S injection microcosm sed.	Van der Welle et al., 2007b
Carex disticha	10–20	AP	SO_4 addition mesocosms	Lamers et al., 1998
Carex disticha	25	LC, RD	H_2S injection microcosm sed.	Lamers, 2001
Carex nigra	10–20	AP	SO_4 addition mesocosms	Lamers et al., 1998
Cladium jamaicense	220/690/920	LE/NP/AD, RD	H_2S in hydroponic culture	Li et al., 2009
Equisetum fluviatile	50/500	AP (unfertilized/fertilized)	SO_4 addition mesocosms	Geurts et al., 2009
Juncus acutiflorus	25/250	RD/AP	H_2S injection microcosm sed.	Lamers, 2001
Juncus alpinoarticulatus (sl)	30–50	AP	Natural production in microcosm	Grootjans et al., 1997
Juncus effusus	500	AP	SO_4 addition mesocosms	Geurts et al., 2009

(Continued)

Table 1 | Continued

Ecotype *Species*	Concentration (μmol L^{-1})	Observation	Method	References
Menyanthes trifoliata	150/>150	AP (unfertilized/fertilized)	SO_4 addition mesocosms	Geurts et al., 2009
Menyanthes trifoliata	>235	AP	Field observation	Armstrong and Boatman, 1967
Panicum hemitomon	630	AP, RD	H_2S in hydroponic culture	Koch and Mendelssohn, 1989
Panicum hemitomon	1000	AP, RA, NU	H_2S in hydroponic culture	Koch et al., 1990
Phragmites australis	1400	AD, SR, B	H_2S in hydroponic culture	Armstrong et al., 1996
Phragmites australis	1500	AP	SO_4 + C addition mesocosms	Howes et al., 2005
Phragmites australis	400	AP	Field observation	Chambers, 1997
Oryza sativa	170	RP, B, RO, NU(Fe), WU	H_2S in anaerobic agar	Armstrong and Armstrong, 2005
Oryza sativa	160–310	AP	H_2S in hydroponic culture	Tanaka et al., 1968
Oryza sativa	30	AP	H_2S in hydroponic culture	Hollis et al., 1972
Oryza sativa (sl)	10–60	NU (acute), RO	H_2S in hydroponic culture	Joshi et al., 1975
Ranunculus lingua	500	AP	SO_4 addition mesocosms	Geurts et al., 2009
Sphagnum cuspidatum	60	AD	SO_4 addition mesocosms	Lamers et al., 1999
Thelypteris palustris	150	AP	SO_4 addition mesocosms	Geurts et al., 2009
Typha domingensis	920	LE, NP, AD, RD	H_2S in hydroponic culture	Li et al., 2009

Concentrations are in μmol L^{-1}, (sl), seedling. Observations: AP, decreased aboveground productivity; AD, aboveground die-off; B, blockage of gas pathways and vascular blockage; LC, leaf chlorosis; LE, decreased leaf elongation rate; NP, decreased net photosynthetic rate; NU, decreased nutrient uptake; RD, root (and rhizome) die-off; RO, decreased radial oxygen loss; RA, decreased root ADH activity; RP, decreased belowground production; SR, stunted roots; WU, reduced water uptake; Y, decreased photosynthetic yield (PAM fluorescence).

species tolerate much higher concentrations. The high tolerance of saltmarsh and mangrove species makes sense, as they grow on soils that are rich in both organic electron donors (derived from decomposition of the large flux of litter) and the alternative terminal electron acceptor sulfate. This may also suggest that early-successional species (including a number of seagrass species) may be more sensitive to sulfide than late-successional species, as the latter generally live on sites with higher organic matter accumulation in the sediment. In addition, different ecotypes of the same species can be expected to exist due to strong selection, each adapted to their specific habitat.

Most of the larger freshwater helophyte species such as *Phragmites australis* and *Typha domingensis* also show tolerance to relatively high sulfide concentrations (500–1500 μmol L^{-1}; Armstrong et al., 1996; Chambers, 1997; Armstrong and Armstrong, 2001; Adema et al., 2003). Sulfur amendment in order to try to control the unbridled expansion of *P. australis* in the USA at the expense of other species, led to sulfide concentrations of 1500 μmol L^{-1}, a level that this species demonstrated survival even at higher salinities (Howes et al., 2005).

In contrast, smaller wetland species and aquatic macrophytes show much lower toxicity thresholds between 10 and 250 μmol L^{-1} (**Table 1**). Some rootless aquatic macrophytes, growing on highly organic soils, such as *Ceratophyllum demersum*, tolerate relatively high concentrations up to 500 μmol L^{-1}. *Oryza sativa* shows intermediate levels of tolerance, although the actual level differs among varieties.

EFFECTS OF EXPERIMENTAL SET-UP AND DIFFERENT FIELD MEASUREMENTS

Concentrations of dissolved sulfide can be measured colorimetrically, with S^{2-} selective electrodes in immediately fixed and alkalized porewater, *in situ* using micro-electrodes, or by gas chromatography analysis after gas stripping of acidified porewater. In addition, sulfide-selective optodes, which neither need additional reagents nor consume sulfide, have been developed for direct sulfide measurement (Choi, 1998). As sulfide is easily oxidized and correct sulfide measurements depends on accurate pH measurements for a number of methods, the analytical methods used may show differences in accuracy.

The interpretation of results from literature is strongly confounded by the myriad of methods used in the field and in experiments. For field observations, low sulfide concentrations may also indicate high tolerance to microbial sulfide production due to high oxidation rates supported by oxygen supply from roots. For laboratory tests, the experimental set-up may therefore well-interfere with toxicity levels and attendant effects. As we will discuss, the ability or inability of plants to generate an oxidized rhizosphere strongly determines their sensitivity to reduced phytotoxic compounds including ammonium (NH_4^+), ferrous iron (Fe^{2+}) and H_2S, (Laan et al., 1989, 1991; Lamers et al., 2012). Therefore, great care has to be taken in the interpretation of hydroponic experiments to the actual effect of the suggested stress conditions under natural conditions. To test the potential toxicity of reduced compounds and separate ecophysiological responses from those related to direct anoxia effects, we therefore stress the importance of an experimental set-up using a realistic substrate in which plants are able to potentially realize a protective rhizospheric environment to cope with both primary (anoxia-related) and secondary (toxicity-related) stress during anaerobiosis. Pezeshki (2001), in his review on wetland plant responses to soil flooding, also pleaded for research differentiating between these effects.

FIGURE 1 | Example of an experimental set-up using rhizotrons showing inflow, outflow, and the tubes of samplers to collect soil porewater (photo: L. Lamers).

On the other hand, the type of soil used in other types of set-up will be very important for the outcome, as this determines the extent of the oxygen sink and diffusion rates. This means that the use of artificial solid substrates, like gels, may also generate experimental artifacts. We therefore suggest using a different approach as an experimental set-up, which includes more realistic edaphic conditions and rhizospheric effects (**Figure 1**). The actual optimal set-up will depend on the particular questions involved (see, e.g., Van der Heide et al., 2012).

SULFUR UPTAKE AND INTERNAL DETOXIFICATION

Sulfur concentrations in shoots of terrestrial plants are, on average 30 μmol g^{-1} (Gruhlke and Slusarenko, 2012), but values may be higher for freshwater wetland plants (35–150 μmol g^{-1}, Van der Welle et al., 2007a,b) and marine plants (100–400 μmol g^{-1}; Holmer and Kendrick, 2013), most probably related to the level of S availability in the different environments, but possibly also as a result of the presence of sulfides in the soil. Sulfate is actively taken up by roots and distributed in the plant, with transport through membranes by proton-sulfate co-transporters driven by a proton gradient (Trust and Fry, 1992; Leustek and Saito, 1999). Studies on the abundance ratios of natural S isotopes in *Spartina alterniflora* revealed that most of the sulfate in these marsh plants was derived from sulfide that had partly been oxidized within the plant (Carlson and Forrest, 1982). For seagrasses including *Zostera marina* and *Thalassia testudinum*, isotopic analysis revealed that 50–96% of the S in plants was derived from different sediment sulfides (Frederiksen et al., 2006; Holmer et al., 2009), even when dissolved sulfide concentrations were low (Holmer and Kendrick, 2013). In small seagrass species, sulfur easily enters the roots, and is transported through rhizomes and stems into the leaves, but in taller species its transport seems to be more limited (Holmer and Kendrick, 2013). It is therefore quite probable that in addition to sulfide oxidation in the rhizosphere and subsequent sulfate uptake, gaseous H_2S is transported to the leaves through the aerenchyma, especially during the night (Pedersen et al., 2004; Holmer and Kendrick, 2013). As the uptake of sulfate after rhizospheric and internal sulfide oxidation generates similar $\delta^{34}S$ values in plants as direct sulfide uptake, it may be difficult to differentiate between both uptake pathways (Trust and Fry, 1992).

Studies related to S uptake and metabolism have generally been conducted with terrestrial plants species, and differences between sulfide and sulfate uptake and their metabolic pathways are not entirely clear yet. The internal toxicity of sulfide will depend on the species' ability to rapidly metabolize this compound to thiols (organosulfur compound, chemical formula R-SH) such as the amino acids cysteine and methionine and, subsequently, glutathione which is the most abundant thiol in plants (Trust and Fry, 1992; Leustek and Saito, 1999; Hawkesford and De Kok, 2006; Nakamura, 2009). Next, S may be built in a range of different plant tissues. A small number of estuarine plant species, including *Spartina* spp. and *Wollastonia biflora* also produce dimethylsulfoniopropionate (DMSP) from methionine, like a number of marine algal species (Stefels, 2000). This compound may act as a constitutive osmoticum, although its concentration in *Spartina* spp. does not respond to changes in salinity. Alternatively, the production may also provide a mechanism to keep cysteine and methionine levels sufficiently low, and redistribute nitrogen to other amino acids (Stefels, 2000; Otte et al., 2004). In addition, a number of secondary metabolites contain sulfur, including antibiotic substances and odorous compounds (giving flavor to garlic, onions and cabbage) (Leustek and Saito, 1999). The capacity to internally detoxify sulfide is therefore related to cysteine synthesis, catalyzed by the enzyme O-acetylserine(thiol)lyase (OAS-TL) that is present in cytosol, plastids and mitochondria, and an as yet unknown other detoxifying mitochondrial mechanism (Birke et al., 2012). Lee (1999) even hypothesized that low sulfide concentrations might be used by plants to generate energy in mitochondria, similar to the process in microbes and animals. In addition, there is a range of reactive sulfur species next to thiols, such as disulfide-S-oxides ($RS(O)_xSR$), sulfenic acids (RSOH), and thiyl radicals (RS) (Gruhlke and Slusarenko, 2012). Although it seems likely that plant hemoglobin (Hb; Igamberdiev et al., 2005) and other metalloproteins may be related to internal sulfide detoxification, similar to Hb in vertebrates and invertebrates (Beauchamp et al., 1984; Weber and Vinogradow, 2001), this is yet to be studied. Next to the metabolic conversion of sulfide, the emission of sulfide from plants, as shown during the exposition to high sulfide concentrations (roots) or SO_2 concentrations (shoots) (Trust and Fry, 1992), may offer protection. For *Spartina alterniflora* it has been shown that leaves show substantial loss of DMSP during high tide (Pakulski and Kiene, 1992), which provides a mechanism to dissipate excess S. Additionally, the loss of dimethylsulfide (DMS), a volatile metabolite of DMSP, may also offer protection against high S accumulation in a number of estuarine plants (Stefels, 2000).

EFFECTS ON NUTRIENT UPTAKE

Sulfide is known to be able to hamper plant nutrient uptake, which is not surprising given its basic disturbance of cell metabolism and energy transfer. In addition, root loss due to die-off and concomitantly decreased root to shoot ratios lead to an unbalanced nutrient uptake. Sulfide can impair the uptake

of nitrogen (N) (Koch et al., 1990), phosphorus (Van der Heide et al., 2012) and Fe (Smolders and Roelofs, 1996; Armstrong and Armstrong, 2005). Depending on the type of nutrient limitation, growth rates may be impaired, while Fe deficiency may lead to lower photosynthetic rates as a result of hampered chlorophyll synthesis. The effects of sulfide on the uptake of Fe and other metals can, however, also be the result of precipitation (Lamers et al., 2012). Although MgS is highly reactive in water and $MgSO_4$ is quite soluble, it has been shown that Mg and Ca concentrations in acid sulfate soils are generally undersaturated and governed by cation exchange rather than by their activities. It is well-known that this phenomenon can lead to Mg and Ca deficiency of *Oryza sativa* growing on these soils (Tanaka et al., 1968; Moore and Patrick, 1989). Next, acid production as a result of sulfide oxidation can lead to loss of Mg and Ca from soil cation exchange sites in the rhizosphere, and concomitant lower availability of these macro-ions. For field measurements, however, negative correlations between nutrient uptake and sulfide do not prove sulfide toxicity, as salinity, soil organic matter concentration, and oxygen and nutrient availability are often changing as well along the gradient. Effects of sulfide on soil biogeochemistry affecting plant performance and fitness will be explained further in sections below.

An interesting, but as yet unknown mechanism of sulfide toxicity on plant nutrient uptake might act through its effects of mycorrhizal activity. Although lead sulfides are known to seriously decrease the vitality of ectomycorrhizae (Fomina et al., 2005), the effect of free sulfide on mycorrhizae, and thereby on plant fitness, remains to be elucidated.

PHYSICO-CHEMICAL PROTECTION: SEQUESTRATION IN THE SOIL AND VOLATILIZATION

Even with high rates of sulfate reduction in the field, the accumulation of dissolved sulfide and its phytotoxic effects can be moderate, or largely absent due to metal sequestration, mainly by Fe. For Fe this leads to the formation of FeS and FeS_2 (pyrite), detoxifying sulfide (**Figure 2**; Smolders et al., 1995; Lamers et al., 2002b; Van der Welle et al., 2006, 2007a; Marbà et al., 2007). This mechanism was proposed for *Spartina alterniflora* already in 1982 by King et al., who showed for marshes on the barrier island Sapelo (GA, USA) that in spite of similar sulfate reduction rates, sulfide accumulation showed large variations related to Fe availability. In the same way, discharge of Fe-rich groundwater in wetlands and aquatic systems effectively protects against sulfide toxicity (Lamers et al., 2002a). In marine systems, where sediment Fe concentrations are generally low, the experimental addition of Fe has been shown to counteract sulfide toxicity to seagrass (*Posidonia oceanica*) in a similar way (Holmer et al., 2003, 2005; Marbà et al., 2007; Ruiz-Halpern et al., 2008). However, even if *total* Fe concentrations (i.e., in destruates) in the soil are high, H_2S accumulation may still occur if the amorphous Fe pool is sulfide-saturated by present or past high S reduction rates. This is clearly indicated by low total Fe:S ratios of the soil. Other metals, including Mn, Zn, Hg, Pb, Cd and Cu, may also precipitate sulfide, but are quantitatively much less important in S biogeochemistry (Bagarinao, 1992). Finally, the accumulation of dissolved sulfide can also be toned down by the activity of microbial communities using nitrate or ferric iron as electron acceptor (Friedrich et al., 2001; see above).

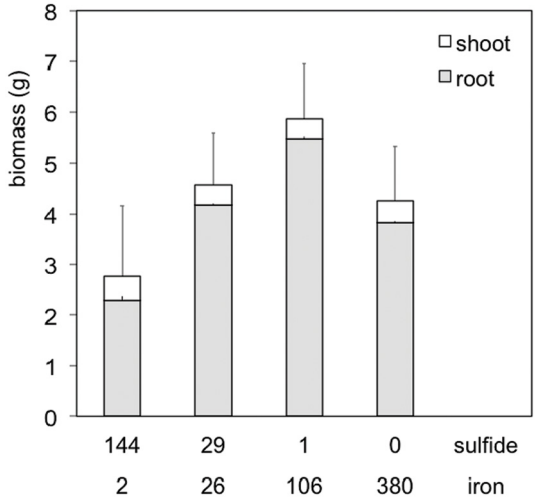

FIGURE 2 | Interacting effects of dissolved sulfide and iron (concentrations in μmol L^{-1}) in the soil porewater on biomass production of *Caltha palustris*. Although both compounds may be toxic for plant growth, they detoxify one another as a result of FeS_x precipitation (quadratic correlation, $p = 0.014$). Adapted from Van der Welle et al. (2006).

As H_2S is a gas, not only sequestration in the soil but also volatilization to the atmosphere determines sulfide concentrations in sediments (Bagarinao, 1992). In addition, sulfide can be methylated in organic marine and freshwater sediments, and released as dimethylsulfide and methanethiol into the atmosphere (Lomans et al., 2002).

BIOLOGICAL PROTECTION: RADIAL OXYGEN LOSS FROM ROOTS

Many flooded or waterlogged plants show radial oxygen loss (ROL) from their roots, and the level and pattern of ROL is determined by photosynthetic rate, root architecture and root morphology (Armstrong, 1979; Jackson, 1985; McKee et al., 1988; Laan et al., 1991; Jackson and Armstrong, 1999; Visser et al., 2003; Frederiksen and Glud, 2006; Visser and Bögemann, 2006; Voesenek et al., 2006; Deborde et al., 2008). During nighttime, sulfide intrusion into roots and rhizomes is highest (Borum et al., 2005). Rhizosphere oxidation provides an obvious potential defense mechanism against the toxicity of reduced components such as sulfide (Pitts et al., 1972; Mendelssohn and McKee, 1988; Armstrong et al., 1996; Smolders and Roelofs, 1996; Hemminga, 1998; Armstrong and Armstrong, 2001, 2005; Holmer and Storkholm, 2001; Deborde et al., 2008), provided that soil aerobic microbial respiration and concomitant consumption of oxygen do not counteract this effect.

Spatial differences in oxygen release can not only be attributed to differences in aerenchyma structure, but also to lignine and/or suberine in the epidermis of the roots of different species, preventing loss of all oxygen in the upper soil layer. As an example, the rush species *Juncus acutiflorus* is able to oxidize its rhizosphere, even for the deeper roots, unlike the sedge species

Carex disticha (Lamers et al., 2012; **Figure 3**). Although both species did release oxygen from their roots, the relatively high ROL in the top layer solely proved to be insufficient to detoxify sulfide for *C. disticha*, leading to almost complete die-off of deeper roots (Lamers, 2001). In contrast, *J. acutiflorus* was able to completely oxidize its rhizosphere, even in deeper layers where a strong O_2 demand results from both soil respiration and S oxidation. Observed root loss correlated well with the differences in spatial ROL patterns for both species (Lamers et al., 2012). Sulfide is even known to induce additional suberization (Armstrong and Armstrong, 2005), which can be either an advantage or a disadvantage depending on the location in the roots. This indicates that the specific pattern of ROL, rather than its overall rate, determines the sensitivity of plant species to reduced phytotoxins such as sulfide. For sufficient ROL, the meristematic oxygen content fuelled by photosynthesis during daytime must also be high enough to prevent oxygen depletion by respiration during nighttime. Particularly at higher temperatures, e.g., as a result of climate change in shallow waters, high respiration rates could exceed photosynthetic O_2 production (Greve et al., 2003).

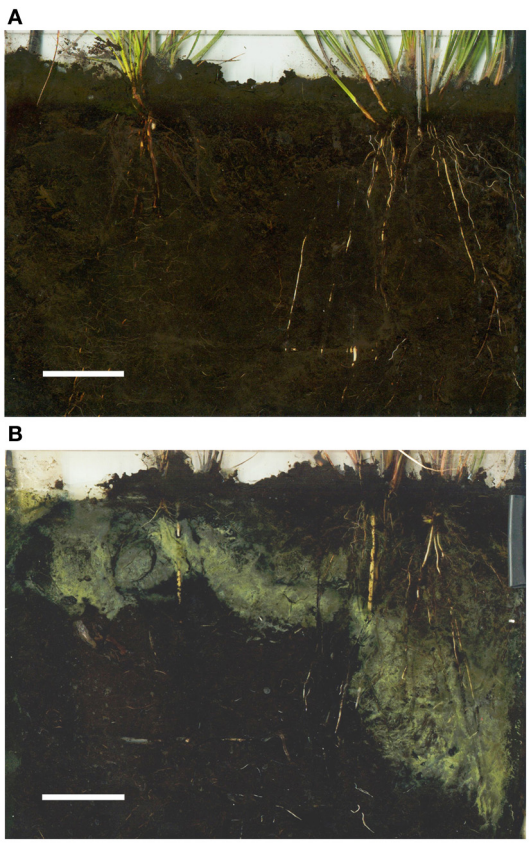

FIGURE 3 | Rhizotron scans for a control (A) and 250 μmol L^{-1} (B) sulfide treatment (darker soil due to FeS_x deposits). The oxidation potential is clearly visible from the yellow-gray halo of metallic sulfur and other products of oxidation. In each scan, *Carex disticha* is positioned left (only shallow S oxidation halos) and *Juncus acutiflorus* right (deep S oxidation halos). On **(B)**, two white soil moisture samplers are visible. Bars represent 40 mm. Adapted after Lamers (2001) and Lamers et al. (2012).

SULFIDE OXIDIZERS AND SOIL FAUNA AS RHIZOSPHERIC GUARDS

As H_2S is readily taken up and causes root toxicity, in contrast to sulfate, the activity of sulfide oxidizing prokaryotes in the rhizosphere is expected to influence both uptake rates and toxicity of S. As the spontaneous chemical oxidation of sulfide is more than 10,000 times slower than biological catalysis (Jørgensen and Revsbech, 1983; Millero, 1986), this rhizosperic sulfur oxidation by prokaryotes (see Friedrich et al., 2001; Ghosh and Roy, 2006) is essential, and the community should comprise large numbers of these organisms living in symbiosis with plants (oxygen supply as a "reward" for detoxification). Sulfur oxidizing microorganisms may be either free living inside or on top of the sediment, in the surface water layer, or associated with roots. Sulfide oxidizers comprise chemolithoautotrophic Proteobacteria such as *Beggiatoa* and *Thiobacillus*, photolithoautotrophic bacteria (e.g., *Rhodovulum, Chromatium*), and chemolitho-autotrophic Archaea (e.g., Sulfolobales) (Ghosh and Dam, 2009). Sulfide-oxidizing prokaryotes may be expected to live inside the root and rhizome aerenchyma as sulfide and oxygen are both present, and *Beggiatoa* presence has indeed been shown inside the rhizomes of seagrass (*Zostera marina*) (Elliott et al., 2006). The oxidation of sulfide in the rhizosphere will, however, also generate acidity in the rhizosphere, slowing down sulfate reduction (Starkey, 1966; Connell and Patrick, 1968) even in the layers beyond the influence of radial oxygen loss by proton diffusion. In this way a second "protective shell" against the adverse effects of sulfate reduction is generated. In addition, the availability of Fe, mobilized by partial FeS_x oxidation, may be higher as a result of lower pH values even at a higher redox potential, although a large part will re-precipitate with sulfide. On the other hand, strong acidification of the rhizosphere may also be detrimental to plant roots, e.g., via NH_4^+ toxicity (Lucassen et al., 2003; Van den Berg et al., 2005). The outcome of these different rhizospheric processes is determined by the interplay between the rates of ROL, oxygen consumption, sulfide oxidation and acid buffering in the soil.

In addition, next to prokaryotes, a range of eukaryote animal species including invertebrates and fish, have been shown to be able to oxidize sulfide in their mitochondria (whether or not ancient endosymbionts; Gray et al., 1999; Emelyanov, 2003; Olsen, 2012), or by sulfide-oxidizing prokaryotes on internal organs, generating energy (Bagarinao, 1992; Ghosh and Dam, 2009). As sediment bioturbation leads to higher rates of oxygen intrusion, sulfate reduction rates are suppressed even though the availability of readily decomposable organic matter may increase, as was shown for the burrow-forming marine polychaete *Arenicola marina* (lugworm; Nielsen et al., 2003). This not only leads to lower concentrations and toxicity of sulfide, but also to higher availability of Fe^{3+} as an alternative electron acceptor (Nielsen et al., 2003). For this effect, however, the level of bioturbation has to be strong enough to affect rhizospheric sulfide concentrations, especially if organic matter is accumulating in burrows as a result of foraging. Even though fiddler crabs (*Uca*

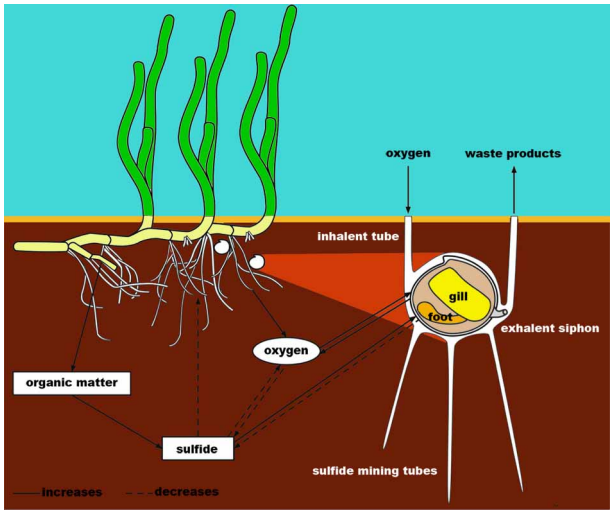

FIGURE 4 | Sulfide-driven coevolution: tripartite mutualistic interactions among seagrasses, lucinid bivalves and sulfide oxidizing bacteria in their gills generate a higher fitness of all species involved under sulfidic conditions. See text for explaining mechanisms. Adapted after Van der Heide et al. (2012).

spp.) were able to oxidize the rhizosphere of young mangrove plants (*Laguncularia racemosa*), sulfide levels remained similar (Smith et al., 2009).

Recently, it was shown in tropical seagrass systems that mutualisms related to rhizospheric S biogeochemistry can be even more complex. Lucinid bivalves containing sulfide-oxidizing symbionts appear to globally occur in tropical and subtropical seagrass meadows (Fisher and Hand, 1984; Van der Heide et al., 2012) and seem strongly associated with these systems ever since seagrasses evolved in the Cretaceous (Van der Heide et al., 2012 and references herein). These lucinids were experimentally shown to play an essential role in seagrass sulfide tolerance, as the sulfide oxidizing prokaryotes living within the gills of the bivalves detoxify sulfide, stimulating seagrass production (**Figure 4**; Van der Heide et al., 2012). ROL by the seagrass species *Zostera noltii* was only able to reduce the added sulfide concentration from 2700 to 2200 μmol L^{-1}, whereas the inclusion of the bivalves led to very low sulfide concentrations of only 15 μmol L^{-1}. Simultaneously, the sulfide oxidizers and their host bivalves benefit from the oxygen supplied by ROL from seagrass, and from its organic matter production. It is very likely that similarly elegant mutualistic symbioses involving multiple species have evolved during evolution enabling other plant species to thrive and have higher fitness under sulfidic conditions. We therefore believe that inclusion of plant-symbiont interactions may be a step forward in our ability to explain sulfide tolerance rather than traditional plant physiology alone.

INDIRECT TOXICITY DURING DROUGHT OF SULFIDIC WETLANDS

Periodic water level fluctuations and severe droughts lead to profound biogeochemical changes in wetlands, due to the strong temporal variation in oxygen concentrations in the soil. Sulfides (free sulfide and metal sulfides) may become toxic in an indirect way in these riparian systems, as the aerobic microbial and chemical oxidation of sulfides generates sulfuric acid (Smolders et al., 2006; Lamers et al., 2012). The actual balance between acid producing and acid consuming processes determines whether this acidification (proton production) leads to an actual drop in pH (increased proton concentration in the porewater). The acid neutralizing capacity (ANC) of soils is determined by the successive extent of bicarbonate and carbonate buffering, the exchange of base cations such as calcium and magnesium at cation binding sites of organic matter and clay, and dissolution of Fe and Al compounds (Scheffer and Schachtschabel, 2002). The total S/(Ca + Mg) ratio of soils may provide an easy proxy to determine the acidification potential of soils during drought (Lucassen et al., 2002). The strong acidification of coastal acid sulfate soils (high concentrations of iron sulfides, low ANC) during droughts, leading to pH values below 4 and concomitant mobilization of aluminum and iron, is a well-known phenomenon, and a problem for rice production and shrimp farming (Dent, 1986; Sammut et al., 1995). Especially in estuarine systems such as marshes, but also in S-rich freshwater systems, massive plant die-off during drought may therefore not only be caused by water deficiency, but also by strong acidification. For the marsh plant *Spartina* spp., it has been shown that the combination of proton toxicity and concomitant mobilization of Al may have contributed to die-off events during droughts (McKee et al., 2004). It has been suggested that acid-tolerant arbuscular mycorrhizal fungi may play an important role in the establishment of pioneer species (grasses, forbs and shrubs) on dry acid sulfate soils (Maki et al., 2008).

OTHER BIOGEOCHEMICAL PROCESSES RELATED TO SULFIDE AFFECTING PLANT GROWTH

The anthropogenically increased availability of sulfate as an electron acceptor in anaerobic freshwater wetland soils potentially results in eutrophication (Lamers et al., 1998). This is not only caused by increased decomposition and nutrient mineralization rates as a result of the increased availability of sulfate as an electron acceptor, but also by the accumulation of sulfide that lowers phosphate binding to iron oxides and iron hydroxide, thereby increasing phosphate availability in the soil (Ohle, 1954; Sperber, 1958; Caraco et al., 1989; Lamers et al., 1998). Enhanced concentrations of ammonium and phosphate may, however, also result from increased decomposition rates due to greater availability of sulfate as an alternative electron acceptor (Roelofs, 1991; Koerselman et al., 1993; Smolders and Roelofs, 1993; Lamers et al., 1998, 2002b; Zak et al., 2006). For *Thalassia hemprichii*, a seagrass species, it was shown that 80% of its P demand was covered by the activity of sulfate reducers (Holmer et al., 2001). Oxidized sulfur may also be recycled and re-reduced in anaerobic parts of the soil, stimulating decomposition. Under fluctuating oxygen conditions, e.g., in riparian wetlands, reduction and oxidation will therefore alternate (Lucassen et al., 2005).

Sulfate reduction rates can be governed either by the availability of electron donors such as acetate and lactate produced by decomposition of organic matter, or by the availability of

sulfate (Lamers et al., 2002b). If, however, high concentrations of a more favorable electron acceptor are available, sulfate-reducing prokaryotes may be partly or completely outcompeted. Wetlands receiving high nitrate loads through discharge of groundwater originating from arable land and fertilized pastures, show low iron and sulfate reduction rates, with concomitantly low phosphate mobilization rates (Lucassen et al., 2004).

In semi-aquatic plants, sulfide toxicity was found to be less pronounced at a higher nutrient availability, possibly as a result of dilution effects by increased growth and increased ROL (Geurts et al., 2009) suggesting that eutrophication may be "masking" sulfide toxicity in polluted areas. For submerged macrophytes, however, eutrophication is expected to aggravate the effects of sulfide, as increased growth of algae and cyanobacteria will directly impair their photosynthetic rates and ability to oxidize the rhizosphere. Filamentous mats of algae on seagrass meadows, resulting from eutrophication, have also been shown to lead to reduced oxygen concentrations in the sediment and increased S uptake and sulfide toxicity in seagrass (Holmer and Nielsen, 2007).

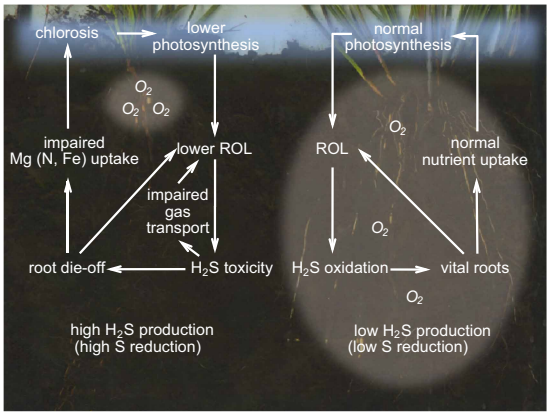

FIGURE 5 | Conceptual model showing positive feedback loops on plant-rhizophere processes under scenarios of sulfide toxicity (left) and non-toxicity (right). See text for further explanation. Unlike in other studies, impaired uptake of N or Fe was not found in this study. Impaired gas transport by sulfide has been shown by Armstrong et al. (1996). Sulfide oxidation is carried out by free-living prokaryotes in sediment and water, symbiotic prokaryotes of roots and soil fauna, and mitochondrial metabolism in soil fauna and plants.

SULFIDE AND INTERSPECIFIC INTERACTIONS: COMPETITION, FACILITATION

Field observations in sulfate-polluted freshwater wetlands suggest that the loss of biodiversity and dominance of a small number of highly competitive plant species may not only be attributed to sulfate-induced eutrophication, but may additionally, or perhaps primarily be triggered by sulfide toxicity (Lamers et al., 2002a). The differential toxicity of hydrogen sulfide provides an additional explanation for changes in competitive strength leading to severe changes in vegetation development in sulfur-loaded wetlands, or in naturally S-rich wetlands that receive higher loads of organic matter. In addition, differences in sulfide accumulation along a gradient may explain vegetation gradients next to salinity effects, e.g., in marshes where *Salicornia* spp. live at the lower, marshes, and high salt marsh species such as *Pucinellia maritime*, *Atriplex patula* and *Festuca rubra* inhabit less sulfidic spots (Ingold and Havill, 1984). Although *Spartina alternifolia* lives at higher marshes than *Salicornia*, the organic content of its sediment is generally higher, potentially leading to higher sulfide accumulation. In freshwater systems, interspecific competition between macrophytes has been shown to depend on the interplay between sulfide and iron in sediments (Van der Welle et al., 2007a). Multiple positive feedback loops therefore increase and stabilize both toxicity and non-toxicity states (**Figure 5**).

The first constraint on the establishment and competitive strength of wetland plants in sulfur-rich areas, naturally or anthropogenically enhanced, may therefore be sulfide toxicity. As a result, plants that are able to provide their root apices with oxygen without losing all oxygen along the root surface, such as a number of larger graminoids, have a strong competitive advantage, especially if high sulfide oxidation rates are sustained by microbial activity in soils and soil macrofauna. If the growth rate is high, the toxicity effects may be "diluted" and ROL is supported by high photosynthetic rates of the highly competitive species. As a result of these feedbacks, eutrophication and sulfide accumulation in concert may rapidly lead to vegetation changes. From their differential responses to sulfide, Li et al. (2009) argued that the undesirable strong expansion of *Typha* in the Florida Everglades, at the expense of *Cladium*, could partially be explained by the high levels of sulfide (250–375 μmol L^{-1}) in this region. These resulted from a combination of high rates of sulfate reduction and low levels of iron to sequester the produced sulfide. For dune slacks it was hypothesized that elevated sulfide concentrations in combination with higher nutrient levels induce a shift to highly productive *Phragmites* stands (Adema et al., 2003). In a recent study on the biogeochemical drivers of species composition in a groundwater-fed freshwater wetland, sulfide appeared to be the most important explaining variable (Simkin et al., 2013). However, in addition to gaining a higher competitive strength, sulfide-detoxifying plant species might also act as ecosystem engineers (sensu Jones et al., 1994) by their facilitation of sulfide-sensitive plants, provided that the latter group is not outcompeted for light by fast-growing species. However, high sulfide levels, in addition to those of other phytotoxins, may have contributed to the large scale *Phragmites* die-back in wetlands loaded with organic compounds (Armstrong and Armstrong, 2001), and to large-scale seagrass die-back events (Carlson et al., 1994; Terrados et al., 1999; Borum et al., 2005). Such massive die-off events have also been shown in constructed wetlands receiving high organic fluxes and showing high sulfide concentrations (above 1000 μmol L^{-1}; Wiessner et al., 2008).

GLOBAL EFFECTS ON WETLANDS

The risks of sulfide toxicity are an important issue at a global scale, as sulfur concentrations have risen in many freshwater waters and wetlands, including natural vegetation types and rice paddies, due to high anthropogenic S emissions (Smith et al., 2011)

and geochemical oxidation processes including the effect of nitrate pollution (Smolders et al., 2010). Although global emissions decreased between 1970 and 2000 due to legislation, they are now increasing significantly again due to the high S emissions of fast-developing regions such as Asia (particularly China) where SO_2 emissions, as a result of the large-scale use of coal as a fuel, may soon equal the combined emissions of North America and Europe (Shah et al., 2000; Smith et al., 2011). In addition, salinization of coastal freshwater wetlands due to the intrusion of saline groundwater or surface water, and salinization due to the increased frequency of drought episodes in more arid regions increase the risk of sulfide-related vegetation changes during anaerobiosis. To determine the exact causes of salinization on vegetation changes, it is, however, important to experimentally test the effects of sulfide and NaCl separately and in concert. In saline systems (in which sulfate is normally not limiting), increased organic loads will stimulate sulfate reduction rates and lead to higher sulfide levels, especially if temperatures become higher (in shallow waters) as a result of global change (Hoffle et al., 2011; Holmer et al., 2011). Accumulated FeS_x in riparian wetlands will massively become oxidized to sulfate during drought (Lucassen et al., 2002), which is prone to renewed reduction during flooding. Even in soils that had not been flooded for more than 10 years, an unexpected diversity of sulfate reducers still appeared to be present and become active after one or two weeks of anaerobiosis (Lamers et al., 1998; Miletto et al., 2008). This shows that the microbial community is very persistent with respect to S biogeochemistry, and able to resuscitate although they have to be classified as "delayed responders" (sensu Placella et al., 2012). As a result, the S legacy of a soil is expected to contribute to sudden die-off of plants in riparian wetlands during anaerobic events.

GENERAL CONCLUSION

As our overview shows that even low concentrations of sulfide are able to 1) affect the ecophysiological functioning individual plants, 2) affect plant competition and facilitation, 3) influence complex rhizospheric mutualisms, and 4) interact with nutrient biogeochemistry, it is clear that sulfide can be a strong driver of ecosystem processes and functioning, also in relation to changing global S balances. Future research should include interactions between plants, microbial communities, soil fauna and soil chemistry, to fully understand and explain differences among plant, vegetation and ecosystem responses to sulfide.

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Tolerance of anaerobic conditions caused by flooding during germination and early growth in rice (*Oryza sativa* L.)

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Rice is semi-aquatic, adapted to a wide range of hydrologies, from aerobic soils in uplands to anaerobic and flooded fields in waterlogged lowlands, to even deeply submerged soils in flood-prone areas. Considerable diversity is present in native rice landraces selected by farmers over centuries. Our understanding of the adaptive features of these landraces to native ecosystems has improved considerably over the recent past. In some cases, major genes associated with tolerance have been cloned, such as *SUB1A* that confers tolerance of complete submergence and *SNORKEL* genes that control plant elongation to escape deepwater. Modern rice varieties are sensitive to flooding during germination and early growth, a problem commonly encountered in rainfed areas, but few landraces capable of germination under these conditions have recently been identified, enabling research into tolerance mechanisms. Major QTLs were also identified, and are being targeted for molecular breeding and for cloning. Nevertheless, limited progress has been made in identifying regulatory processes for traits that are unique to tolerant genotypes, including faster germination and coleoptile elongation, formation of roots and leaves under hypoxia, ability to catabolize starch into simple sugars for subsequent use in glycolysis and fermentative pathways to generate energy. Here we discuss the state of knowledge on the role of the PDC-ALDH-ACS bypass and the ALDH enzyme as the likely candidates effective in tolerant rice genotypes. Potential involvement of factors such as cytoplasmic pH regulation, phytohormones, reactive oxygen species scavenging and other metabolites is also discussed. Further characterization of contrasting genotypes would help in elucidating the genetic and biochemical regulatory and signaling mechanisms associated with tolerance. This could facilitate breeding rice varieties suitable for direct seeding systems and guide efforts for improving waterlogging tolerance in other crops.

Keywords: anaerobic germination, alcoholic fermentation, ALDH, pyruvate dehydrogenase bypass, hypoxia, direct seeding, flooding, submergence tolerance

INTRODUCTION

Waterlogging and floods cause considerable yield losses of major food crops worldwide. For dryland cereals such as maize, wheat, rye and barley, yield losses in rainfed and irrigated areas can reach 20% in some regions (Setter and Waters, 2003). Among cereal crops, rice is unique in being capable of growing well in waterlogged and submerged soils because of its well-developed aerenchyma system that facilitates aeration of the roots and the rhizosphere, thus alleviating most of the stresses experienced under low oxygen (Setter et al., 1997; Jackson and Ram, 2003). However, this escape mechanism is inadequate when floods are partially covering the plants for longer durations of a few weeks to months, as in stagnant, semi-deep (25–50 cm) and deepwater areas (over 50 cm to several meters), or when floods are transient but cause complete inundation for shorter durations of up to 2 weeks as in flash-flood-affected areas. These types of floods are common in rainfed lowlands and flood-prone areas worldwide, and cause considerable losses in grain production each year (Singh et al., 2009, 2011; Mackill et al., 2012; Ismail et al., 2013; Colmer et al., 2014). Floods are therefore considered major challenges for rice production, especially in South and Southeast Asia, where the majority of the world's rice farmers live and depend on rice and rice-based farming as their major source of food, income and livelihood. Moreover, the impacts of these floods seem to be worsening in recent years, due to effects commonly attributed to climate change, such as sea-level rise, the uneven distribution of rains and periodic changes in frequencies and intensities of floods caused by extreme weather events (Coumou and Rahmstorf, 2012). Nonetheless, the enormous diversity and plasticity in adaptation to contrasting hydrological conditions, ranging from aerated soils in uplands to areas with water depths exceeding 5 m in deepwater areas, has made rice one of the most amenable crops for genetic manipulation to develop varieties suitable for excess water conditions (Ismail and Mackill, 2013; Kirk et al., 2014). Several landraces that can withstand different types of floods have been identified and characterized (**Table 1**). Some of these landraces have subsequently been used to breed high yielding modern varieties (Mackill et al., 2012; Ismail et al., 2013).

Table 1 | Examples of rice genotypes identified as tolerant or sensitive of different types of floods and main traits associated with tolerance.

Developmental stage	Type of flood	Tolerant varieties*	Sensitive varieties*	Main traits associated with tolerance	Major QTLs/Genes	References
Germination and early seedling growth	Flash floods/submergence Waterlogging	Khao Hlan On Ma-Zhan (Red) Khaiyan Kalonchi Kharsu Nanhi	IR42 IR64 FR13A	– Fast germination and coleoptile elongation – Ability to break down and use starch under low O_2 – High anaerobic respiration to avoid energy crises when oxygen is low	*qAG7.1* *qAG-9-2*	Ismail et al., 2009, 2012; Angaji et al., 2010; Septiningsih et al., 2013
Vegetative stage	Flash flood Complete submergence	FR13A FR43B Kurkaruppan Goda Heenati Thavalu	IR42 IR64	– Reduced elongation – Slow carbohydrate consumption during submergence – Underwater photosynthesis – Chlorophyll retention underwater – Fast recovery	*SUB1A-1*	Jackson and Ram, 2003; Sarkar et al., 2006; Xu et al., 2006; Mackill et al., 2012; Winkel et al., 2012; Ismail et al., 2013
	Stagnant, medium-deep (30–50 cm), longer duration (weeks to months)	IRRI 119 IRRI 154	Swarna IR64, most modern varieties	– Partial, slow elongation of the shoot – High tillering ability underwater – Strong culms, resistant to lodging – Sufficient leaf area above water – Large fertile panicles	Not genetically characterized	Singh et al., 2011
	Deep-water (>50 cm to > 5 m)	Jalmagna Baisbish Rayada 16-3 Nang Dum To Sudu Gries	All modern lowland varieties	– Fast internode elongation with rising water (> 20 cm per day) – Sufficient leaf area above water – Kneeing ability when water recedes – Large fertile panicles	*SNORKEL1* *SNORKEL2*	Catling, 1992; Hattori et al., 2007, 2009

**Tolerant and sensitive varieties in the table are representative examples. Modified from Ismail and Mackill (2013).*

Notwithstanding its tolerance of waterlogging and shallow floods during the vegetative stage, rice is extremely sensitive to anaerobic conditions during germination (anaerobic germination) and early growth of the embryo (Yamauchi et al., 1993; Ismail et al., 2009; Angaji et al., 2010). Rice seeds can germinate, and to some degree, extend their coleoptiles under hypoxic and even anoxic conditions, but fail to develop roots and leaves (Taylor, 1942; Biswas and Yamauchi, 1997; Ella and Setter, 1999). Tolerance of anaerobic conditions at these early stages is a prerequisite for effective direct-seeded rice in rainfed and flood-affected areas. Soil waterlogging or flooding can be encountered when it rains immediately after seeding or when the land is not well-levelled in irrigated areas (Kirk et al., 2014). In either case severe reductions in or failure of crop establishment can be experienced (Ismail et al., 2012). Varieties that can germinate in flooded soils could be beneficial for direct-seeded systems in these areas and even for intensive irrigated systems, where early flooding can suppress weeds (Ismail et al., 2012). This will consequently result in enormous savings in production costs as opposed to when rice is transplanted. It can also reduce the cost of manual or mechanical weeding or the use of hazardous chemicals for weed control.

Considerable progress has been made in understanding the genetics and physiology of tolerance of flooding during vegetative stage in rice and also during germination as compared with other crops, and this has been comprehensively reviewed over the recent past (e.g., Jackson and Ram, 2003; Magneschi and Perata, 2009; Bailey-Serres and Voesenek, 2010; Bailey-Serres et al., 2010, 2012; Ismail et al., 2012, 2013; Colmer et al., 2014). Genetic variation in the ability to germinate and establish in flooded soils has recently been observed in rice, and a few landraces were identified that are tolerant (Angaji et al., 2010). Here, we attempt to review some of the main physiological and molecular mechanisms studied over the past few decades that can likely explain this genetic variability in tolerance of flooding during germination within rice. We focus on alternative pathways and genes that are suggested to play a role in situations when energy crises arise in germinating seeds or other systems under low oxygen stress. Examples and evidences from studies conducted on rice and other plant species are highlighted in an attempt to recognize the traits and pathways possibly involved in tolerance. We also attempt to pinpoint gaps in knowledge to direct future studies on germination under water. Adequate understanding of the adaptive traits and pathways will help in designing effective breeding strategies to develop tolerant rice varieties for direct-seeding systems, and could guide efforts to improve tolerance of waterlogging in other crop species facing similar challenges.

PLANT RESPONSES TO FLOODING DURING GERMINATION

Several adverse conditions occur in the root zone when plants germinate under water: oxygen becomes scarce hindering root respiration and growth and gases such as CO_2 and ethylene build up. Low oxygen causes a reduction in root growth and function, thus reducing nutrient and water uptake. In addition, several phytotoxic substances such as reduced iron (Fe^{+2}), manganese (Mn^{+2}), hydrogen sulphide (H_2S), and intermediates of anaerobic carbon metabolism—e.g., organic acids—accumulate to toxic concentrations. These alter the soil pH and further affect the availability and uptake of nutrients by the plant. Together, these changes cause injury to roots and the whole plant, and can lead to plant death in severe cases (Drew and Lynch, 1980; Kirk et al., 2014). Even though some genetic diversity in waterlogging tolerance has been found within some crops, as in maize, wheat, barley and rye, this diversity has not been sufficiently exploited through breeding (Drew, 1997; Setter and Waters, 2003).

Plants adapted to waterlogged or submerged conditions during germination and establishment develop different strategies to cope with these adversities. These tactics include morphological adjustments such as mesocotyl root development in *Echinochloa* (Everard et al., 1991), adventitious root development in *Rumex palustris* (Visser et al., 1997), petiole elongation also in *Rumex palustris* (Voesenek et al., 1990) and *Ranunculus sceleratus* (Horton, 1993), and coleoptile elongation in some rice varieties (Pearce and Jackson, 1991; Ismail et al., 2009). The discovery of rice genotypes with better tolerance of flooding during germination facilitated mechanistic studies to decipher some of the traits likely associated with tolerance (Ismail et al., 2009; Angaji et al., 2010; Ella et al., 2010, 2011; Septiningsih et al., 2013). Furthermore, good progress was made in developing breeding lines that are more suitable for direct-seeded systems. Major QTLs (quantitative trait loci) associated with tolerance of flooding during germination have been recently identified and are being targeted for cloning and for use in marker-assisted breeding (Angaji et al., 2010; Septiningsih et al., 2013). Despite this progress, little is known about the molecular basis of tolerance of low oxygen stress in these contrasting rice genotypes.

When flooding occurs just after direct seeding, tolerant rice genotypes germinate faster and their coleoptiles grow at a relatively faster rate to emerge from flooded soils. These genotypes are also capable of forming roots and leaves in shallow water depths (Ismail et al., 2009; Angaji et al., 2010; **Figure 1**). Some progress has been made in unraveling the metabolic processes that are likely associated with tolerance. These include the ability to initiate and maintain carbohydrate catabolism in germinating seeds, anaerobic respiration to sustain energy supply and maintenance of cellular extensibility of the growing embryo. As the seedling elongates to more aerated zones, aerenchyma tissue develops to provide oxygen for the submerged plant parts, especially to roots, through what is frequently referred to as the snorkel effect (Alpi and Beevers, 1983; Kawai and Uchimiya, 2000). Progressive detoxification of oxygen radicals generated in seeds and of other toxins that develop in anoxic soils also helps in preventing further injury (Ismail et al., 2009, 2012; Colmer et al., 2014; Kirk et al., 2014).

COLEOPTILE ELONGATION; AN ESCAPE STRATEGY

Rice can germinate under hypoxic or anoxic conditions, but only tolerant genotypes have the ability of fast coleoptile elongation and root formation under submerged conditions in the field (Ismail et al., 2009) (**Figure 1**). Conversely, the coleoptile growth is slow in sensitive genotypes, and they fail to develop further.

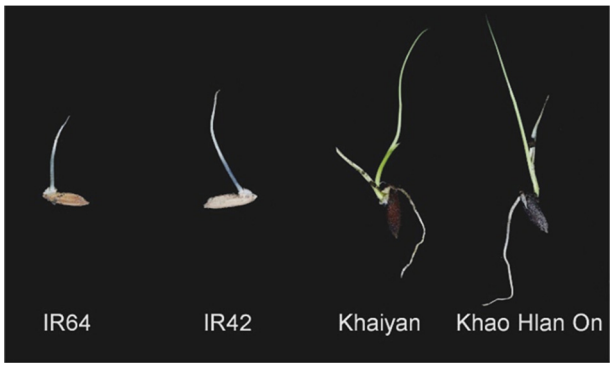

FIGURE 1 | Left to right: seedlings of sensitive varieties "IR64" and "IR42" and tolerant landraces "Khao Hlan On" and "Khaiyan" 7 days after sowing in soil and flooding with 10 cm of water (from image collection of the International Rice Research Institute (IRRI) publicly available at Flickr http://www.flickr.com/photos/ricephotos/21995 44108/in/photostream/).

Coleoptile elongation is usually targeted for selection of tolerant rice genotypes germinating under submergence because it is easy to phenotype. Coleoptile length increases mainly through cell elongation. Cell division is active during the first 48 h of submergence, and that is the period when oxygen is mostly required (Atwell et al., 1982). Since cellular expansion consumes less energy than cell division, the latter is the main process governing elongation under anoxia (Atwell et al., 1982; Magneschi and Perata, 2009). However, no apparent variation in growth has been found between seedlings of tolerant and sensitive genotypes when seeds were pre-soaked or primed through soaking in water and dehydration before sowing and submergence (Ella et al., 2011). Presumably most rice genotypes are capable of initiating germination but cannot elongate further, probably because of bottlenecks in using energy reserves when oxygen is limiting.

Coleoptile elongation is thought to be related to the role of specific expansins under anaerobic conditions for their action in cell wall loosening (Huang et al., 2000; Choi et al., 2003; Ismail et al., 2009; Magneschi and Perata, 2009). Firstly, *EXPA2* and *EXPA4* were found to be expressed in submerged coleoptiles but not in aerobic or anoxic ones (Huang et al., 2000). A later study correlated *EXPA4* with coleoptile and mesocotyl length (Choi et al., 2003). Moreover, *EXPA7* and *EXPB12* were observed to be highly expressed in elongating coleoptiles under anoxia (Lasanthi-Kudahettige et al., 2007). This would agree with previous results that *EXPA2* and *4* are specific to hypoxia and therefore were not detected in the anoxic study by Lasanthi-Kudahettige et al. (2007). Another 3 expansins were also reported to be induced under submergence by Takahashi et al. (2011). These expansins *EXPA1*, *EXPB11,* and *EXPB17* were differentially expressed in the *rad* mutant in comparison with the control, and were affected by deficiency in ADH1. Therefore, these expansins may also be related to elongation under submergence. The importance of expansins for coleoptile extension under submergence seems clearly justified in all these studies. However, all expansins seem equally important and their activity may depend on the kind of stress or genotype being studied.

Another group of enzymes known to be associated with cell wall extensibility are peroxidases (EC 1.11.1), whose activities reduce cell extensibility (Ismail et al., 2009 and citations therein). The association of increased activities of peroxidases with reduction in elongation has been reported in some species, as in mung bean (Goldberg et al., 1987), peanuts (Zheng and van Huystee, 1992) and rice (Ismail et al., 2009). In the latter, higher peroxidase activity was reported to be negatively correlated with seed germination and seedling survival under submergence, and peroxidase concentration was significantly higher in the sensitive genotypes FR13A and IR42. Peroxidase activities, especially the cell bound forms, are negatively correlated with coleoptile elongation (Ismail et al., 2009). Yet other classes of structural proteins and enzymes were recently implicated. The action of tubulin a-1 chain (TUBA1) and actin depolymerizing factor 4 (ADF4) were suggested to be involved in fast coleoptile elongation under anaerobic conditions, as both genes are upregulated under anoxia during germination in rice (Sadiq et al., 2011). Tubulins are involved in the formation of microtubules and are important for both cell division and elongation, and ADF4 is involved in regulating actin assembly (Mayer and Jürgens, 2002; Augustine et al., 2008; Sadiq et al., 2011); therefore both are speculated to play a role in fast growth of the coleoptiles under low oxygen. However, further studies are required to ascertain the actual roles of these genes in coleoptile growth and tolerance of hypoxia or anoxia.

Phytohormones Involved in Germination and Coleoptile Elongation Under Submergence

Mechanisms of tolerance of complete submergence during vegetative stage are fairly established following the discovery of the *SUB1* genes and their further characterization (Bailey-Serres and Voesenek, 2010; Bailey-Serres et al., 2010). *SUB1A*, an ethylene response factor (ERF; Xu et al., 2006) induces "quiescence" state marked by reduction of elongation and slowing of relevant metabolic processes (Bailey-Serres et al., 2010; Schmitz et al., 2013). In addition to *SUB1A*, some wild rice genotypes belonging to the C-genome group showed a similar tolerance mechanism involving a *SUB1* ortholog with high similarity to *SUB1C* (Niroula et al., 2012). Recently, another two ERFs, *SNORKEL1* and *SNORKEL2,* that control fast elongation under deepwater conditions were cloned and characterized (Hattori et al., 2009). Differential response to the content of the gaseous hormone ethylene thus seem to be an important aspect of submergence tolerance, either through inhibiting elongation or through promoting active growth. Indeed ethylene was shown to be involved in internode elongation in deepwater rice (Metraux and Kende, 1983). Coleoptile elongation was also reported to be regulated by ethylene (Atwell et al., 1982; van der Straeten et al., 2001; Zhou et al., 2002; Jackson, 2008). However, an ethylene-independent anoxia response was proposed (Pearce and Jackson, 1991; Pearce et al., 1992), since ethylene and its precursor 1-aminocyclopropane-1-carboxylic-acid (ACC) would not function without oxygen (reviewed in Bailey-Serres et al., 2010). While coleoptile elongation under submergence does not occur under

anoxic, but rather under hypoxic conditions in the field, the action of ethylene may still be relevant in such conditions. Other studies suggested that the importance of ethylene may be genotype dependent (Dubois et al., 2011). Ismail et al. (2009) argued that the involvement of ethylene in coleoptile elongation under submergence probably occurs at the later stages, after active cell division and when cell expansion is predominant. Ethylene could therefore become mainly involved in coleoptile elongation when they are in contact with relatively more aerated layers of the floodwater (Ismail et al., 2012).

The phytohormones ABA and GA also seem to play important roles in submergence tolerance during germination. A recent *in silico* analysis identified patterns of co-expression of different genes under anoxia (Mohanty et al., 2012). This revealed ABA as a positive regulator of rice germination under submergence and its plausible interaction with ethylene for coleoptile elongation. The authors also speculated that GA may be related to inhibition of the coleoptile response to submergence during germination. These results are an apparent contradiction with the known functions of ABA and GA, which are involved in seed dormancy (Hoffmann-Benning and Kende, 1992) and seed germination, respectively. A QTL for tolerance of anaerobic conditions during germination (*qAG12*, Septiningsih et al., 2013) contains candidate genes that are possibly involved in promoting ABA synthesis during early stages of seed development. However, these genes–containing PIL5 (LOC_Os12g40700) and bHLH (LOC_Os12g40730) motifs, were identified within the QTL *qSD12* for seed dormancy (Gu et al., 2010). Both QTLs are located close to another QTL for GA (*qGAR-12*) and for seedling height (*qSPH-12*; Septiningsih et al., 2013). The authors suggested that the QTL for GA may regulate the QTL for anaerobic germination, since GA could promote coleoptile elongation under submergence according to Horton (1991). They also suggested that alleles governing the QTL for anaerobic germination may be different from those of *qSD12* since an opposite function for ABA is expected. Further research on the *qAG12* will identify the genes governing this QTL and inform on the complex role of plant hormones in anaerobic germination. Whether the hormones exchange roles as suggested by Mohanty et al. (2012) or that different alleles with opposite function govern these QTLs as suggested by Septiningsih et al. (2013) will need to be unraveled. Based on Mohanty et al. (2012), if anaerobic germination is positively regulated by ABA, the genotype Nipponbare they studied should not follow an escape strategy, but rather become quiescent under anoxic conditions. Additional results of Mohanty et al. (2012) suggest that sugar, oxygen and ethylene are part of the same activation cascade and also involve ABA and expansins during germination under flooded conditions. A similar network, which also included CIPK15 was invoked by Bailey-Serres and Voesenek (2010), except for the activation of coleoptile elongation. If the ethylene dependent and independent pathways could be linked at the ABA level and not at the shoot elongation level, then the two networks would be rather similar. However, ABA is linked to seed dormancy, suggesting high levels of the hormone would send the seed in a quiescence mode rather than activating growth of the embryo.

These divergent evidences observed in different studies could probably be partially related to experimental conditions, such as the seeds being tested under different oxygen levels or the use of varieties with variable tolerance. For example, varieties with different coping strategies may have different signaling responses involving antagonistic hormones. Such environmental and/or genetic variability could lead to diverse sets of data that should be integrated to predict the most relevant mechanisms. New studies should bring more insights into the regulatory and signaling pathways involving these hormones in different genetic backgrounds adapted to specific environments, yet contrasting in germination and early growth under submergence. According to Ismail et al. (2009, 2012) the roles of different hormones under submergence are difficult to interpret without a standardized phenotyping strategy that addresses the genetic and environmental variables such as O_2 and CO_2 concentrations, temperature and floodwater conditions. Light regime is also an important aspect of the content and interplay between ABA and GA both of which, despite classical associations with dormancy and germination, respectively, are still not definitively associated with the respective process in all plants.

Oxygen deprivation under submergence is one of the key elements that trigger different coping responses to overcome the stress. Direct oxygen signaling, sugar, calcium and pH status are some of the rudiments thought to play major roles in the signaling cascade during germination under submergence (reviewed in Bailey-Serres et al., 2012). Signaling molecules such as nitric oxide (NO), peroxide and reactive oxygen species are also linked to these processes (Finkelstein, 2010; Liu et al., 2010; Hill, 2012). Clearly, several metabolic processes are involved in the tolerant phenotype in rice, which are coordinated in a manner that facilitates germination and fast growth of the embryo to emerge from flooded soils. Since both ethylene-dependent and -independent mechanisms activate a signaling cascade, whether these are truly two independent mechanisms or they are simultaneously coordinated at a specific level remains to be known. Further work is necessary to untangle the regulatory and signaling mechanisms that facilitate these two processes.

OTHER TRAITS ASSOCIATED WITH ANAEROBIC GERMINATION

SELECTABLE TRAITS

One of the main areas of research in anaerobic germination of rice is the identification of traits indicative of the tolerance phenotype. The main trait used is seedling survival after 21 days of submergence under 10 cm water head. Surviving seedlings are those that emerge from the water by fast germination and coleoptile elongation (Ismail et al., 2009; Angaji et al., 2010). Another important trait is the activity of enzymes such as the α-amylases, anaerobic respiration enzymes and others from the TCA cycle, in the anaerobic pathways. Colorimetric reactions for many enzymes including dehydrogenases and peroxidases have been developed for high-throughput screening, making enzyme analysis a valid screening methodology for anaerobic germination. Moreover, new digital imaging techniques interlinking colorimetric assays

with imaging spectroscopy make the screening much more straightforward.

Seed longevity is also related to anaerobic germination as shown by Ella et al. (2010) and Septiningsih et al. (2013). The first paper reported a decrease in germination under submergence when using older seeds. It also suggested increased lipid peroxidation and decreased superoxide dismutase and catalase activities as the reasons for the reduction in seed viability. Septiningsih et al. (2013) discussed the co-location of QTLs for anaerobic germination with QTLs related to seed ageing traits and suggested a relation between seed dormancy, longevity and germination under submergence. Traits associated with seed aging such as the extent of lipid peroxidation, could potentially be used as markers for indirect selection. Other morphological traits worth the attention are coleoptile diameter, days to emergence of first leaf, and the first leaf width and length. Also root characteristics such as root length, diameter, secondary root development and root hairs may be monitored. These traits may be relevant when studying genotypes with intermediate tolerance or when comparing different groups such as *indica*, *aus*, and temperate and tropical *japonica*. However, their relevance as selectable traits still needs to be assessed especially in relation to survival and/or coleoptile elongation.

ENZYMES RELATED TO ENERGY PRODUCTION UNDER LOW OXYGEN

Limitations of energy supply under oxygen deficient conditions caused by submergence are a major bottleneck for seed germination and seedling survival. The main adjustment is the shift from the aerobic to the anaerobic metabolism. The anaerobic fermentative processes normally generate 2–3 ATP per molecule of glucose, compared with the 36–38 molecules generated through aerobic metabolism (Fox et al., 1994; Greenway and Setter, 1996). It is also known that Pasteur Effect, involving accelerated rates of carbohydrate catabolism can provide up to 37.5% of the ATP generated under aerobic conditions in the coleoptiles of tolerant rice genotypes (Gibbs and Greenway, 2003). Hence, supplementing the energy supply through aerobic metabolism via respiration with that from anaerobic metabolism may be useful under hypoxic conditions for coleoptile growth. Some enzymes active under anoxia can use PPi instead of ATP to adapt to the low-energy environment (Carystinos et al., 1995). This suggestion was supported by the results of Huang et al. (2005a), showing up-regulation of nucleoside diphosphate kinase under anoxia, an enzyme associated with coleoptile elongation under submergence.

Another study implicating enzymes of the anaerobic pathway in rice coleoptile growth under anoxic conditions assessed the genetic variation in ATP production in six different rice genotypes; two tolerant ("Khao Hlan On" and "Khaiyan"; Ismail et al., 2009), two moderately tolerant ("Nipponbare" and "Kinmaze") and two mutant lines (*rad* lacking the ADH gene and a *PDC* insertion mutant). The study found that under both hypoxic and anoxic conditions an ATP production rate of about 10% that of the aerated conditions was maintained in the tolerant and moderately tolerant lines. The *PDC* insertion mutant maintained close to 7% and the *rad* mutant only 3.7% of the ATP produced under normoxia (Edwards et al., 2012).

ENZYMES RELATED TO CYTOPLASMIC ACIDOSIS

At the cellular level, changes in cytoplasmic pH have been considered important for cell integrity and function, energy consumption and the activation or inactivation of enzymes involved in anaerobic metabolism (Magneschi and Perata, 2009). These changes have also been considered an adaptive advantage of varieties tolerant of anoxia vs. sensitive varieties (reviewed in Greenway and Gibbs, 2003). Studies comparing pH changes under anoxia in rice and wheat reported that the cytoplasmic pH quickly recovered from low pH, and is therefore somehow regulated (Menegus et al., 1991; Kulichikhin et al., 2009). Vacuolar pH increased in tolerant rice (Menegus et al., 1991; Kulichikhin et al., 2007, 2009), but not in sensitive wheat roots under submergence (Menegus et al., 1991; Kulichikhin et al., 2007). The usual pH under aerobic conditions is about 5.6 in vacuoles of rice coleoptiles, but it becomes increasingly basic under anoxic conditions, reaching 6.0 after 14 h (Menegus et al., 1991). This pH shift in the vacuole in particular was suggested to favor a reduction in cellular energy consumption by decreasing the pH difference between the cytoplasm and the vacuole (Menegus et al., 1993). This is in agreement with later results suggesting the higher vacuolar pH is a response to a new metabolic state of energy crisis, and it is not related to cell injury under anoxia (Felle, 2005; Kulichikhin et al., 2009). Kulichikhin et al. (2009) observed that tolerant tissues under anoxia maintain a stable pH for up to 4 days. Thus, the ability to maintain the cytoplasmic pH and avoid acidosis may be an important component of tolerance of low oxygen stress. Davies et al. (1993) reported that cytoplasmic acidification as a consequence of anoxia would increase the release of energy through PPi, but would have an opposite effect on ATP synthesis. Both energy and pH maintenance in submerged rice coleoptiles have been shown to be directly related to ethanolic fermentation rates (Gibbs et al., 2000), however, association with genetic variation in germination under anaerobic conditions within rice awaits further studies.

A reduction in cytoplasmic pH has been associated with the lactic acid fermentation pathway, where lactate is produced from pyruvate through the activity of lactate dehydrogenase (LDH, EC 1.1.1.27.). The role of lactate in anoxic rice coleoptiles has been discussed and some studies suggested that it is related to a pH decrease that would then activate alcoholic fermentation as in maize root tips (Roberts et al., 1992). This reaction would generate protons that decrease the cytosolic pH by less than 1 unit. However, this role was later rebutted since lactate concentrations alone could not account for the total pH decrease in either rice coleoptiles (Menegus et al., 1991) or maize root tips (Saint-Ges et al., 1991). The ratio of succinate:lactate was also found to be relevant to the final cytoplasmic acidosis and could differentiate between tolerant and non-tolerant species depending on their ability to produce more succinate than lactate to survive anoxia (Menegus et al., 1989). Further, proton-consuming reactions in rice, such as glutamate decarboxylation could maintain the cytoplasmic pH (Menegus et al., 1989). Glutamate decarboxylation was shown to occur under anoxia in rice (Fan et al., 2003). Felle (2005) and Wang et al. (2007) discussed the role of nitrate as a proton acceptor and thus as a pH regulator. Reggiani et al. (1993, 1995) suggested that nitrate is assimilated in rice seeds

germinating under anaerobic conditions. This hypothesis was supported by the studies of Fan et al. (1997) in rice coleoptiles and recently, Greenway et al. (2012) reported that nitrate is converted to ammonium in anoxic rice coleoptiles, which would maintain the pHstat. However, nitrate is quickly reduced in anaerobic soils and its implication in the internal pH balance of rice under anoxic stress is not clear (Greenway et al., 2012). The authors also attempted to assess if a lower pH would compromise plant survival or if anaerobic metabolism would be a durable supplement as suggested by Huang et al. (2005b). However, they found that the pH decrease was balanced by nitrate reduction to ammonium, organic acid pHstat and decreased permeability coefficient of the plasma membrane for protons. The implications of pH regulation under flooding have been reported in various studies, showing that tolerant species are capable of controlling cytoplasmic and vacuolar pH, and can therefore survive longer periods of anoxia (Felle, 2005; Kulichikhin et al., 2009; Greenway et al., 2012). However, the role of this pH regulation in causing higher tolerance within rice needs to be established in contrasting genotypes. Since different substrates may be involved in cytoplasmic acidosis and pH status regulation in submerged rice coleoptiles, a holistic analysis of all reactions that affect cellular pH under anoxia is necessary.

HAEMOGLOBINS IN THE HB/NO CYCLE

The nitrite oxidation product is nitric oxide (NO), which is linked to haemoglobins (Hb) through the Hb/NO cycle. Hypoxic stress activates Hb, probably through nitrite and oxygen levels that increase NO, which may then reduce metabolic activity in stressed cells (Hill, 2012). The NO and Hb are interlinked under hypoxia or anoxia in rice plants and may provide an alternative pathway for the electron transport chain under limiting oxygen conditions (Igamberdiev and Hill, 2004). Furthermore, the Hb/NO cycle maintains energy levels under anoxia and Hb may activate some signaling cascade through NO and ethylene, which in turn could trigger the development of root aerenchyma (Igamberdiev and Hill, 2004). NO may also act as an electron acceptor under oxygen deprivation, since Hb/NO cycle and glycolysis showed similar ATP production rates (15–17 nmol min^{-1} mg^{-1} protein) and that these were 3–5% of the aerobic ATP generated in the mitochondria (Stoimenova et al., 2007). The authors also proposed that ATP is synthesized from succinate. Both Igamberdiev and Hill (2004) and Stoimenova et al. (2007) compared rice with other species such as maize and barley, but there have been no extensive studies within rice on Hb and NO, particularly looking at contrasting rice genotypes under anoxia or hypoxia.

ADJUSTMENTS OF CARBOHYDRATE METABOLISM UNDER LOW OXYGEN

Carbohydrate metabolism is strongly inhibited when oxygen is limiting, especially the steps involved in the breakdown of starch into simple sugars for use in glycolysis, mainly because most of the enzymes involved are less active under low oxygen. A major reason for this inhibition is the activation of energy conserving steps by changing enzyme activities. However, some of these enzymes are active in rice genotypes that are tolerant of hypoxia during germination; among them are α-amylases (especially *RAmy3D*), sucrose synthase and aldolase, but inhibited in sensitive genotypes (Ismail et al., 2009). The second limiting step causing energy crises under low oxygen stress is the breakdown of pyruvate to generate energy through the TCA cycle. This cycle is inhibited by lack of oxygen, which acts as a terminal electron acceptor. Under such conditions, most plants, including rice, activate alternative pathways to supply substrates and sustain energy generation through glycolysis. Fermentative metabolism or anaerobic respiration in submerged coleoptiles uses alcohol, lactate and alanine fermentation pathways to regenerate NAD^+ required for glycolysis. Among the three main pathways, alcoholic fermentation is considered the most important, since about 92% of the pyruvate generated through glycolysis is directed to ethanol production, and only 7% to lactate and 1% to alanine pathways (Kato-Noguchi, 2006). Alcoholic fermentation is strongly activated in rice during germination under oxygen deprivation, as reflected in the induction of the key enzymes, pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The rest of this review will discuss some of the enzymes involved in carbohydrate metabolism that are differentially activated in contrasting rice genotypes germinating under anaerobic conditions, especially those involved in the two critical steps where oxygen is limiting. Alanine metabolism under anoxia is also relevant in rice, especially as related to glutamine and glutamate synthesis for aminoacid accumulation (Reggiani et al., 2000); however, this will not be discussed in this review.

ROLE OF ANAEROBIC RESPIRATION IN SEEDS GERMINATING UNDER AEROBIC AND ANAEROBIC CONDITIONS

In aerobic (normal) conditions, seeds of cereal crops like wheat and rice, have some hypoxic or even anoxic tissues where respiration and therefore energy production become limiting during germination (van Dongen et al., 2004). Rice seeds germinating in aerobic conditions obtain energy from lipid and carbohydrate catabolism. Lipid mobilization is an important part of seed germination in aerobic conditions. Lipid degradation, β-oxidation, glyoxylate cycle and related enzymes were found in germinating rice seeds after 24 h of imbibitions (He et al., 2011). During carbohydrate catabolism, both respiration and alcoholic fermentation are induced, as observed by transcript analysis of particular enzymes in germinating rice seeds (Howell et al., 2006, 2009). This is supposedly to regulate respiration and avoid internal anoxia, which would jeopardize embryo growth (Zabalza et al., 2009). Alcohol dehydrogenase (*adh*) deficient *rad* mutant accumulated pyruvate, which could accelerate respiration and lead to anoxia and irreparable cell damage. The anaerobic pathway may therefore be needed under aerobic conditions to avoid this internal crisis. Also, during the first hours of germination between 0 and 48 h after imbibition, mitochondria will be developed and respiration begins. Until 48 h after imbibition, the pyruvate dehydrogenase (PDH) complex and the tricarboxylic acid (TCA) cycle enzymes are not fully active, and energy is obtained through an alternative NADH dehydrogenase (Howell et al., 2006). A similar pattern was found for the cytochrome c, suggesting that aerobic respiration does not start until 48 h after

seed imbibition. However, under submergence, limited oxygen poses several bottlenecks in the aerobic carbohydrate metabolism. The two major bottlenecks are degradation of starch and complex carbohydrates into simple sugars and pyruvate catabolism to generate energy.

First bottleneck: degradation of starch and complex carbohydrates to soluble sugars

Continuation of carbohydrate metabolism is essential for seed germination and seedling establishment under submergence as has been highlighted in several studies (e.g., Ella and Setter, 1999; Ismail et al., 2009; Magneschi and Perata, 2009). Seeds with large carbohydrate storage reserves have an adaptive advantage under stress and pre-germinated seeds have an advantage over non-germinated seeds when oxygen is limiting (Ella et al., 2011), suggesting that the early processes during germination, especially cell division, are probably more sensitive to low oxygen stress. The only cereal with the necessary set of enzymes needed to break down starch under submergence is rice (Atwell and Greenway, 1987; Perata et al., 1992, 1993; Ismail et al., 2012). Furthermore, Ismail et al. (2009) observed that tolerant rice genotypes had greater ability to break down starch into soluble sugars than sensitive genotypes and therefore, their coleoptiles could sustain higher elongation rates. They further showed that tolerant "Khaiyan" also sustained higher soluble sugar concentrations in germinating seeds. It was later demonstrated that these tolerant landraces also store relatively more soluble sugars in their endosperm than sensitive genotypes (Ismail et al., 2012).

This initial step in starch catabolism provides soluble sugars for glycolysis and subsequent metabolic processes. Seemingly, this step is the one that mostly hinders the ability of intolerant rice varieties and other cereals such as wheat, to germinate under anaerobic conditions (Ismail et al., 2009; Magneschi and Perata, 2009). Two enzymes are considered important for carbohydrate catabolism in rice seeds germinating under low oxygen: sucrose synthase for the breakdown of sucrose, and α-amylases for starch breakdown. Sucrose synthase is functional in rice under submerged conditions at the same rate as in air, but not in wheat and barley seedlings (Magneschi and Perata, 2009). However, there is no apparent difference in the activity of sucrose synthase in seeds of tolerant and sensitive rice genotypes germinating under submergence (Ismail et al., 2009). Therefore, variation in the activity of this enzyme might not have an adaptive significance. Maintenance of higher activity of α-amylases, on the other hand, has been widely reported as an important step in carbohydrate catabolism under submergence, when oxygen is suboptimal (Guglielminetti et al., 1995; Perata et al., 1997; Hwang et al., 1999), and higher activity was observed in seeds of tolerant genotypes germinating in flooded soils. *RAmy3D* was differentially expressed in the tolerant genotype "Khaiyan" compared with the sensitive IR42. In addition, *RAmy3D* expression correlated positively with coleoptile elongation and seedling survival, especially in tolerant genotypes (Ismail et al., 2009, 2012). Since GA is apparently not produced in germinating seeds under anoxia, the GA activated α-amylase *RAmy1A* was not functional. Instead, *RAmy3D* was active in tolerant genotypes under anoxia and this is probably because its activation is not regulated by GA as for its aerobic counterpart *RAmy1A* (Loreti et al., 2003a). That the expression of *RAmy3D* is GA independent was established in a GA mutant capable of germination under anoxia (Loreti et al., 2003b). Additional studies involving expression analysis using rice coleoptiles growing under anoxia also showed positive correlation between the expression of *RAmy3D* and starch degradation (Lasanthi-Kudahettige et al., 2007). This gene is activated during the first 2 days after submergence (Loreti et al., 2003a; Lasanthi-Kudahettige et al., 2007). Collectively, these studies suggest that this gene is important for tolerance of anaerobic conditions during germination in rice. However, since no *RAmy3D* mutant studies have been published so far, we can only speculate how indispensable the enzyme is for tolerance of submergence during germination.

RAmy3D is a glucose and sucrose sensor that is apparently activated by the protein kinase *SnRK1A* under glucose starvation (Lu et al., 2007). The *SnRK1A* in turn, gets activated by calcineurin B-like protein kinase *CIPK15* (Lee et al., 2009; Kudahettige et al., 2010). Its target, the Calcineurin B-like protein 5 (CBL5) is upregulated both at the protein and mRNA levels under anoxia. CBLs contain the common calcium binding domain motif EF-hand. The up-regulation of CBL5 suggests that it may interact with CIPK15 to activate downstream signaling process involved in germination under anoxic conditions (Sadiq et al., 2011). Park et al. (2010) showed that regulation of α-amylases was disrupted by the inhibition of oxidative phosphorylation. They suggested that the removal of repression of the sugar effect on the transcription of *CIPK1* is required for the activation of *SnRK1A* and in turn, the activation of *RAmy3D* during anaerobic germination of rice. Interestingly, *CIPK15* has been shown to act beyond germination. It can sustain its activity until maturity under partial submergence (Lee et al., 2009). However, its role during complete submergence at the vegetative stage is not yet clear, and could either complement or conflict with the *SUB1A* pathway (Kudahettige et al., 2010).

Second bottleneck: metabolism of pyruvate

Three main pathways were identified for anaerobic catabolism of carbohydrates: alcoholic fermentation, lactate fermentation and alanine fermentation. Alcohol fermentation is by far considered the main alternative pathway under anaerobic conditions (Ricard et al., 1994) as the metabolic reactions occurring via the alcoholic or ethanolic fermentation does not decrease the cell pH (Geigenberger et al., 2003). The alcoholic fermentation pathway will be discussed in more details for the remainder of this review.

The first reaction in the alcoholic fermentation pathway is the conversion of pyruvate generated during glycolysis to acetaldehyde by the enzyme pyruvate decarboxylase (PDC, EC 4.1.1.1.). During this irreversible reaction, a CO_2 molecule is generated. Acetaldehyde is metabolized in one of two ways; conversion to ethanol by alcohol dehydrogenase (ADH, EC 1.1.1.1.) or conversion to acetate by mitochondrial aldehyde dehydrogenase (mALDH, EC 1.2.1.3.). When external oxygen decreases (hypoxia or anoxia), aerobic respiration is inhibited in a coordinated response that decreases the adenylate status, the TCA cycle and

glycolysis (Geigenberger et al., 2003). The response to low oxygen (a drop below 5%) has been found to act in two phases, firstly a pre-adaptation response to maintain energy levels through *pdc1*, *pdc2*, *adh1,* and *aldh1* genes, and secondly detoxification of reactive oxygen species through e.g., catalase, superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, glutathione reductase, and superoxide dismutase (Klok et al., 2002). Expression of ADH is sensitive to oxygen concentrations (van Dongen et al., 2009), and is usually the most up-regulated enzyme of the fermentative pathway. ADH quickly metabolizes acetaldehyde to reduce the risk of acetaldehyde-mediated cytotoxicity, and to generate NAD^+ for glycolysis. Ismail et al. (2009) observed that the activity of ADH reached 100 times that of ALDH and about 10 times that of PDC in the tolerant genotype "Khao Hlan On" compared with the sensitive "IR42" (**Table 2**; Ismail et al., 2009; A.M. Ismail, unpublished).

Most reactions of the ethanolic fermentation seem to be substrate regulated to avoid buildup of toxic products, and also to regulate energy production and consumption (Gibbs and Greenway, 2003). For example, depletion of acetaldehyde through the two pathways of ADH and ALDH can swing the ADH reaction in the opposite direction to generate acetaldehyde, the replenishment of which can regulate the activity of PDC. Thus, ADH can indirectly regulate the pyruvate dehydrogenase (PDH) bypass under submergence by substrate regulation of acetaldehyde. Mitochondrial ALDH is activated instead of the cytoplasmic ALDH to avoid competition for NAD^+ with glycolytic enzymes, as the cell stress regulation mechanisms involving energy saving are activated under submerged conditions (Nakazono et al., 2000). The mitochondria sequestered ALDH is not substrate inhibited.

A desaturation mechanism involving pyruvate consumption was invoked to justify the up-regulation of PDC and ADH (Ismail et al., 2009). Similarly the ADH activity was induced in germinating seeds of other cereals, but the highest induction was reported in rice (Guglielminetti et al., 2001; Shingaki-Wells et al., 2011). In anoxia-sensitive maize and pea the ADH activity increase was mostly in roots, whereas in tolerant species like rice and *Echinochloa* it increased in coleoptiles (Cobb and Kennedy, 1987). However, this trend was not found in barley (Kato-Noguchi, 1999).

Plants mutated for or overexpressing ADH and/or PDC can be used to more clearly establish the role of alcohol fermentation during hypoxia or anoxia. For example, the rice cultivar "Taipei 309" over-expressing PDC had higher tolerance of anoxia due to an increase in alcohol metabolism (Quimio et al., 2000). However, over-expression of both PDC and ADH in the same cultivar did not improve anoxia tolerance, probably because of acetaldehyde accumulation (Rahman et al., 2001). Other studies with mutations in *adh* (Matsumura et al., 1995, 1998; Conley et al., 1999; Takahashi et al., 2011; Tougou et al., 2012) suggest that lack of ADH1 creates an energy deficit that affects seed germination, inducing responses similar to that of germination under submergence. Takahashi et al. (2011) concluded that the shortage of energy in the *rad* mutant when germinated under complete submergence affected both cell division and cell extension.

The formation of acetyl CoA and the PDH bypass. The most favorable pathway in anaerobic metabolism is probably the production of acetate to remove toxic acetaldehyde and to recycle carbon for use in other pathways such the glyoxylate cycle, and to feed TCA cycle intermediates. Acetate could then accumulate or be converted into TCA cycle intermediates like malate or citrate (Yamashita and Fujiwara, 1966). Under aerobic conditions, pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase enzymatic complex. However, the first acting enzyme of the complex is not functional under anaerobic conditions, preventing direct production of acetyl-CoA from pyruvate. Thus, an alternative pathway has been suggested to involve the conversion of pyruvate to acetyl-CoA by PDC, mALDH and acetyl-CoA synthase (ACS, EC 6.2.1.1). When oxygen levels decrease NADH accumulates, the pyruvate dehydrogenase complex unit is subsequently inhibited (Yamamoto, 1966) and pyruvate does not feed the TCA cycle, since the complex is feedback regulated by NADH (Randall and Miernyk, 1990). The unit of the complex being inhibited is E_1—pyruvate dehydrogenase (PDH) (Howell et al., 2009). This resulted in reduction of energy production starting after about 2 h of submergence (Vigeolas et al., 2003).

The conversion of acetaldehyde to acetyl-CoA by ALDH and ACS results in the consumption of one NAD^+, one CoA and one ATP molecule, generating a CO_2 molecule, NADH, AMP and PPi. This 3 step alternative pathway results in a loss of two carbon molecules in the anaerobic reaction compared with the 1 step aerobic reaction of the pyruvate dehydrogenase complex. In *Echinochloa* coleoptiles, it has been shown that lipid synthesis and accumulation continue under anoxic conditions (Kennedy et al., 1991). That could be explained by the continuation of fatty acid synthesis through the PDC-ALDH-ACS pathway (**Figure 2**). These authors also found that the mitochondria maintain their integrity and functionality under anoxia. This hypothesis of the PDC-ALDH-ACS pathway has been suggested to be operational in plants as in tobacco (Tadege et al., 1997, 1999), rice (Lu et al., 2005) and *Arabidopsis* (Wei et al., 2009). The pathway was well-studied in yeast, *Saccharomyces cerevisiae* (Klein and Jahnke, 1979; Pronk et al., 1994; van den Berg and de Steensma, 1995). Lu et al. (2005) submerged 10-day-old seedlings of an *indica* variety "Guangluai 4" for durations of 12 h to 3 days, and found an increase in ACS activity in submerged seedlings, confirming the conversion of acetate into acetyl-CoA under submergence. Moreover, they reported an increased expression of *ALDH2a* during the first hours of submergence and that of *ALDH2b* after 2 days of submergence.

Other alternatives for acetyl CoA production may arise from the nitrogen metabolism of ketogenic aminoacids like leucine (Leu), isoleucine (Ile), tyrosine (Tyr), phenylalanine (Phe) and tryptophane (Trp) (**Figure 2**). Accumulation of Leu has been reported to increase under anaerobic conditions in rice (Fan et al., 1997; Narsai et al., 2009; Shingaki-Wells et al., 2011), providing the needed substrate for acetyl CoA production under starved conditions by bypassing the steps of the TCA cycle. Reports on other aminoacids like Tyr, Phe and Trp were inconsistent, and showed either an increase or decrease in different studies. For example, Narsai et al. (2009) reported an increase in Phe and Tyr, whereas Shingaki-Wells et al. (2011) found a decrease in Tyr and

Table 2 | Activities (units min^{-1} mg^{-1} protein) of alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) enzymes in the tolerant "Khao Hlan On" and the sensitive "IR42" from day 0 (dry seeds) to day 10 under aerobic conditions and under 10 cm of submergence (hypoxia) in darkness.

Day	ADH activity				PDC activity			
	Khao Hlan On		IR42		Khao Hlan On		IR42	
	Hypoxia	Aerobic	Hypoxia	Aerobic	Hypoxia	Aerobic	Hypoxia	Aerobic
0	5	5.0	4.0	4.0	0.5	0.50	0.40	0.40
1	14	6.0	5.0	5.0	1.3	0.70	0.70	0.70
2	18	5.0	6.0	5.0	2.0	0.60	1.00	0.60
3	20	5.0	7.0	4.0	2.2	0.50	0.90	0.50
4	21	4.0	8.0	4.0	2.5	0.55	1.00	0.50
5	23	3.0	8.5	3.0	3.0	0.40	0.90	0.40
6	25	3.0	10.0	3.0	2.7	0.40	0.80	0.40
7	24	2.5	9.0	2.5	2.2	0.30	0.85	0.30
8	23	2.0	9.0	2.0	2.2	0.30	0.80	0.30
9	24	1.0	9.5	2.0	2.3	0.35	1.00	0.35
10	24	1.0	10.0	1.5	2.2	0.40	1.00	0.30

Data adapted from Ismail et al. (2009).

no changes in Phe, but also found an increase in Trp, which was not observed in the Narsai et al. (2009) study.

The importance of ALDH in anaerobic germination. Several studies have linked increase in ALDH activity to tolerance of submergence in rice (Nakazono et al., 2000; Tsuji et al., 2003). These studies reported that the mitochondrial ALDH family in particular had the most significant increase in activity in varieties able to germinate under anaerobic conditions (Nakazono et al., 2000; Tsuji et al., 2003). These studies also concluded that *ALDH2a* is more responsive to anaerobiosis and could therefore play a more important role in detoxifying acetaldehyde (Meguro et al., 2006). Similar observations were recently made in a proteomics study by Sadiq et al. (2011) using seeds of the variety "Arborio" imbibed prior to submergence. Lasanthi-Kudahettige et al. (2007) found that *ALDH2a* (*Os02g49720*) was up-regulated by 11-fold and *ALDH2b* (*Os06g15990*) was down-regulated by 22 fold in Nipponbare seeds subjected to anoxia since imbibition. A study by Lu et al. (2005) using the *Oryza sativa* indica cultivar "Guangluai 4" found that the expression of *ALDH2a* was activated between 12 and 72 h after the initiation of anoxia, whereas *ALDH2b* was expressed 48 h after the start of the treatment. Still in another study, Wei et al. (2009) tested *Arabidopsis* single, double and triple mutants for the three family 2 ALDH genes with ^{14}C-ethanol. They developed *Arabidopsis* mutants each lacking one gene (cytosolic *aldh2C4* alleles 1 and 2; mitochondrial *aldh2B4* alleles 1 and 2 and *aldh2B7*) and their possible combinations, and subjected the mutants and wild types to different stress treatments, including 6 h of hypoxia. They found no differences between the mutants for *aldh2C4* and the wild type, suggesting that the cytosolic enzymes are not relevant for the PDC-ALDH-ACS pathway. However, they found lower ^{14}C-ethanol incorporation in the mutants lacking ALDH activity, thus suggesting that the PDH bypass cycle functions in seedlings of *Arabidopsis* when the PDH enzyme is not functional. Among the three ALDH genes, they found that the main ALDH involved in the PDH bypass is *ALDH2B4*, since the ^{14}C-ethanol incorporation rate was the lowest in the *aldh2B4* mutant. They did not, however, find any significant phenotype with the different ALDH mutants (Wei et al., 2009).

Differences in gene expression between the varieties used in these studies may be due to various reasons as shown in Dubois et al. (2011). For example, the level of tolerance may be variable between varieties, even though they have a common phenotype (coleoptile elongation under submergence). Some varieties are either moderately tolerant or sensitive of anoxia during germination; however, they have the ability to extend their coleoptiles in hypoxic conditions (A. M. Ismail, personal communication). Moreover, some varieties showed moderate elongation of their coleoptiles under hypoxia or anoxia imposed prior to seed imbibition and these genotypes may use different survival strategies than that observed in "Khao Hlan On," "Khaiyan" and other tolerant genotypes. Also sensitive varieties may have different responses at the molecular level depending on their strategy, i.e., escape or quiescence. For example in a study with the two tolerant rice genotypes, "Khao Hlan On" and "Khaiyan," and two sensitive genotypes, "FR13A" and "IR42," dry seeds were germinated in 10 cm of water in darkness. Increased activity of both PDC and ADH was observed in all genotypes under submerged (hypoxic) conditions. However, PDC and ADH activities were substantially higher in the tolerant genotypes than in the sensitive ones under submerged conditions (Ismail et al., 2009; **Table 2**). A similar trend was noticed for ALDH2 (A.M. Ismail, pers. communication). This higher activity in the tolerant genotypes under submerged conditions would probably indicate that tolerance of hypoxic conditions during germination is related to the capacity of the plant to metabolize acetaldehyde and recycle carbon. It has previously been reported that alcoholic fermentation toxicity

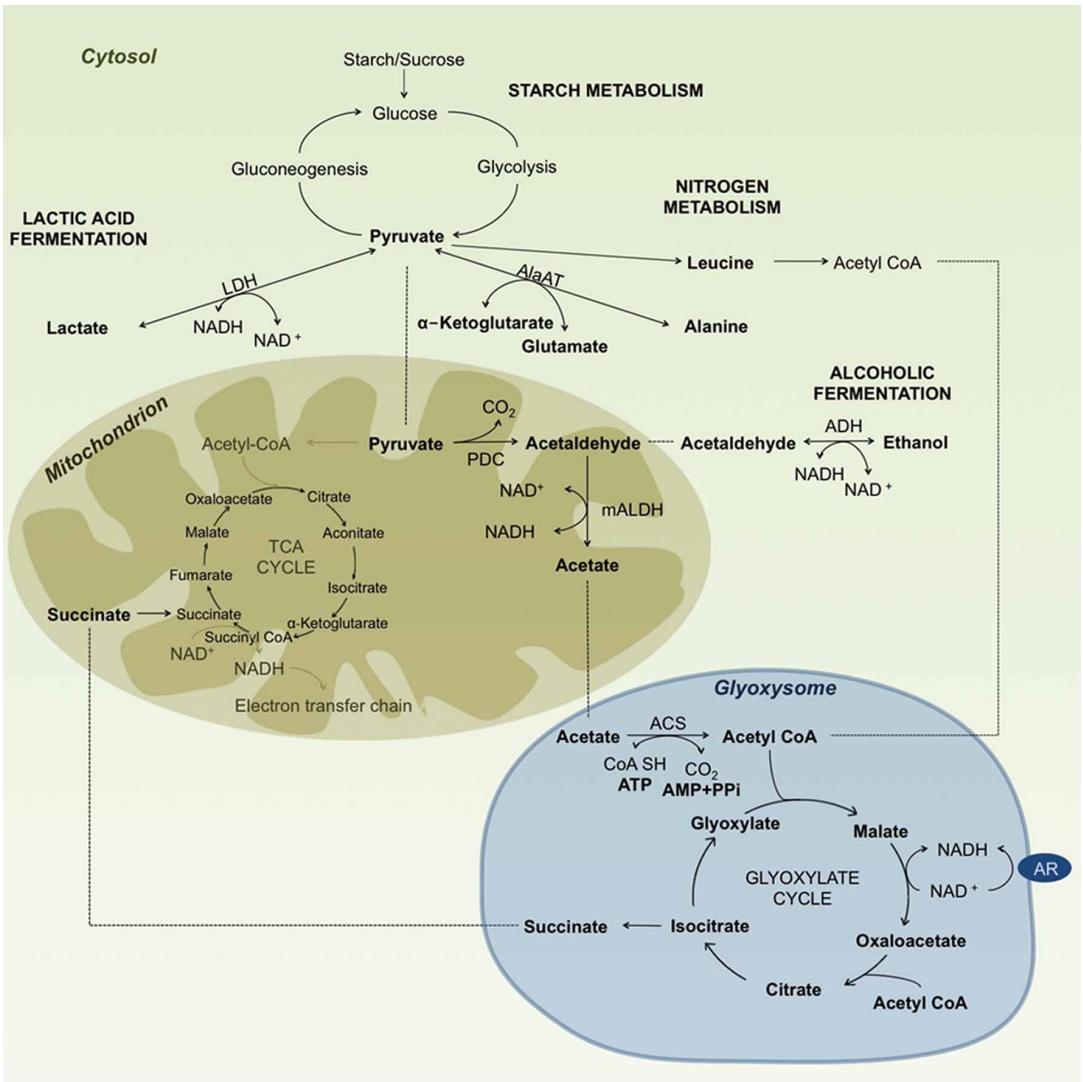

FIGURE 2 | Diagram summarizing carbohydrate metabolism under anoxic/hypoxic conditions in rice. Enzyme abbreviations: AlaAT, alanine aminotransferase (EC 2.6.1.2); LDH, lactate dehydrogenase (EC 1.1.1.27); PDC, pyruvate decarboxylase (EC 4.1.1.1); ADH, alcohol dehydrogenase (EC 1.1.1.1); mALDH, mitochondrial aldehyde dehydrogenase (EC 1.2.1.3); ACS, acetyl-CoA synthetase (EC 6.2.1.1); AR, ascorbate-free radical reductase (EC 1.6.5.4). ·····indicates substrate movement and—indicates substrate conversion/modification. Reaction substrates are highlighted in bold letters.

is not due to the ethanol accumulation per se, but rather to the accumulation of acetaldehyde (Perata and Alpi, 1993, in carrot).

The role of ALDH in detoxifying reactive oxygen species (ROS) has also been suggested in animal systems (Ohsawa et al., 2003; Chen et al., 2007; Xue et al., 2012). These studies found a relationship between ROS accumulation and reduced activity of ALDH2, and vice versa, reduced ROS with increasing ALDH2 activity. They also found that ALDH2 is ethanol activated, leading to the activation of a particular NO synthase in humans. This action happens by reducing ROS accumulation (Xue et al., 2012). Moreover, Chen et al. (2007) suggested that oxidative stress inhibits ALDH2 either by oxidizing the enzyme or its cofactors. In plants, there are some enzymes known to use fermentation products to produce ROS—xanthine oxidase can use acetaldehyde as an electron donor and generate O_2^- and H_2O_2 (Harrison, 2002). Therefore, the direct action of ALDH may not detoxify ROS, but reduction in its production by metabolizing acetaldehyde may regulate ROS content. Additionally, ALDH could inhibit enzymes related to lipid peroxidation and scavenging of ROS (Kotchoni et al., 2006). Since ALDH activity is much higher in "Khao Hlan On" under submergence while ROS concentrations are lower in the same genotype, we hypothesize that ALDH has a detoxifying function for both acetaldehyde and ROS. More research is under way to provide further evidences for the role of ALDH in these processes.

Experiments at the protein level using cultivar "Arborio" (Sadiq et al., 2011) showed the expression of both ALDH2a and ALDH2b under submerged conditions. Different trends were

observed for "Khao Hlan On" and "IR42." "Khao Hlan On" maintained the same levels of both ALDH2a and ALDH2b under submergence, while "IR42" had almost negligible amounts of either protein after day 5 (A.M. Ismail, personal communication). Both rice ALDH2a and ALDH2b and *Arabidopsis* ALDH2B4 and ALDH2B7 were classified very closely in group 2 in a phylogenetic analysis by Kotchoni et al. (2010). This classification showed that rice ALDH2a (OsALDH2B5) and ALDH2b (OsALDH2B1) had similar evolution pathway as that of *Arabidopsis* ALDH2B4 and ALDH2B7. Wei et al. (2009) found that ALDH2B4 is more relevant to the PDH bypass than ALDH2B7. Apparently, further studies are needed to affirm the role of these enzymes in tolerant rice genotypes.

Regulation of alcohol fermentation and its enzymes. The up-regulation of alcoholic fermentation under submergence could be associated with a decrease in oxygen (Nakazono et al., 2000; Ismail et al., 2012) or in the energy levels (Zabalza et al., 2009). It has also been suggested that *Adh1* and *Pdc1* genes are regulated by the concentrations of cytosolic calcium induced by low oxygen conditions (in maize, Subbaiah et al., 1994; in rice, Nakazono et al., 2000; Tsuji et al., 2000). However, the latter authors found that ALDH2a does not share the same mechanism, and there might be two signaling mechanisms under submergence: oxygen dependent and an oxygen independent one. Another hypothesis suggested that the activation of alcoholic fermentation is due to higher pyruvate concentration (discussed in Bailey-Serres and Voesenek, 2008). Based on Roberts et al. (1984) the activation of these enzymes in maize is caused by a decrease in cytoplasmic pH. The regulation of PDC and ADH activities by cellular pH under anoxia was also hypothesized earlier for rice (Davies, 1980), but it was later suggested that alcoholic metabolism is most likely regulated by pyruvate concentration than by cytosolic pH (Kato-Noguchi, 2006). Zabalza et al. (2009) also suggested that high pyruvate concentration activates alcoholic fermentation. They also argued that ADH expression is probably regulated by low oxygen, but later modified by the energy status of the tissue where it is expressed.

Some enzymes have isozyme forms in different organelles and compartmentalization of these isozymes facilitates continuation of some necessary processes in a sequestered manner. For example, the cytosolic enzyme ALDH1 is inhibited under submergence, whereas the mitochondrial enzyme ALDH2 is activated. Carystinos et al. (1995) suggested that the enzyme under stress is less effective than the enzyme active in the absence of stress, because of less efficient translation, less enzyme stability or lower activity. Results similar to those observed in rice showing higher up-regulation of the enzymes involved in alcoholic fermentation in the tolerant genotype were also reported in tolerant *Echinochloa formosensis*, where both PDC and ALDH were activated at much higher rates than in the sensitive *E. praticola* under submergence (Fukao et al., 2003). We recently observed that the 2 isozymes of ALDH are apparently co-regulated by the same activation/deactivation factor at least at the protein level (A.M. Ismail, personal communication), which seems to contradict results from Tsuji et al. (2003) and Sadiq et al. (2011), who suggested that different mechanisms are probably involved in the regulation of ALDH2a vs. ALDH2b, since each enzyme is translated at a different time point. Higher concentration of ALDH2 under submergence suggests this enzyme is probably involved in the tolerance of rice genotypes of submergence during germination and early seedling growth, by catalyzing acetaldehyde detoxification, but further studies will be carried out to affirm this conclusion.

Another important regulatory mechanism relates to substrate concentration and saturation of the different enzymatic reactions involved (Tadege et al., 1999). The higher activities of ADH and PDC observed in the tolerant rice genotypes "Khaiyan" and "Khao Hlan On" than in the sensitive genotypes (Ismail et al., 2009), could possibly be explained by the ability of these tolerant genotypes to metabolize acetaldehyde to acetate, and thus extend the saturation point for acetaldehyde accumulation, which would otherwise be cytotoxic. Besides, tolerant genotypes might have evolved efficient mechanisms to either remobilize or sequester acetaldehyde to avoid both saturation and toxic build-up in the cell cytoplasm. It is also noteworthy mentioning a possible role of ALDH in reducing the ethanol accumulation in cells to avoid post-anoxic damage upon re-aeration. This could involve reactions of ethanol mediated by catalases forming hydrogen peroxide and also oxidation to acetate. As depicted in Meguro et al. (2006), ethanol accumulated in cells during submergence is oxidized to acetaldehyde or hydrogen peroxide when oxygen becomes available upon shifting to aerobic conditions.

Other bottlenecks: the tricarboxylic acid cycle and beyond

Some enzymatic changes also occur in the tricarboxylic acid (TCA) cycle leading to important changes favoring plant survival of anaerobic conditions (Fox and Kennedy, 1991; Fox et al., 1994; Fan et al., 2003; Howell et al., 2006; Nakamura et al., 2012). Fox and Kennedy (1991) reported that 2-oxoglutarate dehydrogenase activity decreased under anoxia and the conversion of 2-oxoglutarate to succinate was identified as the limiting step in the TCA cycle under these conditions. They also found that enzyme activities generally declined after 7 days of anoxia. Conversely, a recent study involving metabolic profiling of rice and wheat coleoptiles under anoxia reported higher concentrations of succinate and also citrate, aconitate, and fumarate under anaerobiosis (Shingaki-Wells et al., 2011). These authors further observed synthesis of the amino acids lysine, methionine, threonine and isoleucine, indicating that the TCA cycle is probably functioning to a certain extent. On the other hand, Wei et al. (2009) suggested that acetyl-CoA is likely converted into fatty acids in the plastid in *Arabidopsis*.

Based on Lu et al. (2005), acetyl-CoA enters the glyoxylate cycle in rice coleoptiles under anaerobic conditions. This cycle is a short version of the TCA cycle that takes place in the glyoxysome, where isocitrate is converted to glyoxylate by isocitrate lyase and glyoxylate to malate by malate synthase. By the action of these two enzymes, the cycle omits two steps of the TCA cycle: from isocitrate to α-ketoglutarate, and then to succinyl-CoA, which is then converted to succinate. By skipping these two steps, the glyoxylate cycle avoids the loss of two carbon molecules, thus giving a net of 4-carbon malate or oxaloacetate (**Figure 2**). To

prove these processes, the authors analyzed mRNA transcripts of the different enzymes involved, and they reported higher expression of isocitrate lyase and malate synthase under submergence. They then concluded that this is the pathway that rice seedlings use to keep their metabolism ongoing under submerged conditions (Lu et al., 2005). Fan et al. (2003) found evidence that the TCA cycle is maintained at least up to the α-oxoglutarate step in rice. However, analyzing the rate of malate production, they also found that malate was mainly being produced by the glyoxylate cycle. Malate could be converted to succinate in this cycle, which could then enter the mitochondrion. Fan et al. (2003) tested pre-germinated seeds of the japonica rice variety "M201" after submergence in darkness. They then followed the metabolic reactions taking place under hypoxia *in vivo* with ^{13}C nuclear magnetic resonance. In this scenario, both cycles maintain carbohydrate catabolism and also support protein biosynthesis. A similar hypothesis was proposed by Tadege et al. (1999) in tobacco pollen. They suggested that both the TCA and glyoxylate cycles were functional, and that the two cycles were simultaneously generating substrates and energy for the cells. In the tolerant "Khao Hlan On," effective ethanol fermentation involving pyruvate decarboxylase, alcohol dehydrogenase and, more importantly, aldehyde dehydrogenase would confer an adaptive advantage for germination under submerged conditions. That could possibly be coupled with the reducing power of the glyoxylate cycle, which could sustain both carbohydrate catabolism and protein biosynthesis.

THE ROLE OF FATTY ACID β-OXIDATION IN AEROBIC CONDITIONS

Glyoxylate cycle in the glyoxysomes carries out lipid metabolism during germination (Donaldson et al., 2001). Conversion of triacylglycerols into sugars by β-oxidation, in the glyoxysomes for energy supply during germination is particularly well-studied in oilseed plants. Rice seeds contain an average of 20% lipids in bran and germ, nearly half that of an oilseed, and could be a possible source of energy under submerged conditions. This pathway can also produce more acetyl-CoA besides generating net energy. This would generate substrates via the glyoxylate cycle to continue with the partial TCA cycle and feed gluconeogenesis metabolism. In turn, it would replenish carbon in the process, thus making metabolism more efficient. The degradation of triglycerides into free fatty acids and glycerol is supposedly carried out under anoxia by ACS (Lu et al., 2005). Moreover, 28 transcripts of proteins involved in lipid metabolism have been found in comparative studies between rice seeds germinating under aerobic and anaerobic conditions (Narsai et al., 2009). However, lipid metabolism in rice germinating under anoxia or hypoxia seems to be shifted toward biosynthesis, since a large proportion of the ATP generated is allocated to maintain membrane integrity (Edwards et al., 2012). In addition, fatty acids do not seem to serve as electron acceptors under submergence in rice coleoptiles (Generosova and Vartapetian, 2005). Other glyoxysome ezymes up-regulated under anoxia, such as the ascorbate peroxidase (metabolizes H_2O_2 into H_2O) and monodehydroascorbate reductase (regenerates NAD^+ from NADH; Donaldson et al., 2001) could have a role in the detoxification of reactive oxygen species (Lasanthi-Kudahettige et al., 2007). Higher activities of superoxide dismutase and catalase were also observed in seeds of tolerant rice genotypes germinating in water (Ella et al., 2011). This was associated with lower lipid peroxidation and higher survival. The definitive association of variation in expression or activities of these genes with flood tolerance in rice awaits further investigation.

CONCLUSIONS AND PERSPECTIVES

Rice is the only cereal capable of germination under submerged conditions, with substantial variation in tolerance of submergence at this stage, within cultivated varieties and landraces. Rice has been used extensively in studies of tolerance of anoxia and hypoxia and also used as a model plant when comparing different crop species. However, the regulatory and signaling mechanisms controlling tolerance of anaerobiosis during germination in tolerant rice genotypes are yet to be revealed. Some progress has been made in uncovering the major traits associated with tolerance of submergence during germination in some rice genotypes, some of which provided evidence supporting the role of the PDC-ALDH-ACS pathway in tolerance. Further studies are needed to elucidate the critical roles of the enzymes of carbohydrate, fatty acid and energy generating metabolic processes associated with tolerance. For example, RAmy3D and ALDH2 are good candidates for studies involving gene silencing or knock-out and complementation. Furthermore, identifying functional alleles associated with tolerance in major anaerobic pathways will help in speeding up breeding programs to develop tolerant rice varieties and varieties of other crops for which waterlogging is a serious problem during crop establishment as in wheat, barley and maize. These findings could also facilitate developing strategies that can help in managing and controlling aquatic weeds and weeds that are becoming problematic in paddy fields because of the evolution of new ecotypes that are more adapted to flooded conditions (Ismail et al., 2012).

Despite the efforts devoted for understanding the different mechanisms associated with tolerance of submergence during germination, development of improved varieties has not yet been possible. Steady progress has, however, been made while bottlenecks continue to be unveiled. One difficulty is the lack of homogeneity of data in the studies available. For example, there is currently little information on integrative data from studies using different approaches or addressing different mechanisms for germination under submerged conditions. Many studies on germination under submergence compare rice to other crops, with rice being the tolerant species. In some cases, studies used rice genotypes that are not particularly tolerant of anaerobic conditions during germination. Only a limited number of comparative studies have included reasonably tolerant rice genotypes, meaning those that germinate under water and develop into mature plants with little or no negative effects on crop establishment. Moreover, the experimental plans for such studies lack a unified approach in most cases, where different testing protocols are followed. Importantly, these protocols do not reflect field conditions, thereby negating the value of the results obtained toward varietal development. These are important obstructs since they lead to a major divergence of opinions in the classification of genotypes for tolerance of submergence

during germination. Furthermore, germination under submergence is a complex trait based on current evidences, which suggests that differences exist between anoxic and hypoxic treatments and different rice genotypes probably respond differently to such treatments. Environmental factors also seem to interact with tolerance, e.g., floodwater temperature, seed and seedbed conditions (Ella et al., 2010, 2011). Hence, variations in the experimental conditions may lead to different results, which are not necessarily contradictory but basically not applicable in standardizing genotype-specific tolerance mechanisms. Future research should be based on appropriately selected genotypes and experimental conditions to provide more insightful results on the mechanisms of tolerance of submergence during germination.

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Reactive oxygen species mediate growth and death in submerged plants

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Aquatic and semi-aquatic plants are well adapted to survive partial or complete submergence which is commonly accompanied by oxygen deprivation. The gaseous hormone ethylene controls a number of adaptive responses to submergence including adventitious root growth and aerenchyma formation. Reactive oxygen species (ROS) act as signaling intermediates in ethylene-controlled submergence adaptation and possibly also independent of ethylene. ROS levels are controlled by synthesis, enzymatic metabolism, and non-enzymatic scavenging. While the actors are by and large known, we still have to learn about altered ROS at the subcellular level and how they are brought about, and the signaling cascades that trigger a specific response. This review briefly summarizes our knowledge on the contribution of ROS to submergence adaptation and describes spectrophotometrical, histochemical, and live cell imaging detection methods that have been used to study changes in ROS abundance. Electron paramagnetic resonance (EPR) spectroscopy is introduced as a method that allows identification and quantification of specific ROS in cell compartments. The use of advanced technologies such as EPR spectroscopy will be necessary to untangle the intricate and partially interwoven signaling networks of ethylene and ROS.

Keywords: reactive oxygen species, adventitious root growth, epidermal cell death, aerenchyma formation, ROS detection, electron paramagnetic resonance spectroscopy, ethylene

INTRODUCTION

In aerobic cells about 1% of metabolically consumed O_2 goes into reactive oxygen species (ROS) generation (Puntarulo et al., 1988). ROS are generated from molecular oxygen by a number of reductive steps. Superoxide anions ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), hydroperoxyl radical ($HO_2^{\bullet}$), and ozone (O_3) are generated by a one-electron to three-electron reduction of oxygen with reductive power being provided by electron carriers in mitochondria and chloroplasts (Blokhina and Fagerstedt, 2010a; Chang et al., 2012; reviewed in Shapiguzov et al., 2012). Hydrogen peroxide (H_2O_2) is a non-radical that can cross membranes by diffusion and it can be transported by specific aquaporins (Bowler et al., 1992; Bienert et al., 2007; Borisova et al., 2012). H_2O_2 is produced by a two-electron reduction of molecular oxygen catalyzed by the respiratory burst NADPH oxidase (RBOH) at the plasma membrane. RBOH proteins in plants are homologs of NADPH oxidase 2 of mammals (Torres et al., 1998) and belong to the cytochrome *b* family. H_2O_2 can also be produced spontaneously by dismutation of either $O_2^{\bullet -}$ or $HO_2^{\bullet}$.

Protection of mitochondria from unwarranted ROS production is provided by the alternative oxidase (AOX) and by an alternative type II, non-proton-pumping, Ca^{2+}-dependent NADPH dehydrogenase (ND; reviewed in Blokhina and Fagerstedt, 2010b). While AOX and ND protect mitochondria from oxidative stress the oxidized state of intermediates of the electron transport chain at the same time results in a decrease in ATP synthesis (Borecký et al., 2006). Antioxidant activity is provided throughout the cell by low molecular mass components such as reduced glutathione, reduced ascorbic acid, tocopherols, tannins, ubiquinol, and phenolic compounds, and by ROS scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX). Non-enzymatic ROS scavenging proteins such as thioredoxin and metallothioneins also contribute to ROS homeostasis. The type of ROS that accumulates is ultimately determined by the balance between ROS producing and ROS scavenging activities. For instance SOD determines the rate of H_2O_2 production and CAT the rate of H_2O_2 metabolism. A change in either activity affects H_2O_2 steady-state levels. Generation and detoxification mechanisms of the main ROS are summarized in **Figure 1A**. This review focuses on ROS as signaling intermediates in submergence adaptation and it summarizes methods used to identify the ROS involved.

SUBMERGENCE-INDUCED AND ROS-MEDIATED GROWTH AND CELL DEATH RESPONSES

As explained above, the balance between production and scavenging of ROS controls cellular ROS levels in plants. Oxidative stress occurs when these processes are imbalanced. High light, heat, pathogen invasion, wounding, low oxygen, and re-aeration after a phase of low oxygen stress increase ROS generation while low light conditions that arise for example during submergence decrease ROS production (Suzuki et al., 2012; Szarka et al., 2012). ROS are generated via enzymatic as well as non-enzymatic reactions. Which of the two mechanisms take place is influenced by

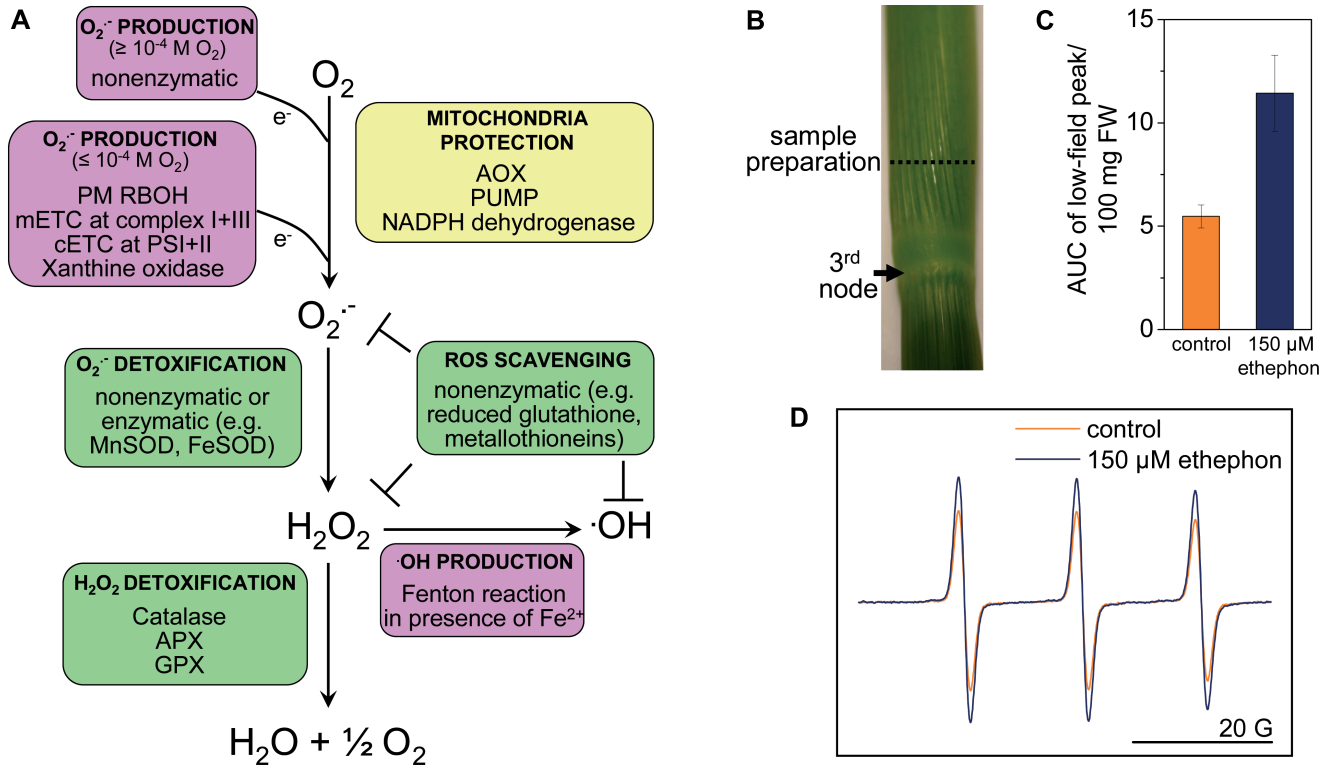

FIGURE 1 | Reactive oxygen species homeostasis in plants. (A) ROS are generated enzymatically or non-enzymatically depending on internal O_2 levels. In photosynthetic tissues, the main sources of ROS are the chloroplasts and peroxisomes (reviewed in Shapiguzov et al., 2012). In photosynthetically active chloroplasts, $O_2^{\bullet-}$ is generated at photosystems I and II. In the dark, at low light conditions or in photosynthetically inactive tissues ROS are produced mainly in mitochondria. $O_2^{\bullet-}$ is produced by the flavin mononucleotide binding subunit of NADH dehydrogenase in complex I and by ubiquinol-cytochrome *bc1* reductase in complex III of the mitochondrial electron transport chain (mETC), or by RBOH at the plasma membrane. $O_2^{\bullet-}$ is dismutated to H_2O_2 non-enzymatically, by a manganese-containing isoform of the superoxide dismutase (MnSOD) in the matrix (Møller, 2001), or by FeSOD in chloroplasts and Cu/ZnSOD in the cytosol. $^{\bullet}$OH is a highly reactive ROS that is produced in the Fenton reaction from H_2O_2 in the presence of metals such as Fe^{2+}. The uncoupling protein (PUMP), the alternative oxidase (AOX) and an alternative NAD(P)H dehydrogenase protect mitochondria from unwarranted ROS production. Antioxidants such as reduced glutathione, metallothioneins; and enzymes such as superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX), and glutathione peroxidase (GPX) exist in different cellular compartments to detoxify ROS. **(B)** Stem sections of deepwater rice cultivar Pin Gaew 56 were treated with 150 µM ethephon or left untreated as a control for 20 h. Internodal tissue was collected 10 mm above the third node for EPR analysis. **(C)** The double integral of the low-field peak (AUC, area under peak) was determined to compare ROS levels detected with the spin probe TMT-H (2,2,6,6-tetramethylpiperidinium) in control sections and after ethephon treatment. **(D)** Typical EPR spectra of the spin probe after the reaction with ROS. Two hundred microliters of a 1-mM spin probe solution were added to 100 mg of tissue and incubated for 10 min. EPR measurements were performed on a Bruker Elexsys E500 spectrometer at room temperature with the following settings: center field 3513 G, sweep width 65 G, microwave frequency 9.84 GHz, microwave power 2 mW, modulation amplitude 1.2 G, conversion time 20.01 ms, time constant 40.96 ms (A. Steffen-Heins, B. Steffens, unpublished).

the cellular oxygen concentration. Non-enzymatic one-electron O_2 reduction occurs at 10^{-4} M and higher concentrations of O_2 while enzymatic reactions take place at lower oxygen concentrations. The same holds true for mitochondrial electron transport and respiration establishing a link between oxygen concentration, mitochondrial ATP production, and oxidative stress.

Soil water logging and partial or complete submergence limit gas diffusion which results on one hand in oxygen shortage and on the other hand in the accumulation of the volatile hormone ethylene in flooded tissues. In rice, ethylene promotes adventitious root growth, death of epidermal cells overlaying adventitious root primordia, and parenchymal cell death which results in aerenchyma formation. All of these responses are mediated by ROS. In deepwater rice, ethylene-induced adventitious root growth is abolished when RBOH activity is inhibited indicating that root growth in response to flooding is controlled by ROS that are generated at the plasma membrane (Steffens et al., 2012). RBOH activity is regulated by small G proteins (Baxter-Burrell et al., 2002; Wong et al., 2007). Inhibition of CAT enhances internal ROS levels and results in growth promotion revealing that either superoxide anion or H_2O_2 are the active ROS. Scavenging of H_2O_2 by potassium iodide partially reduces ethylene-dependent root growth supporting this finding.

Epidermal cells that overlay adventitious root primordia at the stem node of rice plants undergo cell death prior to the emergence of the adventitious root. Epidermal cell death is induced by ethylene which promotes cell death via H_2O_2 (Steffens and Sauter, 2009). The metallothionein MT2b is a non-enzymatic H_2O_2 scavenger in rice. Genetic downregulation of *MT2b* elevates endogenous ROS levels in rice cells (Wong et al., 2004). In

epidermal cells that undergo cell death *MT2b* is downregulated by ethylene suggesting that ethylene promotes ROS accumulation and hence cell death induction via MT2b. In fact, constitutive genetic downregulation of *MT2b* enhances epidermal cell death constitutively showing that modulation of ROS scavenging by MT2b is sufficient to alter cell death rates (Steffens and Sauter, 2009). *MT2b* is downregulated in epidermal cells overlaying adventitious roots not only by ethylene but also by H_2O_2 itself revealing a feedback loop that autoamplifies H_2O_2 accumulation. While induction of adventitious root growth by ethylene is also promoted by ROS, downregulation of *MT2b* does not alter root growth rate suggesting that regulation of epidermal cell death and of adventitious root growth rely on different ROS signaling pathways.

Aside from the formation of adventitious roots, the development of internal gas spaces by way of programmed cell death is another major adaptation that helps plants to cope with flooding stress. Aerenchyma are constitutively formed in deepwater and lowland rice stems and leaf sheaths. Aerenchyma formation is enhanced in internodes of deepwater rice by ethylene which promotes formation of $O_2^{\bullet-}$ (Steffens et al., 2011). In lowland rice varieties aerenchyma formation in leaf sheaths is increased upon submergence (Parlanti et al., 2011). In the lowland rice variety FR13A, the ETHYLENE RESPONSE FACTOR (ERF) SUBMERGENCE 1A (SUB1A) is induced by ethylene during submergence and suppresses ethylene biosynthesis by feedback inhibition (Fukao et al., 2006; Xu et al., 2006). In FR13A, ROS accumulate independent of ethylene signaling but are none the less responsible for submergence-induced aerenchyma formation in leaf sheaths (Parlanti et al., 2011). The lowland rice variety Arborio Precoce does not possess *SUB1A* and ROS do not accumulate during leaf sheath aerenchyma formation. However, Parlanti et al. (2011) postulate that an early transient ROS accumulation that occurs prior to ethylene signaling promotes aerenchyma formation. Hence, aerenchyma formation in response to submergence appears to be controlled by ROS in lowland and deepwater rice varieties. In some but not all varieties ROS accumulation is controlled by ethylene signaling which may influence the timing of cell death induction. In conclusion, ROS are central regulators of plant adaptation to submergence.

ROS HOMEOSTASIS AND SIGNALING IN HYPOXIC PLANTS

At low oxygen conditions, ROS production in *Arabidopsis* occurs predominantly at the plasma membrane through RBOH and in mitochondria. *RbohD* one of the 10 RBOH genes of *Arabidopsis* is induced at low oxygen (Pucciariello et al., 2012). Activation of RBOH occurs furthermore at the protein level by small G proteins such as ROP in *Arabidopsis* (Baxter-Burrell et al., 2002) and OsRac1 in rice (Wong et al., 2007). In mitochondria $O_2^{\bullet-}$, $^{\bullet}OH$, $^1O_2$, $HO_2^{\bullet}$, and O_3 are generated as a result of an overreduction of the redox chain during anoxia (Chang et al., 2012). In *Arabidopsis*, ROS originating in mitochondria activate the mitogen-activated protein kinase MAPK6 to improve survival at hypoxic conditions (Chang et al., 2012). In plant mitochondria, the AOX transfers four electrons from ubiquinone to oxygen thereby preventing ROS production from an overreduced ubiquinone pool (Umbach et al., 2005). AOX is encoded by five genes of the multigene families AOX1 and AOX2 in *Arabidopsis* (Considine et al., 2002; Borecký et al., 2006). Constitutive activation of AOX in *Arabidopsis* or overexpression of *Arabidopsis AOX1a* in tobacco decreases mitochondrial ROS production (Maxwell et al., 1999) while inhibition of AOX increases ROS production (Maxwell et al., 1999; Umbach et al., 2005). In barley roots, AOX activity is elevated at anoxic conditions (Skutnik and Rychter, 2009). Detoxification of ROS serves to prevent oxidative damage but at the same time may alter a ROS signal. Future work is required to consolidate or distinguish between the two pathways.

The dismutation of $O_2^{\bullet-}$ to H_2O_2 is mediated by FeSOD in chloroplasts, MnSOD in mitochondria, and by Cu/ZnSOD in chloroplasts and in the cytoplasm. The enzymatic reaction is 10,000-fold faster than spontaneous dismutation. H_2O_2 is detoxified to H_2O and O_2 by CAT. In addition, soluble, extracellular, or cell wall-associated peroxidases detoxify H_2O_2. Peroxidases also generate $O_2^{\bullet-}$ and H_2O_2 (Mika et al., 2010). Anoxia and hypoxia increase SOD activity in wheat and *Iris pseudacorus* (Monk et al., 1987; Biemelt et al., 1998) but not in barley roots (Szal et al., 2004) while in maize flooded for 7 days $O_2^{\bullet-}$ levels increase due to reduced SOD activity possibly pointing to a regulatory role. In the wetland species *Alternanthera philoxeroides* and *Hemarthria altissima*, SOD and CAT activities are differentially regulated during flooding depending on the survival strategy (Luo et al., 2012). In *Alternanthera philoxeroides* that shows the "escape" strategy (Bailey-Serres and Voesenek, 2008), SOD and CAT activities are downregulated in leaves but recover after de-submergence. *H. altissima* pursues a "quiescence" strategy and displays high SOD and CAT activities in submerged leaves. This differential response is compatible with the view that ROS contribute to shoot growth control.

Lipoxygenases catalyze the hydroperoxidation of polyunsaturated fatty acids. In wheat roots and in corn leaves levels of $O_2^{\bullet-}$ and H_2O_2 increase after re-aeration resulting in elevated lipid peroxidation and loss of membrane integrity (Albrecht and Wiedenroth, 1994). Lipoxygenase activity in anoxia-treated potato cells correlates with the duration of the low oxygen treatment (Pavelic et al., 2000). Lipids are protected from oxidative damage by tocopherols and tocotrienols known as vitamin E. Anoxia-intolerant *I. germanica* has more β-tocopherol as compared to anoxia-tolerant *I. pseudacorus* while α-tocopherol content does not differ (Blokhina et al., 2000). Anoxia induces tocopherol deprivation in both *Iris* species. However, the decline in tolerant *I. pseudacorus* sets in later than in *I. germanica* possibly contributing to the observed tolerance (Blokhina et al., 2000). Along the same line, the submergence-tolerant rice variety FR13A protects lipids during submergence while the anoxia-sensitive variety CT6241 displays enhanced lipid peroxidation (Santosa et al., 2007). The protective mechanism of FR13A is, however, not understood.

In conclusion, regulation of ROS levels in flooded plants relies on the regulation of ROS producing and ROS scavenging mechanisms. It is not always clear if changes in ROS levels exclusively cause or prevent damage or if and how they contribute to signaling. What has become clear, however, is that ROS abundance is regulated at different levels in different plant species. Mechanisms of ROS regulation are numerous and have not been fully analyzed in any one species or been compared stringently between

flooding-resistant and flooding-prone ecotypes. This should be achieved in future research to identify unifying mechanisms that characterize flooding-resistant plants. The following paragraph summarizes and comments on methods currently used to detect ROS.

DETECTION OF ROS BY SPECTROPHOTOMETRICAL AND STAINING METHODS

It is challenging to monitor ROS abundance in plant cells due to their low concentration and short half-life. For example, $^{\bullet}OH$ has a half-life of a few nanoseconds and $O_2^{\bullet-}$ of tenths of microseconds. Another challenge is the spatial resolution as ROS can accumulate in different cell compartments. Detection must be sensitive and specific for defined ROS. Indirect measurement of ROS generation is possible by analyzing lipid peroxidation of unsaturated fatty acids in membranes. This method was used to investigate ROS production under low oxygen stress in oat and wheat roots, Iris rhizomes, and rice seedlings (Blokhina et al., 1999; Santosa et al., 2007), and after re-aeration in rice (Fukao et al., 2011). Methods commonly used for ROS detection are summarized in **Table 1** and described below.

Short-lived $O_2^{\bullet-}$ were measured by irreversible oxidation of epinephrine to adrenochrome (Chance et al., 1979) in hypoxic barley roots (Szal et al., 2004) and during ethylene-induced aerenchyma formation in rice stems (Steffens et al., 2011). In cell cultures, $O_2^{\bullet-}$ concentration was determined by 4-methyl-beta-D-umbelliferyl glucopyranoside (4-MUF-glu) in a fluorometric assay (Kush and Sabapathy, 2001) to analyze a role of the annexin-like protein Oxy5 from *Arabidopsis* in the oxidative stress response. 4-MUF-glu is cleaved by $O_2^{\bullet-}$ to the fluorescent form 4-methylumbelliferone (4-MUF). A common disadvantage of spectrophotometrical methods is the relatively high demand for biological material.

Hydrogen peroxide can be quantified by recording the oxidation of *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), a derivative of dihydro-resorufin in the presence of horseradish peroxidase in an assay that uses plant tissue extract. During the reaction, Amplex Red is converted to the fluorescent resorufin. Amplex Red was used to analyze H_2O_2 production in hypoxic and anoxic wheat roots (Biemelt et al., 2000), in hypoxic and anoxic *Arabidopsis* seedlings (Pucciariello et al., 2012), and to compare differences in H_2O_2 production in two rice cultivars after 3 days of submergence (Parlanti et al., 2011). This probe is useful for *in planta* studies as it is membrane-permeable. This assay does, however, not provide spatial resolution.

Cell type-specific ROS detection is possible with histochemical approaches. Cerium chloride ($CeCl_2$) or 3,3′-diaminobenzidine (DAB, Bestwick et al., 1997; Thordal-Christensen et al., 1997;

Table 1 | Common ROS detection methods.

ROS	ROS detection method	Condition/plant species	Reference
	Spectrophotometrical methods		
$O_2^{\bullet-}$	Irreversible oxidation of epinephrine	Hypoxia, barley roots	Szal et al. (2004)
		Submergence, rice internodes	Steffens et al. (2011)
	Cleavage of 4-MUF-glu	Oxidative stress, *Arabidopsis*	Kush and Sabapathy (2001)
H_2O_2	Oxidation of Amplex Red	Hypoxia and anoxia, wheat roots	Biemelt et al. (2000)
		Hypoxia and anoxia, *Arabidopsis* seedlings	Pucciariello et al. (2012)
		Submergence, rice	Parlanti et al. (2011)
	Histochemical approaches		
$O_2^{\bullet-}$	Oxidation of NBT	Submergence, rice leaves	Fukao et al. (2011)
		Submergence, rice internodes	Steffens et al. (2011)
		Submergence, adventitious roots	Steffens et al. (2012)
		Submergence, nodal epidermis	Steffens and Sauter (2009)
H_2O_2	Cerium perhydroxide	HR, lettuce cells	Bestwick et al. (1997)
	Oxidation of DAB	Submergence, *Alternanthera philoxeroides*, *H. altissima*	Luo et al. (2012)
		Submergence, rice leaves	Fukao et al. (2011)
		Submergence, rice internodes	Steffens et al. (2011)
		Submergence, adventitious roots	Steffens et al. (2012)
		Submergence, nodal epidermis	Steffens and Sauter (2009)
	Live cell imaging		
ROS/RNS	$DCFH_2$-DA	Submergence, rice leaves	Parlanti et al. (2011)
		Arabidopsis roots	Chang et al. (2012)
		Arabidopsis leaves	Umbach et al. (2012)
H_2O_2	Amplex red	Tobacco leaves	Snyrychová et al. (2009)
$^1O_2$	Singlet Oxygen Sensor Green	Wounding, *Arabidopsis* leaves	Flors et al. (2006)

Blokhina et al., 2001) are useful to visualize H_2O_2. In the presence of $CeCl_2$, H_2O_2 produces stable precipitates of cerium perhydroxides with higher electron density that can be observed by transmission electron microscopy. Localization and quantification of H_2O_2 in different cell compartments is possible. DAB reacts with H_2O_2 in a peroxidase-catalyzed reaction resulting in an oxidized insoluble brown precipitate. For the microscopic detection of $O_2^{\bullet-}$, the nitro-substituted aromatic compound nitroblue tetrazolium (NBT) is useful. Oxidized NBT forms precipitates resulting in a blue staining. Detection of H_2O_2 and $O_2^{\bullet-}$ at the cellular level was used to analyze ROS accumulation during submergence in rice leaves (Fukao et al., 2011), ethylene-induced and ROS-mediated epidermal and parenchymal cell death in rice, and adventitious root growth in rice (Steffens and Sauter, 2009; Steffens et al., 2011, 2012). These precipitation techniques usually require removal of chlorophyll and are hence not suited for live cell imaging.

Fluorescent probes such as derivates of dichlorodihydrofluorescein diacetate can non-destructively detect ROS through live cell imaging. The probes permeate membranes in the non-fluorescent uncharged forms and are kept in the charged form in the cytosol, or in organelles after cleavage of the acetate groups by esterases (Kristiansen et al., 2009). Green fluorescence develops due to oxidation of the ROS-reactive charged form by $O_2^{\bullet-}$ or H_2O_2 but also by peroxyl radical ($ROO^{\bullet}$) and peroxynitrite ($ONOO^-$; Tarpey and Fridovich, 2001). The fluorescent probe 2′-7′-dichloro-dihydrofluorescein diacetate ($DCFH_2$-DA) was used for ROS and reactive nitrogen species (RNS) detection in leaf sheath sections of submerged rice seedlings (Parlanti et al., 2011), in roots of *Arabidopsis* seedlings (Chang et al., 2012), and in *Arabidopsis* leaves (Umbach et al., 2012) through confocal laser scanning microscopy. Amplex Red can also be used for specific detection of H_2O_2 in cells by confocal laser scanning microscopy (Snyrychová et al., 2009). For $^1O_2$ detection the fluorescent dye Singlet Oxygen Sensor Green was used to monitor wound-induced production of this highly reactive ROS in *Arabidopsis* leaves (Flors et al., 2006).

Reactive oxygen species likely play an even more important role in the regulation of developmental events than has been recognized so far. The methods presented here will be important in unraveling this role.

ROS DETECTION WITH ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY – A SENSITIVE TECHNIQUE TO ANALYZE ROS *IN PLANTA*

A sensitive technique to identify, quantify and visualize short-lived ROS is electron paramagnetic resonance (EPR) spectroscopy. ROS are detected by EPR using spin traps or spin probes with different properties including lipophilicity, reaction kinetic and stability of adducts. Spin traps including the nitrones DMPO (5,5-dimethyl-pyrroline-N-oxide) and its phosphorylated analog DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) are diamagnetic and form stable adducts with transient radicals to transform them into longer-lived radical species (Bačić and Mojović, 2005). Suitable spin traps are defined by either the ability to exclusively trap one ROS as was shown for EMPO (5-ethoxycarbonyl-5-methyl-pyrroline-N-oxide) and BMPO (5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide) that specifically detect $O_2^{\bullet-}$ (Bačić et al., 2008) or to lead to different specific signature EPR spectra. Improved spin traps like DEPMPO have a longer lifetime than DMPO-adducts, reduced degradation of the spin adducts and a faster reaction kinetic leading to a sufficient trapping of $O_2^{\bullet-}$ and $^{\bullet}OH$. 4-POBN [α(4-pyridyl-1-oxide)-N-tert-butylnitrone] detects specifically $^{\bullet}OH$ and has been used to analyze radicals in the medium surrounding growing maize roots (Liszkay et al., 2004) and in growing cucumber and *Arabidopsis* roots (Renew et al., 2005). In addition, specific EPR spectra of $^{\bullet}OH$ were obtained from defined cucumber root zones (Renew et al., 2005) suggesting that this technique allows for spatial resolution of ROS detection.

Spin probes can be used either as endogenous nitroxides that are reduced by ROS to the EPR-silent hydroxylamines or *vice versa.* Endogenous cyclic hydroxylamines are oxidized by ROS to EPR-active nitroxides. The very fast reaction rates between ROS and hydroxylamine are a major advantage compared with spin traps. The efficiency of hydroxylamines to detect $O_2^{\bullet-}$ is very high so that very low concentrations of the hydroxylamines are necessary to detect $O_2^{\bullet-}$ (1 mM compared with 10–50 mM used in spin traps), and side effects can be minimized (Dikalov et al., 2011). This is mainly due to the high reactivity of radicals so that their reaction site is very close to their generation site (Heins et al., 2007). Since the reaction of hydroxylamines toward ROS is unspecific, ROS must be identified by alternative approaches. Additions of scavengers of defined ROS such as SOD are useful (Dikalov et al., 2011). The spin probe technique has been used to measure $O_2^{\bullet-}$ in *Arabidopsis* roots (Warwar et al., 2011) and in thylakoid membranes (Kozuleva et al., 2011; Borisova et al., 2012). Using the spin probe technique we showed that ethylene enhances ROS levels in rice internodes possibly related to parenchymal cell death and aerenchyma formation (**Figures 1B–D**; Steffens et al., 2011). EPR spectroscopy may turn out as a useful tool to analyze ROS in defined cells and to evaluate their contribution to submergence adaptation.

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Does suberin accumulation in plant roots contribute to waterlogging tolerance?

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Plants that are adapted to waterlogged conditions develop aerenchyma in roots for ventilation. Some wetland plant species also form an apoplastic barrier at the outer cell layers of roots that reduces radial oxygen loss (ROL) from the aerenchyma and prevents toxic compounds from entering the root. The composition of the apoplastic barrier is not well understood. One potential component is suberin, which accumulates at the hypodermal/exodermal cell layers of the roots under waterlogged soil conditions or in response to other environmental stimuli. However, differences in suberin content and composition between plant species make it difficult to evaluate whether suberin has a role in preventing ROL. In this article, we summarize recent advances in understanding apoplastic barrier formation in roots and, between various plant species, compare the chemical compositions of the apoplastic barriers in relation to their permeability to oxygen. Moreover, the relationship between suberin accumulation and the barrier to ROL is discussed.

Keywords: apoplastic barrier, exodermis, hypodermis, suberin, suberin composition, radial oxygen loss, waterlogging

INTRODUCTION

Waterlogging is defined as a condition of the soil in which excess water limits gas diffusion and inhibits plant growth. Oxygen diffusivity in water is approximately 10,000 times slower than it is in air, and the flux of O_2 into soils is approximately 320,000 times less when the soil pores are filled with water than when they are filled with gas (Armstrong and Drew, 2002). Moreover, oxygen in waterlogged soil is consumed rapidly by soil microorganisms and plant roots (Drew and Lynch, 1980). As a result, the growth of anaerobic soil microorganisms is accelerated, leading to the appearance of harmful microbial metabolic products such as organic acids (Jackson and Taylor, 1970; Conrad and Klose, 1999). Soil redox potential is also rapidly decreased, resulting in the accumulation of phytotoxic compounds such as sulfides and reduced forms of minerals (e.g., Mn^{2+} and Fe^{2+}) (Laanbroek, 1990). Plants that are adapted to waterlogged soil conditions form aerenchyma, which permits ventilation between well-aerated shoots and waterlogged roots. Additionally, some waterlogging-tolerant species develop an apoplastic transport barrier at the outer cell layers in the root. This barrier can be also induced by other environmental stresses, such as elevated salinity and drought (Enstone et al., 2003), to reduce of the transport of water, solutes, and gases from the medium to the root and *vice versa*. When the soil is waterlogged, the barrier can minimize the loss of O_2 to the surrounding environment, called radial oxygen loss (ROL), thereby enhancing longitudinal diffusion of oxygen toward the root apex (Armstrong, 1979; Colmer, 2003a). The barrier also impedes the penetration of soil-derived toxins, such as reduced metal ions, into the roots (Armstrong and Armstrong, 2005; Greenway et al., 2006). Mathematical modeling suggests that oxygen diffusion to the apex largely depends on the development of aerenchyma in the plant root (Armstrong, 1979; Armstrong and Beckett, 1987). Therefore, in the presence of extensive aerenchyma, the function of the apoplastic barrier may be more important for restricting the entry of phytotoxins than for improving oxygen diffusion to the apex (Armstrong, 1979).

The apoplastic barrier forms as the result of enhanced accumulation of suberin at hypodermal/exodermal cell walls (**Figure 1**). Suberin is one of the main barrier biopolymers in plants, and is deposited in cell walls to separate living plant tissue from unfavorable environments or to separate different tissues inside the plant during development (Kolattukudy, 2001; Enstone et al., 2003; Schreiber, 2010). Suberin was found to be deposited in the outer cell layers of roots in stagnant deoxygenated medium in several plant species (De Simone et al., 2003; Soukup et al., 2007; Garthwaite et al., 2008; Kotula et al., 2009a).

In this review, we summarize what is known about the induction and function of the apoplastic barrier in plant roots under waterlogged conditions, and examine the relation between the chemical composition of the barrier and its permeability to O_2 in several plant species. Finally, we discuss future directions for understanding the composition of the apoplastic barrier and the potential contribution of the apoplastic barrier to improvement of waterlogging tolerance of crops.

INDUCTION OF APOPLASTIC BARRIER IN PLANT ROOTS

Accumulation of suberin is manifested by the presence of a Casparian strip and the development of suberin lamellae in the hypodermis/exodermis or the endodermis. The maturation processes of the Casparian strip and suberin lamellae in the hypodermis/exodermis differ between plant species and between growth conditions (Enstone et al., 2003). Suberization of the

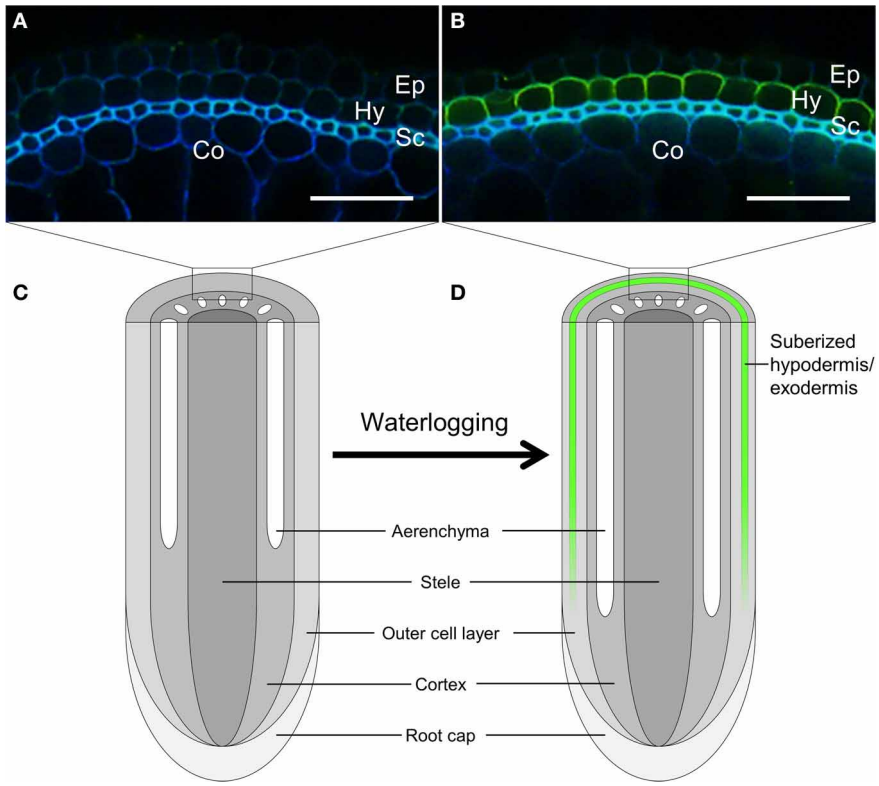

FIGURE 1 | Induction of suberization at the hypodermis/exodermis of rice root under stagnant deoxygenated conditions. Nine-day-old rice plants were grown in aerated solution **(A)** or stagnant deoxygenated solution **(B)** for 14 days. Basal parts (60 mm from root tip) of the adventitious roots were sliced into 80-μm-thick sections. The sections were cleared by incubating them in lactic acid saturated with chloral hydrate at 70°C for 1 h. Suberin was stained yellow green, especially in the hypodermis/exodermis, with Fluorol Yellow 088 at room temperature for 1 h. The section also showed autofluorescence in blue. Ep, epidermis; Hy, hypodermis/exodermis; Sc, sclerenchyma; Co, cortex. Scale bars = 50 μm. **(C,D)** Schematic diagram of longitudinal view of rice root under aerobic conditions **(C)** or under stagnant deoxygenated conditions **(D)**. Suberized hypodermis/exodermis is shown by green line at **(D)**.

outer cell layers in rice (*Oryza sativa*) root occurs under stagnant deoxygenated conditions (which mimic low-oxygen conditions in waterlogged soils) (**Figure 1**; Wiengweera et al., 1997; Kotula et al., 2009a) or after exposure to short-chain carboxylic acids (Armstrong and Armstrong, 2001) or sulfide (Armstrong and Armstrong, 2005). Short-chain carboxylic acids (Conrad and Klose, 1999) and sulfide (Jacq et al., 1991) are known to accumulate around plant roots by reduction reactions and by the activity of anaerobic bacteria under waterlogged soil conditions. Some short-chain carboxylic acids are phytotoxins that damage cell membranes and thus may induce the suberization of plant roots (Armstrong and Armstrong, 2001).

In the absence of suberin, the plant cell wall is porous with pore sizes of 3.5–5.2 nm (Carpita et al., 1979) that allow water, salts, and gases to move freely between the plant and surrounding environment. Increased amounts of suberin and lignin are thought to make strong barriers by reducing the diameter of pores in the apoplastic barriers (Hose et al., 2001). The Casparian strip and suberin lamella restrict apoplastic transfer of Ca^{2+} (hydrated ionic radius: approximately 412 pm; Volkov et al., 1997) and Mg^{2+} (hydrated ionic radius: approximately 428 pm; Volkov et al., 1997) in barley (*Hordeum vulgare*) (Ferguson and Clarkson, 1976) and maize (*Zea mays*) (Maas and Ogata, 1971; Ferguson and Clarkson, 1975). Formation of an apoplastic barrier in the hypodermal/exodermal cells in roots of rice grown in stagnant deoxygenated solution for 17–27 days decreased the permeability of the outer part of the root to water and NaCl (Ranathunge et al., 2011). The decrease of permeability was greater for NaCl than for water, which suggests that the wall pore size in suberized cell walls is large enough to pass water (radius of an H_2O molecule: approximately 137 pm; Zhang and Xu, 1995) but not Na^+ solvated by water molecules (hydrated ionic radius: approximately 358 pm; Volkov et al., 1997). The strengthened apoplastic barriers also appear to prevent the entry of reduced phytotoxic compounds [e.g., Fe^{2+} (approximately hydrated ionic radius 300 pm; Kielland, 1937) and H_2S (approximately radius 193 pm; Kammeyer and Whitman, 1972)] and diffusion of gases [e.g., O_2 (approximately radius 140 pm; Kammeyer and Whitman, 1972)] (Armstrong et al., 2000). However, it should be noted that the size and number of pores in suberized cell walls could be reduced with increasing level of suberization, and that other factors, such as the electrical charge of the cell wall, can limit the flow of substances. The permeability of the apoplastic barrier depends on growth conditions and the length of time in waterlogged soil.

Although suberin accumulation reduces the permeability of the apoplastic barrier to most substances, the tight apoplastic

barrier to ROL in root basal zones does not reduce NO_3^- uptake from aerobic solutions in rice grown in stagnant nutrient solution (Rubinigg et al., 2002). This may be because plasmodesmatal connections, as observed in electron micrographs, are still operational in suberized tissue of the exodermis of adventitious roots from rice grown in hydroponic culture (Clark and Harris, 1981; Rubinigg et al., 2002).

SOME ASPECTS OF THE CHEMICAL COMPOSITION OF APOPLASTIC BARRIER IN RELATION TO ITS ABILITY TO PREVENT ROL

Schreiber et al. (1999) developed a protocol for the analysis of the apoplastic barrier in plant roots consisting of enzymatic digestion of cortical tissue, mechanical isolation of the remaining hypodermal/exodermal tissue, solvent extraction, chemical degradation of lignin and suberin, and analysis of the released monomers by gas chromatography-mass spectrometry (GC-MS). The apoplastic barriers of several plant species have been analyzed with this protocol (De Simone et al., 2003; Soukup et al., 2007; Kotula et al., 2009a). The results of those studies, which also include ROL measurements, are summarized in **Table 1**. In the following, we briefly discuss some aspects of the chemical composition of the barrier in relation to its ability to prevent ROL.

TOTAL SUBERIN CONTENT

In several plant species [*Phragmites australis* (**Table 1**, rows 3–4), *Glyceria maxima* (**Table 1**, rows 5–6), and rice (**Table 1** rows 9–10)], the suberin contents in the peripheral regions at several points along the root (**Table 1**, column 4) were inversely proportional to the ROL at those points (**Table 1**, column 13). On the other hand, if only the data obtained when ROL was not detectable are considered (gray rows in **Table 1**), the species differ dramatically in the amount of suberin. For example, the peripheral region of *Tabernaemontana juruana* root (**Table 1**, row 2, column 4) contained 5.5 times more suberin than that of *G. maxima* (**Table 1**, row 6, column 4), 4 times more suberin than the outer part of rice root (**Table 1**, row 10, column 4), and 1.5 times more suberin than in those tissues of *P. australis* (**Table 1**, row 4, column 4), whereas ROL was not measurable in all cases. This variation may be due to differences in spatial distribution of suberin among cell layers, as well as in chemical composition of suberin deposits in different species (Schreiber et al., 2005a).

SUBERIN COMPOSITION AND ABILITY OF BARRIER TO PREVENT ROL

Among the entries in **Table 1**, where ROL is below the detection limit (rows shaded gray), those with a higher percentage of aromatic suberin (**Table 1**, column 5) tend to have a lower total suberin content (**Table 1**, column 4). Pearson's correlation coefficient for these two variables calculated from the data in **Table 1** (total suberin content 62.1, 40.8, 11.9, 15.7 $\mu g.cm^{-2}$ and percentage of aromatic suberin 4.6, 42.2, 70.0, 77.7% for *T. juruana*, *P. australis*, *G. maxima,* and *O. sativa*, respectively) is −0.982. The absolute value of the coefficient is higher than the critical value for $P = 0.05$ and $n = 4$ (0.950), indicating that total suberin content and percentage of aromatic suberin are negatively correlated.

COMPOSITION OF ALIPHATIC DOMAIN OF SUBERIN

The permeability of suberin to water and solutes was found to be mostly determined by the aliphatic domain of suberin (Hose et al., 2001). In *P. australis* and *G. maxima* (Soukup et al., 2007), as well as in rice (Kotula et al., 2009a), the composition of aliphatic suberin isolated from the peripheral part of the root after incubation in stagnant deoxygenated medium was similar to that of suberin isolated from the peripheral part of the root of well-aerated plants. Most aliphatic monomers that are released after suberin decomposition belonged to one of the following five classes: monocarboxylic fatty acids, α,ω-dicarboxylic fatty acids, ω-hydroxy fatty acids, α-hydroxy fatty acids, and fatty alcohols (**Table 1**, column 7–11). Similar sets of monomers were found in suberin isolated from various herbaceous plant sources, such as wound periderm of potato (Schreiber et al., 2005b; Yang and Bernards, 2006), *Arabidopsis* root tissues (Franke et al., 2005), and *Arabidopsis* and *Brassica napus* seeds (Molina et al., 2006). Among monomer classes, ω-hydroxy fatty acids (**Table 1**, column 9) were the most abundant class of suberin monomers for all plant species presented in **Table 1**, accounting for up to 64.5% of all suberin monomers. The abundances of other classes differ substantially among plant species, which makes it difficult to determine whether the composition of the aliphatic domain of suberin affects the permeability of the barrier to O_2.

DOES LIGNIN CONTRIBUTE TO THE ROL BARRIER?

The tight apoplastic barrier in *T. juruana* is almost exclusively built of suberin. On the other hand, in rice, the outer cell layers have also a significant amount of lignin (5 times higher than those of suberin; **Table 1**, row 10, columns 3–4), although it is unclear whether the lignin contributes to the ROL barrier. In the outer part of the rice root, lignin is predominantly concentrated in lignified sclerenchyma. Lignin content was measured (Kotula et al., 2009a), but whether lignified sclerenchyma can act as the ROL barrier has not been evaluated experimentally. In roots of *P. australis*, sclerenchyma is represented by several layers of cells called a sclerenchymatous ring. Histochemical staining revealed that the walls of these cells were strongly lignified. Nevertheless, the sclerenchymatous ring did not provide a significant barrier to diffusion of periodic acid and is also unlikely to be a barrier to diffusion of smaller molecules such as O_2 (Soukup et al., 2007).

RELATIONSHIP BETWEEN SUBERIN ACCUMULATION AND RESISTANCE TO ROL

Suberin accumulation has been suggested to increase the resistance to O_2 leakage. However, increased resistance to ROL is not always accompanied by an increase of suberin deposition in the plant root. Shiono et al. (2011) reported that when aerobically grown rice plants were treated under stagnant deoxygenated solution for 2 days, a tight ROL barrier was formed in long adventitious roots, whereas suberin and lignin deposits were undetectable by histochemical staining. Subsequently, suberin deposits increased prior to changes in lignin deposits. On the other hand, high-density granules were observed at intercellular spaces between the hypodermal/exodermal cells and also between

Table 1 | Chemical composition of apoplastic barrier to Radial Oxygen Loss in various plant species[a].

Column no.		1	2	3	4	5	6	7	8	9	10	11	12	13	14
					Suberin content and composition										
								Aliphatic suberin fraction, % of aliphatic suberin							
Row no.	Plant species	Root region mm from the root tip	Treatment (S, stagnant; A, aerated)	Lignin content[b] $\mu g \cdot cm^{-2}$	Total suberin, $\mu g \cdot cm^{-2}$	Aromatic suberin $\mu g \cdot cm^{-2}$ (% of total suberin)	Aliphatic suberin $\mu g \cdot cm^{-2}$ (% of total suberin)	Monocarboxylic fatty acids	α, ω-dicarboxylic fatty acids	ω-hydroxy fatty acids	α-hydroxy fatty acids	Fatty alcohols	Other aliphatics	Radial oxygen loss[c], $ng.cm^{-2} \cdot min^{-1}$	References and source of data
1	*Salix martiana*	0–30	S	0.18	24.0	9.30 (38.8)	14.7 (61.2)	17.4	24.0	49.1	3.5	6.0	0.0	Significant ROL (0–30)[d]	De Simone et al., 2003 (Table III, Figure 2)
2	*Tabernaemontana juruana*	0–30	S	0.28	62.1	2.84 (4.6)	59.3 (95.4)	11.7	33.9	43.8	3.8	6.8	0.0	Not detectable (5.0 and above)[d]	De Simone et al., 2003 (Table III, Figure 2)
3	*Phragmites australis*[e]	0–30	S	Not analyzed	7.11	2.67 (37.6)	4.44 (62.4)	12.6	12.6	37.6	18.7	5.0	13.5	84 (10) 81 (20) 64 (30)	Armstrong and Armstrong, 2001 (Figure 4); Soukup et al., 2007 (Figure 3)
4		70–110	S	Not analyzed	40.8	17.2 (42.2)	23.6 (57.8)	9.4	23.0	49.4	14.6	2.4	1.2	Not detectable (70 and above)	
5	*Glyceria maxima*[e]	0–30	S	Not analyzed	3.62	2.22 (61.3)	1.40 (38.7)	4.3	0.0	31.4	27.9	7.9	28.5	29 (10) 15 (20) 2.0 (30)	Soukup et al., 2007 (Figures 3, 5)
6		70–110	S	Not analyzed	11.9	8.33 (70.0)	3.61 (30.0)	1.7	0.0	64.5	15.5	13.0	5.3	0.5 (70) Not detectable (80 and above)	
7	*Oryza sativa* (cv. Azucena)	5–35	A	16.5	1.37	0.99 (72.3)	0.38 (27.7)	35.2	4.0	40.4	12.9	7.5	0.0	14 (10) 17 (20) 22 (30)	Kotula et al., 2009a (Figures 3, 6–9)
8		35–65	A	50.1	12.3	10.0 (81.3)	2.25 (18.7)	30.8	1.5	49.7	15.0	3.0	0.0	17 (40) 14 (50) 5.4 (60)	
9		5–35	S	24.6	4.91	3.35 (68.2)	1.56 (31.8)	33.7	1.3	48.7	11.5	4.9	0.0	50 (10) 17 (20) 2.4 (30)	
10		35–65	S	80.4	15.7	12.2 (77.7)	3.56 (22.3)	31.2	1.5	51.9	13.0	2.3	0.0	Not detectable (40 and above)	

[a] Cases in which a strong barrier to ROL is formed are marked in gray and cases that have significant ROL across the root surface are not marked.

[b] The amount of lignin and suberin has been recalculated and expressed in $\mu g \cdot cm^{-2}$ of root surface where necessary.

[c] Radial Oxygen Loss (except for Tabernaemontana juruana and Salix martiana) is expressed in $ng \cdot cm^{-2} \cdot min^{-1}$. The number in parentheses is the distance (in mm) from the root tip to the point where ROL was measured.

[d] Root cortex oxygen concentration and root surface concentration rather than ROL were measured for Tabernaemontana juruana and Salix martiana (De Simone et al., 2003, Figures 5, 6). Root surface oxygen concentration for T. juruana was above zero just at the region 0–5 mm from the root tip, reaching a maximum of 0.4 $mg.l^{-1}$ at 2 mm from the apex. The surface O_2 concentration in the region above 5 mm from the root tip was below detection limit. Nevertheless, the internal oxygen concentration measured at 10–15 mm from the apex was 2.0 $mg \cdot l^{-1}$. This means that the barrier to ROL was very tight. In the case of S. martiana, the root surface concentration of oxygen was close to its concentration in the root cortex, which shows that if an apoplastic barrier was present, it would not be strong enough to prevent oxygen release from the root to anaerobic environment.

[e] For Phragmites australis and Glyceria maxima the results are for stagnant treatment only. For both species, the aliphatic suberin quantity and composition in plants growing in stagnant conditions were not significantly different from those in plants growing well aerated conditions. The profile of ROL for roots of P. australis plants growing in aerated conditions was similar to that of plants growing in stagnant conditions indicating that P. australis has a constitutive barrier to ROL. On the other hand, roots of G. maxima plants growing in aerated conditions did not form a tight barrier to ROL.

the sclerenchymatous cells in long roots treated under stagnant deoxygenated conditions for 2 days, whereas such granules were absent in roots growing under aerated conditions. Shiono et al. (2011) speculated that microstructural packing of intercellular spaces at the hypodermis/exodermis may be involved in ROL barrier formation and that suberin deposition may be more important than lignin deposition.

In waterlogging-tolerant *Zea nicaraguensis*, stagnant deoxygenated growth conditions induced early development of hypodermal/exodermal suberin lamellae as well as deposition of lignin at the epidermis in adventitious roots (Abiko et al., 2012). On the other hand, in waterlogging-intolerant maize, many of the hypodermal/exodermal cells developed suberin lamellae, but an increase in lignin was not detected (Abiko et al., 2012). The absence of an ROL barrier in spite of suberin deposition in maize roots may be due to the presence of non-suberized passage cells in the hypodermal/exodermal cell layer (Armstrong et al., 2000) and/or the special arrangement of suberin deposition within cell walls (Schreiber et al., 2005a; Ranathunge et al., 2011).

Roots of *G. maxima* grown under stagnant deoxygenated conditions develop an ROL barrier (Soukup et al., 2007). However, the formation of the ROL barrier was not accompanied by an increase of suberin content in roots or by changes in suberin composition, which suggests that the net accumulation of suberin components in the hypodermal/exodermal layers do not necessarily reflect the barrier properties of impregnated cell walls (Schreiber et al., 2005a; Soukup et al., 2007).

Histochemical staining is less sensitive than quantitative chemical analysis (De Simone et al., 2003), and may depend on the composition, molecular and spatial arrangements, and differences in molecular context in suberin lamellae (Soukup et al., 2007). On the other hand, current suberin quantitative and qualitative data (**Table 1**) are insufficient to tell whether the chemical composition of the apoplastic barrier affects ROL. The contributions of lignification and cell wall proteins to the apoplastic barrier remain unclear (Schreiber et al., 2005a). Moreover, the arrangements as well as the contents of suberin monomers may play a role in the formation of the ROL barrier under waterlogged conditions in plants (Ranathunge et al., 2011). Thus, further analyses of the spatial arrangement of suberin deposition within the cell wall are required to elucidate the components of the ROL barrier.

Oxygen consumption by respiration in the outer part of the root also significantly affects ROL (Armstrong et al., 2000; Garthwaite et al., 2008; Kotula et al., 2009b). To determine the real effect of the barrier on ROL, the respiration rate or the number of respiring cell layers in the outer part of the root should be taken into account (Garthwaite et al., 2008; Kotula and Steudle, 2009). For example, when cell respiration was stopped by HCl treatment, the oxygen permeability coefficient of rice root segments was about twice that of non-treated roots (Kotula et al., 2009b), and in the case of *Hordeum marinum*, reducing cell respiration by cooling to 4°C revealed that the cell respiration reduces ROL and improves the apparent tightness of the barrier at 20°C (Garthwaite et al., 2008).

CHALLENGES AND PERSPECTIVES

Although the ROL barrier was first reported almost 50 years ago and was presumed to be due to suberin deposition (Armstrong, 1964, 1967), the chemical nature and induction of the barrier have been studied in detail only in the last 15 years. Suberin deposition in the outer cell layers of roots is presently suspected to take an important part in the formation of the apoplastic barrier to phytotoxins and gases, and thus it can contribute to waterlogging tolerance. However, differences in spatial patterns of suberin deposition and differences in suberin content and composition between plant species make it difficult to determine to what extent suberin deposition contributes to the barrier formation and how the chemical composition of suberin affects barrier properties. In addition, much remains to be learned about the molecular mechanism of suberin accumulation. One approach to answering these questions is to use model plants for which mutants and transformants that differ in suberin contents and compositions can be easily produced. Rice may be useful for this purpose because many molecular and genetic tools have been developed and it forms a tight barrier in roots under stagnant deoxygenated conditions (Colmer, 2003b; Kotula et al., 2009a; Shiono et al., 2011). Thus, genes related to suberin biosynthesis and ROL barrier formation could be identified and analyzed using rice. Another approach is to focus on the strengthening of the apoplastic barrier that can be induced by chemical stimulants, such as sulfides and short-chain organic acids, in the surrounding environment (Armstrong and Armstrong, 2001, 2005). This would allow suberin content and composition to be varied by chemical stimulants, which in turn would make it possible to determine the effects of suberin content and composition on the tightness of apoplastic barrier to ROL.

Many commercially important crops, such as wheat (*Triticum aestivum*) and maize, are not able to form a tight ROL barrier, which can result in a reduction of yield if the soil becomes waterlogged. On the other hand, wild plants, *H. marinum* and *Z. nicaraguensis*, which can be crossed with wheat and maize, respectively, accumulate suberin in response to waterlogged conditions and form a tight ROL barrier (Malik et al., 2011; Abiko et al., 2012). Some amphiploids between wheat (cv. Chinese Spring) and *H. marinum* were able to form an ROL barrier and were more tolerant to waterlogging than wheat (Malik et al., 2011). In addition, genetic analyses using a cross between *Z. nicaraguensis* and maize (inbred line Mi29) have revealed potential quantitative trait loci (QTL) related to waterlogging tolerance (Mano and Omori, 2008, 2009; Mano et al., 2009, 2012). Such genetic analyses provide a promising approach to understanding the mechanism of ROL barrier formation and to improving the waterlogging tolerance of crops.

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Characterization of the phosphofructokinase gene family in rice and its expression under oxygen deficiency stress

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Plants possess two types of phosphofructokinase proteins for phosphorylation of fructose-6-phosphate, the ATP-dependent phosphofructokinase (PFK) and the pyrophosphate-(PPi) dependent pyrophosphate-fructose-6-phosphate-phosphotransferase (PFP). During oxygen deficiency ATP levels in rice seedlings are severely reduced, and it is hypothesized that PPi is used as an alternative energy source for the phosphorylation of fructose-6-phosphate during glycolysis. In this study, we analyzed the expression of 15 phosphofructokinase-encoding genes in roots and aerial tissues of anoxia-tolerant rice seedlings in response to anoxic stress and compared our data with transcript profiles obtained from microarray analyses. Furthermore, the intracellular localization of rice PFK proteins was determined, and the PFK and PFP isoforms were grouped in a phylogenetic tree. Two *PFK* and two *PFP* transcripts accumulated during anoxic stress, whereas mRNA levels of four *PFK* and three *PFP* genes were decreased. The total specific activity of both PFK and PFP changed only slightly during a 24-h anoxia treatment. It is assumed that expression of different isoforms and their catalytic properties differ during normoxic and anoxic conditions and contribute to balanced glycolytic activity during the low-oxygen stress. These characterizations of *phosphofructokinase* genes and the comparison to other plant species allowed us to suggest candidate rice genes for adaptation to anoxic stress.

Keywords: ***Oryza sativa*, anoxia, submergence, phosphofructokinase, pyrophosphate**

INTRODUCTION

The pronounced tolerance of oxygen deprivation during seed germination and vegetative development makes rice plants an interesting subject to elucidate mechanisms of response to low-oxygen stress. Oxygen deficiency results in inhibition of mitochondrial respiration, leading to NADH accumulation as well as ATP deficiency. Glycolytic production of ATP continues, when fermentative enzymes are induced and able to regenerate sufficient amounts of NAD^+ (reviewed in Drew, 1997; de Sousa and Sodek, 2002; Geigenberger, 2003; Gibbs and Greenway, 2003).

Plant cells frequently enhance the rate of sucrose consumption under low-oxygen stress to compensate for the low energy yield during glycolysis (2–4 mol ATP per mol glucose), in comparison to aerobic mitochondrial respiration (30–36 mol ATP per mol glucose) (Summers et al., 2000). We have shown previously that rice plants sustain a higher fermentation rate during anoxia as compared to anoxia-sensitive wheat plants, but ATP levels still drop by two-third (Mustroph et al., 2006a). The decline in cellular ATP content during low-oxygen stress will impact the glycolytic phosphorylation reactions catalyzed by hexokinases and phosphofructokinases (Bouny and Saglio, 1996). Thus, it was hypothesized that plants might use pyrophosphate (PPi) instead of ATP as an alternative energy source for phosphorylation processes during ATP deficiency (Weiner et al., 1987; Stitt, 1998; Huang et al., 2008). PPi is a by-product of many biosynthetic processes like DNA and protein synthesis, and its levels are not changed during oxygen deficiency stress (Dancer and ap Rees, 1989; Mohanty et al., 1993; Mustroph et al., 2005).

Sucrose cleavage and subsequent phosphorylation of hexoses are usually catalyzed by the ATP-dependent invertase/hexokinase reaction, but can be replaced by the PPi-consuming sucrose synthase/UDP-glucose pyrophosphorylase reaction. The activation of this alternative pathway of sucrose catabolism upon oxygen deficiency stress was confirmed for several plant species, and the substitution of the ATP-dependent reactions by the UTP and PPi-dependent pair of enzymes is generally accepted (Springer et al., 1986; Ricard et al., 1991, 1998; Guglielminetti et al., 1995; Perata et al., 1996, 1997; Biemelt et al., 1999; Mustroph and Albrecht, 2003; Albrecht et al., 2004; Bailey-Serres and Voesenek, 2008).

The second phosphorylation step in the glycolytic pathway is the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate via phosphofructokinase. The reaction can be performed by an ATP-dependent phosphofructokinase (PFK) or by the PPi-dependent form pyrophosphate-fructose-6-phosphate-phosphotransferase (PFP). PFP consists of two different subunits (PFP-alpha, PFP-beta) that form a heterotetramer (Wong et al., 1990; Teramoto et al., 2000), whereas the subunit composition of a PFK complex is not known yet.

Abbreviations: ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase; PFK, ATP-dependent phosphofructokinase; PF2K, phosphofructo-2-kinase; PFP, pyrophosphate-fructose-6-phosphate-phosphotransferase; PPi, pyrophosphate.

The contribution of each phosphofructokinase enzyme to the phosphorylation step and their functions during different growth conditions remain unclear. While during hypoxic stress both enzyme activities are induced in wheat and maize roots (Mustroph and Albrecht, 2003), their activities do not change in roots of potato plants (Mustroph et al., 2005). Experiments with transgenic potato plants with a severe decrease in PFP activity revealed no change in aerobic (Hajirezaei et al., 1994) or anoxic metabolism (Mustroph, unpublished results). However, the PFP activity was increased in anoxically germinated rice coleoptiles (Mertens et al., 1990; Kato-Noguchi, 2002) and anoxic rice suspension cells (Mohanty et al., 1993), whereas PFK activity was unaffected.

Studies on genes encoding the ATP- and PPi-dependent phosphofructokinases have been previously limited by the lack of information on coding sequences for plant PFKs, which were only recently described (Mustroph et al., 2007; Winkler et al., 2007). The *Arabidopsis thaliana phosphofructokinase* gene family consists of 11 members, of which four members encode PFPs and seven encode PFKs (Nielsen et al., 2004; Mustroph et al., 2007; Winkler et al., 2007). Here, we identify 15 putative *phosphofructokinase* genes of rice, five encoding PFPs and 10 encoding PFKs, and perform transcriptomic and enzymatic studies to evaluate the contribution of *phosphofructokinase* genes to anoxic metabolism in roots, stems, and leaves of the highly anoxia-tolerant plant. The analysis includes evaluation of *phosphofructokinase* gene expression during darkness and illumination, since it was already demonstrated that illumination greatly enhances the tolerance of plants to anoxic stress due to the contribution of photosynthesis to energy production (Mustroph et al., 2006b). The findings indicate that individual members of the *PFK* and *PFP* gene families are induced by anoxia, although activities of these two enzymes are only slightly increased by the stress. Notably, the induction of *PFP* genes was greater in the shoots and leaves than in roots, leading to the suggestion that the induction of the PPi-utilizing phosphofructokinase may only occur in tissues with sufficient carbohydrate levels for consumption during the stress. During the study, mRNA sequences and intracellular localization of the proteins were analyzed, and several discrepancies to the annotated versions were found.

MATERIALS AND METHODS

PLANT MATERIAL AND ANOXIC TREATMENT

Rice seeds (*Oryza sativa* ssp. *indica* cv. Cigalon) were watered, germinated for 3 days in the dark at 27°C and transferred to pots containing Knop nutrient solution that was continuously aerated (Mustroph and Albrecht, 2003). After growth for 20 days in 16 h-light/8 h-dark cycles and 250 μmoles photons m^{-2} s^{-1} the plants were placed in desiccators, while their roots were immersed in nutrient solution. The gaseous and the aqueous phase (the nutrient solution) in the desiccator were continuously flushed with nitrogen gas and plants were treated for 0.5, 2, 8, or 24 h in the light (250 μmoles photons m^{-2} s^{-1}) or in complete darkness. Additionally, light-grown plants were exposed to 24 h darkness in ambient air to distinguish between light-dependent and anoxia-independent gene expression regulation. For harvest, plants were removed from the desiccator, divided into roots, stem and leaf sheaths, and frozen immediately in liquid nitrogen within 60 s.

ISOLATION OF RNA, cDNA SYNTHESIS, AND SEMI-QUANTITATIVE REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION ANALYSIS

RNA from frozen tissues was extracted using the Trizol reagent (Bioline GmbH, Luckenwalde, Germany). cDNA was synthesized from 15 μg RNA with standard protocols using oligodT primers and MLV reverse transcriptase (Fermentas GmbH, St. Leon-Rot, Germany). The PCR reactions were performed using Taq polymerase (New England Biolabs GmbH, Frankfurt, Germany) according to the purchaser's protocol. The oligonucleotide primers used are listed in Table S1 in Supplementary Material. Because of the high GC content of rice cDNA, for each primer combination the optimal PCR conditions were tested using different concentrations of $MgCl_2$, DMSO, betaine, cycle numbers, and temperatures. The optimal conditions for the semi-quantitative amplification of each fragment from cDNA are summarized in Table S2 in Supplementary Material. PCR products were analyzed by electrophoresis in standard 1% agarose gels. DNA bands were quantified by use of the program AlphaEaseFC (Alpha Innotech Corporation).

CLONING AND SEQUENCING OF PFKs AND PFPs

For intracellular localization of the proteins and confirmation of nucleotide sequences, full-length *PFKs* were amplified from cDNA with VELOCITY DNA polymerase (Bioline, Germany) by use of the primers listed in Table S1 in Supplementary Material and cloned into the vector pDONR221 by use of the Gateway technology (Invitrogen, Germany). The resulting entry clones were completely sequenced by Sanger sequencing and compared to the reference sequences from the rice genome annotation project (Ouyang et al., 2007)[1]. Subsequently, correct clones were transferred via the LR reaction into the vector pEarleyGate 103 (Earley et al., 2006), which had been modified by the addition of one base in order to put the C-terminal GFP into frame. The plasmids were sequenced again to verify the correct frame of the PFK-GFP fusion.

The two sequences *OsPFK07* and *OsPFK08* did not fully match the annotated mRNA sequences. In this case, several clones and fragments of cDNA as well as of genomic DNA were sequenced, not only from the variety Cigalon, but also from other varieties (Nipponbare, M202, FR13A, CT6241, Dongjin, Hwayoung). For *OsPFK07*, a new full-length construct was made based on the truncated version of the Cigalon variety.

After the first localization studies and the observation of aggregate formation in transiently transformed tobacco leaves, we also cloned the N-terminal ca. 100 amino acids to obtain truncated *PFK* sequences by use of the primers listed in Table S1 in Supplementary Material. Here, products were amplified with Phusion DNA Polymerase (Fermentas GmbH, St. Leon-Rot, Germany), and cloned by the Gateway technology into the vector pDONR221 (Invitrogen, Germany). After sequencing, correct sequences were subcloned by the LR clonase into the vector pK7FWG2,0 (Karimi et al., 2002).

For sequencing of *PFPs*, a major piece of each mRNA sequence was amplified by PCR from cDNA by use of the primers listed in Table S1 in Supplementary Material. The PCR products were directly sequenced and compared to the annotated sequences. Nucleotide sequences differing from the annotated versions were submitted to GenBank with the IDs KC620557-KC620559.

[1]http://rice.plantbiology.msu.edu

TRANSIENT TRANSFORMATION OF TOBACCO LEAVES AND INTRACELLULAR LOCALIZATION OF PFKs

Binary expression vectors containing PFK-GFP fusion constructs were transformed into the Agrobacteria strains LBA4404 (full-length constructs) and GV3103 (N-terminal truncations). Tobacco leaves were transiently transformed by infiltration with Agrobacteria suspensions as described in Bendahmane et al. (2000) and Mustroph et al. (2007). About 3–4 days after infiltration, leaf disks were collected and protoplasts were isolated as previously described (Bayley et al., 1992; Mustroph et al., 2007). GFP fluorescence was analyzed on either protoplasts or undigested leaf disks by confocal laser scanning microscopy using Leica TCS SP2 (Leica, Germany, at λ_{ex} 488 nm, λ_{em} 530–555 nm for GFP and 650–720 nm for chlorophyll emission).

ENZYME ACTIVITIES

Plant tissue was ground in liquid nitrogen to a fine powder and extracted in 50 mM Hepes-KOH, pH 6.8 containing 5 mM Mg acetate, 5 mM β-mercaptoethanol, 15% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and 0.1 mM Pefabloc proteinase inhibitor (Boehringer Mannheim, Germany). The homogenate was centrifuged at 13,000 *g* at 4˚C for 15 min. The resulting supernatant was used for spectrophotometric determination of PFK and PFP activities as well as the fermentative enzymes alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) at 340 nm using a UVIKON photometer (Kontron, Germany).

For the assay of PFK (EC 2.7.1.11), the reaction mixture was 0.1 M Hepes-KOH, pH 7.9 with 2 mM $MgCl_2$, 0.15 mM NADH, 7.5 mM fructose-6-phosphate, 1 U aldolase, 1 U triosephosphate isomerase, and 1 U glycerol-3-phosphate-dehydrogenase (Sigma-Aldrich, Germany). The reaction was started by addition of 2.5 mM ATP. For assay of PFP (EC 2.7.1.90), the same reaction mixture was used with the addition of 1 μM fructose-2,6-bisphosphate, and initiation of the reaction with 1 mM NaPPi (modified from Gibbs et al., 2000). PDC (EC 4.1.1.1) was assayed in 50 mM MES, pH 6.8, with 25 mM NaCl, 1 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 2 mM dithiothreitol, 0.17 mM NADH, 50 mM sodium oxamate, 10 U ADH (Sigma-Aldrich, Germany) and the reaction was initiated by addition of 10 mM pyruvate (Waters et al., 1991). ADH (EC 1.1.1.1) was assayed in 50 mM TES buffer, pH 7.5, with 0.2 mM NADH and the reaction was initiated by the addition of 10 mM acetaldehyde (Waters et al., 1991). Protein concentration was measured according to Bradford (1976).

BIOINFORMATICS AND STATISTICS

Nucleotide and protein sequences were analyzed by use of the program Bioedit (Tom Hall, Ibis Biosciences, Carlsbad, USA). ClustalW alignments were done with the website http://www.genome.jp/tools/clustalw/. Phylogenetic trees were constructed by use of the website http://www.phylogeny.fr/version2_cgi/phylogeny.cgi. Expression data from rice microarray experiments were obtained from Mustroph et al. (2010), and heat maps were drawn with TIGR-MeV (Saeed et al., 2006).

Polymerase chain reaction analyses were performed on three independent bioreplicates. Infiltration of tobacco leaves was repeated three to five times for each construct. Enzymatic measurements were done with three independent bioreplicates, and results were statistically analyzed with the package "multcomp" in R by use of the Tukey HSD test.

RESULTS

IDENTIFICATION OF GENES ENCODING PHOSPHOFRUCTOKINASES IN RICE AND COMPARISON OF THEIR SEQUENCES

The *Arabidopsis* phosphofructokinase-encoding gene sequences were used to perform a BLAST-search of the rice genome (Ouyang et al., 2007)[2] for homologous nucleotide sequences. This analysis identified 15 different sequences with high similarity to *Arabidopsis* phosphofructokinase-encoding genes. A phylogenetic tree based on the amino acid sequences was generated, including the sequence of phosphofructo-2-kinase/fructose-2,6-bisphosphatase, which is a similar, but unrelated protein (**Figure 1**). Four of the rice proteins (LOC_Os02g48360, LOC_Os06g22060, LOC_Os08g25720, LOC_Os09g12650) grouped with the two putative *Arabidopsis* PFP-alpha subunits (At1g20950, At1g76550) and known PFP-alpha subunits from other plants. Surprisingly, two rice PFP-alpha protein sequences (LOC_Os08g25720, LOC_Os09g12650) were found to be distinct from the PFP-alpha subunit sequences of dicotyledonous species known so far (**Figure 1**). One rice amino acid sequence (LOC_Os06g13810) showed high homology to the two putative *Arabidopsis* PFP-beta subunits (At1g12000, At4g04040) and the PFP-beta subunits from *Citrus* × *paradisii*, potato and *Ricinus communis*.

The remaining 10 rice phosphofructokinases grouped with seven *Arabidopsis* PFK proteins, indicating that both species have larger families of ATP-dependent than PPi-dependent phosphofructokinases. Based on the sequence similarities among the PFK family, three sub-clades were distinguished (**Figure 1**). One group includes five rice (LOC_Os01g09570, LOC_Os01g53680, LOC_Os05g10650, LOC_Os05g44920, LOC_Os06g05860) and five *Arabidopsis* PFKs, the two other groups have each one *Arabidopsis* member (At2g22480, At5g47810), and two (LOC_Os04g39420, LOC_Os09g30240), or three (LOC_Os08g34050, LOC_Os09g24910, LOC_Os10g26570) rice members, respectively (**Figure 1**). Based on these results, we propose to distinguish the three PFK sub-clades as PFK_A, PFK_B, and PFK_C (**Table 1**). The amino acid alignment reveals distinct sequence patterns with high similarity among the members of each sub-clade that differentiate them from the other two sub-clades (Figure S1 in Supplementary Material). Besides other sequence differences, members of group B have shorter N- and C-termini, whereas members of group C have shorter C-termini compared to group A members. Furthermore, all members of group C are predicted to be localized to plastids (**Table 1**; TargetP, Emanuelsson et al., 2000; pSort, Horton et al., 2006).

EXPRESSION OF PHOSPHOFRUCTOKINASE GENES UNDER AERATION

To study the expression patterns of all representatives of the rice *phosphofructokinase* gene family, transcript abundance was analyzed by use of semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) using total RNA from roots, leaf sheaths, and stems of rice seedlings grown under non-stress conditions in

[2]http://rice.plantbiology.msu.edu

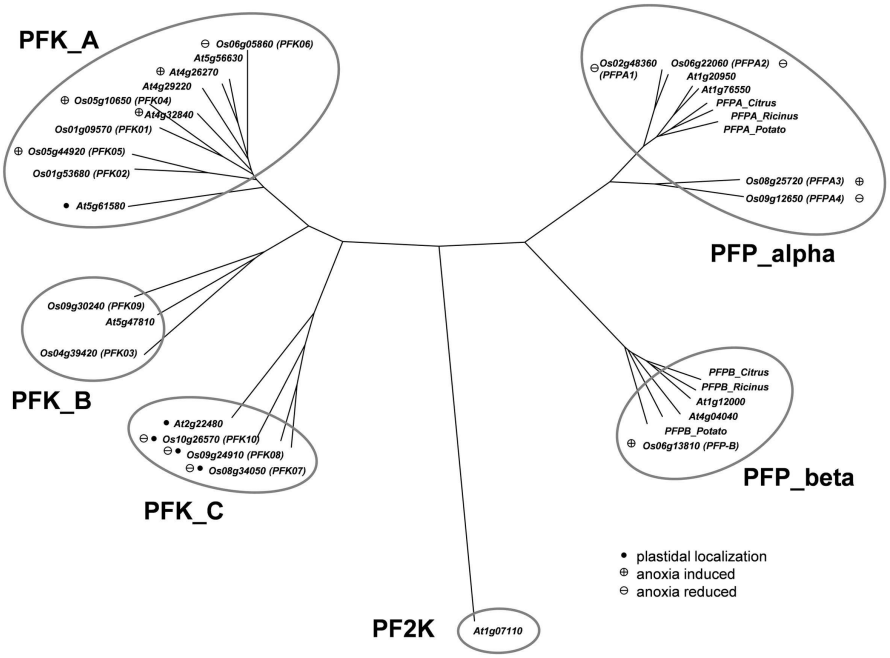

FIGURE 1 | Phylogeny of plant phosphofructokinase genes. Unrooted neighbor joining tree produced from CLUSTALW alignment of amino acid sequences of *Arabidopsis* and rice *phosphofructokinase* genes. Genes marked with a "plus" showed increased transcript levels and names marked with a "minus" showed decreased transcript levels during anoxic stress treatment, respectively. *Arabidopsis* genes marked with a plus are those that were induced by >2-fold in published microarray experiments (Mustroph et al., 2010). Genes marked with a black dot are plastid localized. PF2K: *Arabidopsis phosphofructo-2-kinase* gene (At1g07110) used as an outgroup in the phylogenetic analysis. Accession numbers for other plant *PFPs*: Citrus_Alpha, AAC67587; Potato_Alpha, P21342; Ricinus_Alpha, Q41140; Citrus_Beta, AAC67586; Potato_Beta, P21343; Ricinus_Beta, Q41141.

the light or after transfer to complete darkness for 24 h (labeled as 0 h* in **Figure 2**). The abundance of *actin* mRNA was used as a loading control (Ren et al., 2005). The accumulation of some transcripts encoding PFKs and PFPs were organ-specifically and/or light-dependently regulated, while others exhibited ubiquitous expression patterns. mRNAs for *OsPFK01 – OsPFK03*, *OsPFK05 – OsPFK08*, and *OsPFK10* were detected in all analyzed organs of seedlings exposed to light, as well as transcripts of *OsPFPA1* and *OsPFPA4* (**Figure 2**). *OsPFK04* and *OsPFK09* mRNAs were present at low levels in tissues of non-stressed seedlings during both light and dark exposure (0 h anoxia). *OsPFPA2* was strongly expressed in the stem, and *OsPFPA3* as well as *OsPFP-B* showed lower expression in leaves than in roots and stems. The transfer of seedlings to darkness for 24 h resulted in a decrease in transcripts encoding *OsPFPA4* in all tissues, and *OsPFK07* and *OsPFPA1* in leaves (0 h* in **Figure 2**). *OsPFPA3* transcript levels were slightly enhanced during darkness, whereas transcripts of *OsPFK01 – OsPFK03, OsPFK05, OsPFK06, OsPFK08, OsPFK10, OsPFKPFPA2*, and *OsPFKPFP-B* accumulated to similar extent during dark and light growth.

EXPRESSION OF PHOSPHOFRUCTOKINASE GENES UNDER ANOXIA IN LIGHT AND DARKNESS

To identify the *phosphofructokinase* genes that may play a tissue specific role in the response to anoxic metabolism in rice seedlings, transcript levels of the 10 *PFK* and 5 *PFP* genes were quantified in seedling tissues following exposure to anoxia (30 min to 24 h), in the light or complete darkness. The *pyruvate decarboxylase* (*PDC1, LOC_Os05g39310*) transcript, which is known to significantly increase in abundance under low-oxygen conditions in rice seedlings, was monitored as positive control for anoxic stress (**Figure 2**). Under normoxic conditions, the *PDC1* mRNA was more abundant in roots as compared to stems and leaves. *PDC1* transcript level increased within 30 min of anoxia stress in roots, whereas the increase in stems and leaves was detected after 2 h of stress. *PDC1* transcripts were induced by anoxia in roots and leaves with similar kinetics in seedlings exposed to light or darkness. In stems, the induction of *PDC* was slightly faster in darkness than during light irradiation.

The evaluation of anoxic stress-responsive mRNA accumulation of the rice *PFK* and *PFP* genes identified three groups. Expression of group 1 *phosphofructokinase* genes (*OsPFK01, OsPFK02, OsPFK03*) did not change during stress treatment (**Figure 2**). Group 2 *phosphofructokinase* genes (*OsPFK06, OsPFK07, OsPFK08, OsPFK10, OsPFPA1, OsPFPA2, OsPFPA4*) showed a decrease in transcript levels in response to anoxia. Among these genes, *OsPFK10* and *OsPFPA2* transcripts were reduced in stems only after 24 h anoxia in darkness, whereas the transcript abundance of the other five genes reduced more rapidly. *OsPFK06* transcripts decreased in roots and stems, whereas *OsPFK07, OsPFK08, OsPFPA1*, and *OsPFPA4* transcripts decreased in all analyzed tissues. Interestingly, all *PFK* genes in group 2 that showed reduced expression under anoxia are predicted to be plastid-localized (**Table 1**).

Table 1 | Rice *phosphofructokinase* genes, Locus identifier, length of coding sequence (number of bases), and protein size (number of amino acids).

Locus ID	Name	Length of mRNA	Length of protein	Predicted localization	RNAseq expression level from 16 tissues	
					Maximum	Mean
PFK_A						
LOC_Os01g09570	*OsPFK01*	1596	531	Cytosolic	69.04	27.60
LOC_Os01g53680	*OsPFK02*	1683	560	Cytosolic	44.50	10.24
LOC_Os05g10650	*OsPFK04*	1629	542	Cytosolic	2.89	1.40
LOC_Os05g44922	*OsPFK05*	1704	567	Cytosolic	53.36	14.39
LOC_Os06g05860	*OsPFK06*	1677	558	Plastid (confirmed cytosolic)	118.48	33.60
PFK_B						
LOC_Os04g39420	*OsPFK03*	801	266	Cytosolic	46.80	6.89
LOC_Os09g30240	*OsPFK09*	1398	465	Cytosolic	48.42	5.79
PFK_C						
LOC_Os08g34050	*OsPFK07*	1590 (1608)	529 (535)	Plastid	7.98	2.10
LOC_Os09g24910	*OsPFK08*	1584 (1665)	527 (555)	Plastid	14.83	4.51
LOC_Os10g26570	*OsPFK10*	1575	524	Plastid	41.14	6.07
PFP-ALPHA						
LOC_Os02g48360	*OsPFPA1*	1854	617	Cytosolic	98.49	19.19
LOC_Os06g22060	*OsPFPA2*	1869	622	Cytosolic	171.15	53.64
LOC_Os08g25720	*OsPFPA3*	1854	617	Cytosolic	102.03	42.51
LOC_Os09g12650	*OsPFPA4*	1884 (1722)	627 (573)	Cytosolic	0.68	0.36
PFP-BETA						
LOC_Os06g13810	*OsPFP-B*	1704	567	Cytosolic	110.02	52.99

Numbers for OsPFK07 are for the sequence of the variety FR13A. Numbers in brackets are annotated sizes from the rice genome annotation project (http://rice.plantbiology.msu.edu; Ouyang et al., 2007). Intracellular localization was predicted by use of two online programs (TargetP, Emanuelsson et al., 2000; pSort, Horton et al., 2006). RNAseq expression data are from the Nipponbare variety from 16 tissues, summarized from the rice genome annotation project (http://rice.plantbiology.msu.edu/expression.shtml).

Phosphofructokinase genes of group 3 were induced under anoxia (*OsPFK04, OsPFK05, OsPFPA3, OsPFP-B*). *OsPFK09* is also included in group 3, but displayed a markedly delayed increase in mRNA accumulation in response to stress at an extremely low expression level. *OsPFK04*, predicted to encode a cytosolic enzyme, expresses the strongest anoxia-induced transcript of group 3. *OsPFK04* mRNA content was below the detection level in seedlings grown under aerated conditions, but increased dramatically within 30 min of anoxia in roots resembling the pattern of *PDC1* accumulation. *OsPFK05* mRNA accumulated less dramatically only in leaves after 2–24 h of anoxia. Among the *PFP* genes, only the content of *OsPFPA3* and *OsPFP-B* mRNAs slightly increased in leaves and stems after 2 h of anoxia (**Figure 2**), resembling the expression of *OsPFK05*. The group 2 and group 3 *phosphofructokinase* genes are highlighted in the phylogenetic tree with a minus or plus sign, respectively, for their low-oxygen stress responsiveness (**Figure 1**).

Exposure to light or darkness most dramatically altered mRNA content of group 2 genes of the *phosphofructokinase* family. These genes showed a higher decrease in transcript levels in response to anoxia in darkness as compared to anoxia during illumination. *OsPFPA1* and *OsPFPA4* mRNA content was reduced during dark anoxia most likely in response to the transfer to darkness, as 24 h dark incubation under constant aeration (sample 0 h* in **Figure 2**) caused a similar loss of the mRNA level. On the other hand, *OsPFK06, OsPFK07, OsPFK08, OsPFK10*, and *OsPFPA2* transcript levels were decreased stronger during anoxia in darkness than in light, but not during darkness alone.

SEQUENCE VARIATIONS OF PHOSPHOFRUCTOKINASE GENES

Amplification and sequencing of *PFK* genes revealed in the *OsPFK07* and *OsPFK08* sequences a significant difference to annotated sequences. *OsPFK08* was annotated with two splicing forms (Ouyang et al., 2007)[3]. The longer form *LOC_Os09g24910.1* contained a sequence that was not present in any other sequence of the group PFK_C (**Figure 3A**; Figures S1 and S2 in Supplementary Material). Sequencing of several cDNA clones derived from RNA of different rice varieties revealed the unique presence of the shorter annotated splicing form, *LOC_Os09g24910.2* (**Figure 3A** and data not shown). *OsPFK07* surprisingly revealed the lack of 20 nucleotides in its mRNA sequence in comparison to the annotated version, leading to a frame shift in the translated protein sequence and a premature stop codon (**Figure 3B**). We re-sequenced the *OsPFK07* cDNA and genomic DNA of several rice varieties in order to confirm the initial findings. Genomic clones of *OsPFK07* of all varieties consistently resulted in the complete annotated

[3]http://rice.plantbiology.msu.edu

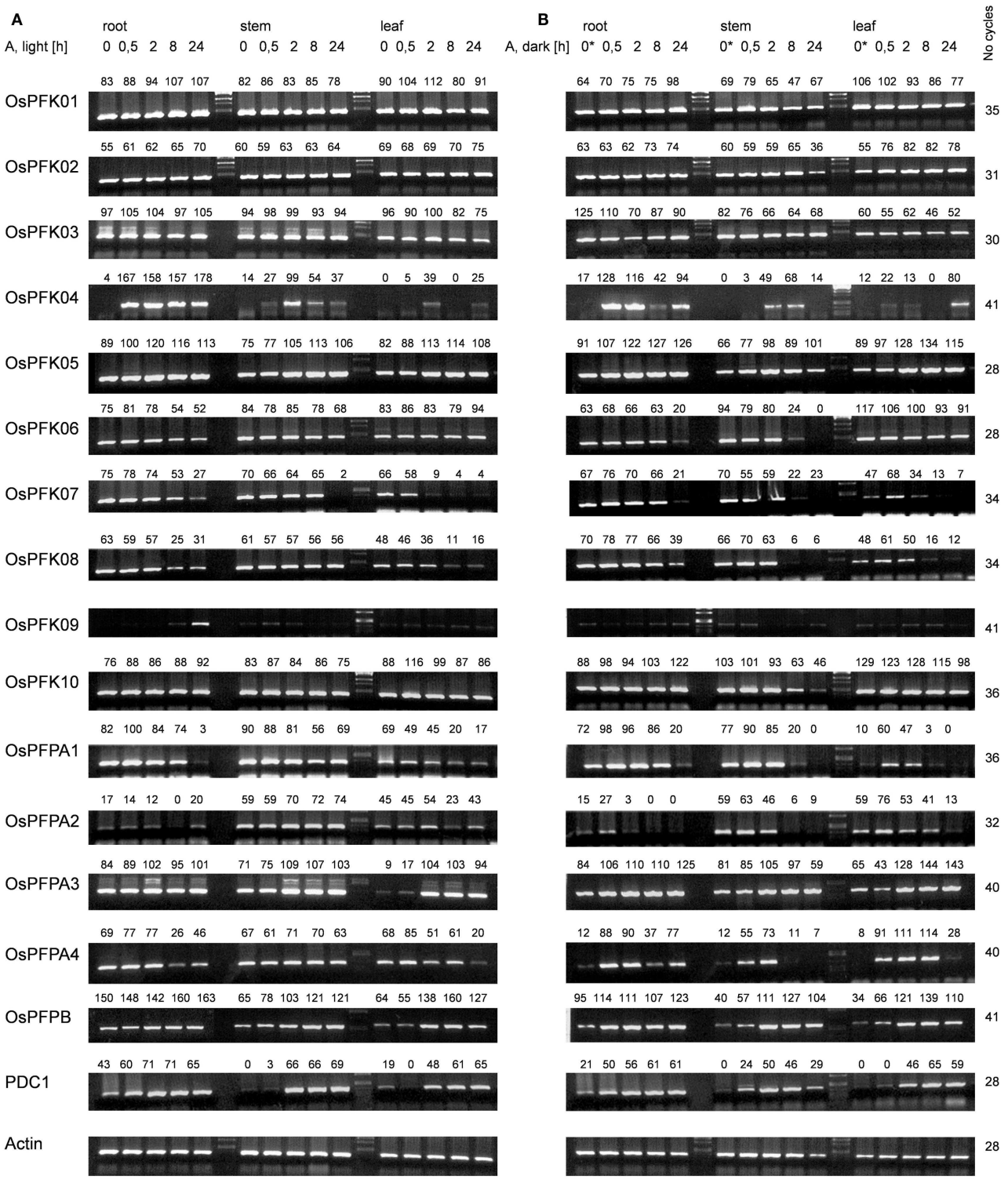

FIGURE 2 | Semi-quantitative RT-PCR analysis of transcript abundance of the rice *phosphofructokinase* genes. Total RNA was extracted from roots, stems, and leaves of 3-week-old rice plants under normoxia or after different durations of anoxic stress. Samples in **(A)** were treated in the light; samples in **(B)** were treated in the dark. The sample 0* was harvested after 24 h in darkness under normoxic conditions. *Actin* and *PDC1* (Os05g39310) transcript levels were used to control for equal loading and to confirm low-oxygen stress, respectively. RT-PCR product levels were quantified by use of the program AlphaEaseFC (Alpha Innotech Corporation), and normalized to the level of actin mRNA in the same sample. The relative transcript level is indicated above each band (% of light control). Gel images are representative of three independent biological replicate experiments. RT-PCR conditions are listed in Table S2 in Supplementary Material.

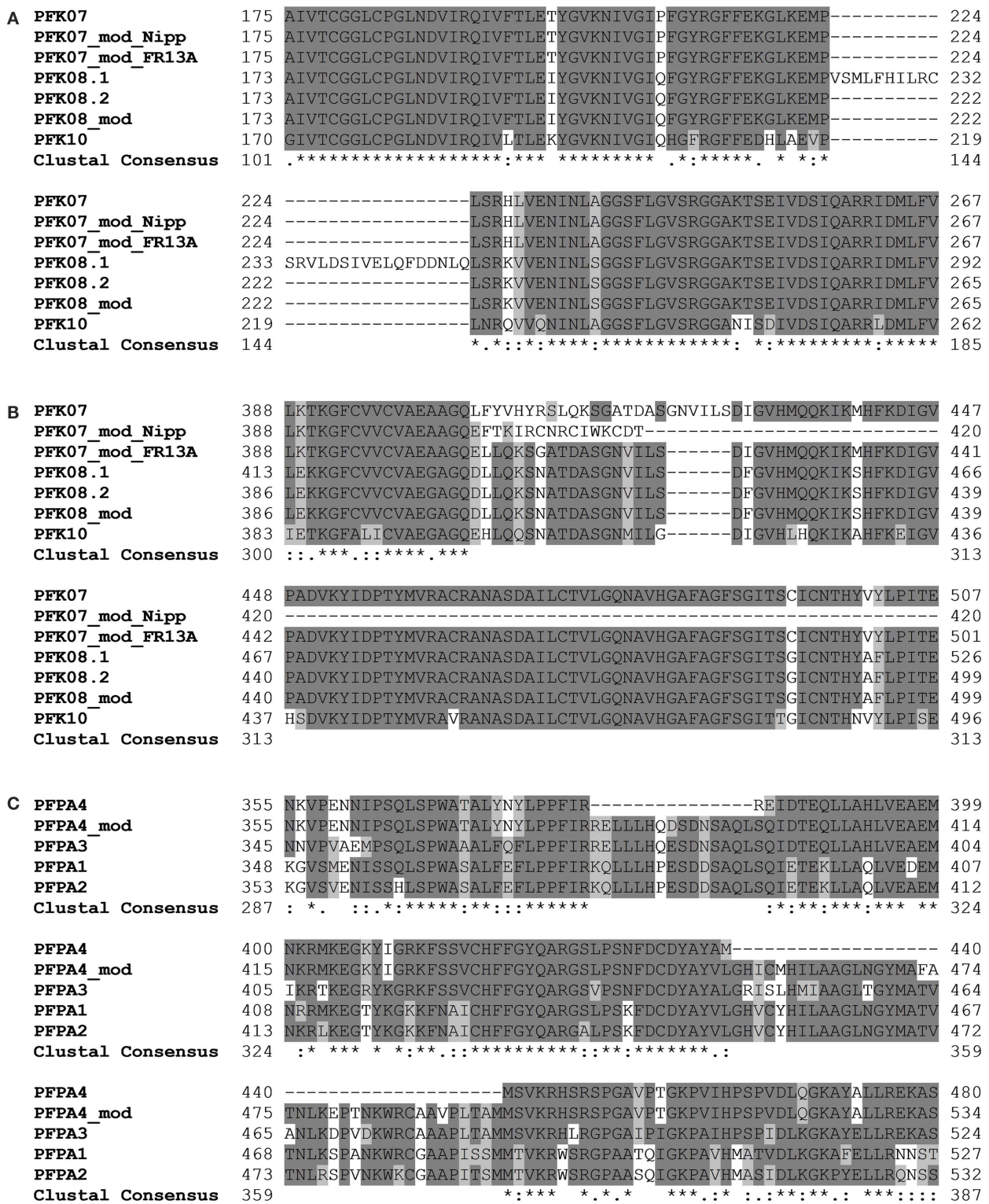

FIGURE 3 | Sections of protein alignments for different phosphofructokinase subgroups, whose sequences differ from the annotated public sequences (http://rice.plantbiology.msu.edu, Ouyang et al., 2007). New sequences that were confirmed by sequencing of PCR products are indicated as "modified" (mod). Full-length nucleotide and protein sequences can be found in Figure S2 in Supplementary Material. **(A)** Two splicing forms of *OsPFK08*; **(B)** modified splicing for *OsPFK07*, leading to a frame shift in the protein, and the modified sequence in the rice variety FR13A; **(C)** modified splicing of *OsPFPA4*.

sequence including the 20 nucleotide region, while sequencing of the *OsPFK07* cDNA of these varieties gave always rise to a truncated sequence lacking this stretch of 20 nucleotides. As result, a truncated protein lacking 120 amino acids at the C-terminus is predicted to be most likely non-functional. Supposedly, this mutation could occur in the rice genome since two other members of the PFK_C are available, while *Arabidopsis* only contains one isoform of this subgroup (**Figure 1**). In consistency with a putative non-functional gene, the expression level of *OsPFK07* is very low in many tissues, as derived for example from RNAseq analyses (**Table 1**). However, this finding was not confirmed in our experiments (**Figure 2**).

Interestingly, two rice varieties, FR13A and CT6241, revealed a second modification of the *OsPFK07* gene, an insertion of two bases in the genomic sequence close to the modified splicing site (Figure S2 in Supplementary Material). This altered sequence restores the reading frame comparable to *OsPFK08* and *OsPFK10* (**Figure 3B**). However, which of the two sequence variants evolved earlier during evolution remains to be explored in future.

Our sequencing revealed also a modified *OsPFPA4* nucleotide sequence. The annotated sequence lacks part of the final coding sequence leading to encoded PFP-alpha subunit with a truncated C-terminus in comparison to the other three isoforms. However, the sequencing results revealed another mRNA sequence that represents an un-annotated alternative splicing form, which more closely resembles the sequence of the other three homologous genes (**Figure 3C**; Figure S2 in Supplementary Material). Interestingly, the expression level of *OsPFPA4* was very low in rice tissues as observed by RNAseq analyses (**Table 1**), but in our analyses *OsPFPA4* was significantly expressed in detectable amounts in seedlings (**Figure 2**).

INTRACELLULAR LOCALIZATION OF PFKs

The expression analysis under anoxia revealed decreased expression of several *PFK* genes, in particular those representatives that encode putative plastid-localized proteins. We therefore aimed to confirm the intracellular localization of PFK isoforms, and applied a similar approach as previously shown for *Arabidopsis* PFKs (Mustroph et al., 2007). Using the leaf infiltration technique for transient transformation of the rice *PFK* genes in tobacco leaves, we showed the clear localization of 5 out of 10 different PFK isoforms in the cytoplasm. Expression of the full-length PFKs of *OsPFK01, OsPFK02, OsPFK04, OsPFK05*, and *OsPFK06* resulted in their cytosolic localization (Figure S3 in Supplementary Material), but the proteins formed cytoplasmic aggregates. Several modified protocols of transient transformation were tested, but resulted always in the production of aggregates: changes in incubation time of transiently transformed tobacco leaves, at different temperatures, use of different Agrobacteria strains and binary vectors, and ultimately stable transformation in *Arabidopsis* (data not shown). However, expression of fusion proteins containing the first 100 amino acids of the respective PFK protein resulted in even distribution throughout the cytosol (**Figure 4**). Surprisingly, *OsPFK06*, which was predicted to be plastid-localized, was clearly cytosolic (**Figure 4E**). As a very short transit peptide of only 10 amino acids was predicted (TargetP, Emanuelsson et al.,

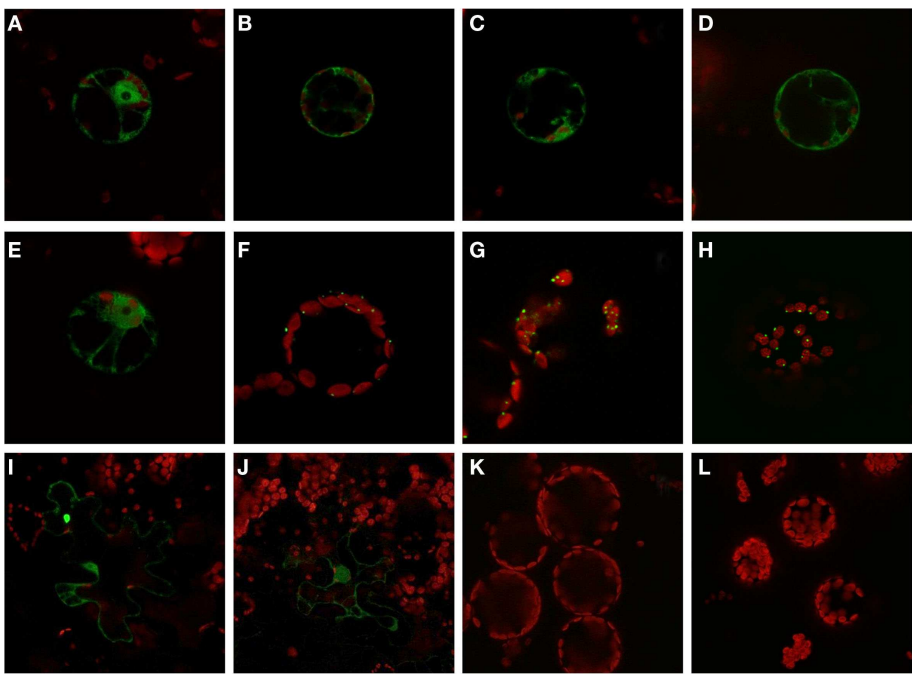

FIGURE 4 | Subcellular localization of PFK isoforms from rice. Full-length mRNA sequences or the N-terminal part of the sequences were cloned in frame in front of a GFP-coding sequence and were transiently transformed into tobacco leaves by Agrobacterium infiltration. After 3–4 days, protoplasts or undigested leaf disks were isolated and analyzed by confocal microscopy. Green color represents GFP fluorescence, red color represents chlorophyll autofluorescence. N-terminal part: **(A)** OsPFK01; **(B)** OsPFK02; **(C)** OsPFK04; **(D)** OsPFK05; **(E)** OsPFK06; **(K)** OsPFK08, **(L)** OsPFK10. Full-length CDS: **(F)** OsPFK07; **(G)** OsPFK08; **(H)** OsPFK10; **(I)** OsPFK03; **(J)** OsPFK09.

2000), it is suggested that the organellar localization is a false prediction.

OsPFK03 and *OsPFK09* are both members of the weakly expressed PFK_B subgroup and are mainly expressed in seeds (Figure S4 in Supplementary Material). Tobacco leaves infiltrated with the truncated or full-length constructs p35S::OsPFK03-GFP and p35S::OsPFK09-GFP did surprisingly not show GFP fluorescence in tobacco mesophyll cells in four independent experiments, although the same vectors, bacterial strains and conditions had been used as for the positive control p35S::OsPFK05-GFP. However, we observed GFP fluorescence in a few epidermal cells that demonstrate cytosolic localization of the proteins (**Figure 4**; Figure S3 in Supplementary Material). It can be concluded that both proteins are less stable during transient overexpression in tobacco leaves than members of the PFK_A group.

All members of the PFK_C group, *OsPFK07, OsPFK08*, and *OsPFK10*, were predicted to be localized in plastids, with a predicted transit peptide consisting of 30–50 amino acid residues. Our assay revealed localization of the full-length proteins in plastids, however associated with the formation of aggregates (**Figure 4**), as previously described for *Arabidopsis AtPFK4* and *AtPFK5* (Mustroph et al., 2007). Still, we observed clear association of the aggregates with chloroplasts, and not with other compartments (**Figure 4**; Figures S3S,T in Supplementary Material). Expression of the N-terminal part did not result in detectable plastidal GFP fluorescence (**Figures 4K,L**).

PHYLOGENETIC ANALYSIS AND EXPRESSION UNDER OXYGEN DEFICIENCY STRESS

The expression analysis of *phosphofructokinase* genes of rice under anoxia revealed differential gene expression of several members in roots and shoots of seedlings. For a wider comparison among species, we first identified *phosphofructokinase* genes in different plant species by use of the Phytozome genome collection[4], and constructed a separate phylogenetic tree for *PFK* and *PFP* sequences (**Figures 5** and **6**). We selected *phosphofructokinase* genes from plant genomes, of which expression data are available under oxygen deficiency stress, and additionally used two other monocotyledonous species, *Sorghum bicolor* and *Brachypodium distachyon*. Comparison of the genome of the green algae *Chlamydomonas reinhardtii* revealed the presence of *PFK_C* genes only, hinting at a complex evolution of *phosphofructokinase* genes in higher plants. Gene expression data from published microarray experiments were added from rice (Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009; Mustroph et al., 2010), *Arabidopsis* (Branco-Price et al., 2008; Hsu et al., 2011; Lee et al., 2011), poplar (Kreuzwieser et al., 2009), cotton (Christianson et al., 2010), and soybean (Nanjo et al., 2011). It was intended to obtain a first qualified overview about the potential role of phosphofructokinases under oxygen deficiency stress.

Interestingly, all analyzed monocotyledonous species only contained one gene for the beta-subunit of PFP, while dicotyledonous species usually contained more isoforms. In general, PFP-beta-encoding genes of either monocotyledonous or dicotyledonous species show only a low induction upon low-oxygen stress, among them the rice *PFP-B*-encoding gene being up to 3.8 times induced in the microarray experiments (Table S3 in Supplementary Material). This induced expression was shown to a similar extent in our experiments (**Figure 2**). Transcript levels of three PFP-alpha subunit-encoding genes were reduced or unchanged under oxygen deficiency stress in rice, while *OsPFPA3* was moderately induced in leaves, as indicated by RT-PCR (**Figure 2**) and microarray analyses (**Figure 5**). This latter gene was grouped into a monocotyledonous-specific sub-clade of PFP-alpha subunit encoding sequences. The genes most similar to dicotyledonous species were down-regulated, similar to the PFP-alpha subunit encoding genes of other plant species, whose expression was hardly induced (**Figure 5**). It would be interesting to analyze if other members of this monocotyledonous-specific subgroup are also induced by oxygen deficiency stress, for example in *Sorghum* or *Brachypodium*, or if this induction is specific to the submergence-tolerant rice plant.

Among the *PFK* gene family, each plant species analyzed contained members that were induced by oxygen deficiency stress (**Figure 6**), indicating an important function in plant metabolism under the stress condition. Most of the induced genes, among them *OsPFK04, OsPFK05, AtPFK3*, and *AtPFK6*, belong to the subgroup PFK_A. The plastidal genes of subgroup PFK_C were generally down-regulated in rice, *Arabidopsis* and soybean (**Figure 6**; Table S3 in Supplementary Material). *OsPFK06* shows the strongest reduced mRNA content and its encoded PFK isoform is localized in the cytosol (**Figure 4E**). *OsPFK06* was grouped with two other genes from monocotyledonous species, whose expression under oxygen deficiency stress remains to be determined.

ACTIVITY OF PHOSPHOFRUCTOKINASES AFTER ANOXIC TREATMENT

To further evaluate the relative importance of PFK and PFP during the transition from aerated to anoxic growth conditions in rice seedlings, we measured the specific activities of these two distinct phosphofructokinases along with the fermentative enzymes ADH and PDC (**Table 2**). In comparison to anoxically treated plants, the ADH showed the lowest basal activity in leaves in ambient oxygen concentration, whereas PDC activity was low in all tissues. Illumination did not affect the basal ADH and PDC activity, but the anoxic induction of these enzymes was more pronounced in illuminated seedlings than in darkness. As expected, a significantly elevated ADH activity was observed after 24 h of anoxia in all tissues. ADH activity increased threefold to ninefold during the 24-h anoxic treatment. Thereby, the greatest increase of activity was observed in leaves relative to the low basal level in this organ. Also for PDC activity, a significant anoxia-induced elevation was determined after 24 h of anoxia (**Table 2**).

Evaluation of PFK activity revealed a decline of 20 and 50% PFK activity after 24 h dark incubation under normoxia in comparison to the illuminated control in stems and leaves, respectively. By contrast, PFP activity was not modified upon the transfer of seedlings to darkness (**Table 2**). In general, PFP and PFK activities did only moderately change during anoxic treatment. PFK activity in stems was significantly higher after 4 and 24 h of anoxia in light and in dark anoxic treatment as compared to the respective control (**Table 2**), which likely results from increased expression of *OsPFK05* in this tissue (**Figure 2**). PFK activity did not change

[4]http://www.phytozome.net/

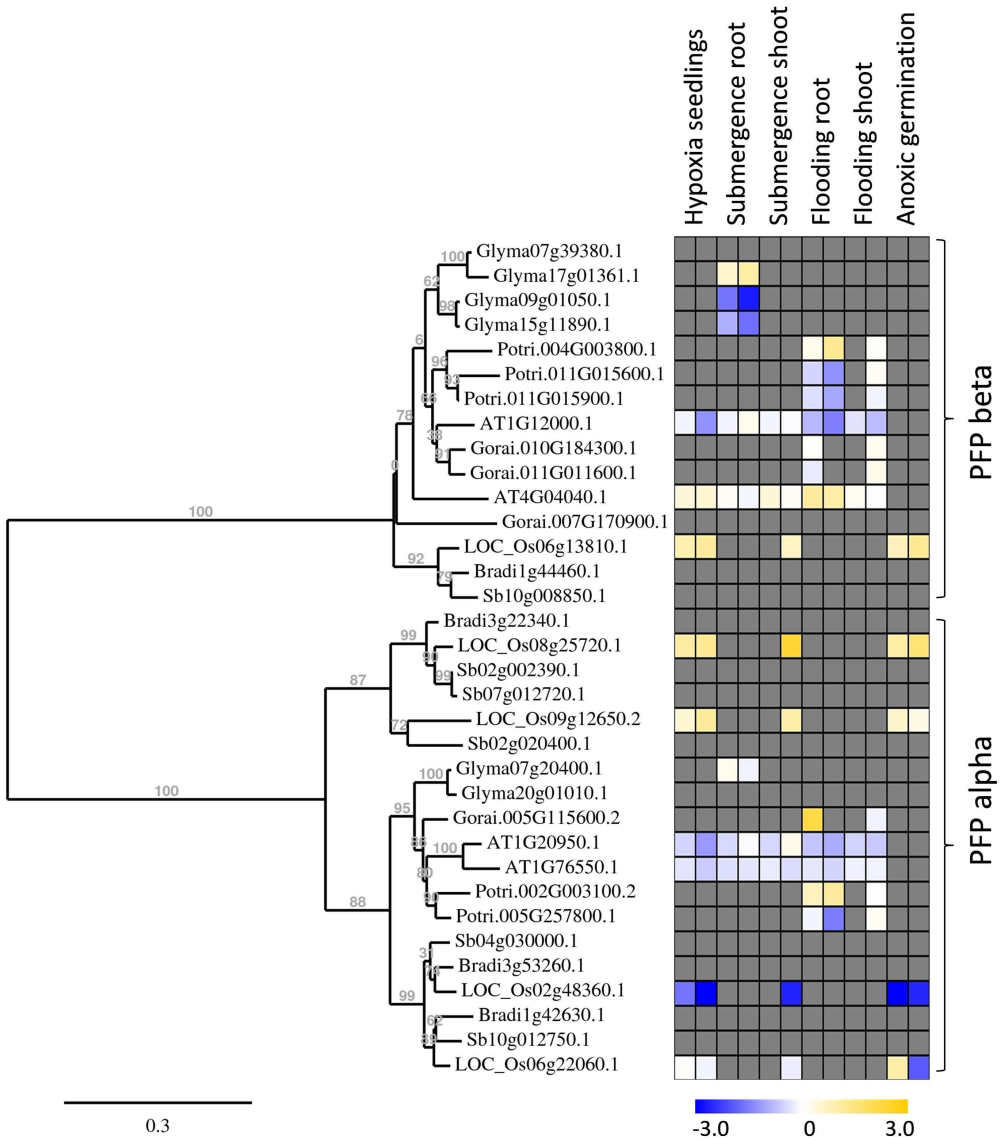

FIGURE 5 | Phylogenetic tree of plant PFP genes, constructed by the platform http://www.phylogeny.fr (Dereeper et al., 2008). Protein sequences were obtained from Phytozome, with modifications after sequencing of rice genes. Gene expression data were collected into a heat map, when available. Data are from the following sources: Hypoxia seedlings: *Arabidopsis* (Branco-Price et al., 2008, 2 and 9 h), rice (Narsai et al., 2009, 3 and 6 h); Submergence root/shoot: *Arabidopsis* (Lee et al., 2011, 7 and 24 h), rice (Mustroph et al., 2010, 24 h shoot); soybean (Nanjo et al., 2011, 6 and 12 h, roots only); Flooding root/shoot: *Arabidopsis* (Hsu et al., 2011, 3 and 12 h), cotton (Christianson et al., 2010, 4 h root and 24 h shoot), poplar (Kreuzwieser et al., 2009, 5 and 24 h root, 168 h shoot); Anoxic germination: rice (Narsai et al., 2009, 24 h; Lasanthi-Kudahettige et al., 2007, 96 h).

significantly in response to anoxia in roots or leaves. The activity of PFP was slightly but not significantly increased in leaves only after 24 h of anoxia in light and darkness (**Table 2**), but not in roots or stems.

DISCUSSION

PHOSPHOFRUCTOKINASE GENES IN RICE AND THEIR EXPRESSION UNDER NORMOXIC CONDITIONS

In this report we analyzed the expression of 15 *phosphofructokinase* genes in rice (**Table 1**). Five gene sequences resemble *PFP* genes derived from other plant species, which have been described earlier (Carlisle et al., 1990; Todd et al., 1995; Kapri et al., 2000; Suzuki et al., 2003). Genes encoding the two different subunits of the heterotetrameric PFP were found in the rice genome (**Figure 1**). Whereas *Arabidopsis* possesses two genes each encoding the PFP-alpha and PFP-beta subunits (Mustroph et al., 2007), available rice sequence data predicts four genes for PFP-alpha subunits, which represent the regulatory PFP subunit (**Table 1**), and one gene encoding the PFP-beta subunit (*PFP-B*, *LOC_Os06g13810*) representing the catalytically active subunit of the PFP complex

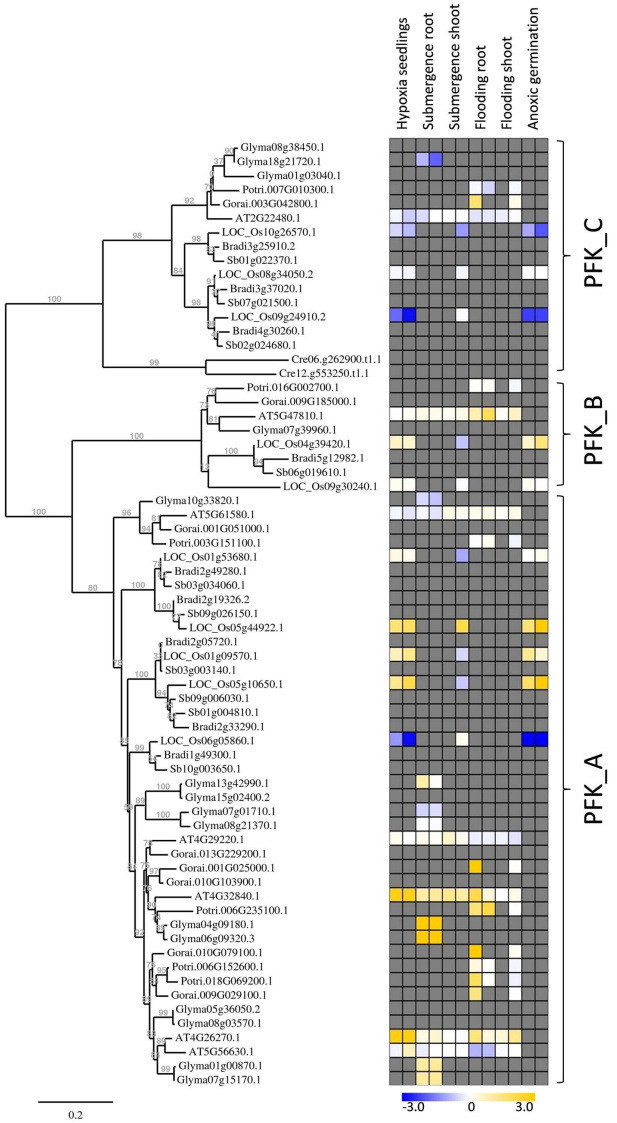

FIGURE 6 | Phylogenetic tree of plant *PFK* genes, constructed by the platform http://www.phylogeny.fr (Dereeper et al., 2008). Protein sequences were obtained from Phytozome, with modifications after sequencing of rice genes. *Chlamydomonas reinhardtii phosphofructokinase* genes were added for comparison. Gene expression data were collected into a heat map, when available. Data are from the following sources: Hypoxia seedlings: *Arabidopsis* (Branco-Price et al., 2008, 2 and 9 h), rice (Narsai et al., 2009, 3 and 6 h); Submergence root/shoot: *Arabidopsis* (Lee et al., 2011, 7 and 24 h), rice (Mustroph et al., 2010, 24 h shoot); soybean (Nanjo et al., 2011, 6 and 12 h, roots only); Flooding root/shoot: *Arabidopsis* (Hsu et al., 2011, 3 and 12 h), cotton (Christianson et al., 2010, 4 h root and 24 h shoot), poplar (Kreuzwieser et al., 2009, 5 and 24 h root, 168 h shoot); Anoxic germination: rice (Narsai et al., 2009, 24 h; Lasanthi-Kudahettige et al., 2007, 96 h).

(Yan and Tao, 1984; Theodorou et al., 1992; Theodorou and Plaxton, 1996). It is suggested that rice uses several regulatory subunits with distinct properties that are activated under different growth conditions. Indeed, the four *OsPFPA* genes show diverse expression patterns in plant tissues and under various light conditions (**Figure 2**). *OsPFPA2* mRNA was detected primarily in stems, whereas *OsPFPA1* and *OsPFPA3* mRNAs were less abundant in leaves than in stems or roots (**Figure 2**). PFP activity was very low in leaf extracts compared to that of roots and stems (**Table 2**). Furthermore, *OsPFPA1* and *OsPFPA4* showed dark-dependent decreases in mRNA accumulation in leaves (**Figure 2**), but the PFP activity after dark incubation was unchanged in comparison to light-grown leaves (**Table 2**). These observations in rice are consistent with the previous report of decreased abundance of two *Arabidopsis PFP* gene transcripts (At1g12000, At1g20950) after transfer to darkness, although no apparent change in PFP activity was determined (Gibon et al., 2004). We predict that the total *in vitro* enzymatic activity from whole cell extracts conceals the biologically significant differences in the assembly of PFP heterotetrameric complexes in response to the cell-specific variation of the expression of PFP isoforms.

The other 10 *phosphofructokinase* genes (**Table 1**) showed homology to the seven *AtPFK* genes (Mustroph et al., 2007) (**Figure 1**). Five of these proteins form a sub-clade, designated the PFK_A group, together with the five AtPFK proteins that are important for cytosolic glycolysis (**Figure 1**). The present study confirms that four of these rice genes are highly expressed in all observed tissues, with the exception of *OsPFK04* (**Figure 2**). Interestingly, the rice genome does not contain a true plastidal isoform in this subgroup, since the predicted plastidal *OsPFK06* of the PFK_A group was clearly cytosolic (**Figure 4E**), while AtPFK4 presents a plastidal isoform in the PFK_A group. The rice PFK_C group members apparently fulfill the sole plastidal PFK function (see below).

The protein sequences of the PFK_B and PFK_C groups markedly differ from the sequences of the group PFK_A (**Figure 1**; Figure S1 in Supplementary Material). *OsPFK03* and *OsPFK09* were closely related to *AtPFK2* (At5g47810), and form the PFK_B group (**Figure 1**). The protein sequences of the PFK_B group are shorter than the other PFK sequences (**Table 1**; Figure S1 in Supplementary Material). *AtPFK2* is specifically expressed in seeds, but scarcely expressed in other tissues, and the same expression pattern was determined for both rice genes (Figure S4 in Supplementary Material; Winter et al., 2007). Nevertheless, in our transcript analysis *OsPFK03* was expressed at considerable amounts, while *OsPFK09* transcripts were present only at very low levels in vegetative tissues (**Figure 2**). All members of this sub-clade are localized to the cytosol (**Figure 4**, Mustroph et al., 2007). It is proposed that PFK_B members are characterized by distinct enzymatic properties for specific tissues, such as seeds and embryos. But, expression of AtPFK2 in tobacco leaves did not induce enhanced PFK activity under our assay conditions (Mustroph et al., 2007). Future analyses of *Arabidopsis* and rice mutants with deficiency in AtPFK2 and OsPFK03/09 expression could shed light on this topic.

The PFK_C subgroup proteins are targeted to plastids and include the rice genes *OsPFK07*, *OsPFK08*, and *OsPFK10* as well as *AtPFK5* (At2g22480; Mustroph et al., 2007; **Figure 4**). Indeed, PFK isoforms of various plant species were found in chloroplasts and in the cytosol (Cawood et al., 1988; Knowles et al., 1990; Turner and Plaxton, 2003). While cytosolic PFKs catalyze

Table 2 | Specific activities of phosphofructokinases (PFK, PFP) and fermentative enzymes (ADH, PDC) (nmol × mg protein^{-1} × min^{-1}) in roots, stems, or leaves of rice seedlings after 4 and 24 h of anoxic stress (A), or under normoxic conditions (C).

		PFK	PFP	ADH	PDC
ROOT					
C	24 h Light	24.12 ± 5.45^{a}	94.98 ± 28.74^{a}	122.26 ± 30.56^{a}	0.00 ± 0.00^{a}
C	24 h Dark	20.98 ± 1.32^{a}	84.27 ± 22.51^{a}	116.90 ± 34.21^{a}	0.00 ± 0.00^{a}
A	4 h Light	20.70 ± 3.42^{a}	110.76 ± 29.55^{a}	212.13 ± 25.83^{a}	1.12 ± 0.98^{a}
A	4 h Dark	23.03 ± 7.13^{a}	120.55 ± 39.30^{a}	256.62 ± 16.24^{a}	0.75 ± 1.30^{a}
A	24 h Light	31.33 ± 4.45^{a}	102.21 ± 11.92^{a}	980.43 ± 152.23^{b}	21.95 ± 2.32^{b}
A	24 h Dark	20.23 ± 0.99^{a}	75.16 ± 12.82^{a}	692.15 ± 121.28^{c}	14.75 ± 3.26^{c}
STEM					
C	24 h Light	16.55 ± 1.46^{bc}	135.34 ± 9.02^{a}	186.27 ± 17.02^{a}	1.45 ± 1.87^{a}
C	24 h Dark	12.44 ± 1.99^{c}	132.30 ± 10.22^{a}	183.65 ± 11.22^{a}	2.29 ± 0.91^{a}
A	4 h Light	23.85 ± 2.72^{a}	141.81 ± 12.72^{a}	325.01 ± 33.06^{b}	5.47 ± 1.17^{a}
A	4 h Dark	18.33 ± 2.53^{b}	131.33 ± 9.61^{a}	207.26 ± 0.86^{a}	4.38 ± 1.50^{a}
A	24 h Light	20.68 ± 1.09^{ab}	143.28 ± 4.50^{a}	599.35 ± 20.47^{c}	16.38 ± 3.08^{b}
A	24 h Dark	17.25 ± 1.73^{bc}	142.75 ± 7.48^{a}	475.82 ± 52.06^{d}	16.63 ± 2.20^{b}
LEAF					
C	24 h Light	11.23 ± 3.46^{ac}	21.52 ± 0.98^{a}	26.58 ± 3.83^{a}	0.00 ± 0.00^{a}
C	24 h Dark	5.28 ± 1.25^{b}	19.94 ± 1.71^{a}	24.69 ± 1.94^{a}	0.00 ± 0.00^{a}
A	4 h Light	12.54 ± 0.94^{ac}	22.16 ± 1.86^{a}	75.60 ± 4.48^{b}	0.17 ± 0.21^{a}
A	4 h Dark	8.58 ± 1.54^{bc}	21.39 ± 2.88^{a}	55.81 ± 6.78^{ab}	0.06 ± 0.11^{a}
A	24 h Light	13.74 ± 1.06^{a}	25.55 ± 3.09^{a}	258.36 ± 31.83^{c}	4.10 ± 0.51^{b}
A	24 h Dark	8.01 ± 0.90^{bc}	23.62 ± 0.21^{a}	156.41 ± 7.41^{d}	2.76 ± 0.34^{c}

Data are mean values from three biological replicate samples ±SD. Values with the same letter within one organ are significantly different at P < 0.05.

a step in the normal cytosolic glycolysis for energy metabolism, plastidal glycolysis using plastidal PFK contributes to starch breakdown and generation of metabolites for biosynthetic processes in dark-adapted or non-photosynthetic plastids (Plaxton, 1996). We propose that plastidal PFKs are light-dependently inactivated to avoid breakdown of photosynthates. But, we found significantly lower PFK activity in 24 h dark-incubated leaves and stems of rice plants exposed to ambient air (50% activity in comparison to light-grown plants, **Table 2**). It is possible that the *in vitro* activity does not reflect the *in planta* activity which could be influenced by redox regulation and phosphorylation (Kachru and Anderson, 1975; Cséke et al., 1982; Heuer et al., 1982).

PFK AND PFP GENE EXPRESSION UNDER ANOXIA

The function of the two different phosphofructokinases in plants is still a matter of debate. It was hypothesized that plants might use PFP instead of PFK for the phosphorylation of fructose-6-phosphate during ATP deficiency (Weiner et al., 1987; Mertens et al., 1990; Stitt, 1998). Our results show that anoxia-tolerant rice plants induce the expression of genes coding for both enzymes, PFK as well as PFP, during anoxia. *OsPFK04* is a *bona fide* inducible gene upon anoxia in all organs, whereas *OsPFK05* transcripts were moderately increased in stems and leaves (**Figure 2**). Induction of *OsPFK04* transcript occurred within 30 min of anoxic stress. This rapid induction resembles that of accumulating *PDC1* mRNA (**Figure 2**). It is suggested that rice has a sensitive and rapid signaling pathway for the detection of low-oxygen levels, most likely via post-translational and oxygen-dependent regulation of group VII ERF transcription factors (Gibbs et al., 2011; Licausi et al., 2011). However, such induction of *PFK* genes was also found for all other plant species observed (**Figure 6**), including low-oxygen-sensitive *Arabidopsis* (At4g26270, At4g32480) and soybean. Therefore, it can be proposed that induction of PFK is important for metabolism under oxygen deficiency in both, sensitive and tolerant plants.

Also *OsPFPA3* and *OsPFP-B* transcripts were clearly increased in rice under anoxia in light and darkness (**Figure 2**), mainly in stems and leaves, which are the tissues with the highest tolerance to anoxia (Mustroph et al., 2006a,b). As stems and leaves store more carbohydrates and ferment them during anoxic periods, their cells survive anoxia better than root cells. Furthermore, these tissues might be able to produce more adaptive proteins, including PFP against stress through enhanced availability of photosynthetic energy. Interestingly, while *PFPAs* encoding the regulatory subunit were not induced in low-oxygen sensitive *Arabidopsis* or soybean plants, the *OsPFPA3* was stronger induced in anoxic rice seedlings than the catalytic subunit *OsPFP-B* (**Figures 2** and **5**) indicating a high importance for modulation of enzyme activity under oxygen deficiency (**Figure 5**). These results favor the idea that PFP plays a role in the reorganization of metabolism during low-oxygen stress in anoxia-tolerant leaves, but not in sensitive *Arabidopsis* plants or rice roots. However, the particular role of PFP in plant metabolism remains open. This question should be addressed in transgenic rice plants displaying reduced PFP activity.

Transcripts of four *PFK* genes were found to be less abundant during anoxia compared to aeration (*OsPFK06, OsPFK07, OsPFK08, OsPFK10*; **Figure 2**). Remarkably, three of the four

PFK genes are plastidic and belong to group PFK_C (**Table 1**). Also *Arabidopsis*, soybean and poplar PFK_C members were not induced under oxygen deficiency stress and hypothetically hint at reduced plastidal starch degradation and biosynthetic processes under oxygen deficiency. However, rice seeds are able to germinate under anoxia by making use of the amylase-degraded starch (Guglielminetti et al., 1995; Perata et al., 1997). It is not entirely excluded that either the degradation products of starch are translocated from plastids as hexoses, or plastidal PFK activity is mainly post-translationally stimulated despite the transcriptional reduction. Thus, more detailed studies are needed to elucidate the link between reduced *OsPFK* transcription of plastid-localized isoforms and the response and adaptation to anoxic stress.

Illumination during the anoxic period greatly enhances the plant survival rates due to photosynthesis-driven ATP production (Mustroph et al., 2006b). In our recent analysis, we did not find dramatic differences in the induction of *phosphofructokinase* genes (**Figure 2**) or enzyme activities (**Table 2**) in light versus dark anoxia. This suggests that phosphofructokinase activity has not a major role in the positive effect of illumination during oxygen deficiency stress. But, all *phosphofructokinase* genes with reduced expression during an anoxic stress period showed an even stronger decrease in darkness than in light, especially in stems and leaves (**Figure 2**) suggesting an energy-dependent decrease of transcription in anoxia. It is reasonable to speculate on the avoidance of unwanted transcription and translation to save valuable ATP. It is reported that the highly energy-consuming translation of house-keeping genes is especially tightly regulated under oxygen deficiency stress (Branco-Price et al., 2008; Mustroph et al., 2009). In consistency, ADH and PDC activities did not increase as much during anoxia in darkness as in light (**Table 2**; Mustroph et al., 2006a,b).

Complete analyses of the expression of *phosphofructokinase* gene family under oxygen deficiency are only now possible after the identification of the entire gene family in rice. Previously, the effect of oxygen deficiency on the content of a single *phosphofructokinase* transcript was studied by RNA blot analysis in rice (Umeda and Uchimiya, 1994; Minhas and Grover, 1999). Based on our classification of the *phosphofructokinase* genes, both research groups monitored *OsPFP-B* and concluded that the transcript level is strongly increased upon oxygen deprivation. This is in agreement with our results showing a two- to threefold increase in the *OsPFP-B* mRNA content (**Figure 2**). Data from recent microarray analyses using rice coleoptiles exposed to anoxic stress (Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009), and of rice leaves to submergence (Mustroph et al., 2010) confirmed the highest accumulation of *OsPFK04* and *OsPFK05* transcript levels among the *phosphofructokinase* genes (**Figure 6**; Table S3 in Supplementary Material). The microarray data also show a moderate induction of *OsPFPA3* and *OsPFP-B* (**Figure 5**), as shown in our experiments (**Figure 2**). Furthermore, the reduced accumulation of *OsPFK06, OsPFK08, OsPFK10*, and *OsPFPA1* transcripts during anoxia in comparison to the aerated controls was congruently presented in the microarray profiles and our studies. When expression patterns, for example from *OsPFK01* and *OsPFPA4,* differ between reported and our own studies, these differences likely refer to the use of different plant tissues and rice cultivars as well as different growth conditions and stress applications.

INDUCED EXPRESSION OF PHOSPHOFRUCTOKINASE GENES DOES MODIFY ENZYME ACTIVITIES ONLY SLIGHTLY

Despite the fact that some *PFK* and *PFP* genes were strongly expressed during anoxia in rice compared to aeration, the activities of PFK and PFP in cell extracts were only slightly increased in response to anoxic treatment (**Table 2**). PFP activity increased slightly but not significantly after 24 h of anoxia in leaves, which correlated with increased *OsPFPA3* transcript levels, while PFK activity was slightly induced in stems during illuminated anoxia. Although the translational activity of single mRNAs and the stability of individual isoforms are not known, it is proposed that the simultaneous increase of transcript amounts for two *PFK* and two *PFP* genes and the decrease of transcript amounts for four *PFK* and three *PFP* genes contribute to the maintenance of the activity of both enzymes during anoxia (**Figure 2**). However, rice shoots, the most tolerant tissue in this study, showed an increase in PFP enzyme activity during long-term anoxia. This elevated activity was never obtained for the anoxia-sensitive *Arabidopsis* plants (data not shown) and could presumably partly contribute to anoxia tolerance.

Increased activities of PFP, but not of PFK were reported for rice coleoptiles (Mertens et al., 1990; Kato-Noguchi, 2002) or suspension cells (Mohanty et al., 1993) in response to oxygen deficiency. Two reasons could explain the lower activation of enzyme activity in the present experiments. First, plant organs respond differently to oxygen deficiency stress. Coleoptiles possess the highest anoxia tolerance among the different plant tissues and can survive several days of anoxia. Therefore, it is possible that among other factors this tolerance is due to strong increase of PFP activity. Second, the experimental conditions differ in the studies. Rice suspension cells were exposed to 12 and 24 h of anoxia in darkness with carbohydrate addition, and a sixfold PFP activity increase was determined, but no increase of PFK activity (Mohanty et al., 1993). Without additional sugar supply, dark-incubated rice seedlings do not tolerate anoxia for more than 24 h before they die (Mustroph et al., 2006a). It is likely that the sugar supplement enables the strong increase in enzyme activity in the previous study (Mohanty et al., 1993).

We conclude that, although the overall *in vitro* activity of PFK and PFP was only slightly modified during anoxic treatment, the induction and repression of several of the *PFK* and *PFP* genes contributes to changes of the *in planta* metabolic activity. The encoded phosphofructokinases could have different affinities to substrates and cofactors, such as fructose-6-phosphate, ATP, and PPi. Furthermore, PFK and PFP activities are highly regulated by other metabolites like magnesium or phospho*enol*pyruvate (summarized in Plaxton, 1996). Another possible factor is phosphate, a potent activator of cytosolic PFK activity that functions as an inhibitor of plastidic PFK activity (Kelly and Latzko, 1977). In conclusion, the distinct upregulation of *PFP* genes during anoxia in rice provides a means for the use of PPi instead of ATP for the conversion of fructose-6-P to fructose-1,6-BP. An increase in *PFP* transcript levels and PFP activity during anoxia in rice likely alleviates the energy crisis. These regulatory mechanisms were

not observed in low-oxygen sensitive plants, such as *Arabidopsis*, soybean, or poplar.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2013.00125/abstract

Figure S1 | Alignment of amino acid sequences of PFKs from Arabidopsis and rice. The sequences were aligned by use of the ClustalW method (http://www.genome.jp/tools-bin/clustalw). Similar amino acids are marked with shadows. The letters A, B, and C mark the three PFK subgroups. For the alignment, the corrected protein sequences for LOC_Os08g34050 (*OsPFK07*) and LOC_Os09g24910 (*OsPFK08*) were used (see Figure S2 in Supplementary Material).

Figure S2 | Modified nucleotide and protein sequences for LOC_Os08g34050 (OsPFK07), LOC_Os09g24910 (OsPFK08), and LOC_Os09g12650 (OsPFPA4) after sequencing of several PCR products and comparison to the annotated sequences. For *OsPFK07*, two versions were found in different varieties, one for Nipponbare (as well as Cigalon, M202, Dongjin, Hwayoung), and one for FR13A (as well as CT6241).

Figure S3 | Subcellular localization of PFK isoforms from rice. Full-length mRNA sequences or the N-terminal part of the sequences were cloned in frame in front of a GFP-coding sequence and were transiently transformed into tobacco leaves by Agrobacterium infiltration. After 3–4 days, protoplasts or undigested leaf disks were isolated and analyzed by confocal microscopy. Green color represents GFP fluorescence, red color represents chlorophyll autofluorescence. Full-length CDS: **(A)** OsPFK01; **(B)** OsPFK02; **(C)** OsPFK04; **(D)** OsPFK05; **(E)** OsPFK06; **(I,J)** OsPFK03; **(O)** OsPFK09; **(S)** OsPFK08; **(T)** OsPFK10. N-terminal part: **(F–H)** OsPFK03; **(K–N)** OsPFK09; **(P–R)** OsPFK05.

Figure S4 | Expression of the PFK_B group members in different organs. eFP browser pictures were obtained through http://bar.utoronto.ca (Winter et al., 2007). Red color intensity shows high expression level in the respective tissue type.

Table S1 | Primers used for the experiments. Actin primers were those used by Ren et al. (2005).

Table S2 | Semi-quantitative RT-PCR analysis of rice phosphofructokinase genes: conditions for the PCR reactions.

Table S3 | Data for differential gene expression of phosphofructokinase genes in rice under oxygen-deficient conditions, obtained by microarray analyses with the Affymetrix rice Microarray Chip (Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009; Mustroph et al., 2010). The signal-log-ratio (SLR) of all probe-sets is shown for each sample set. Data had been analyzed previously by GC-RMA.

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Barley responses to combined waterlogging and salinity stress: separating effects of oxygen deprivation and elemental toxicity

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Salinity and waterlogging are two major factors affecting crop production around the world and often occur together (e.g., salt brought to the surface by rising water tables). While the physiological and molecular mechanisms of plant responses to each of these environmental constraints are studied in detail, the mechanisms underlying plant tolerance to their combined stress are much less understood. In this study, whole-plant physiological responses to individual/combined salinity and waterlogging stresses were studied using two barley varieties grown in either vermiculite (semi-hydroponics) or sandy loam. Two weeks of combined salinity and waterlogging treatment significantly decreased plant biomass, chlorophyll content, maximal quantum efficiency of PSII and water content (WC) in both varieties, while the percentage of chlorotic and necrotic leaves and leaf sap osmolality increased. The adverse effects of the combined stresses were much stronger in the waterlogging-sensitive variety Naso Nijo. Compared with salinity stress alone, the combined stress resulted in a 2-fold increase in leaf Na^+, but a 40% decrease in leaf K^+ content. Importantly, the effects of the combined stress were more pronounced in sandy loam compared with vermiculite and correlated with changes in the soil redox potential and accumulation of Mn and Fe in the waterlogged soils. It is concluded that hypoxia alone is not a major factor determining differential plant growth under adverse stress conditions, and that elemental toxicities resulting from changes in soil redox potential have a major impact on genotypic differences in plant physiological and agronomical responses. These results are further discussed in the context of plant breeding for waterlogging stress tolerance.

Keywords: salinity, waterlogging, microelement toxicity, barley, breeding, manganese, potassium, sodium

INTRODUCTION

Excessive soil salinization is a major ecological and agronomic problem throughout the world. According to the FAO Land and Plant Nutrition Management Service (2008), more than 800 Mha of land throughout the world is affected by salinity, which accounts for over 6% of the world's total land area. Apart from natural salinity, secondary (human-induced) salinization of arable land has become a serious threat to agricultural production owing to improper cultivation practices and irrigation (Pannell and Ewing, 2004). Excessive quantities of ions (mainly Na^+ and Cl^-) in the soil solution decrease soil osmotic potentials and the availability of water to plant roots. When accumulated in the shoot, these ions induce ion toxicity by disrupting the structure of enzymes, damaging cell organelles and interfering with cell metabolism (Maathuis and Amtmann, 1999). Salinity stress also results in significant ROS accumulation, both in roots (Xie et al., 2011) and leaves (Tanou et al., 2009), and interferes with K^+ homeostasis (Maathuis and Amtmann, 1999; Shabala and Cuin, 2008), triggering accelerated cell death (Shabala, 2009; Joseph and Jini, 2010).

Waterlogging occurs over a vast region of the world, adversely affecting about 10% of the global land area (Setter and Waters, 2003) and reducing crop yields by as much as 80% (Shabala, 2011). Under waterlogging condition, soil gas exchange is severely impeded. This results in a significant depletion of free oxygen (O_2) and accumulation of carbon oxide (CO_2) due to microbial and root respiration (Bailey-Serres and Voesenek, 2008). As soon as the free O_2 surrounding the roots is depleted, hypoxia stress occurs, causing a transfer from aerobic to anaerobic metabolism in roots, with dramatic restrictions to ATP synthesis (Barrett-Lennard, 2003; Teakle et al., 2006). Waterlogging also causes a sharp decrease in the soil redox potential, resulting in very significant changes to the soil chemical profile. Effects include a changed availability of mineral substances, reduction of manganese (Mn^{4+}), iron (Fe^{3+}), and sulfate (SO_4^{2-}), increased solubility of potentially toxic metals and production of toxic compounds by plant roots and microbial anaerobic metabolism (Kozlowski, 1997; Shabala, 2011). As a consequence of these changes, plants show altered membrane transport, decreased stomatal conductance and leaf water potentials, enhanced root

senescence, reduced root and shoot growth, and eventually, death of the whole plant (Barrett-Lennard, 2003).

In recent years, an impressive amount of knowledge has accumulated on plant physiological and molecular responses to salinity or waterlogging stresses. However, studies dealing with the combined effects of these two stresses are much rarer and often controversial [reviewed by Barrett-Lennard (2003)]. Nonetheless, the occurrence of combined salinity and waterlogging stress is increasing throughout the world This is due to intensive irrigation in agricultural production systems (Smedema and Shiati, 2002), rise of saline water tables (Hatton et al., 2003), and seawater intrusion in coastal environments (Carter et al., 2006). When combined with waterlogging, salinity can cause even greater damage to plants, so having a major impact on agricultural production (Barrett-Lennard, 2003). Only a very few crop species can tolerate the combination of salinity and waterlogging (Bennett et al., 2009), and the physiological and molecular mechanisms conferring this tolerance remain elusive.

Barley (*Hordeum vulgare* L.) is one of the most important crop species in the world. Barley is tolerant to salinity (Chen et al., 2007), so is a good candidate for use in saline discharge areas. However, barley is sensitive to waterlogging (Zhou et al., 2012), and the co-occurrence of waterlogging with salinity may seriously decrease this potential. In this study, the physiological and ionic characteristics of two contrasting barley varieties, grown in two soil types, were investigated in response to combined salinity and waterlogging stress. Our results suggest that hypoxia is not the only factor affecting plant growth under adverse stress conditions and that elemental toxicities resulting from changes in the soil redox potential has a major impact on genotypic differences in plant physiological and agronomical responses.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

Two barley (*Hordeum vulgare* L.) varieties contrasting in both waterlogging and salinity tolerance (CM72, tolerant to salinity and with medium tolerance to waterlogging, and Naso Nijo, sensitive to both waterlogging and salinity; Chen et al., 2007; Pang et al., 2007), were used in this work. Seeds were obtained from the Australian Winter Cereal Collection and multiplied using the TIA facilities in Launceston. Seeds were surface sterilized with 10% commercial bleach (NaClO 42 g/L, Pental Products, Shepparton, Australia), thoroughly rinsed with tap water, then sown at a 10 mm-depth in 2 L-pots. Two different types of growth media—sandy loam and vermiculite—were used. Sandy loam soil, which was taken from the University of Tasmania farm near Cambridge in southeast Tasmania, was first air-dried and then sieved through a 5 mm sieve. The soil was fertilized by adding all the essential macronutrients at the optimal field application rates, taking into account the pot factor (in g/10 L: 5.13 NH_4NO_3, 13.57 NaH_2PO_4, 4.88 K_2SO_4, 3.1 $CaSO_4$, and 1.42 $MgCl_2$) and watered to field capacity with tap water during the experiment. Plants grown in vermiculite were watered with half-strength Hoagland's nutrient solution. After germination, barley seedlings were thinned to 10 uniform and healthy plants in each pot. Plants were grown under controlled glasshouse condition (with a day-length of 14 h; light/dark temperatures, 25/15°C; and relative humidity, 65%) at the University of Tasmania (Hobart, Australia). The experiment was repeated twice in April and June 2012.

Treatments were imposed when plants were at the fully expanded first leaf stage (∼1 week old). For treatments with waterlogging, pots were placed into large black tanks (6 pots in each tank). Four treatments were given: control (no NaCl; well drained), salinity (referred as "NaCl"; well drained pots watered with 200 mM NaCl solution), waterlogging ("WL"; submerged pots; no NaCl) and combined waterlogging and salinity ("NaCl/WL"; pots submerged in 200 mM NaCl solution). Waterlogged conditions were created by using either tap water or half-strength Hoagland's nutrient solution, depending on the soil type (i.e., whether sandy loam or vermiculate). The entire

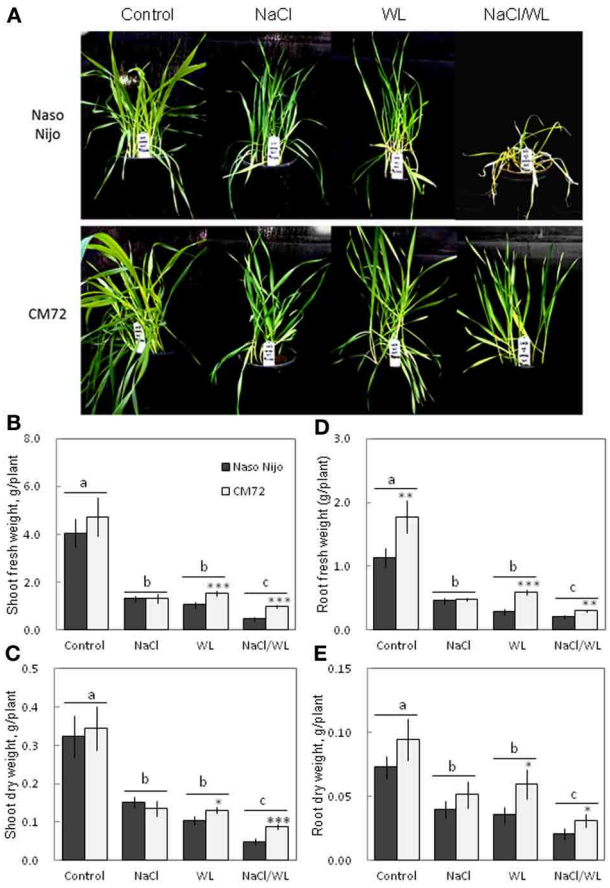

FIGURE 1 | Effects of separate and combined salinity and waterlogging stresses on growth of two contrasting barley cultivars in sandy loam. Dark bars, Naso Nijo; light bars, CM72. One-week old barley seedlings were subjected to one of four treatments: Control (no NaCl; well drained), NaCl (well-drained pots watered with 200 mM NaCl solution), WL (submerged pots; no NaCl), NaCl/WL (pots submerged in 200 mM NaCl solution). Plants were harvested for morphological **(A)** and biomass analysis **(B–E)** 14 days after the onset of treatment. For growth condition and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within treatment at $^{*}P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$.

experiment was carried out in a split-plot design with tanks as the main plots and barley varieties as subplots. Six replicates were set up for each treatment × variety combination.

Daily irrigation for drained treatments was artificially maintained with the same amount (150 mL) of different solutions, as described above. The water level of waterlogged treatments was kept 15 mm above the soil surface. Plants were subjected to different treatments for 14 days, after which the genotypic variance was clearly distinguished visually. The seedlings were then sampled randomly for the following analyses.

SPAD AND CHLOROPHYLL FLUORESCENCE

Leaf chlorophyll content and chlorophyll fluorescence were measured on the middle part of the oldest fully expanded leaves (which had been subjected to the treatment for a full 2 weeks), prior to sampling for other parameters. Leaf chlorophyll content was measured with a SPAD meter (SPAD-502, MINOLTA, Japan). Chlorophyll fluorescence was measured with a OS-30p chlorophyll fluorometer (OPTI-Sciences, Hudson, USA). Plants were dark-adapted for 30 min prior to measurement. The maximum quantum efficiency of photosystem II ($Fv/Fm = (Fm–Fo)/Fm$) was recorded at a saturating actinic light (660 nm) intensity of 1100 μmol/m^2/s. Twelve replicates were randomly taken for each treatment × variety combination.

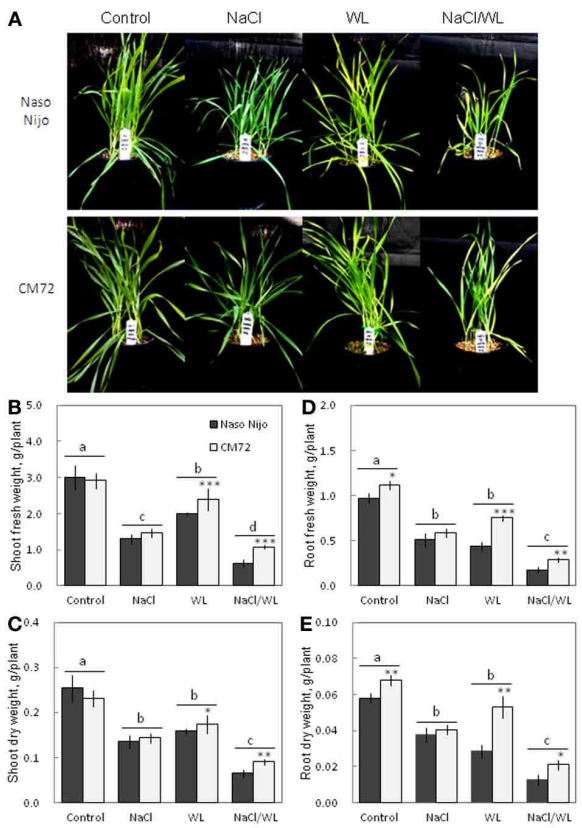

FIGURE 2 | Effects of separate and combined salinity and waterlogging stresses on growth of two contrasting barley cultivars in vermiculite. Dark bars, Naso Nijo; light bars, CM72. One-week old barley seedlings were subjected to one of four treatments: Control (no NaCl; well drained), NaCl (well-drained pots watered with 200 mM NaCl solution), WL (submerged pots; no NaCl), NaCl/WL (pots submerged in 200 mM NaCl solution). Plants were harvested for morphological **(A)** and biomass analysis **(B–E)** 14 days after the onset of treatment. For growth conditions and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within treatment at $^*P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$.

RATIO OF CHLOROTIC AND NECROTIC LEAVES

Prior to harvest for biomass, the number (no.) of chlorotic, necrotic, and total leaves from each plant were counted. The ratio of chlorotic (or necrotic) leaves was then calculated according to the equation: ratio of chlorotic (or necrotic) leaves = no. of chlorotic (/necrotic) leaves/no. of total leaves. Twelve replicates were randomly taken for each treatment × variety combination.

BIOMASS

For harvesting, plant roots were gently washed with running tap water, rinsed with distilled water, then blotted dry with soft tissue. Five replicates were taken for each treatment × variety combination, and three plants were bulked together for each replicate. The fresh weights of shoots and roots were measured as soon as the seedlings were separated and the dry weights were measured after drying in a Unitherm Drier (Birmingham, England) for 2 days at 65°C.

LEAF WATER CONTENT

Leaf fresh and dry weights were used to calculate the leaf water content (WC) on a fresh weight basis using the following equation: WC% = (fresh weight - dry weight)/fresh weight × 100%. Fresh leaf blades of whole seedlings were collected and weighed immediately for their fresh weights. Dry weights were determined after drying for 2 days at 65°C. Five replicates were taken for each treatment × variety combination.

Na^+ AND K^+ CONTENTS AND OSMOLALITY IN PLANT TISSUE

Na^+ and K^+ contents were determined from the leaf and root sap. After harvesting as described above, plant roots and the oldest fully expanded leaves, which had been subjected to the treatment for a full 2 weeks, were collected and immediately stored in a 1.5 ml microcentrifuge tube at −20°C. Five replicates were taken for each treatment × variety combination. The sap from roots and leaves was extracted by the freeze-thaw method (Cuin et al., 2008). After centrifuged at 10,000 g for 3 min, the extracted sap sample was diluted 500 times with double distilled water and analyzed for its Na and K content using a flame photometer (PF97, VWR International, Murarrie, Australia).

The extracted leaf sap was also analyzed for its osmolality using a vapor pressure osmometer (Vapro, Wescor Inc. Logan, Utah, USA).

ROOT ATP CONTENT

Two weeks after commencing treatments, roots were harvested for ATP extraction according to a modified method of Yang

et al. (2002). Briefly, 0.2 g of liquid nitrogen-homogenized root powder was rapidly mixed with 0.5 mL 0.0005% (v/v) $HCl.O_4$ and heated in a boiling water bath for 10 min. After cooling on ice, the extraction mixture was centrifuged for 5 min at 10,000 $\times$ g at 4°C. The supernatant was used for the ATP assay. The ATP content was quantified using the ATP Colorimetric Assay Kit (ab83355, ABCAM, Cambridge, UK) with a microplate reader (SPECTROstar Nano, BMG LABTECH, Mornington, Australia). The reaction mix contained 4% (v/v) ATP probe, 4% (v/v) ATP converter and 4% (v/v) developer in the ATP Assay Buffer (ab83355, ABCAM, Cambridge, UK). ATP standards (0–5 nmol range) and extracted samples were placed into a 96-well plate. The reaction mix was added at 1:1 (v/v) ratio, and plates were incubated at the room temperature in the dark for 30 min. The extraction solution without root tissue was used as a negative control. ATP concentrations in the samples were calculated by plotting the measured absorbance at 570 nm ($OD_{570\ nm}$) vs. the standard linear curve.

NET ION FLUXES FROM THE ROOT EPIDERMIS

Net K^+ and H^+ fluxes were measured from the root epidermis of barley seedlings using non-invasive ion-selective vibrating microelectrodes (the MIFE technique, University of Tasmania, Hobart, Australia), essentially as described in our previous publications (Chen et al., 2007; Cuin et al., 2008). Barley seedlings were grown in an aerated Basic Salt Media (BSM) solution (0.5 mM KCl + 0.1 mM $CaCl_2$, pH 5.6) in the dark for 3 days at room temperature (25 ± 1°C). At that stage, 1 mM $MnCl_2$ was added to the BSM, and plants were grown for up to 3 more days in the presence of Mn. Roots of intact seedlings were mounted in a 10 mL perspex measuring chamber filling with the appropriate solution (BSM for control; BSM + 1 mM $MnCl_2$ for Mn treatment) 1 h prior to measurement. Ion-selective microelectrodes were position 40 μm above the root surface, with their tips separated by ca. 2 μm. Ion fluxes were measured by a slow (5 s half-cycle) square-wave movement of electrodes between two positions, close to (40 μm) and away from (80 μm) the root surface. Net ion fluxes were measured from the mature root epidermis ~20 mm from the root tip. The potential difference between two positions was recorded by the MIFE CHART software and converted to the electrochemical potential difference using the calibrated Nernst slope of the electrodes. Net ion fluxes were calculated from the electrochemical potential difference using cylindrical diffusion geometry by the MIFEFLUX program. Net ion fluxes were measured for 10 min, and the

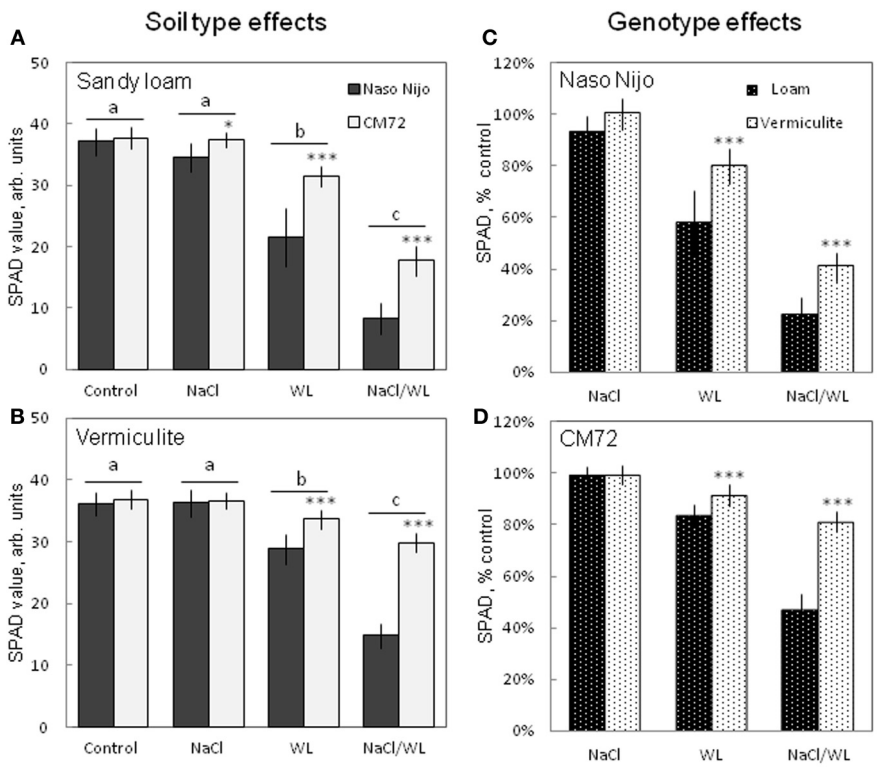

FIGURE 3 | Effects of separate and combined salinity and waterlogging stresses on chlorophyll content (SPAD values) of two contrasting barley cultivars. Measurements were taken 14 days after the onset of treatment. **(A,B)** Soil type effects. Dark bars, Naso Nijo; light bars, CM72. **(C,D)** Genotype effects. Dark bars, sandy loam; light bars, vermiculite. For growth conditions, details of treatments, and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 12$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within the treatment at $^*P < 0.01$ and $^{***}P < 0.0001$.

steady-state fluxes were calculated by averaging the values over the last 5 min.

SOIL REDOX POTENTIAL

Soil redox potential (or oxidation-reduction potential, ORP) was measured using an ORP electrode connected to a Handheld Multi-Parameter (LabNavigator, Forston Labs, Colorado, USA) before treatment ("drained control"), and after 3 and 14 days of waterlogging, both in the presence and absence of NaCl. To measure the ORP for drained controls, a 5 cm-deep hole was dug in the soil and the ORP electrode was carefully placed in the hole with the diaphragm touching the humid soil surface. All measurements were taken as close to roots as practically possible, without causing any damage. Each measurement was recorded for 180 s with one reading per second, and a reliable ORP value was achieved by averaging the readings from the last 120 s. These measurements were conducted about 1 h after watering.

Fe AND Mn CONCENTRATIONS IN THE SOIL SOLUTION

Soil solutions for determining Fe and Mn concentrations were sampled at the time of ORP measurement. For the WL and NaCl/WL treatments, soil solution was taken from five spots for each container, and combined into one collective sample for Fe and Mn determination. To determine Fe and Mn concentrations in the drained controls, a 1:2.5 (v/v) soil:solution mix was made (using tap water for clay, and ½ Hoagland nutrient solution for vermiculite), stirred for 30 min at room temperature, then decanted. All samples were then filtered, and Fe and Mn concentrations were determined using the Atomic Absorption Spectroscopy (AAS) technique (Avanta Σ, GBC Scientific Equipment, Braeside, Australia). Five replicates of collective samples were taken for each treatment.

STATISTICAL ANALYSIS

Statistical analysis was performed by the statistical package, IBM SPSS Statistics 20 (IBM, New York, USA). All data in the figures and tables are given as means ± SD. The Multivariate General Linear Model was used to confirm the significance of the factors (soil types, treatments, and varieties). Significant differences between treatments on the mean of the two varieties were compared using Duncan's multiple range tests.

RESULTS

PLANT GROWTH

Plant growth was significantly reduced by all stress treatments (**Figures 1**, **2**). The combined NaCl/WL treatment showed the most severe effect on plant growth. When grown in sandy loam, the average shoot FW and DW of the two varieties treated by NaCl/WL were reduced by 81 and 76% relative to the control, respectively, with the corresponding values for roots being 85

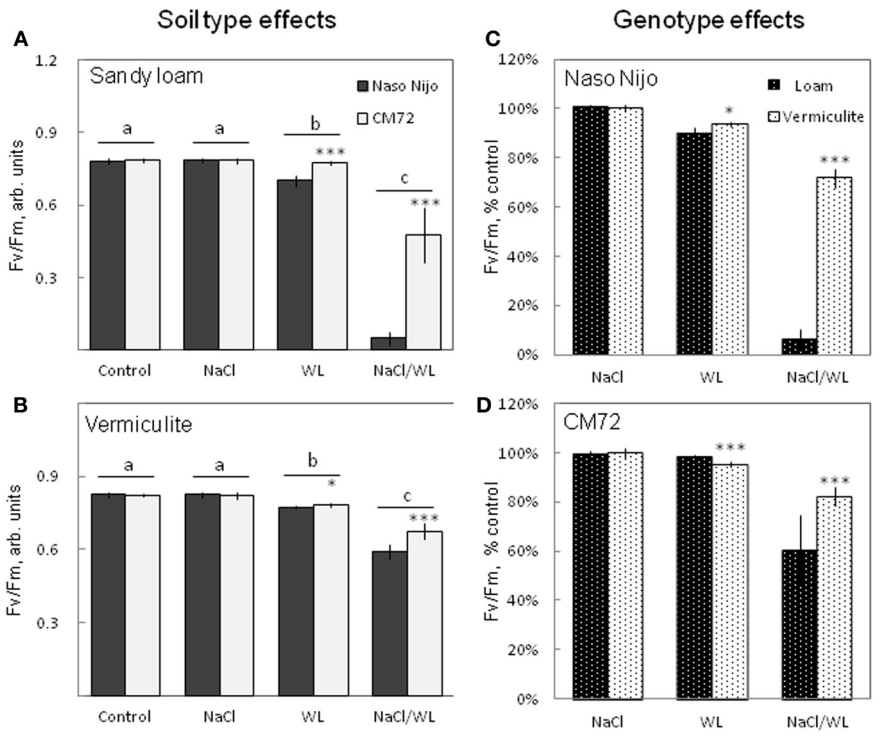

FIGURE 4 | Effects of separate and combined salinity and waterlogging stresses on maximum photochemical efficiency of PSII (Fv/Fm chlorophyll fluorescence values) of two contrasting barley cultivars. Measurements were taken 14 days after the onset of treatment. **(A,B)** Soil type effects. Dark bars, Naso Nijo; light bars, CM72. **(C,D)** Genotype effects. Dark bars, sandy loam; light bars, vermiculite. For growth conditions, details of treatments, and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 12$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within the treatment at $^{*}P < 0.01$ and $^{***}P < 0.0001$.

and 77% (**Figures 1B–E**). Plants grown in vermiculite performed better under combined NaCl/WL treatment, with a 71 and 32% reduction in the shoot FW and DW and 78 and 73% reduction in root FW and DW, respectively (**Figures 2B–E**). The genotypic difference in tolerance to the combined stress was clearly visible when plants were grown in sandy loam. After 14 days of NaCl/WL treatment, most Naso Nijo plants had died, and the plant FW was only 12% of that of control (**Figures 1A,C**). However, in the CM72 plants, only some moderately stressed symptoms were observed and 25% FW of the control was maintained.

CHLOROPHYLL CONTENT AND FLUORESCENCE

After 2 weeks of treatment, NaCl alone had no significant (at $P < 0.01$) effect on the leaf chlorophyll content (SPAD value) for either variety grown in either growth media. However, WL and NaCl/WL treatments caused a massive reduction in the chlorophyll content (**Figure 3**) in both varieties. Sandy loam-grown plants were affected more than plants grown in vermiculite (ca. 40% more reduction in NaCl/WL treatment, **Figures 3C,D**). The combined NaCl/WL treatment was more detrimental compared with WL alone in both varieties (**Figures 3A,B**). The observed decline in chlorophyll content was genotype-specific, with chlorophyll content in CM72 plants being significantly higher than that in Naso Nijo plants for each treatment, except for the control (**Figures 3A,B**).

The maximum photochemical efficiency of PSII (chlorophyll fluorescence *Fv*/*F*m value) was also significantly affected by WL and NaCl/WL treatments (**Figure 4**). A substantial decline in *Fv*/*F*m value was reported for both treatments (**Figures 4A,B**). None of these parameters, however, was affected by NaCl alone (at $P < 0.01$, **Figures 4A,B**). On averaging the two varieties, the effect of the combined NaCl/WL stress was much more severe than WL alone (at $P < 0.01$, **Figures 4A,B**). CM72 plants were less sensitive to both WL and combined NaCl/WL stresses than Naso Nijo (**Figures 4A,B**). Stress effects were strongly influenced by the growth conditions (sandy loam vs. vermiculite), with a much stronger decline in *Fv*/*F*m observed in sandy loam-grown plants (66% more reduction for Naso Nijo and 22% more for CM72 under sandy loam conditions than those grown in vermiculite, **Figures 4C,D**).

LEAF CHLOROSIS AND NECROSIS

Leaf chlorosis and necrosis are important visible symptoms associated with abiotic stresses. As shown in **Figure 5**, leaf chlorosis and necrosis were induced by WL treatment and exacerbated by the combined NaCl/WL stress. Variety CM72 performed much better compared with Naso Nijo. As such, no chlorotic or necrotic leaves were present in CM72 plants under WL treatment, while a substantial percentage of Naso Nijo leaves were affected by WL (**Figure 5A**). Under combined NaCl/WL stress, half the CM72 leaves showed visual stress symptoms, while in the more sensitive Naso Nijo, over 90% leaves were either chlorotic or nectrotic (**Figure 5A**). Visual stress symptoms were more pronounced in the sandy loam-grown plants (**Figure 5A**) compared with those grown in vermiculate (**Figure 5B**). Taking the combined NaCl/WL treatment as an example, Naso Nijo had over 70% necrotic leaves when grown in sandy loam but only 18% in vermiculite; for CM72 the corresponding values were 22 and 0% (**Figure 5**).

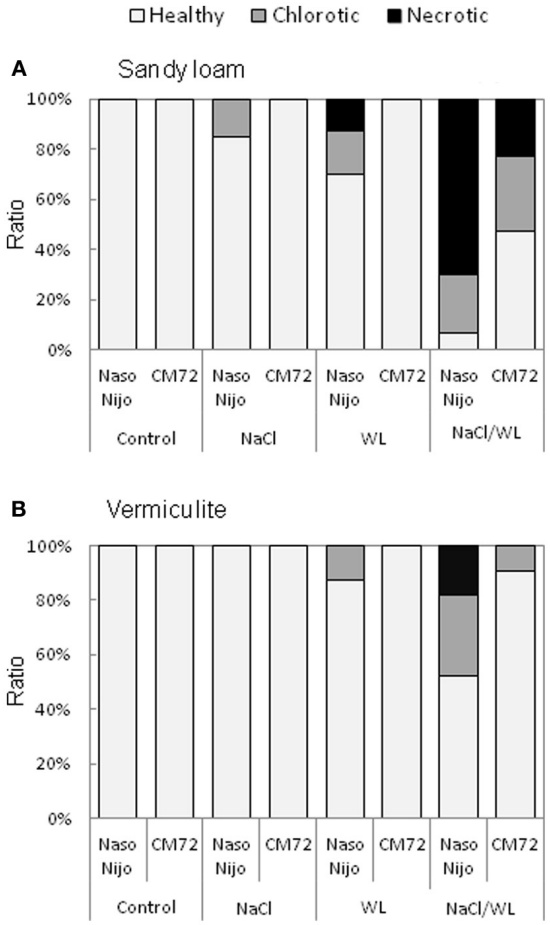

FIGURE 5 | Effects of separate and combined salinity and waterlogging stresses on the ratio of chlorotic and necrotic leaves of two contrasting barley cultivars. Ratio of chlorotic and necrotic leaves were calculated according to the numbers of total, chlorotic and necrotic leaves on plants grown in sandy loam **(A)** and vermiculite **(B)** 14 days after the onset of treatment (dark bars, Naso Nijo; light bars, CM72). For growth conditions, details of treatments, and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 12$).

LEAF WATER CONTENT AND SAP OSMOLALITY

Leaf WC was significantly decreased by NaCl and NaCl/WL treatments under both growth conditions (**Figure 6**). At the same time, WL treatment did not significantly affect leaf WC under sandy loam conditions (at $P < 0.01$, **Figure 6A**), and even slightly increased leaf WC in plants grown in vermiculite (**Figure 6B**). A significant (at $P < 0.01$) difference in leaf WC between the contrasting varieties was observed for each treatment except the control in this experiment (**Figures 6A,B**). The growth conditions

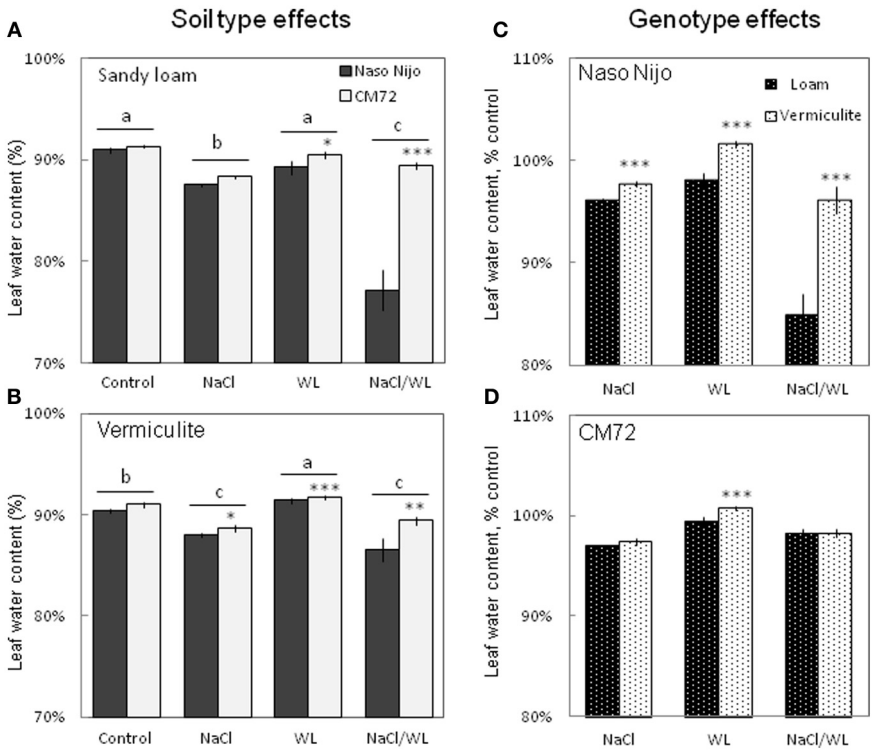

FIGURE 6 | Effects of separate and combined salinity and waterlogging stresses on leaf water content of two contrasting barley cultivars. **(A,B)** Soil type effects. Dark bars, Naso Nijo; light bars, CM72. **(C,D)** Genotype effects. Dark bars, sandy loam; light bars, vermiculite. For growth conditions, details of treatments, and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within the treatment at $^{*}P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$.

(sandy loam vs. vermiculite) showed a significant impact on leaf WC, but only in the sensitive variety Naso Nijo (**Figures 6C,D**).

To a large extent, changes in the leaf WC were mirrored by changes in leaf sap osmolality (**Figure 7**). Much higher leaf sap osmolality (significant at $P < 0.01$) was measured in plants grown in the presence of NaCl (i.e., in both NaCl and NaCl/WL treatments), while no significant change in leaf osmolality was found for WL treatment as compared to the control. The combination of waterlogging and salt stress not only induced a massive increase in leaf sap osmolality, but also allowed a clear differentiation between the contrasting varieties, with about a 2-fold difference (significant at $P < 0.001$) between them (**Figures 7A,B**). No significant (at $P < 0.01$) effect of growth media (sandy loam vs. vermiculate) on leaf sap osmolality was found (**Figures 7C,D**).

SAP Na^+ AND K^+ CONTENTS

As expected, NaCl treatment caused dramatic increases in the Na^+ content (**Figure 8**) but a decrease in the K^+ content (**Figure 9**) for both the leaf and root (at $P < 0.01$). Compared with NaCl alone, the combined NaCl/WL treatment further increased the leaf Na^+ content (about a 1.7–2-fold increase on average of the two varieties, **Figures 8A,B**), but decreased leaf K^+ content (about 27 to 44% decrease on average of the two varieties, **Figures 9A,B**). However, the combined NaCl/WL treatment did not cause further changes to root Na^+ (**Figures 8C,D**) and K^+ (**Figures 9C,D**) contents. WL treatment induced a significant decrease in leaf K^+ content and a significant increase in root K^+ content (at $P < 0.01$, **Figure 9**) as compared to control, while no significant change was found in Na^+ content (at $P < 0.01$, **Figure 8**). A clear differentiation in Na^+ and K^+ contents between the contrasting varieties appeared under the combined NaCl/WL treatment. Relative to Naso Nijo, the tolerant variety CM72 had a 27–33% lower Na^+ content (at $P < 0.01$, **Figures 8A,B**) and 77–88% higher K^+ content (at $P < 0.0001$, **Figures 9A,B**) in leaves. For roots, the corresponding values were 15–18% (at $P < 0.0001$, **Figures 8C,D**) and 94–150% (at $P < 0.0001$, **Figures 9C,D**). The growth conditions (sandy loam vs. vermiculite) showed a significant impact on both Na^+ and K^+ contents. The vermiculite-grown plants contained much more Na^+ and K^+ in both the leaves and roots compared to those grown in sandy loam under saline conditions (both NaCl and NaCl/WL treatments) (**Figures 8**, **9**). Taking CM72 in the combined NaCl/WL treatment as an example, plants grown in vermiculite had a 54% higher leaf Na^+ and 32% higher root Na^+ contents than in sandy loam (**Figure 8**). For K^+, the corresponding values were 50 and 27% (**Figure 9**).

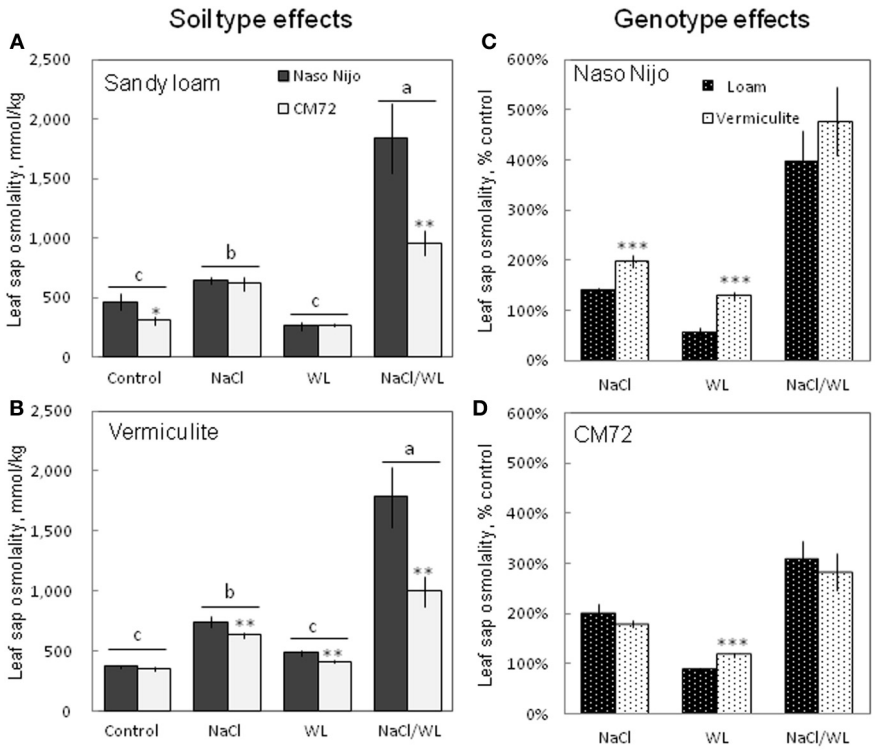

FIGURE 7 | Effects of separate and combined salinity and waterlogging stresses on leaf sap osmolality of two contrasting barley cultivars. (A,B) Soil type effects. Dark bars, Naso Nijo; light bars, CM72. **(C,D)** Genotype effects. Dark bars, sandy loam; light bars, vermiculite. For growth conditions, details of treatments, and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within the treatment at $^{*}P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$.

ROOT ATP CONTENT

Under normoxic conditions, the ATP content in plant roots was not significantly (at $P < 0.05$) different between genotypes (**Figure 10**). ATP content was also not significantly (at $P < 0.05$) different between two soil types, ranging from 42 to 51 nmol ATP/g FW (**Figure 10**). Two weeks of hypoxia resulted in a 2–3-fold decrease in the root ATP content. No significant ($P < 0.05$) effect of soil type on ATP decline was observed (**Figure 10**). Hypoxia-exposed CM72 plants were capable of maintaining a slightly higher (significant at $P < 0.05$) root ATP level compared with Naso Nijo variety.

CHANGES IN SOIL CHEMISTRY

Development of hypoxia was accompanied by a progressive decline in the soil redox potential (ORP), from 391 ± 1.5 mV to less than 50 mV, depending on the soil type and duration of waterlogging (**Figure 11**). No significant (at $P < 0.05$) differences were found for the ORP values between two soil types after 3 days of WL stress. When pots were submerged for 2 weeks however, ORP values were significantly ($P < 0.01$) lower in sandy loam compared with vermiculite (80 ± 4.4 mV vs. 151 ± 4.5 mV, respectively). The presence of salt further reduced the appropriate ORP values for each treatment by 30–40 mV, but the general trends remained the same.

Consistent with the decline in the ORP values, waterlogging induced a very substantial increase in both Mn and Fe content in the sandy loam soil (**Figure 12**). After 2 weeks of waterlogging, the Mn content rose from 2.8 ± 0.01 to 15.4 ± 0.06 ppm in waterlogging treatment alone, and to 21.8 ± 0.08 ppm in combined NaCl/WL treatment, exceeding the threshold level considered to be toxic for cereals (10 ppm; Setter et al., 2009; shown as a dotted line). Also dramatic was the increase in solution Fe content (from 0.23 ± 0.02 ppm to 16.8 ± 0.05 ppm). No physiologically relevant increase in either Mn or Fe content was found in waterlogged vermiculite (**Figure 12**). The presence of NaCl in the media consistently increased the Mn and Fe content in the soil solution by around 2-fold for each treatment.

ROOT ION FLUX CHANGES

A strong net K^+ uptake of 130–180 nmol $m^{-2}s^{-1}$ (depending on gentotype) was measured from the barley root epidermis in the control (**Figure 13A**). After 1 days of root treatment with 1 mM Mn, K^+ fluxes turned to a net efflux of 35–70 $m^{-2}s^{-1}$ and remained negative (net K^+ loss) for at least a few more days (**Figure 13A**). This Mn-induced K^+ efflux was 2-fold higher in the waterlogging-sensitive Naso Nojo variety (significant at $P < 0.05$). Changes in H^+

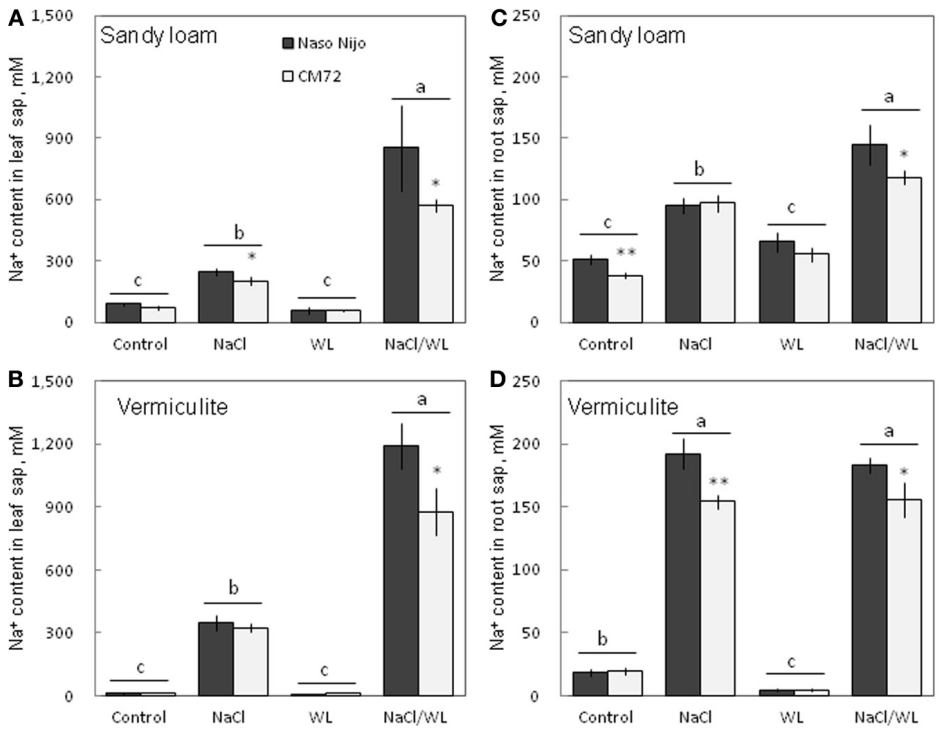

FIGURE 8 | Effects of separate and combined salinity and waterlogging stresses on tissue Na^+ content of two contrasting barley cultivars. (A,B) Soil type effects. Dark bars, Naso Nijo; light bars, CM72. **(C,D)** Genotype effects. Dark bars, sandy loam; light bars, vermiculite. For growth conditions, details of treatments, and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within treatment at $^*P < 0.01$, $^{**}P < 0.001$.

flux mirrored the changes in the K^+ flux. A strong net H^+ efflux of around 50 nmol $m^{-2}s^{-1}$ was measured from the control roots (**Figure 13B**). This efflux was sensitive to 1 mM vanadate, a known blocker of H^+-ATPase (data not shown). Mn treatment reversed the H^+ efflux into a net H^+ influx after 1 day (**Figure 13B**). At day 3 of Mn treatment, the CM72 genotype was capable of restoring its net H^+ extrusion (albeit at a reduced rate), while the WL-sensitive variety Naso Nijo still displayed a net H^+ influx (**Figure 13B**).

DISCUSSION

DISTURBANCE TO K^+/NA^+ HOMEOSTASIS IS CENTRAL FOR THE SEVERE EFFECTS OF COMBINED SALINITY AND WATERLOGGING STRESS

In this study, a much more dramatic increase in Na^+ and decrease in K^+ content were observed in both roots and leaves of barley plants grown under a combined salinity and waterlogging stress compared to plants grown with salinity under drained condition (**Figures 8**, **9**). Intracellular K^+/Na^+ homeostasis is often named as a key determinant of plant salinity tolerance (Maathuis and Amtmann, 1999; Shabala and Cuin, 2008). The key players in this process are the plasma membrane SOS1 Na^+/H^+ antiporters that actively expel Na^+ from the cytosol (Shi et al., 2002) and depolarization-activated outward-rectifying (GORK in Arabidopsis; Ache et al., 2000) channels, responsible for K^+ retention in the cytosol (Shabala and Cuin, 2008). The operation of both of these transporters is critically dependent on oxygen availability. Indeed, SOS1 activity relies on existence of steep H^+ gradients across the plasma membrane, fueled by the plasma membrane H^+-ATPase activity (Palmgren and Harper, 1999). The H^+-pump is also the main electrogenic factor essential for maintaining a highly negative membrane potential value, thus keeping GORK channels closed. Salinity by itself causes a substantial membrane depolarization (by 60–80 mV; Shabala and Cuin, 2008). As the oxygen is gradually used up (under waterlogged conditions), root O_2 deficiency would restrict aerobic respiration, so the production of ATP would be dramatically reduced (from 30 to 36 mol ATP via mitochondrial oxidative phosphorylation to 2–4 mol ATP via glycolysis per hexose, Bailey-Serres and Voesenek, 2008). Indeed, the ATP content dropped 2–3-fold in waterlogging-affected roots (**Figure 10**). This reduction will compromise a plant's ability to fuel H^+-ATPases, with major implications to both Na^+ exclusion and K^+ retention, as discussed above. Overall, unfavorable Na^+/K^+ ratios in plant will affect plant metabolism, resulting in a significant decrease in chlorophyll content (**Figure 3**) and photochemical efficiency of PSII (**Figure 4**); drastic increases in the number of chlorotic and necrotic leaves (**Figure 5**) and leaf sap osmolality

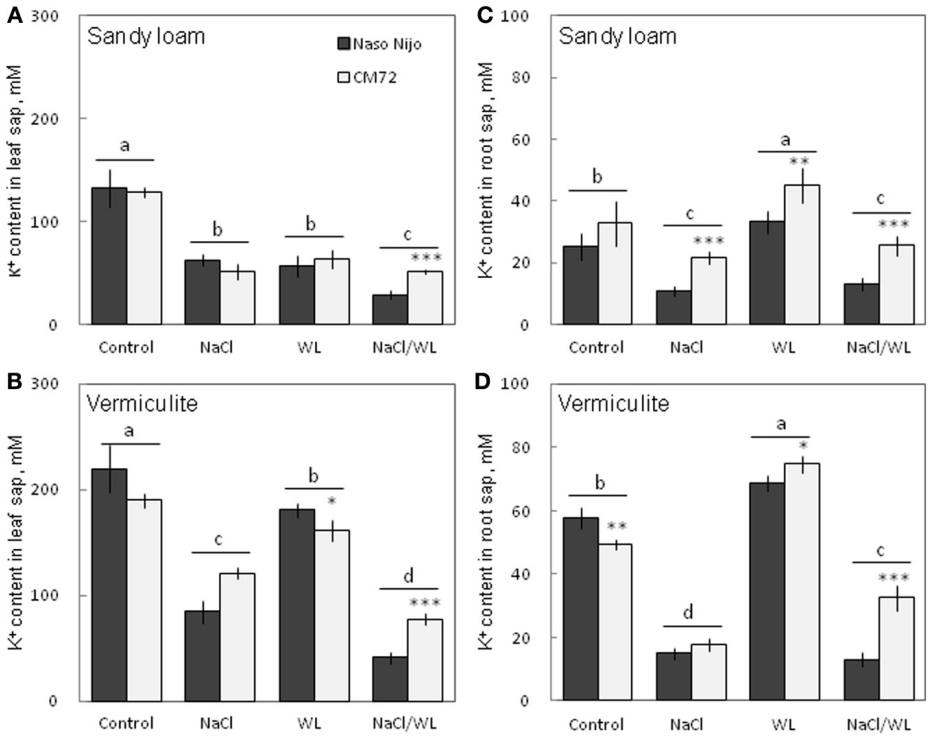

FIGURE 9 | Effects of separate and combined salinity and waterlogging stresses on tissue K^+ content of two contrasting barley cultivars. (A,B) Soil type effects. Dark bars, Naso Nijo; light bars, CM72. **(C,D)** Genotype effects. Dark bars, sandy loam; light bars, vermiculite. For growth conditions, details of treatments, and media composition, refer to the Materials and Methods section. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisks indicate the significant difference between cultivars within treatment at $^*P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$.

(**Figure 7**); and ultimately reduced growth of roots and shoots (**Figures 1**, **2**).

SOIL TYPE AFFECTS THE ADVERSE EFFECTS OF COMBINED WATERLOGGING AND SALINITY STRESS

As the soil redox potential is reduced under waterlogged conditions, manganese dioxide (Mn^{4+}) and the insoluble ferric (Fe^{3+}) hydroxides are reduced to the soluble manganous (Mn^{2+}) and ferrous (Fe^{2+}) ions (Khabaz-Saberi et al., 2006; Setter et al., 2009; Hernandez-Soriano et al., 2012). In our case, a substantial increase in both Mn and Fe content in the soil solution was observed for the sandy loam, but not the vermiculite soil (**Figure 12**). Despite its importance as an essential micronutrient, excess Mn is damaging to the photosynthetic apparatus, interferes with uptake of other nutrients and may cause oxidative stress, resulting in chlorosis and necrosis in leaves and subsequently death of whole plants (Millaleo et al., 2010). Visual symptoms of Mn toxicity such as chlorosis and necrosis were observed in barley leaves of hydroponically-grown plants at concentrations as low as 50 μM (i.e., <5 ppm; Führs et al., 2010). In field experiments, the toxic threshold for cereals grown in drained soils was reported to be around 10 ppm of Mn (Setter et al., 2009). In our case, the Mn content in the sandy loam soil solution exceeded the latter threshold by day 14 for WL alone treatment, and was close to it around day 3 in the combined NaCl/WL treatment (**Figure 12**). Mn treatment also had major implications for K^+ retention in barley roots within 24 h of exposure (**Figure 13**). Consequently, the dramatic increase in Mn concentration that

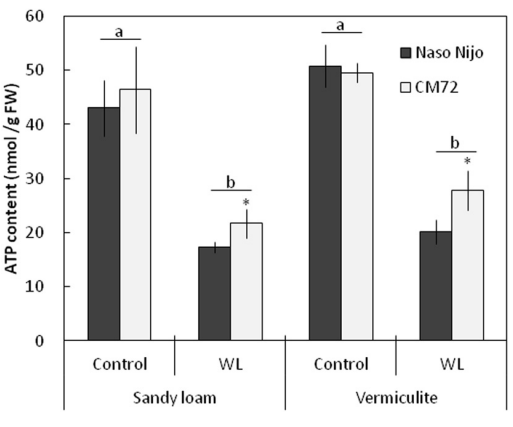

FIGURE 10 | ATP content in barley roots grown under normoxic and hypoxic conditions for 14 days. Closed bars—Naso Nijo; open bars—CM72. Mean ± SD ($n = 4$ plants). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.01$. Asterisk indicate the significant difference between cultivars within the treatment at $^*P < 0.05$.

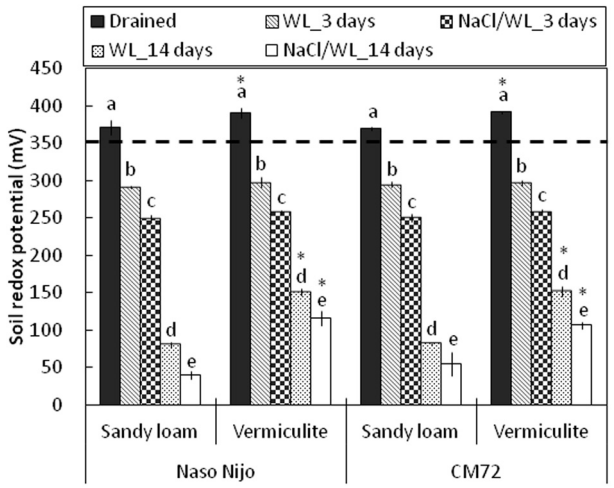

FIGURE 11 | Soil redox potential during waterlogging (submerged; no NaCl) and combined salinity and waterlogging (submerged in 200 mM NaCl solution) treatments. Soil redox potential measurements were taken before treatment (drained), and 3 days and 14 days after commencing the treatments. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference between treatments within soil type at $P < 0.01$. Asterisk indicates the significant differences between sandy loam and vermiculite within variety a $P < 0.01$. Horizontal dashed line indicates the threshold level for anaerobic condition (below 350 mV).

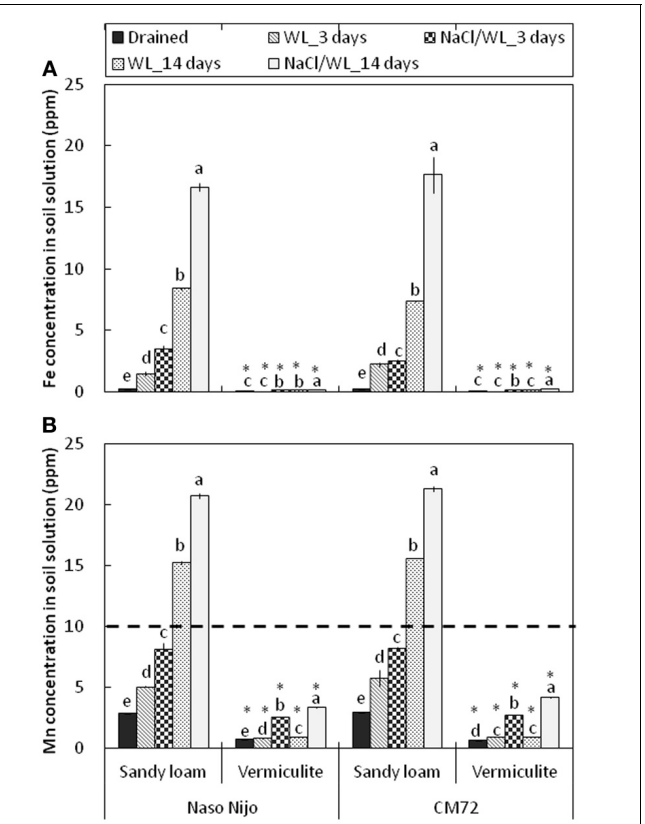

FIGURE 12 | Concentrations of Fe (A) and Mn (B) in the soil solution in pots subjected to waterlogging (submerged; no NaCl) and combined salinity and waterlogging (submerged in 200 mM NaCl solution). Soil samples were taken on days 3 and 14 since commencement of treatment and compared with those taken from control treatment. Mean ± SD ($n = 5$). Different lower case letters indicate the significant difference within the soil type at $P < 0.01$. Asterisk indicates the significant differences between sandy loam and vermiculite within variety at $P < 0.01$.

we found in sandy loam (up to 20 ppm, **Figure 12**) under waterlogged conditions would likely induce a high risk of Mn toxicity to barley plants. Interestingly, the presence of NaCl in the soil exacerbated the effect of waterlogging on Mn availability, in a full agreement with the ORP data (**Figure 11**). The Fe content in the soil solution also increased steadily, approaching toxic levels. Together, when delivered to the shoot by the transpiration flow, these two toxic micronutrients could lead to increased ROS formation in green tissues (Millaleo et al., 2010; Keunen et al., 2011), impairing the photosynthetic machinery (**Figure 4**), reducing chlorophyll content (**Figure 3**) and enhancing the senescence process (**Figure 5**). Such elemental toxicity may be an additional (to unfavorable Na^+/K^+ balance in leaf tissues) factor contributing to the poor plant performance under combined WL/NaCl conditions in sandy loam. Elemental toxicity, however, is not a factor for vermiculate-grown plants, explaining their much better performance (compared with loam-grown plants) under combined stress conditions (**Figures 1**, **2**).

So far, most studies dealing with the combined effects of salinity and waterlogging stress have been conducted with deoxygenated solution (stagnant agar solution or N_2 aerated solution) or waterlogged sand (Barrett-Lennard, 2003; Teakle et al., 2006, 2010; Rogers et al., 2011). Being very useful to clarify the underlying mechanisms of this interaction, these results may be at some discrepancy with the real situation in the natural stressed environment, due to the lack of the factor of elemental toxicity, typically present in waterlogged soils. Therefore, for accurately evaluating and efficiently improving the tolerance of germplasm to combined salinity and waterlogging stress, it is necessary to carry out investigations and screening with the soil from the target environment rather than with artificial growing methods.

GENOTYPIC DIFFERENCES IN RESPONDING TO COMBINED WATERLOGGING AND SALINITY STRESS AND THEIR POTENTIAL UTILIZATION IN SALTLAND

It is estimated that over 370,000 barley germplasms are preserved as *ex situ* collections in worldwide representative genebanks (Saisho and Takeda, 2011). The genetic diversity of either salinity or waterlogging tolerance has been well proved amongst cultivated and even wild barley germplasms (Wu et al., 2011). However, little information about the genetic variation in a combined waterlogging and salinity stress has been accumulated. In the present study, a significant genetic difference in response to combined waterlogging and salinity stress was observed between CM72 and Naso Nijo. Regardless of the soil type, CM72 showed a much higher tolerance to combined WL/NaCl stress than Naso Nijo, as reflected by the lower reduction in root and shoot growth (**Figures 1**, **2**), higher chlorophyll content (**Figure 3**)

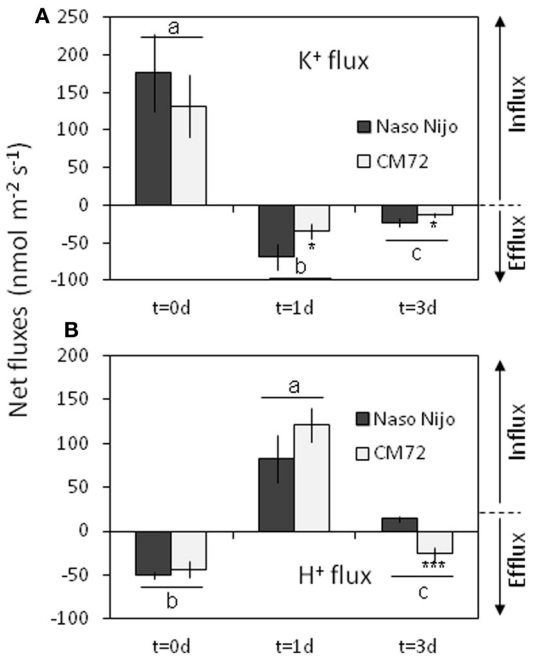

FIGURE 13 | Net steady-state K^+ (A) and H^+ (B) fluxes measured from mature barley root epidermis in control (day 0) and after one and three days exposure to 1 mM Mn. Mean ± SD ($n = 6$). Different lower case letters indicate the significant difference between treatments (averaged for both genotypes) at $P < 0.05$. Asterisks indicate the significant difference between cultivars within treatment at $^*P < 0.05$ and $^{***}P < 0.001$.

and fluorescence (**Figure 4**), fewer chlorotic and necrotic leaves (**Figure 5**), higher leaf WC (**Figure 6**), lower Na^+, and higher K^+ content in root and leaf sap (**Figures 8**, **9**). The vast collection of barley germplasms and the recent completion of the barley genome sequencing (The International Barley Genome Sequencing Consortium, 2012) makes it possible to thoroughly explore the potential mechanisms of tolerance of different barley cultivars to individual or combined stress conditions and utilize the more tolerant lines to breed suitable varieties for the corresponding environment. Up to now, saltland agriculture has relied heavily on the use of pasture halophylic species such as *Atriplex*, *Maireana*, *Puccinellia*, *Thinopyrum*, *Lotus*, and *Melilotus* (Teakle et al., 2006; Bennett et al., 2009; Rogers et al., 2011). Barley is a crop that can be used not only for animal feed, but also for alcoholic and non-alcoholic beverages and human food. It is already known for its tolerance to stresses such as cold, drought, and salinity (Saisho and Takeda, 2011). It can be predicted that barley will be a potential food source in future for the increasing world population under deteriorating environments. To achieve this goal, truly tolerant varieties capable of performing without, or with only little yield reduction, under hostile soil conditions, such as combined salinity and waterlogging, should be selected or created by breeders.

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How unique is the low oxygen response? An analysis of the anaerobic response during germination and comparison with abiotic stress in rice and Arabidopsis

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Plants face a variety of environmental stresses and have evolved molecular mechanisms to survive these challenges. One of these stresses is low oxygen conditions, which can occur under flooding conditions. Rice (*Oryza sativa*) is somewhat unique for its ability to tolerate and even germinate under low to no oxygen conditions. In this study, we examined global transcriptomic responses over the course of germination and in response to low oxygen and other abiotic stress in rice and Arabidopsis (*Arabidopsis thaliana*). Over 150 microarray datasets were analyzed in parallel to determine just how unique the low oxygen response is in rice. Comparison of aerobic germination in rice and Arabidopsis, with anaerobic germination in rice revealed conserved transcriptomic responses that are not only conserved across both species but also occur in the absence of oxygen in rice. Thus, these genes may represent functions necessary for the developmental progression of germination, whether or not oxygen is present in rice. Analysis of genes that responded differently in rice compared to Arabidopsis revealed responses specific to anaerobic germination in rice, including the down-regulation of genes encoding redox functions and up-regulation of receptor kinases. Comparison of a range of hypoxia/anoxia studies within and across Arabidopsis and rice revealed both conserved and species specific changes in gene expression (e.g., Arabidopsis specific up-regulation of WRKYs and rice specific down-regulation of heme), unveiling unique transcriptomic signatures of the low oxygen response. Lastly, a comparison of the low oxygen response with cold, salt, drought and heat stress revealed some similarity with the response to heat stress in Arabidopsis, which was not seen in rice. Comparison of these heat-responsive, abiotic stress marker genes in Arabidopsis with their rice orthologs revealed that while low oxygen may be perceived as an abiotic stress in Arabidopsis, this is not the case in rice.

Keywords: low oxygen, transcriptomes, rice, Arabidopsis, microarrays

INTRODUCTION

Plants can experience low oxygen conditions at various times during the plant life cycle, from early development (such as germination) due to diffusional resistance to oxygen resulting from anatomical restrictions, or more commonly due to partial or complete submergence under flooding conditions. Several species of plants, including some varieties of rice (*Oryza sativa*), have the ability to germinate, grow and survive under oxygen limiting conditions. However, most other plant species including Arabidopsis (*Arabidopsis thaliana*) are highly intolerant of low oxygen conditions. Interestingly, despite the different levels of low oxygen tolerance across different plant species, common responses to oxygen limitation are also often observed (Mustroph et al., 2010) including the shift to fermentation from mitochondrial respiration, as well as other multi-level molecular changes that help limit energy demanding processes in an attempt to prolong survival (Bailey-Serres and Voesenek, 2008; Magneschi and Perata, 2008; Mustroph et al., 2010). By far the most well-characterized response to low oxygen is the activation of fermentation involving an increase in alcohol dehydrogenase abundance (Sachs et al., 1980). Additionally, an increase in glycolytic flux, lactate dehydrogenase and pyruvate dehydrogenase are also seen under low oxygen stress in plants (Rivoal et al., 1991; Gibbs et al., 2000). Sucrose degradation is also modified under low oxygen, whereby sucrose is metabolized by sucrose synthase in an attempt to conserve ATP (Ricard et al., 1991; Magneschi and Perata, 2008). Similarly, a switch to pyrophosphate (PPi) linked enzymes is also made as PPi is adopted over ATP as high-energy donor molecules under low oxygen conditions (Huang et al., 2008).

Abbreviations: ABA, abscisic acid; AGI, Arabidopsis gene identifier; CAREs, *cis*-acting regulatory element(s); CHO, carbohydrate; DEG, differentially expressed genes; ERF, ethylene response factor; FA, fatty acid; FDR, false discovery rate; GCRMA, GC content based Robust Multi-array Average; GI, gene identifier; HSF, heat shock factor; MSU, Michigan State University (rice database); PPDE, Posterior Probability of Differential Expression; PPR, pentatricopeptide repeat; ROS, reactive oxygen species; TAIR, The Arabidopsis Information Resource; TF, transcription factor.

In both plants and animals, nitrite is also reduced to form nitric oxide (NO) under low oxygen conditions (Sturms et al., 2011). Studies in plants have implied a role for cytochrome oxidase, cytochrome bc1, and non-symbiotic hemoglobins in this reaction (Igamberdiev and Hill, 2009; Igamberdiev et al., 2010; Igamberdiev and Kleczkowski, 2011; Sturms et al., 2011). While the role of non-symbiotic hemoglobins has not been fully elucidated, there is increasing evidence for a function in NO scavenging, with a recent study even confirming significantly faster rates of hemoglobin activity under low oxygen conditions in rice plants compared to animals (Igamberdiev and Hill, 2009; Sturms et al., 2011). Thus, these changes in NO metabolism and ATP synthesis present an alternative method for supporting the redox and energy balance under low oxygen conditions in rice.

Given this general response of limiting energy demanding processes under low oxygen conditions, it is unique that rice has the ability to germinate under the complete absence of oxygen. Germination under aerobic conditions has been well-characterized at the transcript and protein levels in rice (Howell et al., 2006, 2009) and Arabidopsis (Gallardo et al., 2002; Nakabayashi et al., 2005; Narsai et al., 2011a). As a high energy demanding process, germination is often characterized by the significant up-regulation of mitochondrial respiratory chain and glycolysis components (Howell et al., 2007, 2009) in order to produce the energy required for development. While it may be expected that germination occurs more slowly under low oxygen conditions, in fact, under anaerobic germination in rice, morphological changes such as accelerated shoot elongation is observed and aerenchyma is developed to efficiently deliver oxygen from the shoot to the submerged organs (Magneschi and Perata, 2008). In addition to these morphological adaptations, the typical increases in alcohol dehydrogenase, pyruvate decarboxylase and lactate dehydrogenase also occurs (Magneschi and Perata, 2008). Thus, significant metabolic and molecular reprogramming must occur to generate the energy required for both germination and rapid shoot elongation under low oxygen in rice.

It has previously been shown that a significant amount of transcriptomic re-programming occurs under low oxygen conditions in various species (Mustroph et al., 2010; Narsai et al., 2011b). Thus, it is not surprising that transcription factors, such as ethylene response transcription factors (ERFs) have been shown to have a key role in the response to low oxygen in plants. The Sub1A locus encoding an ERF was the first gene shown to confer submergence tolerance in certain rice cultivars by altering the expression of specific low oxygen responsive genes including the alcohol dehydrogenase I encoding genes (Xu et al., 2006). Similarly, it was shown that group VII ERFs, containing a conserved N-terminal motif have an important role in oxygen sensing and mediating the low oxygen response in Arabidopsis (Gibbs et al., 2011). It was shown that the N-end rule pathway of targeted proteolysis acts as an oxygen sensor, where plants lacking the constituents of this pathway were more tolerant to hypoxia (Gibbs et al., 2011). This tolerance was also shown to occur as a result of an increase in the expression of core hypoxia response genes (Gibbs et al., 2011). Considering these findings, it is clear that transcriptional reprogramming is a core component of the hypoxia response in plants, with many studies observing substantial transcriptomic changes in response to low oxygen in rice (Lasanthi-Kudahettige et al., 2007; Magneschi and Perata, 2008; Narsai et al., 2009), Arabidopsis (Loreti et al., 2005; Branco-Price et al., 2008; Christianson et al., 2009, 2010; Banti et al., 2010) and several other plant species (Mustroph et al., 2010; Banti et al., 2013).

While significant transcriptomic changes do occur under low oxygen conditions, it is important to note that many of these are not exclusively due to low oxygen stress. In fact, transcription factors can often regulate responses that confer tolerances to multiple abiotic stresses. For example, WRKY transcription factors, such as WRKY18 and WRKY60 have been shown to have a role under both salt and osmotic stress (Chen et al., 2010). Similarly, it has been shown that while the Sub1A locus confers submergence tolerance in rice, it also has a role in drought tolerance (Fukao et al., 2011). Likewise, it has been shown that the heat shock factor, HsfA2 also, has a role in enhancing anoxia tolerance in Arabidopsis (Banti et al., 2010). Notably, this gene was identified when the transcriptomic responses to heat and anoxia were compared to identify common responses (Banti et al., 2008, 2010).

In the present study, we aimed to gain insight into the specificity of the transcriptomic response to low oxygen in rice. Over 150 microarrays from various studies were analyzed in parallel, comparing the transcriptomic responses to low oxygen conditions within and across Arabidopsis and rice. Firstly, core transcriptomic changes during germination were examined under aerobic and anaerobic conditions in rice (Howell et al., 2009; Narsai et al., 2009) as well as under aerobic germination in Arabidopsis (Narsai et al., 2011a). Therefore, developmental responses could be identified and separated from the specific anaerobic responses during germination. Also, core oxygen dependent developmental responses over the course of anaerobic germination were identified. Furthermore, multiple hypoxia/anoxia datasets were combined for both rice and Arabidopsis to reveal common and distinct functional categories affected under low oxygen conditions between both species. Lastly, the expression of Arabidopsis abiotic stress marker genes were examined in response to hypoxia and cold, drought, salt and heat, revealing common responses between low oxygen and other abiotic stresses. The results presented here reveal unique responses to low oxygen conditions over germination compared to other abiotic stresses.

RESULTS

OXYGEN INDEPENDENT TRANSCRIPTOMIC CHANGES DURING RICE GERMINATION

In order to identify common changes in the transcriptomes of rice and Arabidopsis, we compared three germination studies; one from Arabidopsis (Narsai et al., 2011a), as well as aerobic germination (Howell et al., 2009) and anaerobic germination in rice (Narsai et al., 2009) (**Supplementary Tables 1**, **2** and **Supplementary Figure 1**). As these were all time course studies, step-wise differential expression analysis was carried out comparing each time point with the previous time point, only genes significantly differentially expressed by two or more fold were included ($p < 0.05$, PPDE > 0.96) (**Figure 1A**). These were

then analyzed for over-representation of functional categories using the Pageman tool (Usadel et al., 2006) and the over-represented functional categories are shown for each comparison in **Supplementary Table 1**. By comparing the over-represented functional categories in each of these studies, it was possible to identify functions that showed a common response across all three germination studies (**Figures 1B,C**).

Common regulation of genes encoding several different functional categories was seen across these studies including the conserved down-regulation of transcripts encoding protein degradation functions, abscisic acid (ABA) responsive proteins, storage proteins, and late embryogenesis abundance proteins (**Figure 1C**). For some of these functions, it was seen that this regulation occurred at the same time in both aerobic and anaerobic germination in rice, denoted with an asterisk (**Figure 1C**). For example the up-regulation of protein synthesis functions such as protein elongation occurred between 3 and 12 h during rice germination (denoted with asterisk; **Figure 1C**; **Supplementary Table 3**). Similarly, the down-regulation of protein degradation functions also occurred between 3 and 12 h during rice germination (denoted with asterisk; **Figure 1C**; **Supplementary Table 3**). Note that the down-regulation of these functions is also well-known in other plant species during germination (Catusse et al., 2008; Sreenivasulu et al., 2008). However, while the same up-regulation and down-regulation patterns were seen for these during germination in Arabidopsis as well, these occurred between 12 and 48 h of cold, dark stratification in Arabidopsis (Narsai et al., 2011a). Thus, the exact timing and conditions must be considered in these comparisons.

It is important to note that a number of light responsive genes were differentially expressed during Arabidopsis germination, whilst the rice orthologs to these did not show this expression pattern during rice germination, given that the rice germination studies were carried out in the dark. Nevertheless, some photosystem I polypeptide subunits were seen to be induced during germination in both rice and Arabidopsis (**Figures 1A,C**). However, closer examination reveals that this induction occurs between 3 and 12 h in rice, whilst this largely occurs after 12 h into the light (after 48 h stratification) in Arabidopsis. Thus, any common induction of light-responsive genes is likely to be due to a small amount of light exposure on the rice seeds, possibly occurring during the sample collection process. Whereas, if rice germination had occurred under light conditions, this induction may have taken place later, upon, and after radicle emergence (as is seen in Arabidopsis). Interestingly, despite the fact that rice is a starch seed and Arabidopsis is an oil seed, a conserved

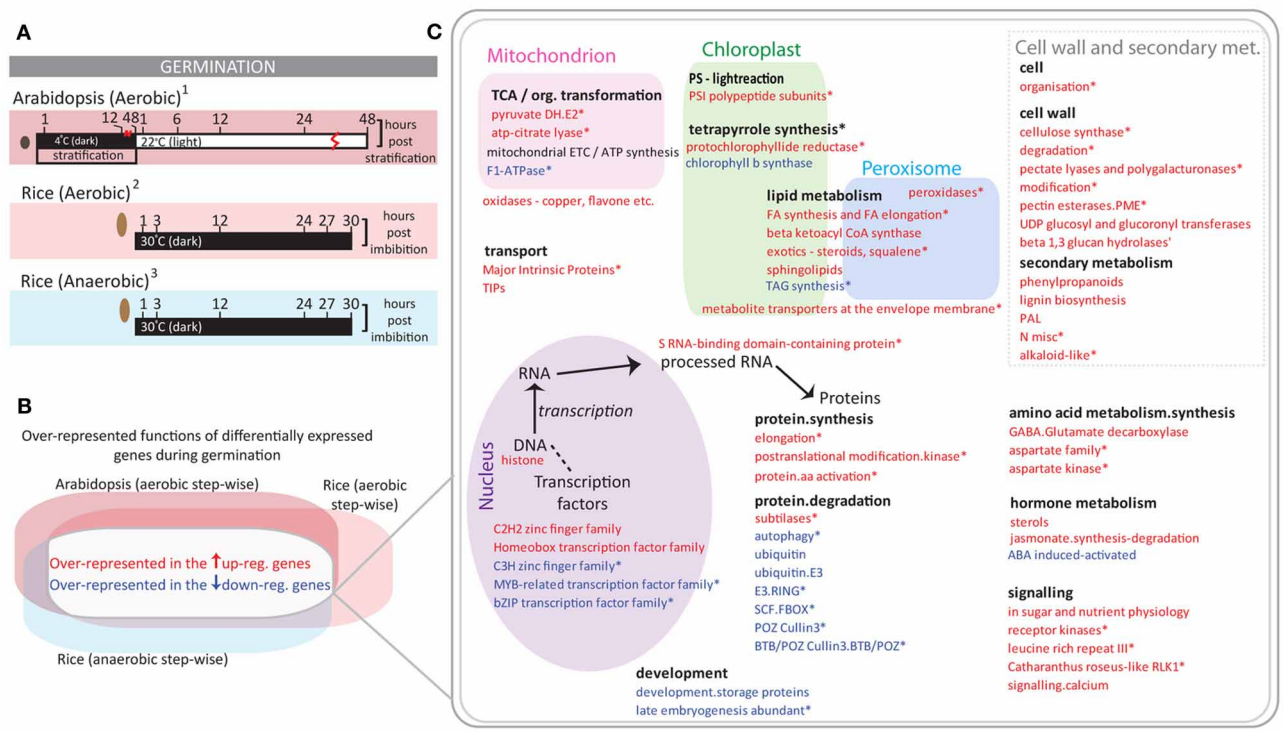

FIGURE 1 | Oxygen independent transcriptomic changes during rice germination. (A) Step-wise comparisons were carried out over the course of aerobic germination in Arabidopsis ([1]Narsai et al., 2011a) aerobic germination in rice ([2]Howell et al., 2009) and anaerobic germination in rice ([3]Narsai et al., 2009). **(B)** The differentially expressed genes (>2-fold, $p < 0.05$, PPDE > 0.96) were analyzed for common over-representation of functions in the up/down-regulated gene-sets. **(C)** Functional categories seen to be commonly up/down-regulated across all three germination studies are shown. Red font indicates that genes encoding these function(s) are over-represented in the up-regulated gene-sets and blue indicates over-representation of functions in the down-regulated gene-sets. Categories responsive at the same time in both aerobic and anaerobic germination in rice are denoted with an asterisk.

up-regulation of lipid metabolism functions was also seen, for example, genes encoding fatty acid (FA) synthesis and elongation functions, sphingolipid metabolism and peroxidases were up-regulated in both Arabidopsis and rice (aerobic and anaerobic germination) (**Figure 1C**). Thus, finding these functions to be responsive in the same manner under anaerobic conditions (and in the dark) in rice indicates that irrespective of the presence of oxygen, these represent conserved transcriptomic changes that may be necessary for germination progression.

IDENTIFYING TRANSCRIPTOMIC CHANGES UNIQUE TO ANAEROBIC GERMINATION

After comparing the over-represented functional categories in each of the germination datasets, it also became apparent that certain functional categories were only over-represented in each of the individual germination studies. Specifically, we were interested to see which functions may be responsive under anaerobic germination only. To do this, another dataset was included for comparison which encompassed a comparison of aerobic vs. anaerobic germination (**Supplementary Table 4**), as well as each of the step-wise germination comparisons (**Figure 2A**). These combined analyses revealed functional categories that were over-represented not only compared to aerobic germination, but also over the course of anaerobic germination, showing core up-regulated and down-regulated functions specific to anaerobic germination (**Figure 2A**) (**Supplementary Table 5**). Fold-changes compared to aerobic germination, as well as over the course of anaerobic germination are shown (**Figure 2B**). In this way, it was revealed that there are a number of unique responses observed only during anaerobic germination in rice (**Figure 2B**).

For example, genes encoding redox functions showed significant down-regulation, not only over the course of anaerobic germination but also compared to aerobic germination indicating that the suppression of these occurs specifically during anaerobic germination (**Figure 2B**). Notably, when these categories were compared with those seen during Arabidopsis germination (Narsai et al., 2011a), it was seen that while redox functions were down-regulated in rice; several orthologous Arabidopsis genes even showed up-regulation during germination. This was particularly notable for the ascorbate glutathione cycle functions, for example while the rice gene encoding a monodehydroascorbate reductase (LOC_Os08g44340.1) was down-regulated nearly 9-fold during anaerobic germination, its Arabidopsis ortholog (At3g09940) was up-regulated 30-fold (**Figure 2B**). Similarly, a rice gene encoding an L-ascorbate peroxidase (LOC_Os04g51300.1) was down-regulated over 5-fold under anaerobic germination, while its Arabidopsis ortholog (At4g09010) was up-regulated 32-fold (**Figure 2B**). In contrast, while genes encoding various major CHO metabolism and glycolysis functions showed unique up-regulation under anaerobic germination (compared to aerobic germination) in rice, these functions were also up-regulated during germination in Arabidopsis (denoted with ^ in **Figure 2B**). The up-regulation of major CHO metabolism and glycolysis functions specifically during anaerobic rice germination was not surprising as it a well-known response to anaerobic conditions in rice (Magneschi and Perata, 2008). Similarly, the up-regulation of protein folding functions as well as receptor kinases was also seen in Arabidopsis, but was unique to anaerobic germination in rice (**Figure 2B**). Interestingly, transcripts encoding pentatricopeptide repeat (PPR) containing proteins, which are involved in organelle RNA processing functions were seen to be specifically up-regulated during anaerobic germination in rice (**Figure 2B**). This was particularly interesting given that many PPR genes are known to be essential in Arabidopsis, where knocking out these genes often results in embryo lethality (Tzafrir et al., 2003; Khrouchtchova et al., 2012). Furthermore, it has been shown that these are transiently expressed over the course of germination in Arabidopsis, whereby a strong increase in expression is seen in the early hours after germinating seeds are transferred into light, and this expression decreases substantially by 6 h into the light (Narsai et al., 2011a) (# indicates both up- and down-regulation of genes encoding these functions in Arabidopsis; **Figure 2B**). Thus, the specific expression of these genes during anaerobic germination in rice may be indicative of unique RNA processing demands necessary under anaerobic conditions.

HOW CONSERVED IS THE LOW OXYGEN RESPONSE?

It has been shown that there is cross kingdom conservation of specific responses to anaerobic conditions, such as the up-regulation of the fermentative pathway (Mustroph et al., 2010). Further evidence for this was shown in **Figure 2B**, whereby, despite some rice genes showing specific expression not seen during aerobic germination, these expression patterns were seen for some genes during (aerobic) Arabidopsis germination. Thus, in order to examine how conserved the low oxygen response is, we compiled data from multiple studies and comparisons and analyzed the transcriptomic responses to low oxygen within and between Arabidopsis and rice (studies outlined in **Table 1**; data shown in **Supplementary Tables 4**, **6**). For the rice analysis; four sets of comparisons looking at the anoxia response were used from three studies, whilst five sets of comparisons of the hypoxia/anoxia response were used from five studies in Arabidopsis (references listed in **Table 1**). Over-representation analysis of functional categories within the differentially expressed datasets were compared and matched, and functional categories over-represented in one or both species were identified (**Figure 3**). In this way, it was possible to see the expected up-regulation of genes encoding pyruvate decarboxylase (involved in glycolysis) across multiple studies within and across both Arabidopsis and rice (**Figure 3**). A closer look at some other components of glycolysis revealed differences in the magnitude of responses between Arabidopsis and rice, for example, the pyruvate dikinase encoding gene (LOC_Os03g31750.1), was up-regulated 117-fold during anaerobic germination, and over 360-fold in the anaerobic coleoptile; (Lasanthi-Kudahettige et al., 2007), while the Arabidopsis ortholog (At4g15530) only shows between a 2- and 16-fold induction in response to low oxygen in Arabidopsis (**Supplementary Table 2**). In addition to these, a conserved up-regulation of protein ubiquitination functions was also identified in both species (**Figure 3**). In contrast, cell wall and secondary metabolism functions were seen to be over-represented in one or more down-regulated gene sets in both species (**Figure 3**). Note that several of these trends were confirmed both in the individual

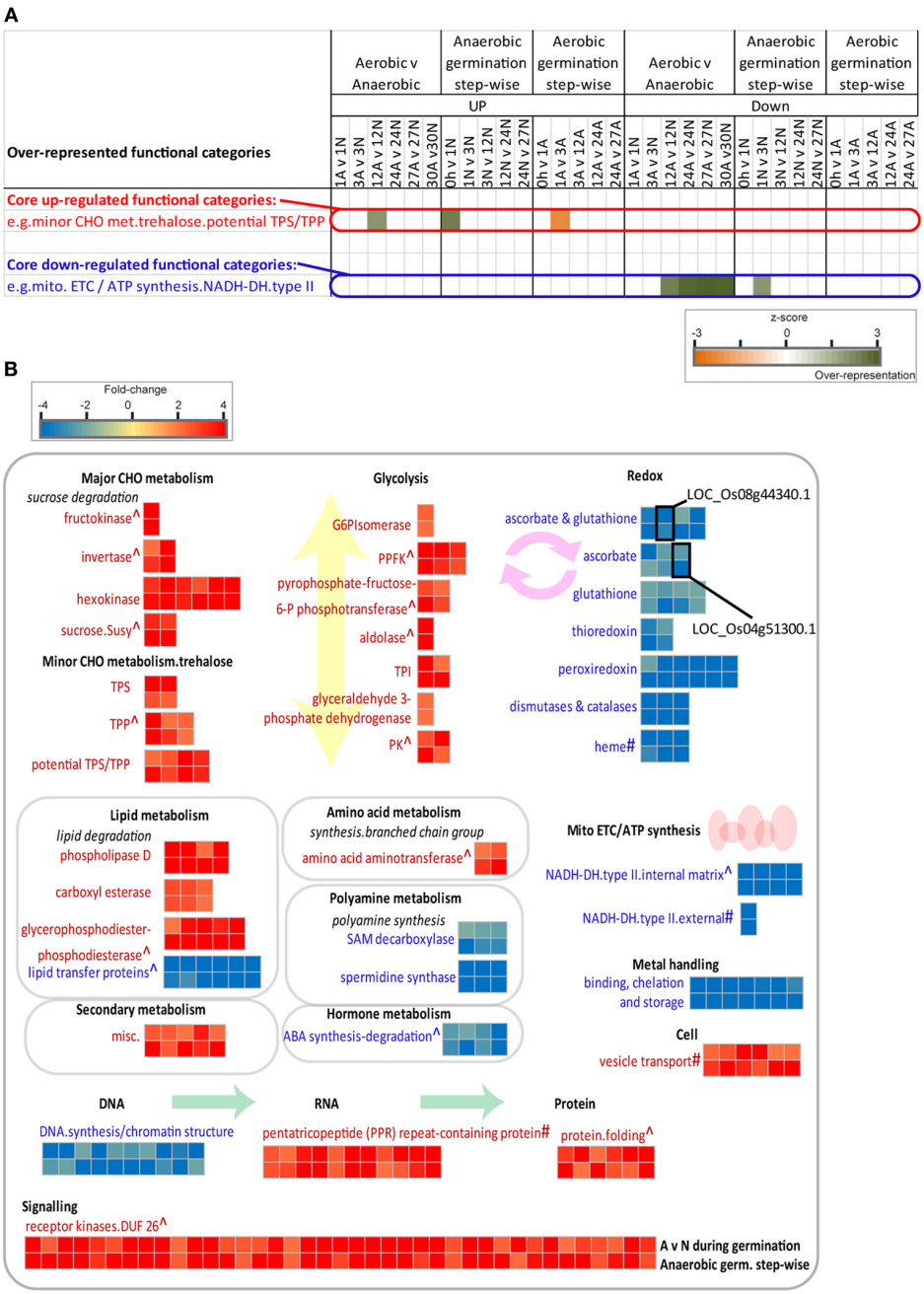

FIGURE 2 | Transcriptomic changes specific to anaerobic germination in rice. (A) Core up/down-regulated genes exclusive to anaerobic germination were identified by isolating genes encoding functions that were over-represented in comparison to aerobic germination (Aerobic v Anaerobic), as well as over the course of anaerobic germination (step-wise), whilst not being over-represented over the course of aerobic germination (step-wise). **(B)** Differential expression of genes encoding core over-represented functions unique to anaerobic germination only [as defined in **(A)**]. Significant fold-changes (>2-fold, $p < 0.05$, PPDE > 0.96) are shown as a heatmap for genes both in comparison to aerobic germination (A v N—top rows) as well as over the course of anaerobic germination (step-wise—bottom rows). Categories showing similar responses during Arabidopsis germination are indicated with ^ and categories containing genes that showed transient responses in Arabidopsis e.g., up then down or down then up, are denoted with #.

studies that presented these microarray data (**Table 1**) and in the cross-kingdom analysis of responses to anoxia (Mustroph et al., 2010).

Thus, while there was some conservation in the response to low oxygen within and across Arabidopsis and rice, we also identified functional categories that appeared only to be specifically over-represented in only one species. These were highlighted as species specific when over-representation was seen in three out of four of the rice comparisons, or four out of five of the Arabidopsis comparisons (highlighted in **Figure 3**).

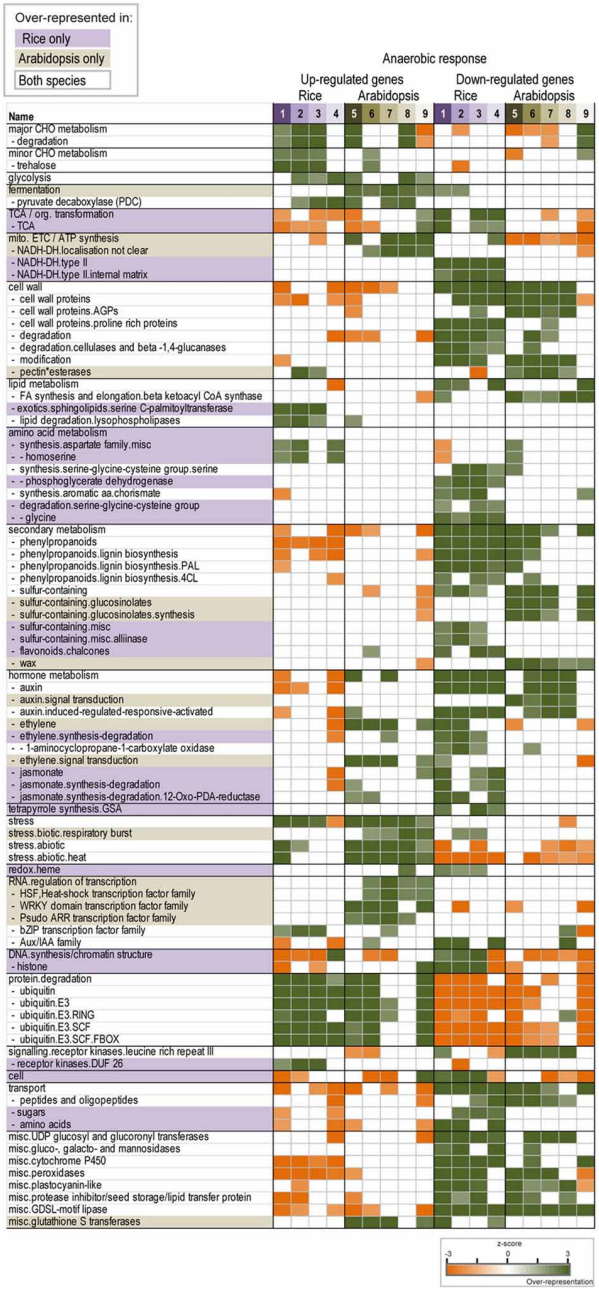

FIGURE 3 | Defining the core low oxygen response. Pageman over-representation analysis was carried out for the significantly differentially expressed genesets (>2-fold, $p < 0.05$, PPDE > 0.96) in response to low oxygen in rice and Arabidopsis. The gene sets used included four different comparisons in rice and five different comparisons in Arabidopsis of control vs. anaerobic treatment (details in **Table 1**). Only the functional categories over-represented in rice only, Arabidopsis only or both species are shown (*z*-scores indicate over/under-representation, indicated by the green/yellow colors, respectively).

For example, genes encoding NADPH type II dehydrogenases, phosphoglycerate dehydrogenase, glycine degradation (amino acid metabolism) and heme (redox) functions were seen to be specifically down-regulated in rice, whilst this was not seen for Arabidopsis (**Figure 3**). Of these, the opposite responses of heme (i.e. non-symbiotic hemoglobin) encoding genes was particularly noteworthy, as these are thought to have a role in NO scavenging and redox balance maintenance under low oxygen conditions in rice (Igamberdiev and Hill, 2009; Sturms et al., 2011). Specifically, the two genes encoding non-symbiotic hemoglobins were down-regulated by ∼10-fold (LOC_Os03g12510.1) and ∼90-fold (LOC_Os03g13140.1), whilst their Arabidopsis ortholog (At2g16060) was induced up to 34-fold under low oxygen conditions (**Supplementary Tables 4**, **6**). In contrast, sphingolipid metabolism, aspartate and homoserine metabolism, as well as DUF26 receptor kinases were only enriched in the up-regulated gene-sets in rice (**Figure 3**). Whereas, genes encoding pectin esterases and wax related functions were only significantly over-represented in the down-regulated gene-sets from Arabidopsis, but this was not seen in rice (**Figure 3**). One of the most interesting observations was that the up-regulation of biotic stress-respiratory burst functions, heat shock factors, WRKY transcription factors and ARR transcription factors was also unique to the Arabidopsis response to hypoxia/anoxia (**Figure 3**). Given that these are typically up-regulated under various stress conditions, finding these specifically induced in Arabidopsis under low oxygen conditions suggests that the perception of low oxygen stress in Arabidopsis may overlap with its perception of other abiotic/biotic stresses.

HOW DOES THE LOW OXYGEN RESPONSE COMPARE TO THE ABIOTIC STRESS RESPONSES?

Given that the response to low oxygen stress showed some similarity to abiotic stress in Arabidopsis, we overlapped the lists of differentially expressed genes in response to low oxygen stress with abiotic stress including drought, salt, cold and heat stress. All data was analyzed in the same manner using the abiotic stress datasets outlined in **Table 1** (Jain et al., 2007; Kilian et al., 2007; Hu et al., 2009). For each of the four anoxia comparisons individually in rice, the numbers of genes overlapping were mostly similar with the highest overlap seen with drought stress for three out of the four studies (**Figure 4A**). In contrast, the transcriptomic response to heat showed the highest number of overlapping genes across each of the five individual hypoxia/anoxia comparisons in Arabidopsis (**Figure 4B**). Given that this overlap is observed with heat stress in Arabidopsis, and the known link between the heat and anoxia response in Arabidopsis, we pursued this further by extracting at the heat responsive genes in Arabidopsis and examining these more closely. In an effort to see whether low oxygen conditions is perceived as an abiotic stress in Arabidopsis, particularly for heat stress, we extracted the differentially expressed genes in response to heat that were also known stress markers (Gadjev et al., 2006; Lu et al., 2007; Rasmussen et al., 2013) and viewed the expression changes in parallel with the responses to anoxia/hypoxia as well as other abiotic stresses (cold, drought, salt; **Figure 4Ci**). Specifically, these included the genes identified as abiotic stress marker genes (Lu et al., 2007) including, cold, drought, salt, and heat specific stress marker genes (Rasmussen et al., 2013) as well as oxidative stress markers genes (Gadjev et al., 2006). In this way 80 Arabidopsis genes are shown that are responsive to both low oxygen as well as abiotic

Table 1 | Microarray data used for analysis.

Experiment	No. of arrays	Treatment	Up-reg. (>2 FC)	Down reg. (>2 FC)	Age	References	Accession
RICE LOW OXYGEN							
1. Rice germination	39	1, 3, 12, 24 h anoxia	3377	4927	1, 3, 12, 24 h	Howell et al., 2009; Narsai et al., 2009	E-MEXP-1766, E-MEXP-2267
2. Rice A to N	12	27, 30 h anoxia	2292	3290	27, 30 h	Narsai et al., 2009	E-MEXP-2267
3. Rice N to A	12	27, 30 h anoxia	3218	2453	27, 30 h	Narsai et al., 2009	E-MEXP-2267
4. Rice coleoptile	4	4 days anoxia	3779	3631	4-day old	Lasanthi-Kudahettige et al., 2007	GSE6908
ARABIDOPSIS LOW OXYGEN							
5. AT hypoxia	8	2 h and 9 h hypoxia	2309	3097	1-week old	Branco-Price et al., 2008	GSE9719
6. AT hypoxia	4	5 h hypoxia	1788	2070	3-week old	Christianson et al., 2010	GSE21504
7. AT anoxia	4	6 h anoxia	587	923	4-day old	Banti et al., 2010	GSE16222
8. AT anoxia	4	6 h anoxia	457	804	4- day old	Loreti et al., 2005	GSE2133
9. AT hypoxia	4	4 h hypoxia	4912	3535	3-week old	Christianson et al., 2009	GSE14420
AT germination	30	10 time points, dry seed to 48 h			0–48 h	Narsai et al., 2011a	GSE30223
ARABIDOPSIS ABIOTIC STRESS							
Cold	12	0.5, 1, 3 h	989	987	16-day	Kilian et al., 2007	GSE5621
Drought	12	0.5, 1, 3 h	1068	571	16-day	Kilian et al., 2007	GSE5624
Salt	12	0.5, 1, 3 h	985	953	16-day	Kilian et al., 2007	GSE5623
Heat	12	0.5, 1, 3 h	1585	2972	16-day	Kilian et al., 2007	GSE5628
RICE ABIOTIC STRESS							
Cold	6	3 h	1323	1272	7-day	Jain et al., 2007	GSE6901
Drought	6	3 h	3701	4259	7-day	Jain et al., 2007	GSE6901
Salt	6	3 h	2899	2290	7-day	Jain et al., 2007	GSE6901
Heat	6	10 h	3634	4244	14-day	Hu et al., 2009	GSE14275

For rice and Arabidopsis, various datasets examining the hypoxia/anoxia responses as well as abiotic stress responses were analyzed in parallel. The number of arrays involved in each of the comparisons/experiments, the treatments involved and the number of significantly differentially expressed genes (>2-fold) after false discovery rate correction ($p < 0.05$, PPDE > 0.96) are shown (up/down-regulated). The age of the plants, publications referring to these arrays and array accession numbers are shown.

stress (**Figure 4Ci**). Using sequence similarity [Gramene; (Jaiswal et al., 2006)] and Inparanoid (Ostlund et al., 2010), the 135 rice orthologs to the 80 Arabidopsis genes were also analyzed and shown in the same way (**Figure 4Cii**).

As expected, many of the rice orthologs were also induced under abiotic stress in rice (**Figure 4Cii**). However, it is also apparent that while many of these stress marker genes are also induced under the hypoxia/anoxia comparisons in Arabidopsis, this is not seen for rice, where many are in fact down-regulated in response to hypoxia (**Figure 4C**). Three examples of genes showing different/opposite responses between Arabidopsis and rice are indicated in the green boxes (**Figure 4Cii**) and closer examination of these is shown in **Figures 4Di,ii**, respectively. One of these genes encodes the cold-responsive marker gene in Arabidopsis (cold-regulated 47—COR47; At1g20440) (Guo et al., 1992; Lu et al., 2007), which is orthologous to LOC_Os02g44870.1 in rice and shows significant induction in both species under cold and other abiotic stresses (**Figure 4D**). However, while this gene was induced 2.5-fold under hypoxia in Arabidopsis, its ortholog was down-regulated between 2.5 and 4-fold in response to anoxia in rice (**Figure 4D**). Similarly, an oxidative stress marker gene in Arabidopsis (At4g24570) encoding a dicarboxylate carrier was induced under hypoxia (2.6-fold), anoxia (3.7-fold), cold (454-fold) and drought stress (40-fold) in Arabidopsis while its rice ortholog (LOC_Os08g37370.1) was unchanging under abiotic stress and down-regulated up to 12-fold under anoxia (**Figure 4D**). This was particularly interesting as this gene, among others (**Figure 4Ci**), was down-regulated 15-fold under heat-stress in Arabidopsis (**Figure 4Di**). Similarly another gene that was down-regulated 6-fold under heat stress, but up-regulated after hypoxia/anoxia (up to 6-fold), cold (31-fold), drought (41-fold), and salt (5-fold) stress in Arabidopsis was the jasmonate zim-domain containing gene (At1g19180) (**Figure 4Di**). Interestingly, the Gramene database (Jaiswal et al., 2006) shows eight zim-domain containing rice genes as orthologs to this Arabidopsis gene (**Figure 4Dii**). While seven out of eight of these were induced under abiotic stress and three were briefly induced in response to switching to anoxia, all eight genes were down-regulated in two or more anoxia comparisons (**Figure 4Dii**).

It was particularly interesting to find that the rice orthologs to the Arabidopsis abiotic stress markers, which were down-regulated under heat stress in Arabidopsis, are in fact down-regulated under anoxia in rice (**Figures 4C,D**). Specifically, it was evidenced that oxidative stress marker genes (flu, O_3, and

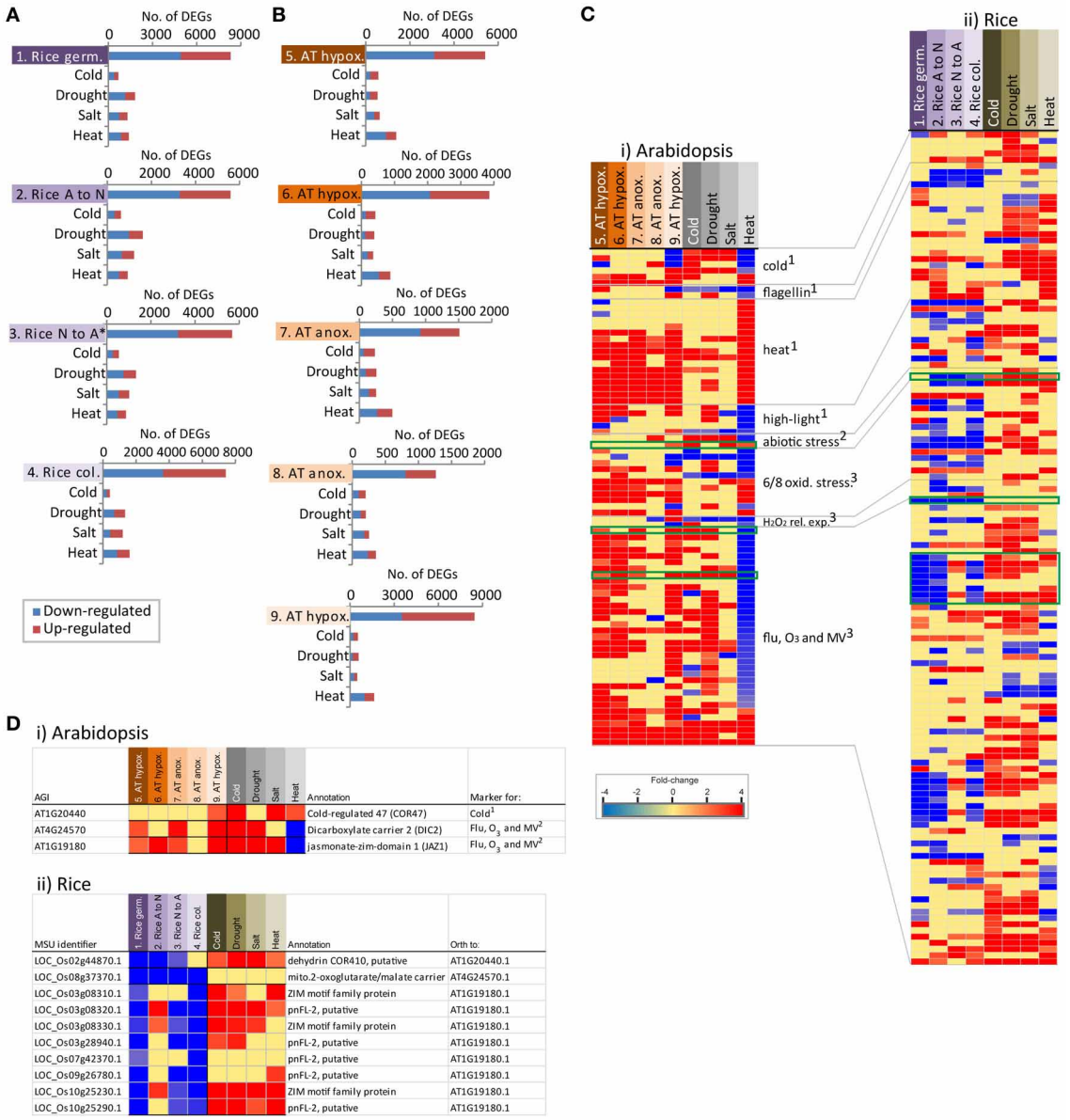

FIGURE 4 | Overlapping responses between low oxygen and abiotic stress in rice and Arabidopsis. (A) Differentially expressed genes from each of the four low oxygen transcriptome datasets in rice (Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009) were overlapped with the differentially expressed genes in response to cold, drought, salt, and heat stress (Jain et al., 2007; Hu et al., 2009). The numbers of genes showing overlapping responses are shown. The * indicates that the fold-changes were inversed for this comparison to show the response to anoxia. **(B)** Differentially expressed genes from each of the five low oxygen transcriptome datasets in Arabidopsis (Loreti et al., 2005; Branco-Price et al., 2008; Christianson et al., 2009, 2010; Banti et al., 2010) were overlapped with the differentially expressed genes in response to cold, drought, salt, and heat stress (Kilian et al., 2007). The number of genes showing overlapping responses is shown. (**Ci**) A heatmap showing differential expression of the 80 genes that were both significantly responsive to heat in Arabidopsis, as well as being previously identified as abiotic stress marker genes [by Gadjev et al. (2006), Lu et al. (2007), and Rasmussen et al. (2013) in Arabidopsis]. Expression is shown in response to low oxygen and abiotic stress. (**Cii**) A heatmap showing the expression of the 135 rice orthologs to the 80 Arabidopsis abiotic stress responsive genes (from **Ci**). (**Di**) The expression of three Arabidopsis genes representing examples of common transcriptomic responses to low oxygen and one or more abiotic stress in Arabidopsis. (**Dii**) Expression of the rice orthologs to the three Arabidopsis genes, showing that these differ in the transcriptomic responses to low oxygen and one or more abiotic stress in rice.

MV) in particular were down-regulated under heat stress and up-regulated under hypoxia/anoxia in Arabidopsis (**Figure 4Ci**), whilst the rice orthologs to these genes are down-regulated under anoxia (**Figure 4Cii**). It has previously been shown that under heat stress, there is a significant number of oppositely responsive orthologous genes between rice and Arabidopsis (Narsai et al., 2010). Specifically, it was shown that under heat stress, genes encoding redox functions are up-regulated in rice

and down-regulated in Arabidopsis (Narsai et al., 2010). Thus, it was particularly interesting to see these genes were up-regulated under low oxygen conditions in Arabidopsis, whilst their rice orthologs are down-regulated (**Figure 4C**).

Using these parallel datasets to analyse the low oxygen response and other abiotic stress responses, it was possible to isolate and identify low oxygen marker genes in rice and Arabidopsis. To do this, the significantly differentially expressed genes were filtered to identify genes showing the highest fold induction in response to low oxygen in rice (>50-fold) in two or more out of the four low oxygen response studies/comparisons, whilst not showing up-regulation under abiotic stress (**Table 2**). Similarly, the significantly differentially expressed genes in Arabidopsis were filtered to identify genes showing the highest fold induction in response to low oxygen (>50-fold) in three or more out of the five low oxygen response studies/comparisons (**Table 2**). In this way, highly responsive low oxygen markers were identified in both rice and Arabidopsis (**Table 2**). Notably, pyruvate decarboxylase was identified as a marker of low oxygen stress in Arabidopsis, showing between a 17- and 586-fold induction under all five low oxygen comparisons (At4g33070; **Table 2**). Similarly, in rice, pyruvate decarboxylase was also induced under low oxygen, however, this was to a lower extent (not meeting the criteria as a marker) (**Supplementary Table 4**). Additionally, it was not surprising that an alpha amylase encoding gene (LOC_Os09g28340.1) was identified to be a low oxygen marker in rice, as these have been shown to have a crucial function in the metabolic changes that occur under low oxygen conditions in rice (Loreti et al., 2003). Similarly, it was not surprising to see a glycosyl hydrolase (LOC_Os11g47560.1) as a low oxygen marker gene in rice, as these are also known to be responsive under low oxygen conditions in rice (**Table 2**). Interestingly, no Arabidopsis orthologs were detected for the aforementioned alpha-amylase and glycosyl hydrolase using the Inparanoid method (Ostlund et al., 2010) or based on sequence homology using Gramene (Jaiswal et al., 2006), suggesting a more specific role for these in rice.

Notably, the Arabidopsis gene At1g35140 encoding the phosphate induced (exordium-like EXL1) gene was also identified as a low oxygen marker (**Table 2**). This was particularly interesting given that Arabidopsis mutants of this gene have recently been shown to have reduced hypoxia tolerance (Schroder et al., 2011). Interestingly, it can be seen that while eight out of the 18 Arabidopsis low oxygen markers had rice orthologs that were also induced under low oxygen (indicated by an asterisk in **Table 2**), only two of the 12 low oxygen markers in rice had Arabidopsis orthologs showing conserved inductions under low oxygen (asterisk next to rice genes in **Table 2**). In addition, it was also seen that two rice genes had orthologs that were in fact down-regulated under low oxygen in Arabidopsis (**Table 2**). Namely; the fascilin domain containing gene LOC_Os03g57490.1 (induced between 18 and 116-fold; **Table 2**) is orthologous to At2g35860.1 (64% sequence similarity) which is down-regulated over 2.5 fold under low oxygen in two of the five Arabidopsis studies (**Supplementary Table 6**). Similarly, while the UDP-glucoronosyl transferase encoding gene At1g05680 was identified as a low oxygen induced marker in Arabidopsis (induced between 7 and 606-fold; **Table 2**), its rice ortholog LOC_Os04g12980.1 (41% sequence similarity) is down-regulated over 2.2-fold in response to anoxia in all four rice studies (**Supplementary Table 4**).

DISCUSSION

Germination is a crucial, energy demanding stage of the plant life cycle. Rice can not only survive under low oxygen conditions but can also germinate in the complete lack of oxygen (Howell et al., 2007; Bailey-Serres and Voesenek, 2008; Magneschi and Perata, 2008). Despite significant differences in the life cycle lengths or storage reserves (e.g., oil seed vs. starch seed) of different plant species, the process of germination often occurs rapidly. When germination under aerobic conditions in Arabidopsis (Narsai et al., 2011a), aerobic conditions in rice (Howell et al., 2009) and anaerobic conditions in rice were compared (Narsai et al., 2011a), core functions was altered in the transcriptomic responses, revealing conserved down-regulation of ABA responsive proteins, seed storage proteins and protein degradation, as well as the up-regulation of protein synthesis, lipid metabolism and cell wall functions (**Figure 1**). Thus, even under anaerobic conditions in rice, we found that there are several conserved transcriptomic responses that are likely to be crucial for germination progression in plants.

During anaerobic germination, in addition to the morphological adaptations including coleoptile elongation, it is known that carbohydrate metabolism is altered and fermentation is activated (Magneschi and Perata, 2008). As expected, closer examination under anaerobic germination also revealed an early induction of carbohydrate metabolism and glycolysis functions specific to anaerobic germination (**Figure 2**). Notably, in some cases, even when the induction of specific genes was seen between both species, the magnitude differed significantly. For example, while pyruvate decarboxylase was identified as a marker of low oxygen stress in Arabidopsis, showing a greater than 50-fold induction (**Table 2**), the induction of pyruvate decarboxylase in rice was much smaller. In contrast, pyruvate dikinase was also induced under low oxygen conditions in both species; however, this induction was much larger in rice compared to Arabidopsis. This was not entirely surprising, given that under low oxygen conditions in rice, it has been proposed that pyrophosphate may be used as an alternative energy currency over ATP, acting as a high-energy donor molecule (Huang et al., 2008; Igamberdiev and Kleczkowski, 2011). Hence, this alteration may help to maintain the energy balance, and tolerance of rice under low oxygen conditions.

Interestingly, a number of genes encoding PPR domain containing proteins were also found to be specifically induced under anaerobic germination in rice. In the past few years, the roles of PPR proteins have been better elucidated revealing functions in organelle RNA processing and editing (Saha et al., 2007). Interestingly, during Arabidopsis germination (i.e., under aerobic conditions) it was revealed that several PPR encoding genes show germination specific expression (Narsai et al., 2011a) and many of these are embryo lethal when a loss-of-function occurs (Tzafrir et al., 2003). Thus, the specific induction of these PPRs during anaerobic germination may be indicative of a crucial role for these PPRs during anaerobic germination in rice as well.

Table 2 | Low oxygen marker genes in rice and Arabidopsis.

Probeset ID	MSU identifier	MSU putative function	1. Rice germ.	2. Rice A to N	3. Rice N to A	4. Rice coleop.	Cold	Drought	Salt	Heat
Os.16456.1.S1_at	LOC_Os07g25690.1	subtilisin N-terminal Region family	161	64	−209	227				
Os.54891.1.S1_at	LOC_Os02g18650.1	pectinesterase-2 precursor	331	41	−157	68				
Os.28566.1.S1_at	LOC_Os03g19840.1	cysteine-rich repeat secretory protein	81	44	−94	121				
Os.25125.1.A1_at	LOC_Os03g57490.1^	fasciclin domain, putative, expressed	116	18	−70	67				
Os.12488.1.S1_at	LOC_Os04g43650.1	L-allo-threonine aldolase	263	119	−412	10			−3	
Os.36919.1.S1_at	LOC_Os01g62010.1*	hydrolase, alpha/beta fold family protein	74	58	−61	9		−2	−2	
OsAffx.17379.1.S1_at	LOC_Os08g37470.1	cupin, RmlC-type, 2-aminoethanethiol dioxygenase	113	56	−105	8				
Os.17474.1.A1_at	LOC_Os02g11790.1*	OsFBK4: F-box domain/kelch repeat domain	64	27	−65	24				
Os.50837.1.S1_at	LOC_Os11g47560.1	glycosyl hydrolase, putative, expressed	82	29	−11	197				
Os.28903.1.S1_at	LOC_Os01g52900.1	expressed protein		31	−171	172				
OsAffx.6201.1.S1_at	LOC_Os09g08100.1	cysteine proteinase inhibitor precursor	60	62	−49					
Os.11391.1.S1_at	LOC_Os10g02750.1^	Ser/Thr protein phosphatase family protein	161	94	−45	3				
Os.33211.1.S1_at	LOC_Os09g28430.1	alpha-amylase isozyme 3C precursor	87	2	−55			−19		

Probeset ID	AGI	TAIR annotation	5. AT hypox.	6. AT hypox.	7. AT anox.	8. AT anox.	9. AT hypox.	Cold	Drought	Salt	Heat Heat
264846_at	AT2G17850*	Rhodanese-like	445	137	3	116	56				
267024_s_at	AT2G34390^; AT2G29870	lactate transmembrane transporter/ MIP family protein	623	467	20	318	685				
245173_at	AT2G47520	AP2 domain-containing transcription factor	113	460	56	37	449				
250464_at	AT5G10040	similar to unknown protein	595	422	14	338	511				
263231_at	AT1G05680^	UDP-glucoronosyl/UDP-glucosyl transferase	61	606	64		7				
260741_at	AT1G15040*	glutamine amidotransferase-related	87	4	62		502				
245757_at	AT1G35140*	PHI-1 (PHOSPHATE-INDUCED 1)	85	137			128		−11	−13	−425
262124_at	AT1G59660	nucleoporin family protein	108	71	10	17	145				
261545_at	AT1G63530	similar to hydroxyproline-rich glycoprotein	324	55	13	3	127				
258930_at	AT3G10040*	transcription factor	64	15		59	140				
252746_at	AT3G43190*	SUS4;UDP-glycosyltransfer./suc.synthase	153	26	13	180	89				
255807_at	AT4G10270*	wound-responsive family protein	93	8	3	61	53				
254200_at	AT4G24110	similar to unnamed protein product	158	48		80	168				
253616_at	AT4G30380	EXLB2 (EXPANSIN-LIKE B2 PRECURSOR)	58	64			141				
253416_at	AT4G33070*	pyruvate decarboxylase, putative	566	39	17	586	91				
253060_at	AT4G37710*	VQ motif-containing protein	6	880	50	16	279				
249384_at	AT5G39890	similar to unknown protein	96	19	2	87	90				

*For rice and Arabidopsis, various datasets examining the hypoxia/anoxia responses as well as abiotic stress responses were analyzed in parallel (details in **Table 1**). Top induced genes in response to low oxygen in rice and Arabidopsis were identified and the significant fold-changes after false discovery rate correction ($p < 0.05$, PPDE > 0.96) are shown. For rice, genes induced >50-fold in two or more of the rice anoxia studies are shown. For Arabidopsis, genes induced >50-fold in three or more hypoxia/anoxia studies are shown. These were also filtered exclude genes up-regulated under abiotic stress. An asterisk next to a gene indicates that the orthologous gene(s) from the other species (rice/Arabidopsis) is/are also up-regulated under low oxygen, whereas a ^ indicates that the orthologous gene is down-regulated.*

In addition, a specific suppression of genes encoding redox related functions were identified during anaerobic germination in rice, where these genes were not only down-regulated compared to aerobic germination but also down-regulated over the anaerobic germination time-course. This is particularly interesting given that cross-talk between NO and reactive oxygen species signaling has been shown to have a role in the light- and hormone-specific regulation of seed development and germination in plants [reviewed in Sirova et al. (2011)]. It has also been proposed that plant hemoglobins may modulate the effects of hormones that use NO as a signal transduction component (Hebelstrup et al., 2007). Thus, the specific suppression of redox functions, including hemoglobins in rice, may be part of the transcriptomic response to these signals coordinating anaerobic germination. Additionally, recent studies in rice are providing evidence supporting a crucial role of hemoglobins in NO scavenging, even reporting increased activity of these proteins in rice compared to their human counterparts under low oxygen conditions (Igamberdiev and Hill, 2009; Sturms et al., 2011). Furthermore, when the expression of hemoglobin genes were compared under low oxygen conditions in rice, Arabidopsis and poplar (which is also a flood tolerant species), it was seen that while the genes encoding non-symbiotic hemoglobins are suppressed in rice and poplar, they are significantly induced in Arabidopsis (Narsai et al., 2011b). This implies a controlled response to low oxygen, whereby suppressing hemoglobin gene expression may be more characteristic of low oxygen tolerant species.

Recent studies have shown a link between redox functions, anaerobiosis and heat stress in Arabidopsis (Pucciariello et al., 2012a). In 2008, it was shown that pre-treating Arabidopsis plants with heat stress resulted in the induction of heat shock factors and enabled greater hypoxia tolerance (Banti et al., 2008). Comparative analysis of the transcriptomic response to heat and anoxia/hypoxia in this study also confirmed this greater overlap with heat stress, compared to salt, drought and cold stress. In addition, a more Arabidopsis specific induction of heat shock factors was seen under the five Arabidopsis low oxygen studies, compared to rice (**Figure 3**). Confirmation for the role of heat shock factors in the low oxygen response also came when it was shown that HsfA2 enhances low oxygen tolerance by altering the expression of its target genes in Arabidopsis (Banti et al., 2010). Similarly, although the SUB1A locus was first described for its role in anoxia tolerance in rice (Xu et al., 2006), a more recent study has also revealed a role in drought tolerance (Fukao et al., 2011). Thus, there is cross-talk between the low oxygen and other abiotic stress responses.

Interestingly, when the expression of known heat-responsive abiotic stress marker genes in Arabidopsis (Gadjev et al., 2006; Lu et al., 2007; Rasmussen et al., 2013) were examined under low oxygen conditions in this study, strong induction of these was also seen under low oxygen conditions in Arabidopsis, whilst this was not seen for their rice orthologs. Specifically, it was seen that oxidative stress marker genes [flu, O_3, and MV—identified in Gadjev et al. (2006)] were down-regulated under heat stress and up-regulated under low oxygen in Arabidopsis, whilst the rice orthologs to these genes were down-regulated under anoxia. These divergent expression responses for oxidative stress marker genes, suggests differences in oxidative/ROS signaling under low oxygen conditions between Arabidopsis and rice. In Arabidopsis, it has been shown that ROS are produced under heat stress and low oxygen conditions, and a mechanism linking ROS signaling with ERFs has been also been shown (Pucciariello et al., 2012a,b).

Since the identification of the role of the SUB1A ERF in anoxia tolerance in rice (Xu et al., 2006), a link between anoxia and ERFs has been founded. Recently, a role for group VII ERFs (containing a conserved N-terminal motif) in oxygen sensing and mediating the low oxygen response was shown in Arabidopsis (Gibbs et al., 2011). It was determined that under low oxygen conditions in Arabidopsis, the N-end rule pathway of targeted proteolysis acts as an oxygen sensor, where Arabidopsis plants lacking the constituents of this pathway were more hypoxia tolerant (Licausi et al., 2011). Notably, in comparison to rice, genes encoding ethylene signaling functions were more specifically induced in Arabidopsis, supporting the role for ethylene signaling in the Arabidopsis hypoxia response.

CONCLUSIONS

In this study, core transcriptomic responses during germination in rice and Arabidopsis were identified. Comparison with other abiotic stress revealed some overlap with the low oxygen stress response, particularly for heat stress in Arabidopsis. Also, sets of low oxygen responsive markers were identified, both for Arabidopsis and rice, with two of the identified Arabidopsis markers (pyruvate decarboxylase—At4g33070 and phosphate induced EXL1—At1g35140) already known to function in the hypoxia response in Arabidopsis (Kursteiner et al., 2003; Schroder et al., 2011). The induction of HSFs and WRKY TFs were seen under heat and low oxygen stress in Arabidopsis, supporting the demonstrated role of these HSFs in heat and anoxia stress (Banti et al., 2008, 2010). While HSF functions were also first characterized for their role under other abiotic stresses, the crucial role of WRKY TFs under abiotic stress is also well-known (Chen et al., 2012). Thus, it is also worthwhile considering a potential role for these TFs in hypoxia tolerance in Arabidopsis as well. Overall, we have shown the different responses to low oxygen stress in rice and Arabidopsis and demonstrated the core transcriptional reprogramming that occurs as part of the hypoxia response in plants, including divergent responses between Arabidopsis and rice. Specifically, these findings revealed an interesting link between ROS and the anoxia response.

A ROLE FOR ROS IN THE ANOXIA RESPONSE

While many plants, and indeed organisms, display common responses to low or no oxygen conditions, these common responses alone are not sufficient to explain anaerobic tolerance. A comparison of the unique responses in rice over germination with Arabidopsis, reveal that the suppression of a variety of genes associated with redox balance is unique to rice. Furthermore, heat treatment in Arabidopsis, also results in a suppression of genes associated with redox functions, and heat has been shown to increase tolerance to anaerobic conditions (Banti et al., 2008, 2010). In recent years it has been shown that ROS play essential roles not only in stress responses, but in also maintaining growth (Foreman et al., 2003). A ROS gradient from

root tips defines proliferation and differentiation (expansion) (Tsukagoshi et al., 2010; Wells et al., 2010). To date this role is largely defined in roots, but the role of ROS, and different ROS species defining proliferation and expansion position them as key mediators between environmental stress signaling and growth promoting pathways. ROS have also been implicated in development in animals from Dictyostelium to mammals (Aguirre and Lambeth, 2010). From the analysis carried out above it is proposed that low oxygen conditions is not sensed as a stress in rice, in comparison to Arabidopsis where it is perceived as a stress. In Arabidopsis, this leads to an induction of stress and anti-oxidant defence systems, resulting in a cessation of growth. In rice, the suppression of transcripts for anti-oxidant defence systems will mean that ROS (or RNS) can still exist and act as an essential signal to drive the morphological changes that occur in rice under low oxygen conditions, thus, enabling growth to continue.

MATERIALS AND METHODS

PUBLICALLY AVAILABLE MICROARRAY DATASETS

Publically available microarrays were downloaded from the Gene Expression Omnibus or MIAME Array Express Databases (for each species) and these were normalized together. Affymetrix Expression Console software was used to first obtain present, absent, marginal calls for gene expression following MAS5.0 normalization. Genes that were called present ($p < 0.05$) were kept for further analysis. Data was GC-RMA normalized using Partek Genomics Suite and this was used for the differential expression analysis. The microarrays used for the germination analyses included an aerobic germination time course in rice—E-MEXP-1766 (Howell et al., 2009), anaerobic germination in rice—E-MEXP-2267 (Narsai et al., 2009) and aerobic germination time course in Arabidopsis—GSE30223 (Narsai et al., 2011a). For the hypoxia/anoxia datasets only the control (air) v hypoxia/anoxia microarrays were analyzed for both species (details, references and accession are shown in **Table 1**). For the differential expression analysis in response to abiotic stress in rice, these appear as shown in (Narsai et al., 2010), which compared the responses to drought, salt, cold and heat stress in Arabidopsis and rice. Details are also shown in **Table 1**.

DIFFERENTIAL EXPRESSION ANALYSIS

After all the arrays were pre-processed and normalized, differential expression analysis was carried out using the Cyber-T method (Baldi and Long, 2001; Long et al., 2001), as done in previous studies (Narsai et al., 2010, 2011a). Using the Cyber-T software for differential expression analysis, a gene was identified as significantly differentially expressed when $p < 0.05$ and the false discovery rate is less than 5% (PPDE > 0.96). For all the analyses shown in this study, this was further filtered to only include genes that were significantly differentially expressed by greater than 2-fold.

PAGEMAN ANALYSIS

All the differentially expressed genes were used for Pageman over-representation analysis (Usadel et al., 2006) as done in previous studies (Narsai et al., 2011a). ORA Fisher's test 2.0 was used to determine significant over-representation which calculates a *z*-score showing the over/under-representation of specific functional categories. *Z*-scores greater than 1.96 indicate significant over-representation at $p < 0.05$.

ORTHOLOGY BETWEEN RICE AND ARABIDOPSIS GENES

In order to identify rice orthologs to the Arabidopsis genes, two methods were employed based on; (1) sequence identity [extracted from Gramenemart; Jaiswal et al. (2006)] and (2) the Inparanoid method (Ostlund et al., 2010). If one or both of these methods identified a gene as orthologous to the Arabidopsis gene, these were considered rice orthologs.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: www.frontiersin.org/Plant_Physiology/10.3389/fpls.2013.00349/abstract

Supplementary Figure S1 | Pageman analysis of germination in rice (aerobic germination; Howell et al., 2009, anaerobic germination; Narsai et al., 2009) and Arabidopsis (Narsai et al., 2011a) using step-wise comparisons. Over-representation analysis was carried out for genes significantly differentially expressed (>2-fold, $p < 0.05$, PPDE > 0.96) over the course of germination. Common over-represented functional categories across the three germination studies are shown. The *z*-scores indicating over/under representation of genes in each functional category are indicated as a heatmap where red indicates over-representation and blue indicates under-representation.

Supplementary Table 1 | The step-wise comparisons used for rice under aerobic (Howell et al., 2009) and anaerobic (Narsai et al., 2009) germination. All microarrays for both species were GC-RMA normalized and analyzed in the same manner for differential expression using the Cyber-T method ($p < 0.05$, PPDE > 0.96).

Supplementary Table 2 | The step-wise comparisons used for Arabidopsis germination (Narsai et al., 2011a). All microarrays for both species were GC-RMA normalized and analyzed in the same manner for differential expression using the Cyber-T method ($p < 0.05$, PPDE $>$ 0.96).

Supplementary Table 3 | Pageman analysis of rice aerobic and anaerobic germination was carried out together and then matched with the Pageman analysis of Arabidopsis germination to show represented functional categories over the course of germination. All microarrays for both species were normalized and analyzed in the same manner for differential expression (i.e., FC = 2-fold, $p < 0.05$, PPDE > 0.96) and the same parameters were used for the Pageman analysis (i.e., ORA Fisher using 2.0 as a cut-off).

Supplementary Table 4 | The low oxygen and abiotic stress comparisons and used for rice from the sources outlined in Table 1. All microarrays for both species were GC-RMA normalized and analyzed in the same manner

for differential expression using the Cyber-T method ($p < 0.05$, PPDE > 0.96).

Supplementary Table 5 | Pageman analysis (ORA Fisher using 2.0 as a cut-off) of rice aerobic vs. anaerobic germination, as well as step-wise germination was carried out to show represented functional categories unique to anaerobic germination. All microarrays were normalized and analyzed in the same manner for differential expression (i.e., FC = 2-fold, $p < 0.05$, PPDE > 0.96). Genes from these are shown in **Figure 2**.

Supplementary Table 6 | The low oxygen and abiotic stress comparisons and used for Arabidopsis from the sources outlined in Table 1. All microarrays for both species were GC-RMA normalized and analyzed in the same manner for differential expression using the Cyber-T method ($p < 0.05$, PPDE > 0.96).

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