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RESEARCH PAPER

Regulatory interplay of the Sub1A and CIPK15 pathways in the regulation of α-amylase production in flooded rice plants

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ABSTRACT

Rice (*Oryza sativa* L.) can successfully germinate and grow even when flooded. Rice varieties possessing the submergence 1A (*Sub1A*) gene display a distinct flooding-tolerant phenotype, associated with lower carbohydrate consumption and restriction of the fast-elongation phenotype typical of flooding-intolerant rice varieties. Calcineurin B-like interacting protein kinase 15 (CIPK15) was recently indicated as a key regulator of α-amylases under oxygen deprivation, linked to both rice germination and flooding tolerance in adult plants. It is still unknown whether the Sub1A- and CIPK15-mediated pathways act as complementary processes for rice survival under O_2 deprivation. In adult plants Sub1A and CIPK15 may perhaps play an antagonistic role in terms of carbohydrate consumption, with Sub1A acting as a starch degradation repressor and CIPK15 as an activator. In this study, we analysed sugar metabolism in the stem of rice plants under water submergence by selecting cultivars with different traits associated with flooding survival. The relation between the Sub1A and the CIPK15 pathways was investigated. The results show that under O_2 deprivation, the CIPK15 pathway is repressed in the tolerant, *Sub1A*-containing, FR13A variety. CIPK15 is likely to play a role in the up-regulation of *Ramy3D* in flooding-intolerant rice varieties that display fast elongation under flooding and that do not possess *Sub1A*.

INTRODUCTION

Flooding is a widespread environmental constraint that has a dramatic effect on the growth and yield of agronomically important crops not adapted to a submerged environment (Perata & Voesenek 2007). It can severely affect food availability in several regions of South and South-East Asia that are prone to water submersion during the rainy season. In the context of crops, all cereals apart from rice (*Oryza sativa* L.) are flooding intolerant. The rice plant originates from a semi-aquatic environment and has evolved specialised functions to adapt to different types of flooding event, either deepwater or flash (Nagai *et al.* 2010). Two opposite strategies involving unique adaptations were recently classified as low oxygen quiescence syndrome (LOQS), where the rice shoot does not elongate upon submergence but re-grows after de-submergence, and low oxygen escape syndrome (LOES), characterised by rapid shoot extension under flooding to reach the water surface (Bailey-Serres & Voesenek 2008; Colmer & Voesenek 2009).

The restriction of oxygen (O_2) availability due to partial or complete water submergence inhibits aerobic respiration of plants, thus leading to an energy deficit (Perata & Alpi 1993). Rice is characterised by a broad range of metabolic and morphological adaptations to flooding and can successfully germinate and grow even when O_2 supply is limited (Alpi & Beevers 1983; Perata & Alpi 1993). Rice can also germinate in the absence of O_2, thanks to the successful induction of the enzymes needed to degrade the starch reserves present in the endosperm (Perata *et al.* 1992, 1996; Guglielminetti *et al.* 1995a). Of the enzymes required for starch degradation, α-amylases are required in order to initiate degradation of starch granules (Dunn 1974; Sun & Henson 1991). In rice, α-amylases are encoded by a large multigene family (Huang *et al.* 1990), and while the gibberellin (GA)-induced *Ramy1A*-encoded isoform plays a major role during aerobic rice germination, the *Ramy3D*-encoded isoform is predominant during anaerobic germination (Loreti *et al.* 2003a; Lasanthi-Kudahettige *et al.* 2007). *Ramy3D* is not regulated by GA, whose synthesis requires O_2, but is induced by sugar starvation that is a consequence of the fast anaerobic use of soluble sugars (Loreti *et al.* 2003a,b). *Ramy3D* is therefore transcriptionally regulated by sugar signaling, through a sugar response complex (SRC) situated on the promoter region and activated by the MYBS1 transcription factor under sugar starvation (Lu *et al.* 1998; Yu 1999). The calcineurin B-like interacting protein kinase 15 (CIPK15) has been proposed as a key regulator of the sensing cascade for successful rice germination under flooding (Lee *et al.* 2009). CIPK15 seems to act through positive regulation of the sucrose non-fermenting 1 related protein kinase (SnRK1A) and MYBS1 to control α-amylase abundance under O_2 deprivation (Lu *et al.* 2002, 2007; Lee *et al.* 2009). It has been suggested that the CIPK15 effect goes beyond the seedling stage, thus playing a positive role in growth of the mature rice plant under partial flooding (Lee *et al.* 2009).

However, the survival of established rice plants when submerged is believed to be largely dependent on the presence of the submergence 1A (*Sub1A*) ethylene-responsive factor (ERF) gene, which is limited to a subset of *indica* varieties (Xu *et al.* 2006). Submergence tolerance is in fact strongly correlated with the presence of the *Sub1A-1* allele (Xu *et al.* 2006), which activates ethanolic fermentation and negatively regulates carbohydrate catabolism, including α-amylase-dependent starch degradation (Fukao *et al.* 2006). The flooding induction of *Sub1A* results in a quiescence status that prevents rapid elongation of the submerged plants and depletion of carbohydrate reserves, thus enabling a fast recovery after de-submergence (Bailey-Serres & Voesenek 2008).

The physiology of germinating seedlings and established seedlings is different during complete submergence (Xu *et al.* 2006; Lee *et al.* 2009; Magneschi & Perata 2009). Sub1A- and CIPK15-mediated pathways could complement each other at different developmental stages and under different growth conditions (Lee *et al.* 2009). However, at present it is unknown whether the Sub1A- and CIPK15-mediated pathways act as complementary processes for rice survival under O_2 deprivation. Sub1A is unlikely to play a role during anoxic germination (Magneschi & Perata 2009) while the CIPK15 pathway appears to be essential to this growth stage. In adult plants Sub1A and CIPK15 may perhaps play an antagonistic role in terms of carbohydrate consumption, with Sub1A acting as a starch degradation repressor and CIPK15 as an activator.

In this study, we analysed sugar metabolism in stems of rice plants under water submergence by selecting cultivars with different traits associated with flooding survival. The relation between the Sub1A and the CIPK15 pathways was investigated. The results show that under O_2 deprivation, the CIPK15 pathway is repressed in the tolerant *Sub1A*-containing FR13A variety (Ellis & Setter 1999; Xu *et al.* 2006). On the other hand, CIPK15 is likely to play a role in the up-regulation of *Ramy3D* in flooding-intolerant rice varieties that display fast elongation under flooding and do not possess *Sub1A*.

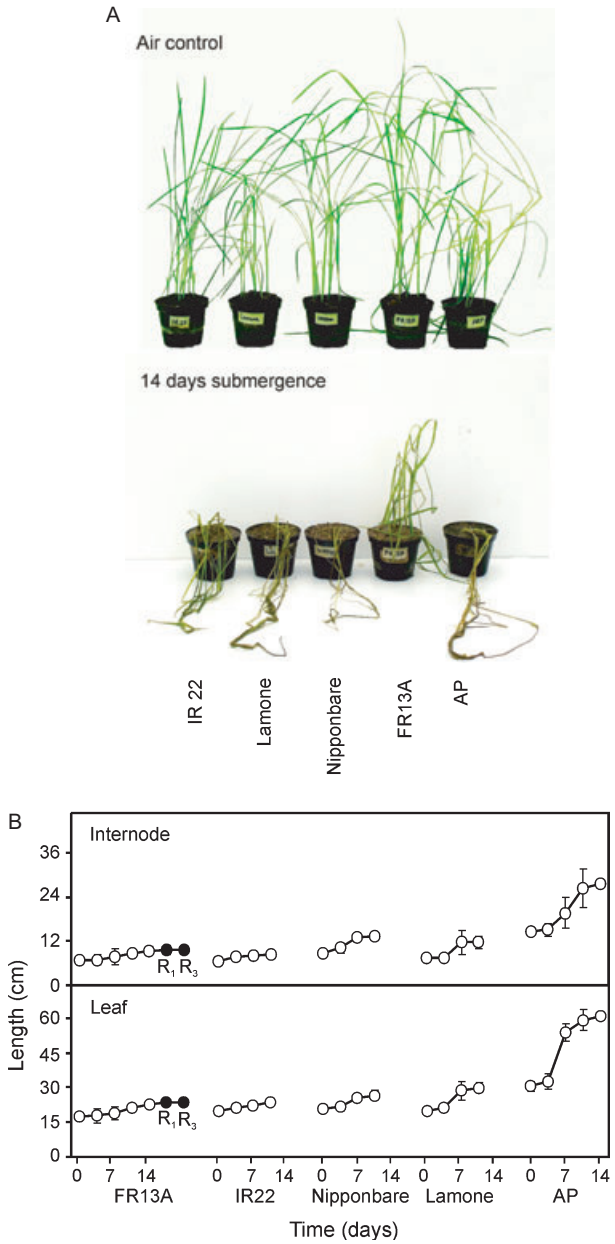

Fig. 1. Growth behaviour of rice varieties under complete submergence stress. Fourteen-day-old rice seedlings were grown in pots filled with soil and peat (approximately 10-cm deep) and completely submerged in water, 1 m above the soil surface. Rice plants were grown in a growth chamber for 17 days after flooding, at 26 ± 2 °C in a photoperiod of 15-h light. A: Plant phenotype of rice varieties grown in air and after 14 days of complete submergence. B: Shoot elongation of rice varieties under complete submergence (empty circles) and after 1 and 3 days of recovery after de-submergence (filled circles R_1 and R_3). Stem elongation and leaf elongation were taken from the base of the plant to the upper collar and from there to the edge of the longest leaf, respectively. Data are expressed as the mean of 30 measurements ± SD.

MATERIALS AND METHODS

Plant material and submergence treatment

Seeds of *O. sativa* varieties FR13A, IR22, Nipponbare, Lamone and Arborio Precoce (AP) were obtained from our institute's farm. Seeds were soaked on sterile water wetted filter paper in Petri dishes, at 28 ± 2 °C in dark conditions for 3–4 days. Pre-germinated healthy seedlings were then transferred to plastic pots filled with soil and peat (1:1) and kept in growth chambers for 14 days at 26 ± 2 °C with a 15-h photoperiod (light intensity: 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Fourteen-day-old seedlings (V4 stage, Counce *et al.* 2000) of all five varieties were subjected to complete submergence in distilled water, as described in the legend to Fig. 1.

In the experiment specifically carried out using the FR13A and AP varieties, the seedlings were germinated as described above and grown in a sand medium with the following complete nutrient solution: 4.5 mM $(CaNO_3)_2$, 0.8 mM $MgSO_4$, 2.6 mM KH_2PO_4, 9.0 mM KNO_3 and 0.2 mM K_2SO_4. Seven-day-old seedlings (two leaf stage) were subjected to complete submergence, as described in the legend to Fig. 3.

For the experiment with an external sugar source, de-hulled AP and FR13A seeds were sterilised in 70% ethanol

for 1 min, followed by 6% sodium hypochlorite for 15–20 min. Seeds of each variety were then placed on half-strength Murashige and Skoog medium, containing 0.8% agar, in 5-l glass bottles at 26 ± 2 °C and a 15-h photoperiod (light intensity: 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The glass bottles and media where sterilised before use. Seven-day-old seedlings (V2 stage, Counce *et al.* 2000) were subjected to complete submergence, using sterile 50 mM sucrose or 50 mM mannitol solutions for 14 days, followed by 7 days of recovery after de-submergence. The O_2 concentration in the media was measured after 0, 10 and 14 days of submergence with a dissolved oxygen meter (Jenway 9071, Jenway, Staffordshire, UK). Solution contamination was screened on bacterial nutrient agar (at 35 °C for 24 h) by inoculating plates with media used to submerge the rice plants.

Screening for *Sub1A* gene presence

Genomic DNA of FR13A and AP stems was prepared using GenElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich, St Louis, MO, USA), following the manufacturer's protocol. The PCR reaction mixture was prepared in 20 µl total volume using Red Taq Master mix (Invitrogen, Carlsbad, CA, USA), 0.25 uM primers and 100 ng genomic DNA. PCR was performed using *Sub1A* genomic specific primers in accordance with Xu *et al.* (2006) (Table S1).

RNA extraction, cDNA synthesis and real-time reverse transcription PCR

Total RNA was extracted from rice stems, subjected to DNase treatment and reverse-transcribed to produce cDNA, as previously described (Lasanthi-Kudahettige *et al.* 2007). Transcript abundance was analysed by real-time reverse transcription PCR, using TaqMan probes (Table S2) and qPCR MasterMix Plus for SYBR® green I (Eurogentec, Liège, Belgium) with specifically designed primers (Table S3). The relative expression level of each gene was quantified as previously described (Lasanthi-Kudahettige *et al.* 2007), using glyceraldehyde-3-phosphate dehydrogenase (*Os08g03290*) as an internal reference.

Semi-quantitative RT-PCR analysis

One microgram of RNA, treated as previously indicated, was reverse-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. RT-PCR was performed using GoTaq® Green Master mix (Promega, Madison, WI, USA) in a reaction volume of 50 µl, containing 0.2 µM forward and reverse primers and 100 ng cDNA template. The sequence and conditions of *RAmy 3C*, *3D*, *3E* and *actin1* (used as control) were as described in Fukao *et al.* (2006), *RAmy 1A* was designed as described in Hwang *et al.* (1999) (Table S4). The resulting PCR products were visualised with ethidium bromide staining on 2% agarose gels after electrophoresis.

Assay of α-amylase activity

α-Amylase activity was determined in rice stems using the amylopectin azure method, as described in Magneschi *et al.* (2009). Total protein content was measured using the Biorad Protein Assay based with the Bradford (1976) method.

Extraction and determination of soluble sugars and starch

Samples (0.1–0.3 g fresh weight) were extracted as described by Tobias *et al.* (1992). Analyses of sucrose, fructose and glucose were carried out as previously described (Guglielminetti *et al.* 1995b). The starch-containing pellet was extracted using 10% KOH, the neutralised supernatant was treated with 2.5 units amyloglucosidase (from *Rhizopus niger*) for 3 h to release glucose. Starch was quantified on the basis of the glucose units released after amyloglucosidase treatment (Magneschi *et al.* 2009).

RESULTS

Screening of rice cultivars for sensitivity to complete submergence stress

We analysed the flooding response of five rice varieties (FR13A, IR22, Lamone, Nipponbare and AP) known to possess different ability to survive flooding or to elongate when submerged (Gibbs *et al.* 2000; Xu *et al.* 2006; Magneschi *et al.* 2009). AP had an unhealthy appearance after 14 days of complete submergence, and the plants collapsed when de-submerged. IR22, Lamone and Nipponbare did not survive beyond 10 days of submergence. Only FR13A, a *Sub1A*-harbouring variety (Xu *et al.* 2006), survived 14 days of submergence (Fig. 1A). When submerged, AP showed the highest elongation rates, whereas FR13A had the lowest (Fig. 1B). Even though IR22 displayed limited shoot elongation under flooding, comparable to FR13A, it did not survive flooding, thus suggesting that reduced shoot elongation does not necessarily lead to submergence tolerance. According to Gibbs *et al.* (2000), IR22 possesses insufficient glycolysis substrate supply to survive anoxia.

The expression of fermentation-related genes was analysed in rice stems. FR13A had the highest relative expression levels of alcohol dehydrogenase 2 (*ADH2*) and pyruvate decarboxylase 1 (*PDC1*), reaching a maximum after 3 days of flooding (Fig. 2). All the other varieties failed to accumulate these transcripts during submergence (Fig. 2). Lamone and AP, but not FR13A, displayed *ADH1* transcript accumulation (Fig. 2). AP had the highest *SUS1* expression after 3 days of complete submergence, followed by Lamone and Nipponbare after 7 days of flooding (Fig. 2). The expression of *SUS1* was negligible in FR13A and IR22 (Fig. 2).

AP and FR13A had opposite responses to submergence, in terms of survival, elongation rates and gene expression. These two varieties were therefore chosen for further analyses. Differences in shoot elongation were already visible after 3 days of submergence (Fig. 3A). The expression of *Sub1A-1* increased rapidly in submerged FR13A seedling stems but was not expressed in AP (Fig. 3B). PCR analysis of genomic DNA indicated that this gene is absent in AP (data not shown). In agreement with the known effects of *Sub1A-1* (Fukao *et al.* 2006; Xu *et al.* 2006), FR13A showed significantly higher *PDC1* and *ADH2* mRNA accumulation than AP, while the *ADH1* transcript accumulated in both stems of varieties during submergence (Fig. 3B). Differences in the

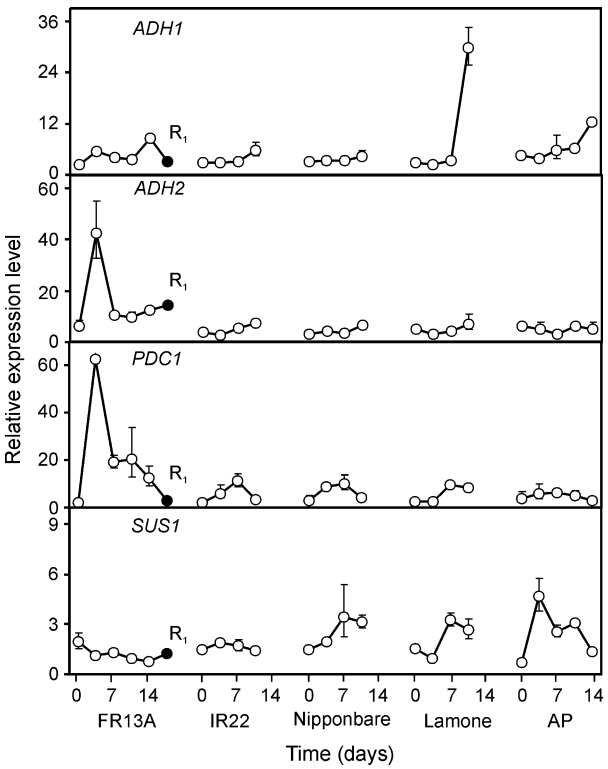

Fig. 2. Gene expression patterns of transcripts involved in fermentation and carbohydrate catabolism in the rice variety stems under complete submergence stress (empty circles) and after 1 day of recovery after de-submergence (filled circles R_1). The expression level was measured on the basis that Nipponbare control air = 1. Data are the mean of three replicates ± SD.

expression of *ADH* genes under O_2 deprivation are of interest and suggest that tissue-specific expression of *ADH* genes might play a role in the metabolic adaptation of rice plants to submergence (Xie & Wu 1989).

Surviving complete submergence requires available carbohydrates

Carbohydrate availability might affect rice ability to survive submergence. To verify this possibility we submerged FR13A and AP for 14 days in either mannitol (osmotic control) or sucrose. Recovery of plants was tested 7 days after de-submergence. AP submerged in a mannitol solution showed a high rate of shoot elongation, chlorotic leaves and high mortality (more than 75% of seedlings; Fig. 4). Of the AP seedlings, 20% had stunted growth with a low probability of producing healthy plants after recovery, and only 5% of these seedlings survived (Fig. 4). FR13A seedlings, on the other hand, were able to survive when submerged in mannitol (100% seedling survival; Fig. 4). When seedlings were submerged in sucrose solution, both FR13A and AP seedlings had less chlorosis, higher stem rigidity and reduced leaf death. Interestingly, AP also showed 100% survival when submerged in sucrose (Fig. 4), and resumed normal growth after de-submergence. No differences in elongation due to the carbohydrate treatment were noticed for AP (stem length: 22.6 ± 2.34 and 21.27 ± 2.30 cm, n = 20, for sucrose and

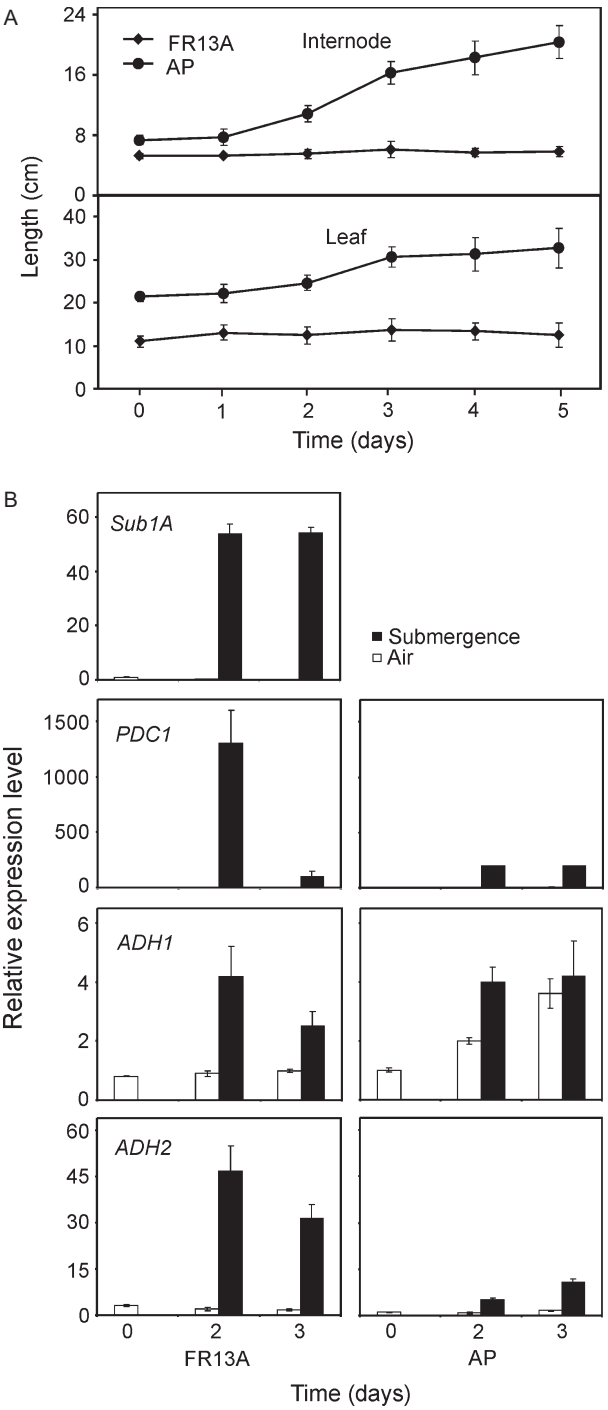

Fig. 3. FR13A and AP under complete submergence stress. Seven-day-old rice seedlings were grown in pots filled with sand (approximately 5-cm deep) and completely submerged in water, 1 m above the sand surface. Rice plants were grown in a growth chamber for 5 days after flooding, at 26 ± 2 °C in a photoperiod of 15-h light. A: Shoot elongation of rice varieties under complete submergence. Stem elongation and leaf elongation were taken from the base of the plant to the upper collar and from there to the edge of the longest leaf, respectively. Data are the means of 30 measurements ± SD. B: FR13A and AP stem gene expression patterns of *Sub1A* transcripts and genes involved in fermentation grown in air and in complete submergence stress. The expression level was measured based on AP control air at day 0 = 1. Data are the mean of three replicates ± SD.

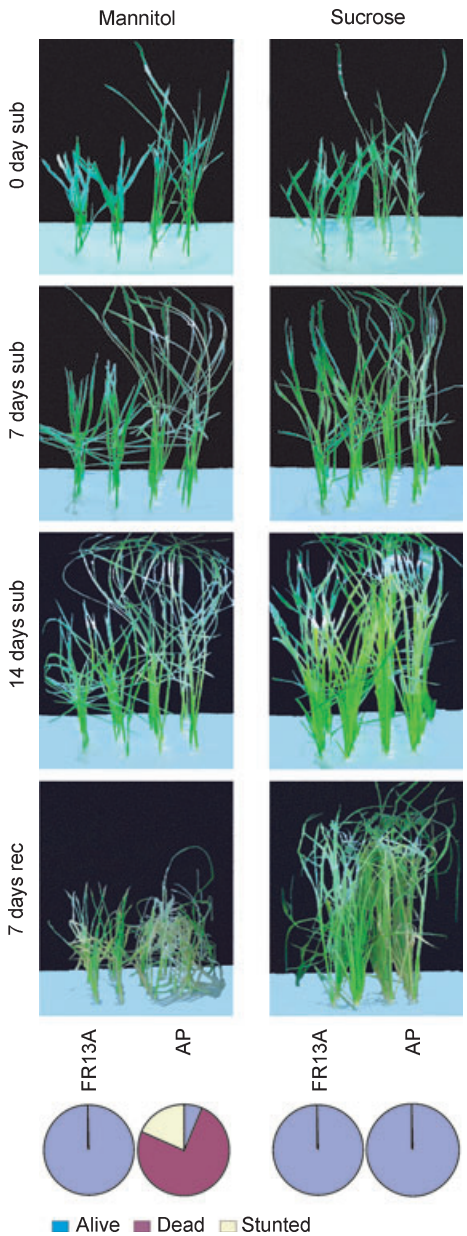

Fig. 4. FR13A and AP after 0, 7 and 14 days of complete submergence (sub) followed by 7 days of recovery (rec), by providing an external sugar source. Seedlings were grown in *in vitro* conditions and subjected to complete submersion in 50 mM sucrose or 50 mM mannitol (osmotic control). The pie charts indicate the viability of plants after 7 days of recovery (n = 20). Survival criteria correspond to re-growth capability after a recovery period.

mannitol, respectively). FR13A displayed 100% survival in both sucrose and mannitol, but showed a significant difference in elongation between the two treatments (stem length: 12.48 ± 1.28 and 6.90 ± 0.38 cm, n = 20, for sucrose and mannitol, respectively).

Carbohydrate metabolism in submerged rice plants

The metabolism of carbohydrates is strongly affected by the presence or absence of the *Sub1A* gene (Fukao *et al.* 2006).

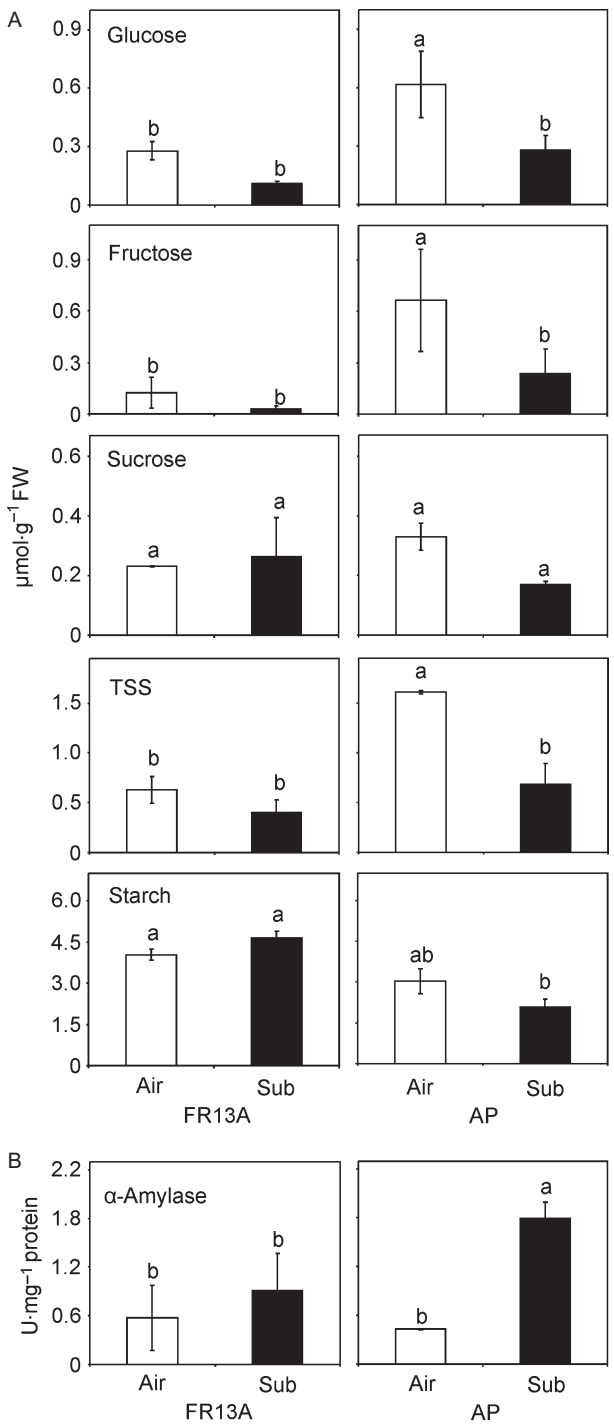

Fig. 5. Sugar availability in FR13A and AP stems. A: Total soluble sugars and starch concentration in stems of rice varieties grown in air and after 3 days of submergence stress. The starch content is expressed as glucose equivalents. Data are the means of three replicates ± SD. B: α-Amylase activity in stems of rice varieties grown in air and after 3 days of submergence stress. Data are the means of three replicates ± SD. Different letters indicate significant differences between treatments (0.05 significance level) based on an LSD multiple pair-wise comparison test.

AP showed constitutively higher total soluble sugars (TTS) when compared with FR13A (Fig. 5A). When submerged, however, the level of TTS dropped significantly only in AP

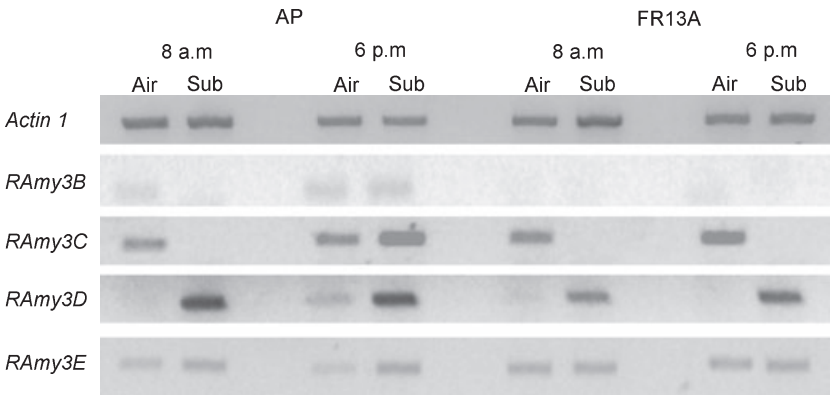

Fig. 6. Differential expression of AP and FR13A rice stem α-amylase genes under complete submergence stress analysed using RT-PCR at 08:00 h and 18:00 h, in air and after 3 days of complete submergence. *Actin1* was used as internal control. Data are from a representative experiment.

(Fig. 5A). Starch content was unaltered in submerged FR13A, while a slight reduction was observed in AP (Fig. 5A). In this context, α-amylase activity increased notably in AP during submergence (Fig. 5A).

In rice, α-amylases are encoded by a multigene family (Huang *et al.* 1990). We investigated the expression pattern of α-amylase genes in rice stems from aerobic and submerged plants. Expression of the *RAmy1A* gene was unaffected by submergence (data not shown), while an altered pattern of genes in the *RAmy3* sub-family was observed. Expression of *RAmy3B* was undetectable in FR13A, while the low mRNA level of this gene already present in AP under air was slightly induced in submerged AP at 18:00 h (Fig. 6). *RAmy3C* was always expressed in aerobic samples, but its expression declined under submergence, with the exception of AP (18:00 h; Fig. 6). *RAmy3D* displayed clear submergence-dependent expression (Fig. 6) in both AP and FR13A, although its induction was higher in AP. *RAmy3E* expression was slightly induced in submerged AP, whereas FR13A did not show any increase in *RAmy3E* mRNA level (Fig. 6).

RAmy3D is regulated by a complex, sugar starvation-dependent pathway (Lu *et al.* 2002, 2007; Lee *et al.* 2009). We therefore analysed the consequences of a starvation-inducing treatment (submergence in mannitol), compared with submergence in a sucrose solution, which prevents the submerged seedlings from starving. Sucrose supply in the water is likely to increase the CO_2 concentration due to increased respiration and, as a consequence, the submergence water O_2 level might rise due to increased photosynthesis (Setter *et al.* 1989; Waters *et al.* 1989). To test this possibility, we measured the O_2 concentration in the mannitol and sucrose solutions in which the plants were submerged. We did not find any significant difference between the treatments, but there was a reduction in the O_2 concentration from 7.5 ± 0.42 and 7.7 ± 0.35 $mg \cdot l^{-1}$ on 0 day, to 4.2 ± 0.78 and 4.1 ± 0.82 on 14 day ($n = 3$), for sucrose and mannitol, respectively. *Sub1A* expression in submerged FR13A was high in mannitol-submerged plants, however its induction was abolished in the sucrose-submerged plants (Fig. 7). The *Sub1A* gene was absent from AP. *ADH2* was induced by submergence in both varieties, although the response was higher in FR13A, however sucrose reduced its induction (Fig. 7). Notably, while submergence in mannitol elicited a marked induction of genes involved in the starvation-dependent *RAmy3D* pathway (*CIPK15*, *MYBS1*) in AP, this was not observed in FR13A (Fig. 7). Our analysis of genomic DNA indicated that *CIPK15* and *MYBS1* are present in FR13A (data not shown). *SnRK1A* expression was only slightly induced by submergence, both in mannitol and sucrose, in AP but not in FR13A (Fig. 7). Submergence in sucrose prevented or strongly reduced the induction of the *CIPK15–MYBS1–RAmy3D* signalling cascade in AP.

DISCUSSION

The molecular mechanisms that allow rice to germinate, grow and survive when submerged have remained elusive until recently, when the discovery of *Sub1A*, *SNORKEL1/SNORKEL2* and *CIPK15* genes revealed the molecular physiology of submerged rice (Xu *et al.* 2006; Hattori *et al.* 2009; Lee *et al.* 2009). Different rice groups activate distinct mechanisms to survive flooding. The *indica* group of rice comprises several cultivated lowland rice varieties that overcome submergence stress by minimal shoot elongation and a drastic reduction in most metabolic activities, thus activating a 'quiescence' strategy. *Sub1A-1* has been identified as the key determinant of submergence tolerance based on the 'quiescence' strategy. This is achieved by controlling the accumulation of transcripts involved in ethanolic fermentation and repression of genes associated with the carbohydrate catabolism (for a review see Perata & Voesenek 2007). Rice plants belonging to the *japonica* group do not possess the *Sub1A-1* and fail to survive prolonged submergence. Deepwater rice varieties, on the other hand, are characterised by the 'escape' strategy, based on rapid internode elongation promoted *via* GA during flooding (Voesenek & Bailey-Serres 2009), regulated by the two ERF genes *SNORKEL1* and *SNORKEL2* (Hattori *et al.* 2009).

Rice seed germination and early seedling growth under hypoxia rely instead on the ability to induce α-amylase under low O_2 conditions, thus allowing starch degradation and sugar availability in submerged seedlings (Perata *et al.* 1993). The CIPK15-dependent pathway was recently indentified as a key regulator of α-amylase abundance under limited O_2 (Lee *et al.* 2009). It is largely unknown whether Sub1A- and CIPK15-based tolerance mechanisms can co-exist. This would be illogical, since Sub1A-1 actually represses amylase activity, while CIPK15 activates the flooding-dependent *Ramy3D* gene.

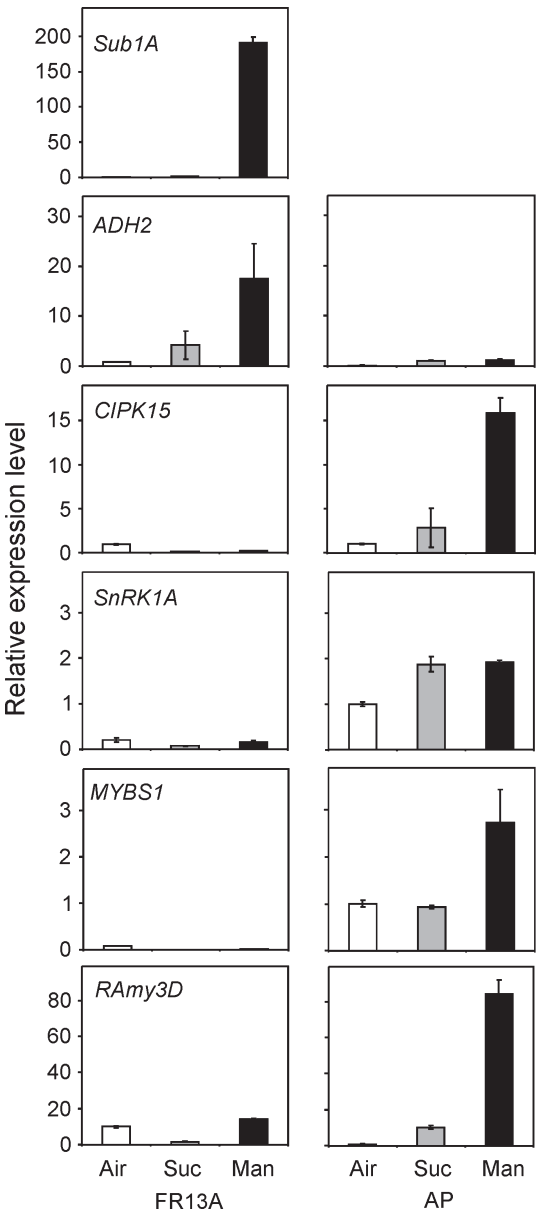

Fig. 7. Analysis of transcript levels of *Sub1A*, *ADH2*, *CIPK15*, *SNRK1*, *MYBS1* and *RAmy3D* genes in stems of both FR13A and AP grown in air and after 3 days of submergence, with sucrose 50 mM or mannitol 50 mM. The expression level was measured based on AP control air at day 0 = 1. Data are the mean of three replicates ± SD.

We analysed the responses of two rice varieties that differed in their ability to survive submergence because of presence or absence of the *Sub1A* gene. FR13A is a submergence-tolerant variety displaying the typical Sub1A response (limited elongation when submerged). AP, on the other hand, was selected because of its fast-elongating phenotype under submergence in the absence of *Sub1A* (Fig. 1A and B). When submerged, FR13A showed the highest accumulation of *ADH2* and *PDC1* transcripts (Fig. 2). Both ADH and PDC play important roles in plant adaptation to low O_2 conditions (Agarwal & Grover 2006). The high *ADH1* expression observed in Lamone and AP, on the other hand, did not correlate with survival.

Long-term submersion may cause extensive carbohydrate consumption leading to energy starvation (Jackson & Ram 2003). The amount of carbohydrate reserves has been positively correlated with the level of submergence tolerance (Setter & Laureles 1996; Greenway & Gibbs 2003; Jackson & Ram 2003; Das *et al.* 2005). Previous results on submergence-induced rapid internode elongation in deep-water rice showed the mobilisation of starch from internodes accompanied by enhancement of amylolytic activity (Raskin & Kende 1984).

Although AP had a higher abundance of soluble sugars when compared to FR13A (Fig. 5A), a significant reduction in TSS together with a reduction in starch content was observed after submergence only in AP. A drop in starch and carbohydrate content was observed by Fukao *et al.* (2006) in leaves of the intolerant line M202 when compared to the tolerant M202 (Sub1A). The reduced α-amylase and sucrose synthase (SUS) mRNA accumulation suggests that the preservation of carbohydrate content in *Sub1A*-containing varieties is related to the lower activity of starch and sucrose degrading enzymes (Fukao *et al.* 2006).

We showed that tolerance to submergence was greatly enhanced by exogenous sucrose, even in a submergence-intolerant variety such as AP (Fig. 4). Sucrose improved the elongation of FR13A, bypassing the suppressive role played by the presence of the *Sub1A* gene. Interestingly, while FR13A displayed clear induction of both *Sub1A* and *ADH2* when submerged in mannitol, a reduction of these mRNAs was observed when sucrose solution was used to submerge the seedlings (Fig. 7). These results suggest that there might be an interaction between the Sub1A–dependent pathway and sugar signalling.

Sub1A-1 down-regulates α-amylases (Fukao *et al.* 2006) and it is also known that regulation of the major anaerobic amylase (*RAmy3D*) is independent of hormonal control (Mitsui & Itoh 1997) but relies on a starvation-sensing mechanism (Loreti *et al.* 2003a). We showed that in FR13A, *Ramy3D* is repressed by a mechanism that acts upstream of *CIPK15* and *MYBS1*, which are both positive regulators of *RAmy3D* (Lee *et al.* 2009). In the case of *SnRK1A* expression, a component upstream of *MYBS1*, we did not observe any differences between the treatments (Fig. 7). This was expected, since the regulation of SnRK1A is at a post-transcriptional level (Lu *et al.* 2007). The MYBS1 transcription factor is known to mediate sugar regulation of α-amylase gene expression (Lu *et al.* 2002). It has high transactivation ability to activate TATCCA containing a *cis*-element, present in the *RAmy3D* promoter in two tandem repeats (Lu *et al.* 1998). Although it would be logical to speculate on the absence of a starvation signal in FR13A, which, thanks to *Sub1A*, retains an adequate level of carbohydrates and prevents activation of the *CIPK15/MYBS1/Ramy3D* cascade, this hypothesis is not entirely satisfactory. In AP, in fact, *CIPK15*, *SnRK1A*, *MYBS1* and *RAmy3D* are expressed, although at very low levels, even in the sucrose-submerged seedlings (Fig. 7).

We conclude that sucrose availability modulates the expression of *Sub1A* and of its downstream gene *ADH2*, also leading to a high elongation rate in FR13A plants when submergence is not associated with sugar starvation. In addition, *RAmy3D* is up-regulated by starvation in AP but not in

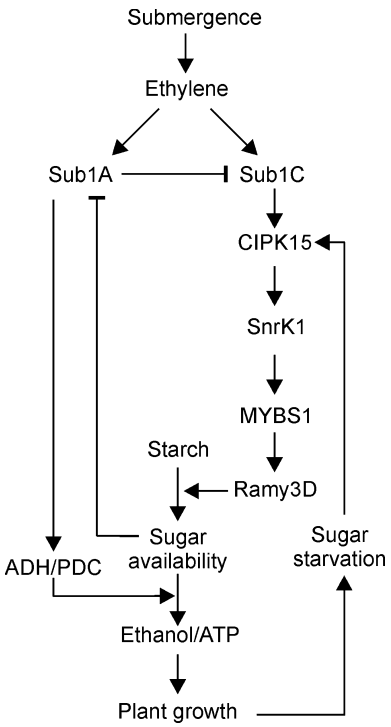

Fig. 8. Proposed model for *Sub1A* and *CIPK15* pathway cross-talk in the response to submergence of established plants. Submergence promotes ethylene production and accumulation within the plant resulting in activation of the *Sub1A* and *Sub1C* genes (Fukao *et al.* 2006). *Sub1A* limits ethylene accumulation through a feedback mechanism and represses *Sub1C* transcript accumulation. *Sub1C* controls the *Ramy3D* gene (Fukao *et al.* 2006), likely through induction of the *CIPK15* pathway, described as a *Ramy3D* regulator in rice seedlings under flooding (Lee *et al.* 2009). The presence of the *Sub1A* gene leads to repression of the *Sub1C–CIPK15–Ramy3D* pathway, resulting in limited growth under submergence. When sugar is available in excess, it represses the *Sub1A* gene, activating growth, even under submergence. In the absence of *Sub1A*, plant growth can lead to subsequent starvation, which further promotes the *CIPK15* pathway to activate *Ramy3D* and promote starch degradation.

FR13A, suggesting cross-talk between the Sub1A and CIPK15 pathways (Fig. 8). Further work is required to understand how sugar sensing and signalling interferes with Sub1A-1, and the possible negative interplay between the Sub1A-1 and CIPK15 pathways.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequences of primers used for *Sub1A* gene presence screening.

Table S2. Sequences of TaqMan primers and probes used for quantitative (real-time) PCR analysis.

Table S3. Sequences of SYBR green primers used for quantitative (real-time) PCR analysis.

Table S4. Sequences of primers used for semi-quantitative RT-PCR analysis.

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REFERENCES

Agarwal S., Grover A. (2006) Molecular biology, biotechnology and genomics of flooding-associated low O_2 stress response in plants. *Critical Reviews in Plant Sciences*, **25**, 1–21.

Alpi A., Beevers H. (1983) Effects of O_2 concentration on rice seedlings. *Plant Physiology*, **71**, 30–34.

Bailey-Serres J., Voesenek L.A.C.J. (2008) Flooding stress: acclimation and genetic diversity. *Annual Review of Plant Biology*, **59**, 313–339.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, **72**, 248–254.

Colmer T.D., Voesenek L.A.C.J. (2009) Flooding tolerance: suites of plant traits in variable environments. *Functional Plant Biology*, **36**, 665–681.

Counce P.A., Keisling T.C., Mitchell A.J. (2000) A uniform, objective, and adaptive system for expressing rice development. *Crop Science*, **40**, 436–443.

Das K.K., Sarkar R.K., Ismail A.M. (2005) Elongation ability and non-structural carbohydrate levels in relation to submergence tolerance in rice. *Plant Science*, **168**, 131–136.

Dunn G. (1974) A model for starch breakdown in higher plants. *Phytochemistry*, **13**, 1341–1346.

Ellis M.H., Setter T.L. (1999) Hypoxia induces anoxia tolerance in completely submerged rice seedlings. *Journal of Plant Physiology*, **154**, 219–230.

Fukao T., Xu K., Ronald P.C., Bailey-Serres J. (2006) A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. *Plant Cell*, **18**, 2021–2034.

Gibbs J., Morrell S., Valdez A., Setter T.L., Greenway H. (2000) Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. *Journal of Experimental Botany*, **51**, 785–796.

Greenway H., Gibbs J. (2003) Mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. *Functional Plant Biology*, **30**, 999–1036.

Guglielminetti L., Yamaguchi J., Perata P., Alpi A. (1995a) Amylolytic activities in cereal seeds under aerobic and anaerobic conditions. *Plant Physiology*, **109**, 1069–1076.

Guglielminetti L., Perata P., Alpi A. (1995b) Effect of anoxia on carbohydrate metabolism in rice seedlings. *Plant Physiology*, **108**, 735–741.

Hattori Y., Nagai K., Furukawa S., Song X.J., Kawano R., Sakakibara H., Wu J., Matsumoto T., Yoshimura A., Kitano H., Matsuoka M., Mori H., Ashikari M. (2009) The ethylene

response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water. *Nature*, **460**, 1026–1030.

Huang N., Sutliff T.D., Litts J.C., Rodriguez R.L. (1990) Classification and characterization of the rice α-amylase multigene family. *Plant Molecular Biology*, **14**, 655–668.

Hwang Y.S., Thomas B.R., Rodriguez R.L. (1999) Differential expression of rice α-amylase genes during seedling development under anoxia. *Plant Molecular Biology*, **40**, 911–920.

Jackson M.B., Ram P.C. (2003) Physiological and molecular basis of susceptibility and tolerance of rice plants to complete submergence. *Annals of Botany*, **91**, 227–241.

Lasanthi-Kudahettige R., Magneschi L., Loreti E., Gonzali S., Licausi F., Novi G., Beretta O., Vitulli F., Alpi A., Perata P. (2007) Transcript profiling of the anoxic rice coleoptile. *Plant Physiology*, **144**, 218–231.

Lee S.C., Lan W.Z., Kim B.G., Li L., Cheong Y.H., Pandey G.K., Lu G., Buchanan B.B., Luan S. (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. *Proceedings of the National Academy of Sciences USA*, **104**, 15959–15964.

Lee K.W., Chen P.W., Lu C.A., Chen S., Ho T.H.D., Yu S.M. (2009) Coordinated responses to oxygen and sugar deficiency allow rice seedlings to tolerate flooding. *Science Signaling*, **2**, ra61.

Loreti E., Yamaguchi J., Alpi A., Perata P. (2003a) Sugar modulation of alpha-amylase genes under anoxia. *Annals of Botany*, **91**, 143–148.

Loreti E., Yamaguchi J., Alpi A., Perata P. (2003b) Gibberellins are not required for rice germination under anoxia. *Plant and Soil*, **253**, 137–143.

Lu C.A., Lim E.K., Yu S.M. (1998) Sugar response sequence in the promoter of a rice α-amylase gene serves as a transcriptional enhancer. *The Journal of Biological Chemistry*, **273**, 10120–10131.

Lu C.A., Ho T.H.D., Ho S.L., Yu S.M. (2002) Three novel MYB proteins with one DNA binding repeat mediate sugar and hormone regulation of α-amylase gene expression. *Plant Cell*, **14**, 1963–1980.

Lu C.A., Lin C.C., Lee K.W., Chen J.L., Huang L.F., Ho S.L., Liu H.J., Hsing Y.I., Yu S.M. (2007) The SnRK1A protein kinase plays a key role in sugar signaling during germination and seedling growth of rice. *Plant Cell*, **19**, 2484–2499.

Magneschi L., Perata P. (2009) Rice germination and seedling growth in the absence of oxygen. *Annals of Botany*, **103**, 181–196.

Magneschi L., Kudahettige R.L., Alpi A., Perata P. (2009) Comparative analysis of anoxic coleoptile elongation in rice varieties: relationship between coleoptile length and carbohydrate levels, fermentative metabolism and anaerobic gene expression. *Plant Biology*, **11**, 561–573.

Mitsui T., Itoh K. (1997) The α-amylase multigene family. *Trends in Plant Science*, **2**, 255–261.

Nagai K., Hattori Y., Ashikari M. (2010) Stunt or elongate? Two opposite strategies by which rice adapts to floods. *Journal of Plant Research*, **123**, 303–309.

Perata P., Alpi A. (1993) Plant response to anaerobiosis. *Plant Science*, **93**, 1–17.

Perata P., Voesenek L.A.C.J. (2007) Submergence tolerance in rice requires *Sub1A*, an ethylene-response-factor-like gene. *Trends in Plant Science*, **12**, 43–46.

Perata P., Pozueta-Romero J., Akazawa T., Yamaguchi J. (1992) Effect of anoxia on starch breakdown in rice and wheat seeds. *Planta*, **188**, 611–618.

Perata P., Geshi N., Yamaguchi J., Akazawa T. (1993) Effect of anoxia on the induction of alpha-amylase in cereal seeds. *Planta*, **191**, 402–408.

Perata P., Guglielminetti L., Alpi A. (1996) Anaerobic carbohydrate metabolism in wheat and barley, two anoxia-intolerant cereal seeds. *Journal of Experimental Botany*, **47**, 999–1006.

Raskin I., Kende H. (1984) Effect of submergence on translocation, starch content and amylolytic activity in deep-water rice. *Planta*, **162**, 556–559.

Setter T.L., Laureles E.V. (1996) The beneficial effect of reduced elongation growth on submergence tolerance of rice. *Journal of Experimental Botany*, **47**, 1551–1559.

Setter T.L., Waters I., Wallace I., Bhekasut P., Greenway H. (1989) Submergence of rice. I Growth and photosynthetic response to CO_2 enrichment of flood water. *Australian Journal of Plant Physiology*, **16**, 251–263.

Sun Z.T., Henson C.A. (1991) A quantitative assessment of the importance of barley seed alpha-amylase, beta-amylase, debranching enzyme, and alpha-glucosidase in starch degradation. *Archives of Biochemistry and Biophysics*, **284**, 298–305.

Tobias R.B., Boyer C.D., Shannon J.C. (1992) Alterations in carbohydrate intermediates in the endosperm of starch-deficient maize (*Zea mays* L) genotypes. *Plant Physiology*, **99**, 146–152.

Voesenek L.A.C.J., Bailey-Serres J. (2009) Genetics of high-rise rice. *Nature*, **460**, 959–960.

Waters I., Armstrong W., Thompson C.J., Setter T.L., Adkins S., Gibbs J., Greenway H. (1989) Diurnal changes in radial oxygen loss and ethanol metabolism in roots of submerged and non-submerged rice seedlings. *New Phytologist*, **113**, 439–451.

Xie Y., Wu R. (1989) Rice alcohol dehydrogenase genes: anaerobic induction, organ specific expression and characterization of cDNA clones. *Plant Molecular Biology*, **13**, 53–68.

Xu K., Xu X., Fukao T., Canlas P., Maghirang-Rodriguez R., Heuer S., Ismail A.M., Bailey-Serres J., Ronald P.C., Mackill D.J. (2006) *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature*, **442**, 705–708.

Yu S.M. (1999) Cellular and genetic responses of plants to sugar starvation. *Plant Physiology*, **121**, 687–693.