

Distinct mechanisms for aerenchyma formation in leaf sheaths of rice genotypes displaying a quiescence or escape strategy for flooding tolerance

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- **Background and Aims** Rice is one of the few crops able to withstand periods of partial or even complete submergence. One of the adaptive traits of rice is the constitutive presence and further development of aerenchyma which enables oxygen to be transported to submerged organs. The development of lysigenous aerenchyma is promoted by ethylene accumulating within the submerged plant tissues, although other signalling mechanisms may also co-exist. In this study, aerenchyma development was analysed in two rice (*Oryza sativa*) varieties, 'FR13A' and 'Arborio Precoce', which show opposite traits in flooding response in terms of internode elongation and survival.
- **Methods** The growth and survival of rice varieties under submergence was investigated in the leaf sheath of 'FR13A' and 'Arborio Precoce'. The possible involvement of ethylene and reactive oxygen species (ROS) was evaluated in relation to aerenchyma formation. Cell viability and DNA fragmentation were determined by FDA/FM4-64 staining and TUNEL assay, respectively. Ethylene production was monitored by gas chromatography and by analysing *ACO* gene expression. ROS production was measured by using Amplex Red assay kit and the fluorescent dye DCFH₂-DA. The expression of *APX1* was also evaluated. AVG and DPI solutions were used to test the effect of inhibiting ethylene biosynthesis and ROS production, respectively.
- **Key Results** Both the varieties displayed constitutive lysigenous aerenchyma formation, which was further enhanced when submerged. 'Arborio Precoce', which is characterized by fast elongation when submerged, showed active ethylene biosynthetic machinery associated with increased aerenchymatous areas. 'FR13A', which harbours the *Sub1A* gene that limits growth during oxygen deprivation, did not show any increase in ethylene production after submersion but still displayed increased aerenchyma. Hydrogen peroxide levels increased in 'FR13A' but not in 'Arborio Precoce'.
- **Conclusions** While ethylene controls aerenchyma formation in the fast-elongating 'Arborio Precoce' variety, in 'FR13A' ROS accumulation plays an important role.

Key words: *Oryza sativa*, rice, submergence, *Sub1A*, *ACO* expression, lysigenous aerenchyma, ethylene, reactive oxygen species, ROS.

INTRODUCTION

Flooding events of various depths and durations are the principal environmental cause of a shortage of oxygen (O₂) in plants. Rice is one of the few crops able to withstand periods of partial or even complete submergence (Colmer and Voesenek, 2009; Licausi and Perata, 2009; Nagai *et al.*, 2010). One of the adaptive traits of rice is the constitutive presence of aerenchyma which enables O₂ to be transported to the submerged organs (Armstrong, 1971; Kawase and Whitmoyer, 1980). Although this tissue is present even in well-drained conditions, it develops further when the soil becomes waterlogged (Armstrong, 1971; Pradhan *et al.*, 1973; Das and Jat, 1977). In rice, aerenchymatous areas are formed by cell lysis and seem to be co-ordinated by a programmed cell death (PCD) mechanism (Kawai *et al.*, 1998). In mature leaf sheaths of rice, lysigenous aerenchyma is linked to stomata, allowing gases to flow from leaves to the basal part of the plants (Matsukura *et al.*, 2000), and it is interrupted by septa

composed of stellate cells that have large intercellular spaces and which thus allow the passage of O₂ (Hoshikawa, 1989).

The mechanisms responsible for aerenchyma formation have not yet been fully elucidated (Shiono *et al.*, 2008); however, it is known that this process involves ethylene, which accumulates in submerged organs (Kavase, 1972, 1978; Könings and Jackson, 1979; Justin and Armstrong, 1991; He *et al.*, 1996; Zhou *et al.*, 2002; Geisler-Lee *et al.*, 2010; Lenchova *et al.*, 2009). In hypoxic roots of maize, exogenous ethylene applications induce the aerenchymatous areas and ethylene inhibitors repress their formation (Drew *et al.*, 1981; Jackson *et al.*, 1985; Könings, 1982). In addition, both 1-aminocyclopropane-1-carboxylate synthase (ACC) synthase activity and ACC concentrations are high in hypoxic maize roots (Atwell *et al.*, 1988; He *et al.*, 1994; Geisler-Lee *et al.*, 2010).

Aerenchyma formation, however, does not always require ethylene, as is described for lysigenous aerenchyma formation in the root of the wetland plant *Juncus effusus* (Visser and Bögemann, 2006).

Although the formation of maize root aerenchyma under waterlogging and hypoxia is stimulated by enhanced ethylene biosynthesis and increased endogenous ethylene concentration (Drew *et al.*, 1981; Könings, 1982; Jackson *et al.*, 1985; Atwell *et al.*, 1988; He *et al.*, 1994; Geisler-Lee *et al.*, 2010), this is not the case when aerenchyma formation is induced by nutrient-starvation (Drew *et al.*, 1989; He *et al.*, 1992). While temporary deprivation of N or P greatly enhances the sensitivity of ethylene-responsive cells of the maize root cortex leading to cell lysis and aerenchyma formation (He *et al.*, 1992, 1994), sulfate deprivation shows altered levels of reactive oxygen species (ROS) in the aerenchymatous areas (Bouranis *et al.*, 2003, 2006), suggesting a role for ROS in inducing aerenchyma formation.

In rice, early studies reported that aerenchyma formation in adventitious roots was not controlled by ethylene (Jackson *et al.*, 1985). Ethylene was later shown to have a role in promoting aerenchyma, but differences were found in cultivar responses (Justin and Armstrong, 1991). Recently, a study by Steffens *et al.* (2011) on aerenchyma formation in rice stems in response to ethylene and hydrogen peroxide (H_2O_2) showed that both compounds promote its formation in a dose-dependent manner. The production of lysigenous aerenchyma in *Arabidopsis thaliana* under hypoxia requires both ethylene and H_2O_2 signalling (Mühlenbock *et al.*, 2007). ROS have been proposed to be the central component of a plant's adaptation to both biotic and abiotic stresses, by exacerbating oxidative damage and by acting as signal molecules in protective responses (Mittler *et al.*, 2004). ROS signalling is involved in the metabolic adaptation to low O_2 (Baxter-Burrell *et al.*, 2002) and also acts as a trigger for the induction of *HsfA2* transcription factors, thus conferring anoxia tolerance (Banti *et al.*, 2010).

The ability to grow when submerged is another trait which may help to allow the efficient transport of O_2 to the submerged organs. The formation of aerenchyma is crucial for submerged organs, but if the growth of the plant is unable to compete with rises in water levels, the plant dies. In rice, the type of growth response to submergence strongly depends on the genotype; rice varieties containing the *Sub1A* gene adopt a quiescence strategy with growth restriction, while other varieties grow very rapidly, to attempt to maintain at least the leaf tips above the water level (Bailey-Serres and Voeselek, 2008; Colmer and Voeselek, 2009; Bailey-Serres *et al.*, 2010; Nagai *et al.*, 2010). Extraordinary internode elongation induced under deep water via gibberellin is observed in deepwater rice varieties, and is triggered by the ethylene responsive factors SNORKEL (SK) 1 and 2 (Hattori *et al.*, 2009).

Sub1A is an ethylene responsive factor that is induced by ethylene signalling under submergence (Fukao *et al.*, 2006; Xu *et al.*, 2006). *Sub1A* represses further ethylene synthesis (Bailey-Serres and Voeselek, 2010), and may hamper aerenchyma formation in *Sub1A* varieties. *Sub1A* varieties do not grow in response to submergence. This would not make aerenchyma important in deep flooding to have access to O_2 above the water level, but rather to rely on O_2 produced and stored during photosynthesis in the underwater organs, or retained by the leaf surface gas film (Colmer and Pedersen, 2008; Pedersen *et al.*, 2009). However, under shallow submergence it is unclear how to reconcile the *Sub1A*-dependent inhibition of ethylene synthesis with the formation of aerenchyma.

In this paper, aerenchyma formation was examined in 'FR13A', a *Sub1A* variety, and in 'Arborio Precoce' ('AP'), a non-*Sub1A* variety displaying fast shoot elongation when submerged. The results showed the presence of constitutive aerenchyma in both 'AP' and 'FR13A' varieties, which further increased under submergence. Submergence-induced ethylene synthesis was observed in 'AP' only, 'FR13A' did not show any increase in ethylene production. The results suggest that aerenchyma formation in 'FR13A' is independent of ethylene signalling, and ROS appear to be important to regulate aerenchyma formation in this *Sub1A* variety.

MATERIALS AND METHODS

Plant material and submergence treatment

Oryza sativa seeds of the varieties 'FR13A' and 'AP' were water-soaked in Petri dishes for 3 d ($28 \pm 2^\circ\text{C}$, dark conditions). Germinated seedlings were grown in 50-mL plastic pots filled with sand and transferred to a growth chamber for 7 d ($26 \pm 2^\circ\text{C}$, 15-h light photoperiod; PAR approx. $50 \mu\text{mol m}^{-2} \text{s}^{-2}$ provided by white fluorescence lamps). The following complete nutrient solution was used: $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (4.5 mM), MgSO_4 (0.8 mM), KH_2PO_4 (2.6 mM), KNO_3 (13.5 mM), K_2SO_4 (0.2 mM) and Chelamix (30 mg L^{-1} ; Valagro, Chieti, Italy). Submergence treatments were carried out for up to 21 d, as detailed in Fig. 1 ($26 \pm 2^\circ\text{C}$, 15-h light photoperiod; PAR approx. $50 \mu\text{mol m}^{-2} \text{s}^{-2}$).

Fresh leaf sheath samples were harvested after 2 and 3 d of complete submergence to observe sampling and microscopic events following the treatment. For each time-point, three biological repetitions, each consisting of a composite sample from three different plant leaf sheaths, were immediately processed or stored at -80°C for further molecular and biochemical analysis. Other analyses were repeated as described in figure legends.

Aerenchyma observation and quantification

Transverse sections (80 μm) of leaf sheaths, were prepared using samples from rice plants that had been flooded for 3 d as well as from control plants using a vibratory microtome (Vibratome 1000 Plus; Vibratome, St Louis, MO, USA). Sections were observed for aerenchyma formation and photographed with a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan). The percentage of aerenchyma was determined using the Imag-pro Plus version 6.2 (Media Cybernetics, Bethesda, MD, USA) and was calculated on total tissue cross-sectional area.

Viability staining

The cell viability of rice leaf sheath sections was determined by staining with fluorescein diacetate (FDA) ($2 \mu\text{g mL}^{-1}$ in phosphate buffer saline; Sigma-Aldrich, St Louis, MO, USA) for 15 min followed by FM4-64 (20 μM in phosphate buffer saline; Molecular Probes, Carlsbad, CA, USA) for 3 min (Fath *et al.*, 2001). The sections were examined using a Nikon Eclipse Ti-S microscope (Nikon) equipped with EGFP (λ_{ex} 450–490, dichroic 495, λ_{em} 500–550 nm) and

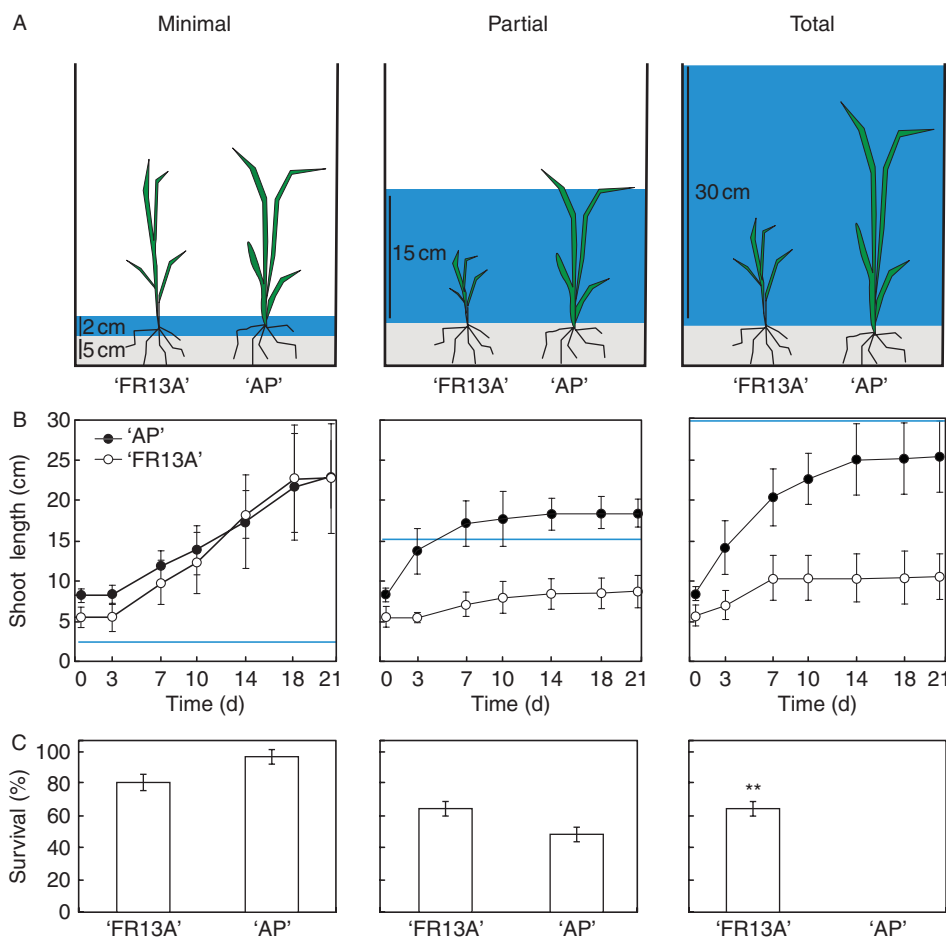


FIG. 1. 'Arborio Precoce' ('AP') and 'FR13A' plants under submergence. (A) Diagram showing how the rice varieties were submerged in water. One-week-old rice seedlings were grown in pots and flooded with water, 2 cm (minimal), 15 cm (partial) or 30 cm (total) above soil surface. The drawing depicts the plants at the end of the experiment. (B) Shoot length of the rice plants under different submergence conditions. The blue lines indicate the water level. Data are expressed as mean \pm s.d., $n = 12$. (C) Percentage of plant survival after 21 d of submergence followed by 7 d of recovery under well-drained conditions. Data are expressed as mean \pm s.e., $n = 12$; **, $P < 0.01$ according to Student's t -test.

TRITC (λ_{ex} 505–565, dichroic 550, λ_{em} 580–630 nm) filter blocks for FDA (λ_{ex} 488, λ_{em} 502–540 nm) and FM4-64 (λ_{ex} 515, λ_{em} 625 nm) signals, respectively. Images were captured by a QICAM digital CCD camera (QImaging, Surrey, BC, Canada).

In situ detection of DNA fragmentation (TUNEL assay)

'FR13A' and 'AP' leaf sheath from 3-d flooded and control plants were fixed in 4% (w/v) paraformaldehyde in a phosphate buffer saline (pH 7.4). After dehydration through an ethanol series, samples were embedded in Paraplast Plus (Paraplast, Sherwood Medical Industries, St Louis, MO, USA). Sections (10 μ m) were cut and stretched onto poly-lysine-coated slides. The sections were then dewaxed in xylene and rehydrated before examination. A TUNEL assay was performed using the 'In situ cell death detection kit' (Promega, Madison, WI, USA), according to the manufacturer's instructions. To facilitate the introduction of the TdT enzyme into the tissue sections, the slides were treated with proteinase K (20 mg mL⁻¹) for 20 min. The labelling reaction was performed at 37 °C in a humidified

chamber in the dark for 1 h. A negative control was included in each experiment by omitting TdT from the reaction mixture. As a positive control, permeabilized sections were incubated with DNase I (10 U mL⁻¹) for 10 min before the TUNEL assay. The yellow-green fluorescence of incorporated fluorescein-12-dUTP was examined using the microscope equipment previously described, using filter blocks for EGFP (λ_{ex} 450–490, dichroic 495, λ_{em} 500–550 nm). Experiments were repeated three times and each time, five slides were labelled for both the control and treated plants. A counter stain was done with DAPI (1 mg mL⁻¹).

Ethylene experiments

Ethylene production was measured by enclosing samples in airtight glass containers (30 mL). Each sample consisted of three rice leaf sheaths picked from separate plants submerged for 3 d before performing ethylene measurements. Gas samples (2 mL each) were taken from the headspace of the containers with a hypodermic syringe after 1 h incubation at room temperature. The ethylene concentration in the sample

was measured by gas chromatography (HP5890; Hewlett-Packard, Menlo Park, CA, USA) using a flame ionization detector, a stainless steel column (150 × 0.4 cm diameter packed with Hysep T), column and detector temperatures of 70 °C and 350 °C, respectively, and nitrogen carrier gas at a flow rate of 30 mL min⁻¹. Quantification was performed against an external standard and results were expressed on a fresh weight basis (nL g⁻¹ f. wt h⁻¹).

For the experiments designed to test how inhibiting ethylene biosynthesis affects aerenchyma formation, rice leaf sheaths were brushed with 500 μM aminoethoxyvinylglycine (AVG) (Fluka, Sigma-Aldrich, St Louis, MO, USA) in an aqueous solution every 24 h for 3 d before and during the water submersion.

ROS experiments

Leaf sheaths excised from control and 3-d flooded plants were used to measure H₂O₂ production using the Amplex Red H₂O₂/peroxidase assay kit (Molecular Probes) according to the manufacturer's instructions. Fifty milligrams of frozen ground tissue was used for the extraction, and was mixed with 200 μL of 20 mM sodium phosphate buffer (pH 6.5), according to the protocol developed by the Schachtman Laboratory (Shin and Schachtman, 2004). After centrifugation at 9000 g for 10 min (4 °C), 50 μL of supernatant were used for the Amplex Red assay.

ROS visualization was performed on 'FR13A' and 'AP' leaf sheath sections using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA; Sigma-Aldrich) (λ_{ex} 488, λ_{em} 525 nm). Sections were obtained and immediately immersed in 10 mM DCFH₂-DA in 10 mM Tris-KCl buffer (pH 7.4) in dark conditions for 30 min, followed by a 15-min washing step with a buffer. Negative controls were only incubated with the buffer. Imaging was performed with the microscope equipment previously described using filter blocks for EGFP (λ_{ex} 450–490, dichroic 495, λ_{em} 500–550 nm).

For the experiments designed to test how inhibiting ROS production affects aerenchyma formation, 10 μM diphenyleneiodonium (DPI) (Sigma-Aldrich) in an aqueous solution (3% DMSO) was injected in the central cavity of the leaf sheaths of rice every 24 h for 3 d before and during the water submersion.

Molecular analysis

Genomic DNA of 'FR13A' and 'AP' leaf sheaths was prepared using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich), 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 mM primers and 100 ng genomic DNA. PCR was performed using *Sub1A*, *SK1* and *SK2* specific primers according to Fukao *et al.* (2006) and Hattori *et al.* (2009; Table S1 in Supplementary data, available online).

For gene expression analysis, total RNA was extracted using a RNAqueous kit (Applied Biosystems/Ambion, Foster City, CA, USA), according to the manufacturer's instructions, and subjected to DNase treatment using TURBO DNA-free kit (Ambion). Five micrograms of RNA were reverse-transcribed

using the High-Capacity cDNA Archive kit (Applied Biosystems). Transcript abundance was analysed by real-time reverse transcription PCR, using qPCR MasterMix Plus for SYBR® green I (Eurogentec, Liège, Belgium) with specifically designed primers (Table S1 in Supplementary data), using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The relative expression level of each gene was quantified with the comparative threshold cycle method, as described in the ABI PRISM 7000 Sequence Detection System User Bulletin No 2 (Applied Biosystems), using rice glyceraldehyde-3-phosphate dehydrogenase as internal reference. PCR reactions for each of the three biological replicates were performed in duplicate.

RESULTS

'AP' and 'FR13A' showed a different sensitivity to partial and total submergence

Sub1A varieties such as 'FR13A' can tolerate complete, short duration submergence thanks to their growth restriction strategy. The rapid growth of submerged plants can, however, be an advantage when growth is fast enough to allow the plant to reach the water surface and transport air to the submerged organs through aerenchyma. 'FR13A' and 'AP' differ greatly in their growth responses to submergence and we therefore tested whether their tolerance to submergence changed depending on the water level used to submerge the plants (Fig. 1A).

When plants were submerged with only 2 cm of water, most of the aerial parts of the plants were aerobic and both varieties survived well (Fig. 1B). However, 'FR13A' showed a percentage of survival lower than 'AP' (approx. 20%), due probably to the sensitivity of this variety to stagnant water (Datta and Banerji, 1973). Submergence in 15 cm of water implies that both 'AP' and 'FR13A' were completely submerged at time 0. In this case, while 'FR13A' elongation was not sufficient for emergence, rapid growth allowed 'AP' plants to emerge between day 3 and day 7 of submergence. This variety did not show amplification with *SK1* and *SK2* primers (data not shown), suggesting the absence of the genes as already described for other *japonica* rice species (Hattori *et al.*, 2009). The growth of 'AP' was very rapid when submerged with 15 cm of water and slowed down after the leaf tip of 'AP' had reached the water surface (Fig. 1B). Both varieties suffered from submergence; however, approx. 50% of plants survived, without any significant differences between the two varieties (Fig. 1C). When submerged under 30 cm water, 'AP' plants grew longer than plants submerged under 15 cm of water, but growth was not enough to reach the water surface (Fig. 1B). 'FR13A' growth was, as expected (Xu *et al.*, 2006), minimal (Fig. 1B), but survival was good (Fig. 1C), whereas 'AP' plants were all dead by the end of the treatment (Fig. 1C).

'AP' and 'FR13A' leaf sheath aerenchyma formation under complete submergence

'AP' leaf sheaths showed more constitutive aerenchyma than 'FR13A' (Fig. 2A, B; Air), that increased following submergence in both 'AP' and 'FR13A' (Fig. 2A, B; Sub).

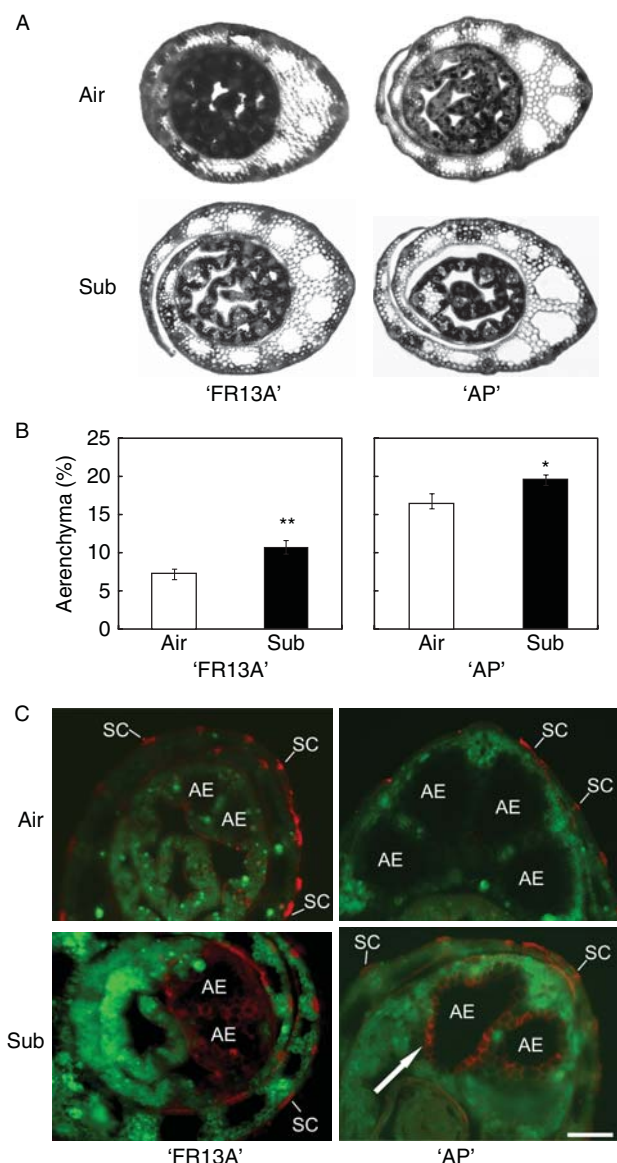


FIG. 2. Aerenchyma formation in leaf sheath of 'AP' and 'FR13A' under air and after 3 d of total plant submergence (Sub). (A) Representative fresh cross-sections of the leaf sheath rice varieties under air and submergence. (B) Percentage of aerenchymatous area in leaf sheath sections of the two varieties. Data are expressed as mean \pm s.e., $n = 9$; * $P < 0.05$ and ** $P < 0.01$ according to Student's t -test. (C) Viability of 'AP' and 'FR13A' leaf sheath cells showing aerenchyma formation using double staining with FM4-64 and FDA. Viable cells stained green by FDA indicate intact plasma membranes. Absence of green colour and substantial red cell staining with FM4-64 indicates the loss of membrane integrity (arrow). Abbreviations: AE, aerenchyma; SC, schlerenchyma. Scale bar = 0.2 mm.

In rice, aerenchyma develops through lysigeny (Hoshikawa, 1989; Matsukura *et al.*, 2000), resulting from the selective death of root and shoot cortex cells (Kawai *et al.*, 1998).

The viability of leaf sheath cells was examined using a double-labelling procedure with FDA/FM4-64 (green/red) fluorescent dyes (Lombardi *et al.*, 2007; Schapire *et al.*, 2008). FDA stains viable cells green, while FM4-64 stains cell plasma membranes, with increased red fluorescence in the cells where the membrane is damaged (Samaj *et al.*, 2005).

Both 'FR13A' and 'AP' showed dead cells (stained red) localized on the border region of the constitutive aerenchyma, mostly in samples from submerged plants (Fig. 2C). The cell death pattern (Fig. 2C) indicated progressive enlargement of aerenchyma following submergence (Fig. 2C; AE). Red fluorescent cells were also observed along schlerenchyma (Fig. 2C; SC).

To determine whether DNA fragmentation, a process associated with PCD (Wang *et al.*, 1996), occurred in the nuclei, thus revealing dying cells, transverse sections of 'FR13A' and 'AP' leaf sheaths were processed for a TUNEL assay. TUNEL-positive nuclei were present in both 'FR13A' and 'AP' aerobic sections, in the tissue surrounding the expanded dead regions of the parenchymatic cells detected with the FDA/FM4-64 dyes (Fig. 3). DNA degradation became more evident in the submerged samples of both varieties, as demonstrated by the presence of a higher quantity of green-fluorescent TUNEL-positive nuclei (Fig. 3). The blue-fluorescent nuclei detected with DAPI staining showed the presence of an intact nucleus in the other regions of the leaf sheath sections (Fig. 3).

Only 'AP' showed submergence-dependent ethylene production

The growth responses described in Fig. 1 suggested that 'AP' does not contain the *Sub1A* gene. This prediction was experimentally confirmed by the lack of *Sub1A* PCR product in DNA samples of 'AP', a *japonica* group rice variety (data not shown). In 'FR13A', a typical *Sub1A* variety, the mRNA level of *Sub1A-1* increased rapidly during submergence (Fig. 4).

Since *Sub1A* has been reported to reduce ethylene synthesis (Fukao *et al.*, 2006), a plant hormone with a predominant role in aerenchyma formation (Shiono *et al.*, 2008), we checked whether 'AP' and 'FR13A' differed in their ability to produce ethylene when submerged.

The mRNA level of ACC oxidase (ACO), a key enzyme for ethylene biosynthesis, increased in 'AP' under flooding stress, but not in 'FR13A' (Fig. 4A). In agreement with the molecular evidence, ethylene production was significantly higher in submerged 'AP' leaf sheaths (Fig. 4B), while in 'FR13A' no increased ethylene synthesis/entrapment (Voeselek *et al.*, 1993) was observed following submergence (Fig. 4B).

Treating the plants with the ethylene biosynthesis inhibitor AVG prevented the submergence-dependent increase in aerenchyma formation in 'AP' but no change was observed in 'FR13A' (Fig. 4C), suggesting that ethylene is not involved in leaf sheath underwater aerenchyma formation in this *Sub1A* variety.

'FR13A' displayed increased ROS accumulation under submergence

The lack of ethylene synthesis as a consequence of submergence (Fig. 4B), together with the increased aerenchymatous areas in 'FR13A' (Fig. 2) prompted us to verify whether an increased ROS production could compensate for the lack of ethylene as a signal triggering underwater aerenchyma formation in 'FR13A'.

A significantly increased level of H_2O_2 was detected in submerged 'FR13A' leaf sheaths (Fig. 5A), while in 'AP' the H_2O_2 level was unchanged (Fig. 5A).

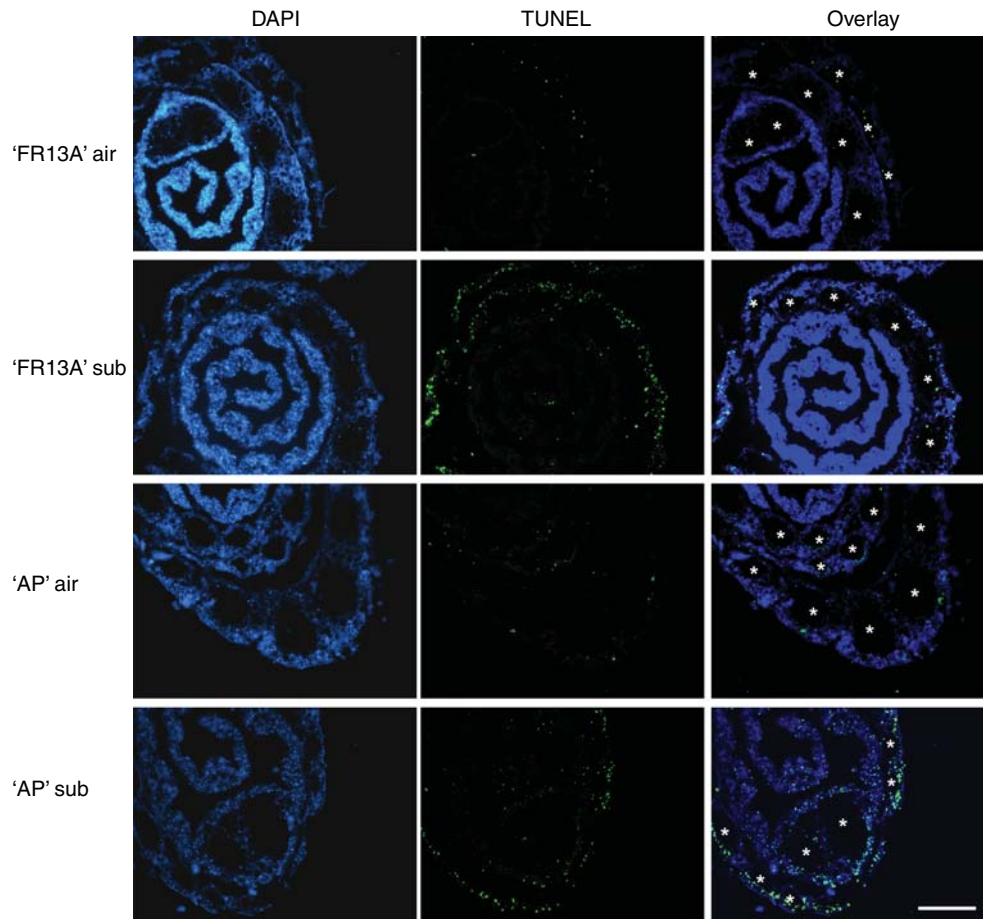


FIG. 3. DNA fragmentation in 'AP' and 'FR13A' leaf sheaths under air and after 3 d of total plant submergence (sub), detected by TUNEL staining. DAPI-stained nuclei were used as positive controls. Scale bar = 0.2 mm.

A remarkable induction of ascorbate peroxidase 1 (*APX1*), a H_2O_2 -induced mRNA (Karpinsky *et al.*, 1997, 1999), was also observed in 'FR13A' under submergence, while no variation in the *APX1* expression was detectable in 'AP' (Fig. 5B).

ROS production, detected using the fluorescent dye H_2 -DCFDA, was highest in 'FR13A' leaf sheath sections in submerged plants, in the internal immature tissue that had not yet been directly affected by the aerenchymatous areas (Fig. 5C). A H_2 -DCFDA green-fluorescence signal, indicating ROS accumulation, was also observed in both the varieties around the vascular tissue system towards the xylem (Fig. 5C).

Treating the plants with DPI, an inhibitor of the ROS-producing NADPH oxidase, prevented the submergence-dependent increase in aerenchyma formation in both 'AP' and 'FR13A' (Fig. 5C). This suggests that a NADPH oxidase-dependent ROS signalling is involved in leaf sheath aerenchyma formation under submergence.

DISCUSSION

Rice is a semi-aquatic plant, which is able to survive prolonged submergence. The molecular basis behind two adaptation mechanisms for surviving flooding have been identified in different rice genotypes, highlighting the existence of profound intra-specific variations in plant survival strategies

to submergence (for reviews, see Bailey-Serres and Voisenek, 2008, 2010; Colmer and Voisenek, 2009; Bailey-Serres *et al.*, 2010; Nagai *et al.*, 2010).

Lowland rice varieties, belonging to the *indica* group and harbouring the ethylene-responsive factor *Sub1A*, respond to submergence by adopting a quiescence strategy, which includes reduced growth (Fukao *et al.*, 2006; Xu *et al.*, 2006). Deepwater rice varieties display an opposite strategy of fast internode elongation when submerged (Kende *et al.*, 1998), in an attempt to reach the water surface and avoid O_2 deprivation (Hattori *et al.*, 2009). This strong growth is controlled by the two ethylene-responsive factors, *SK1* and *SK2*, which are absent in all the non-deepwater rice varieties evaluated to date, but are present in some wild *Oryza* species that show deepwater responses (Hattori *et al.*, 2009). The escape strategy is also activated in lowland rice lacking *Sub1A*; however, it is often unsuccessful if the plant is unable to reach the water surface early enough to avoid prolonged O_2 deprivation, before a rapid depletion of carbohydrate reserves has taken place (Bailey-Serres and Voisenek, 2008).

It was observed that both 'AP', a variety lacking *Sub1A*, *SK1* and *SK2*, and 'FR13A', a variety containing *Sub1A*, can survive moderate submergence, while 'FR13A' can additionally survive complete submergence, through the *Sub1A* tolerance mechanism. Under partial and total submergence, 'AP'

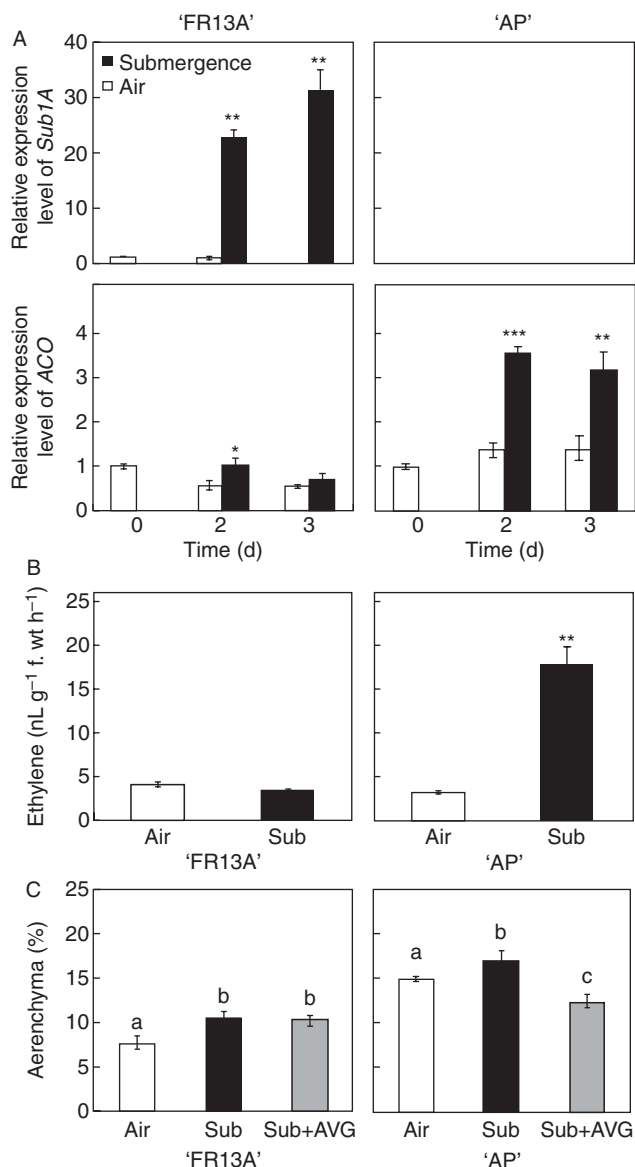


FIG. 4. Ethylene synthesis in 'AP' and 'FR13A' leaf sheaths during aerenchyma formation. (A) Expression of *Sub1A* and *ACO* genes. The expression level was measured based on 'FR13A' air control at day 0 = 1. Data are mean \pm s.d., $n = 3$. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ according to Student's *t*-test. (B) Ethylene production after 3 d air and after 3 d of submergence treatment. Data are mean \pm s.d., $n = 3$. ** $P < 0.01$ according to Student's *t*-test. (C) Percentage of aerenchymatous area in leaf sheath sections of 'AP' and 'FR13A' after the submergence treatment, with or without the ethylene biosynthesis inhibitor AVG (500 μ M), every 24 h for 3 d before and during submergence. Data are mean \pm s.e., $n = 9$. Different lower-case letters indicate significant differences between treatments (0.05 significance level) based on LSD multiple pairwise comparison test.

showed fast growth with an energy cost which is probably exceedingly high, as demonstrated by the low percentage of survival (Fig. 1). Although the two varieties differ profoundly in their tolerance mechanisms, they both display increased aerenchyma formation when submerged, indicating that not only fast-elongating varieties, but also *Sub1A* varieties rely on O_2 transport to the underwater organs when submergence is shallow. Aerenchyma is very important in deep flooding

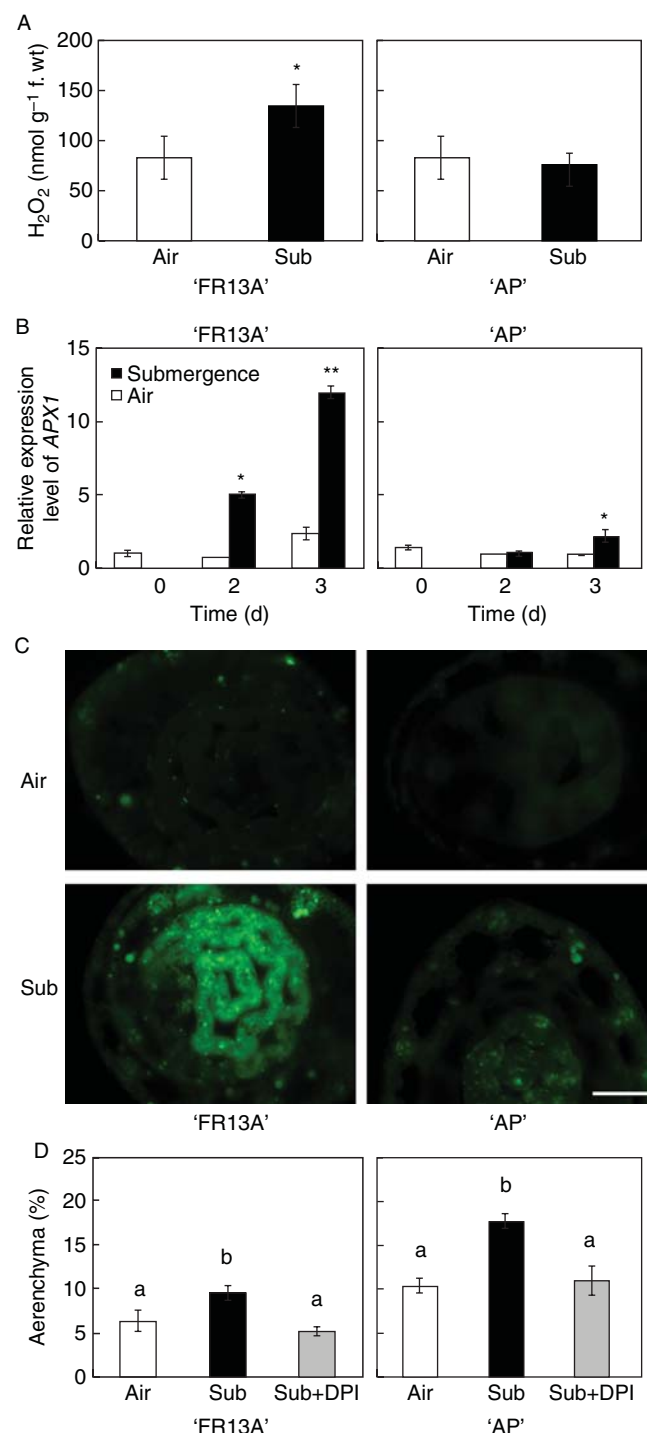


FIG. 5. H_2O_2 production in 'AP' and 'FR13A' leaf sheaths. (A) H_2O_2 production after 3 d of submergence treatment and in well-drained conditions (air). Data are the mean \pm s.d., $n = 3$; * $P < 0.05$ according to Student's *t*-test. (B) Expression pattern of *APX1*. The expression level was measured based on 'FR13A' control air at day 0 = 1. Data are the mean \pm s.d., $n = 3$; ** $P < 0.01$ and * $P < 0.05$ according to Student's *t*-test. (C) Leaf sheath sections of 'AP' and 'FR13A' treated with the green fluorescent dye H_2 -DCFDA for the detection of ROS. Scale bar = 0.2 mm. (D) Percentage of aerenchymatous area in leaf sheath sections of 'AP' and 'FR13A' after the submergence treatment, with or without DPI (10 μ M), every 24 h for 3 d before and during submergence. Data are mean \pm s.e., $n = 3$. Different lower-case letters indicate significant differences between treatments (0.05 significance level) based on LSD multiple pairwise comparison.

conditions, allowing the plant to access the alternative O₂ sources, i.e. submergence water, underwater photosynthesis and leaves surface gas film (Colmer and Pedersen, 2008; Pedersen et al., 2009).

The major problem that a plant has to confront when submerged is a dramatic reduction in gas exchanges (Bailey-Serres and Voesenek, 2008). A decline in O₂ to a concentration that limits aerobic respiration, leads to reduced ATP synthesis (for a review, see Gibbs and Greenway, 2003). Aerenchyma formation is one of the plant's morphological adaptations that helps to increase gas circulation inside the underwater organs (Evans, 2003).

By analysing the leaf sheaths of the two rice varieties 'FR13A' and 'AP', it was found that both displayed constitutive aerenchyma when not submerged which increased following submergence (Fig. 2). Increased aerenchyma likely helps both 'AP' and 'FR13A' to survive minimal submergence better (Fig. 1). The mechanism of aerenchyma formation appears to be similar in 'AP' and 'FR13A' leaf sheaths and is based on progressive cell-death along the peripheral tissues of the aerenchymatous tubes under submersion (Figs 2C and 3). TUNEL-positive nuclei that are peripheral to the FM4-64 fluorescent dead cells suggest the activation of a DNA-degradation mechanism prior to the final disruption of the nucleus under autolysis (Bouranis et al., 2003).

The signalling events leading to aerenchyma formation were very different. In 'FR13A' the activation of the *Sub1A* gene (Xu et al., 2006) probably limited the submergence-induced ethylene production. On the other hand, 'AP', which does not possess the *Sub1A* gene, displayed a remarkable induction of *ACO*, probably leading to the observed high ethylene synthesis in submerged plants. Therefore, although ethylene is known to be a key component in the lysigenous aerenchyma formation under waterlogging through PCD (for a review, see Shiono et al., 2008), it does not explain the formation of aerenchyma in 'FR13A'. In agreement with our hypothesis that ethylene plays only a minor role in aerenchyma formation in 'FR13A', treatment with the ethylene biosynthesis inhibitor AVG resulted in a significant reduction in aerenchyma in 'AP' only (Fig. 4C).

Recently, aerenchyma formation in rice stems was found to be dependent on both ethylene and ROS accumulation (Steffens et al., 2011). Moreover, in arabidopsis hypocotyls, both ethylene and ROS are involved in aerenchyma formation under hypoxic conditions (Mühlenbock et al., 2007). A clear increase in H₂O₂ concentrations was found in submerged 'FR13A' plants (Fig. 5A), together with the up-regulation of *APX1* expression (Fig. 5B), H₂O₂ regulated in higher plants (Karpinsky et al., 1997, 1999; Volkov et al., 2006). In addition, the analysis of ROS presence in the 'AP' and 'FR13A' leaf sheath sections revealed a higher H₂O₂ level in submerged 'FR13A', preferentially localized in the internal tissues that had not yet been affected by aerenchyma development (Fig. 5C). Similarly, high fluorescence due to the ROS-staining DCFH₂-DA was observed in intact, sulfate-starved cells in maize roots forming aerenchyma (Bouranis et al., 2003). In the present experiments, treating the leaf sheaths with DPI, an inhibitor of the the ROS-producing NADPH oxidase enzyme, resulted in reduced aerenchyma formation in both 'FR13A' and 'AP'. It has been proposed that the timing of ROS signalling occurs ahead to

ethylene signalling in aerenchyma development in arabidopsis (Mühlenbock et al., 2007). In our hypothesis, a ROS transient production occurs ahead to ethylene signalling in rice as well and is not detectable in 'AP' after 3 d of flooding.

In 'FR13A', the ethylene synthesis response is blocked when plants are submerged (Fukao et al., 2006) and ROS-dependent signalling is likely to substitute it in promoting further aerenchyma production.

Jung et al. (2010) reported the activation of genes related to the removal of ROS in M202(*Sub1*) plants after submergence, suggesting that *Sub1A* is a positive regulator in ROS scavenging, in agreement with the higher *APX1* expression that was observed in 'FR13A' (Fig. 5B).

It is concluded that ethylene signalling is not involved in aerenchyma formation in 'FR13A'. The two cultivars investigated do not differ in the presence of *Sub1A* only, thus the observed difference can be due to several factors. However, it is likely that *Sub1A* rice varieties, that rely on a limited production of ethylene when submerged to restrict their growth response, have developed alternative signals to enhance aerenchyma formation, which presumably help the plant to survive shallow flooding. The production of ROS in submerged *Sub1A* rice varieties, such as 'FR13A', may be that signal. Further analyses are under way to evaluate the possible role of the *Sub1A* gene in activating ROS signalling and to determine its relationship to aerenchyma formation under submergence.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: sequences of primers used for real-time PCR analysis and for gene screening.

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