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

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INTRODUCTION

Flooding events of various depths and durations are the principal environmental cause of a shortage of oxygen \( (O_2) \) in plants. Rice is one of the few crops able to withstand periods of partial or even complete submergence (Colmer and Voeseck, 2009; Licausi and Perata, 2009; Nagai et al., 2010). One of the adaptive traits of rice is the constitutive presence 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.

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; König and Jackson, 1979; Justin and Armstrong, 1991; He et al., 1996; Zhou et al., 2002; Geisler-Lee et al., 2010; Lenochova 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; König, 1982). In addition, both 1-amino-1-cyclopropane-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 and others on aerenchyma formation in rice stems in response to submergence with 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 ± 2°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 ± 2°C, 15-h light photoperiod; PAR approx. 50 μmol m⁻² s⁻¹ provided by white fluorescence lamps). The following complete nutrient solution was used: Ca(NO₃)₂·4H₂O (4.5 mM), MgSO₄ (0.8 mM), KH₂PO₄ (2.6 mM), KNO₃ (13.5 mM), K₂SO₄ (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 ± 2°C, 15-h light photoperiod; PAR approx. 50 μmol m⁻² s⁻¹).

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 μg 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 (λex 450–490, dichroic 495, λem 500–550 nm) and
TRITC (λex 505–565, dichroic 550, λem 580–630 nm) filter blocks for FDA (λex 488, λem 502–540 nm) and FM4-64 (λex 515, λem 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 μm) 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 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 (λex 450–490, dichroic 495, λem 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 submergence.

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) (λex 488, λem 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 (λex 450–490, dichroic 495, λem 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 submerison.

Molecular analysis

Genomic DNA of ‘FR13A’ and ‘AP’ leaf sheaths was prepared using GenEluteTM 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).
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 sclerenchyma (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. 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-I 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 (Voesenek 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. 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 (Voesenek et al., 1993) was observed following submergence (Fig. 4B).

‘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 for underwater aerenchyma formation in ‘FR13A’.

A significantly increased level of H2O2 was detected in submerged ‘FR13A’ leaf sheaths (Fig. 5A), while in ‘AP’ the H2O2 level was unchanged (Fig. 5A).
A remarkable induction of ascorbate peroxidase 1 (APX1), a H₂O₂-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 H₂-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 H₂-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 Voesenek, 2008, 2010; Colmer and Voesenek, 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 O₂ 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 O₂ deprivation, before a rapid depletion of carbohydrate reserves has taken place (Bailey-Serres and Voesenek, 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’
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 O2 transport to the underwater organs when submergence is shallow. Aerenchyma is very important in deep flooding...
conditions, allowing the plant to access the alternative O\textsubscript{2} 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 exchange (Bailey-Serres and Voeseenk, 2008). A decline in O\textsubscript{2} 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 periphercal 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 posses 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 both ‘AP’ and ‘FR13A’, 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 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 Sub1 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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