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Ethylene influences in vitro regeneration frequency in the FR13A rice harbouring the *SUB1A* gene

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Abstract Many studies have examined the effects of ethylene on in vitro plant growth and development, often with controversial results. Ethylene accumulates in culture vessels due to both the release from the tissues and the physical entrapment due to the need for closed containers. This hormone has several effects on plant regeneration, depending on the plant species and even the cultivar. A prerequisite for ethylene use for in vitro culture is thus to formulate a specific protocol for the genotype of interest. In rice, ethylene is a key regulator of adaptation strategies to low oxygen environments. In particular, the *SUBMERGENCE1A* (*SUB1A*) gene, when present, drives the acclimation response which when activated by ethylene produced by submerged plants leads to adaptation through reduced plant growth and ethanolic fermentation enhancement. This gene is restricted to a limited number of rice for which a very specific response to ethylene is expected, whatever the source. This paper reports the regeneration differences between a *SUB1A* rice landrace (*indica-aus*, FR13A) and a non-*SUB1A* variety (*japonica*, Nipponbare). Our results suggest that regeneration protocols with exogenous ethylene precursors supply are required for the FR13A rice harbouring the *SUB1A* gene to overcome the problem of low regeneration efficiency.

Keywords Ethylene · In vitro culture · *Oryza sativa* · Rice · *SUB1* gene

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Introduction

A large number of studies have examined the effects of ethylene on in vitro plant culture and reported controversial results, its use for plant regeneration and transformation is thus not systematic. Ethylene accumulates to a different extent in culture vessels due to both the release from the tissues and the need to use closed containers to avoid microbial contamination. This inevitably leads to ethylene entrapment (Biddington 1992). Additionally, during the initial steps of in vitro culture, plant tissues are often exposed to a combination of stress factors, such as wounding and low or high light intensity, which can result in the accumulation of stress related compounds, of which ethylene is the predominant (Fehér et al. 2003). An increase in ethylene may modify the physiological and morphogenetic processes that influence the success of the plant regeneration technique (Biddington 1992; Kępczyńska et al. 2009). Indeed, ethylene has been found to have a diverse influence on in vitro plant culture, depending not only on the plant species (Auboiron et al. 1990; Chi et al. 1990; Chraibi et al. 1991; Biddington 1992; Mensuali-Sodi et al. 1995; Hu et al. 2006; Santana-Buzzy et al. 2006), but also on the cultivar (Jha et al. 2007). Thus, a prerequisite for ethylene use is to formulate a specific protocol for the genotype of interest.

As a plant hormone, ethylene regulates a broad range of cellular and developmental processes and it is also involved in plant responses to various stresses. For instance, ethylene is a central component of the molecular response to submergence, and more in general, to low oxygen stress (Bailey-Serres and Voesenek 2010). Ethylene is a key regulator of rice adaptation strategies to floods, governing ethylene responsive factors (ERFs), which

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activate specific adaptive pathways to various types of flood (Nagai et al. 2010).

The *SUBMERGENCE1A* (*SUB1A*) gene drives a mechanism of adaptation to flooding based on the induction of a "quiescence" status through a reduction of growth in submerged rice plants, by avoiding excessive energy consumption associated with plant elongation and thus allowing re-growth when water recedes (Fukao et al. 2006; Xu et al. 2006). The discovery of the *SUB1A* locus and the centrality of the *SUB1A-1* allele in the molecular and physiological response of rice varieties to low oxygen allows the transfer of submergence resistance to otherwise sensitive but highly yielding rice varieties (Fukao et al. 2006; Xu et al. 2006; Septiningsih et al. 2009; Singh et al. 2010). *SUB1A* is only present in a limited number of rice varieties and wild rices (Xu et al. 2006; Niroula et al. 2012), which may tend to respond in very specific ways to any source of ethylene. The induction of *SUB1A-1* expression by ethylene under submergence limits the gibberellin-driven elongation of the plants underwater, but also activates the transcript levels and enzymatic activities such as *PDC* and *ADH*, which are both related to the ethanolic fermentation (Fukao et al. 2006; Fukao and Bailey-Serres 2008). The intimate relation between ethylene production and the modulation of fermentation related genes and proteins may have physiological consequences whenever the level of ethylene is altered, with rice plants that harbour the *SUB1A* gene expected to respond differently.

Chatfield and Raizada (2008) showed that there is a correlation between plant regeneration efficiency and ethylene signalling. In *Arabidopsis*, they found that the shoot regeneration rate was reduced in ethylene-insensitive mutants (*etr1-1*, *ein2-1*, *ein4*, *ein7*), while it was enhanced in plants with a constitutive ethylene response (*ctr1-1*, *ctr1-12*). In addition, an expression analysis of *Arabidopsis* under regeneration (Che et al. 2006) revealed that some genes with putative roles in ethylene biosynthesis or signalling, including *RAP2.6L,* were up-regulated during early shoot development.

Genetic transformation is an important tool for crop improvement and molecular studies. A prerequisite for efficient plant transformation is a high rate of regeneration from tissue culture (Goldstein and Kronstad 1986). Since the successful *Agrobacterium* transformation of *japonica* rice varieties (Hiei et al. 1994), many protocols have been proposed to reduce the timing and improve the efficiency of regeneration in both *japonica* and *indica* rice groups (Toki 1997; Uze et al. 1997; Nishimura et al. 2006; Toki et al. 2006; Karthikeyan et al. 2009; Sahoo et al. 2011). However, the high genotype-specificity has a negative impact on the technique, with most *indica* varieties showing a lower rate of callus growth and regeneration efficiency (Nishimura et al. 2006).

In this paper, we report on some regeneration differences in a rice landrace harbouring *SUB1A* (i.e. *indica-aus* FR13A) compared with the *japonica* variety Nipponbare. Our results suggest that protocols that include exogenous ethylene precursors are required for this *SUB1A* rice landrace.

Materials and methods

Plant material and embryogenic callus induction

Mature dry seeds of the *indica-aus* landrace FR13A (harbouring *SUB1A*) and the *japonica* variety Nipponbare (not harbouring *SUB1A*) (International Rice Research Institute accession IRGC-117267 and IRGC-117274, respectively) were used in this study. Seeds were de-hulled and surface sterilized with 70 % ethanol (v/v) for 1 min, followed by 20 % bleach (v/v) for 10 min, thoroughly washed several times in sterile distilled water and dried on sterile filter paper. Sterilized seeds were cultured in N6D medium (Toki et al. 2006) containing 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.4 % Gelrite (w/v, Sigma-Aldrich) and pH adjusted to 5.8 before autoclaving. Seeds were then incubated at 28 ± 2 °C under continuous light.

After 7 days, scutellar embryogenic calli were collected and maintained on the same fresh medium for a further 21 days under the same conditions for callus induction. For the callus growth experiment, the medium was modified adding the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC, 5 μM) or the ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG, 10 μM). For regeneration, healthy embryogenic calli were transferred to Murashige Skoog's medium (Murashige and Skoog 1962), supplemented with kinetin (12.5 μM) and different concentrations of ACC (0, 2.5, 5, 10 μM) or AVG (10 μM), where they were maintained for 45 days at 28 ± 2 °C under continuous light.

Indexes of regeneration

To compare the capability of calli to regenerate under different conditions, three performance indexes were used: the number of days required for green spot induction on calli, the percentage of calli with at least one plantlet after 45 days of in vitro culture, and the ratio between the number of regenerated plants and the number of calli.

Ethylene measurement

Ethylene production was measured by enclosing samples in 30 cm^3 airtight glass bottles (Pyrex, France), containing the same culture medium used for regeneration and closed with

holed plastic screw caps provided with rubber septa. Each sample consisted of three calli picked 1, 3, and 7 days after adding ACC (5 μM) and AVG (10 μM). Gas samples (1 cm^3 each) were taken from the headspace of the containers with a hypodermic syringe after 1 h of incubation at room temperature. The ethylene concentration in the sample was measured by gas chromatography (HP5890, Hewlett-Packard, Menlo Park, California), using a flame ionization detector (FID), a metal column (i.d. 150 × 0.4 cm) packed with Hysep T, and column and detector temperatures of 70 and 350 °C, respectively. A nitrogen carrier gas at a flow rate of 30 mL min^{-1} was used. Quantification was performed against an external standard, and subtracting the empty plate ethylene content. The results were expressed on a dry weight basis (pL g^{-1} DW h^{-1}). Gas losses and abiotic contribution to ethylene production was estimated according to Mensuali-Sodi et al. (1992).

Gene expression analysis

For the gene expression analysis, total RNA was extracted using an RNAqueous kit (Applied Biosystems/Ambion, Foster City, California), according to the manufacturer's instructions, and subjected to DNase treatment using TURBO DNA-free kit (Ambion, Foster City, California). Five micrograms of RNA were reverse-transcribed using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, California, USA). Transcript abundance was analyzed by Real-Time Reverse Transcription PCR, using qPCR MasterMix Plus for SYBR® green I (Eurogentec, Liège, Belgium) with specifically designed primers (Kudahettige et al. 2011), using a ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, California, USA). 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 Number 2 (Applied Biosystems, Foster City, California, USA), using rice glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as an internal reference. PCR reactions for each of the three biological replicates were performed in duplicate.

Results and discussion

We used a rice regeneration protocol (Toki et al. 2006) to induce shoots from calli of the rice landrace FR13A and the Nipponbare variety, the first of which harboured the ethylene-responsive gene *SUB1A*, but not the second. Unlike Nipponbare, FR13A showed a very low production of regenerable calli using this protocol (Fig. 1). The growth environment of plants regenerating in vitro is often hypoxic, which could affect regeneration efficiency when comparing rice varieties displaying large differences in their ability to survive low oxygen conditions. Considering the importance of ethylene for growth responses under low oxygen, the media for callus maintenance and regeneration were supplied with either ACC, the precursor of ethylene synthesis, or AVG, an ethylene synthesis inhibitor (Fig. 2).

Callus induction and growth were evaluated in the two varieties using either a control medium (N6 containing 2,4-D) or the same medium supplemented with either ACC or AVG (Table 1). The fresh weight increment after 7 days of treatment was significantly higher in the FR13A variety, irrespectively of the presence/absence of the ethylene modulators. Overall, no significant differences were found when comparing the 7-day AVG and ACC treatments with the control conditions, regardless of the variety used. This suggests a general higher ability of the FR13A callus to proliferate, as well as the absence of a direct effect of ethylene modulation by ACC and AVG on the proliferation process, a result that would seem to rule out this in vitro

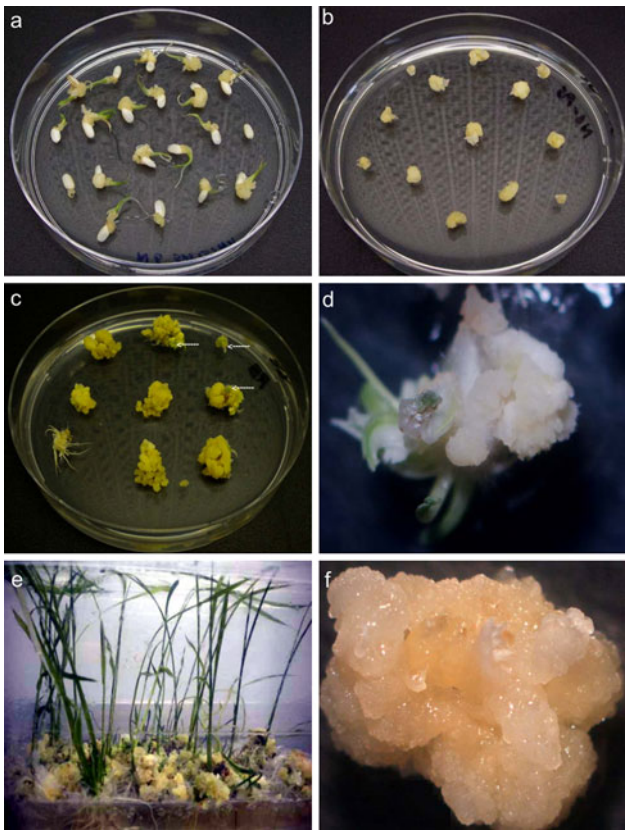

Fig. 1 Regeneration of Nipponbare and FR13A plants from scutellum tissue (Toki et al. 2006). **a** Seven-day Nipponbare scutellum-derived calli; **b** seven-day Nipponbare scutellum detached calli; **c** induced shoot formation on 21-day Nipponbare calli; **d** detail of Nipponbare shoot formation; **e** forty-five-day regenerated Nipponbare plantlets; **f** not regenerable FR13A callus

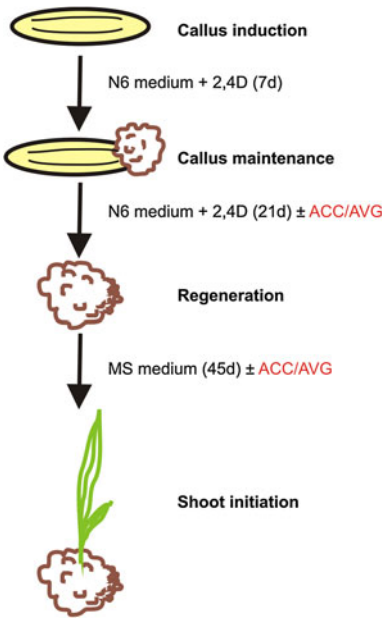

Fig. 2 The regeneration protocol of FR1A and Nipponbare rice, adding ACC and AVG to the callus maintenance or to the regeneration media

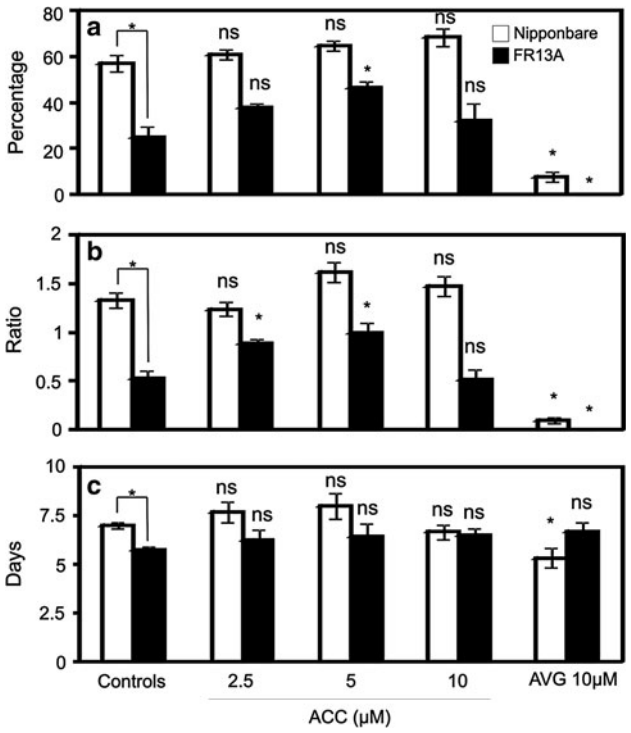

Fig. 3 Regeneration performance indexes of 28-day calli of Nipponbare and FR13A in the presence of ACC or AVG. **a** Percentage of calli with plantlets after 45 days of regeneration; **b** ratio between the number of regenerated plantlets and calli; **c** days required for the induction of green spots on calli. Student's *t* test significance (*$p < 0.05$) was calculated for each variety by comparison between the treated samples and respective controls. For the control samples only, significance was calculated between the two varieties

phase from being responsible for the very low production of regenerable calli in FR13A.

Regeneration performance was then evaluated for both FR13A and Nipponbare grown in a regeneration medium modified with ACC/AVG supply (Fig. 3). In control conditions, significant differences between the two genotypes were always found ($p < 0.05$), with FR13A showing lower values for all the performance indexes evaluated. However, only in FR13A the treatment with ACC resulted in a significant increase in the percentage of calli with plantlet and the ratio between regenerating plants and calli. This suggested that an increase in endogenous ethylene production by feeding ACC positively affects the regeneration performance of FR13A alone (Fig. 3a, b).

When AVG was added to the regeneration medium, both FR13A and Nipponbare showed a lower performance, suggesting that endogenous ethylene production is indispensable for regeneration whatever the variety (Fig. 3a, b). Interestingly, the number of days to induce green spots on calli was not influenced by ACC supply, and AVG only had a minor effect (Fig. 3c). However, the number of regenerating plants for calli was affected by both treatments (Fig. 3b). This suggested that ethylene quantitatively affects shoot initiation and development without modifying the regeneration pathway. In some early studies, ethylene was reported to have increased shoot formation in rice, an effect that seems to be increased by the presence of CO_2 (Cornejo-Martin et al. 1979). A more recent study on tomato in vitro organogenesis showed that the inhibition of ethylene production may decrease the percentage of explants with shoots and the mean number of shoots per

Table 1 Callus growth of Nipponbare and FR13A

	Initial	7-day control	7-day ACC treatment		7-day AVG treatment	
Nipponbare	34 ± 8	64 ± 11	58 ± 9	ns	53 ± 14	ns
FR13A	43 ± 12	89 ± 7	97 ± 13	ns	107 ± 21	ns
	ns	*	*		*	

Seven-day old calli were grown on N6 medium containing 2,4-D ± AVG (10 μM) or ACC (5 μM) and then cultivated for an additional 7 days. Data points represent the pooled FW average ± SD of three biological replications with seven calli in each replication. Student's *t* test significance ($p < 0.05$) was calculated between the varieties (columns), and between the 7-day treated samples and the 7-day control group (rows)

explant, and that ethylene supplementation may enhance shoot development (Trujillo-Moya and Gisbert 2012). Other studies have shown that although ethylene promotes callus growth, it may inhibit shoot production (Biddington 1992; Panizza et al. 1997). In fact, Grady and Bassham (1982) found that shoot-forming and non-shoot-forming tobacco callus cultures showed significant differences in the endogenous content of ACC, with a higher amount in the non-shoot-forming ones.

To test whether ethylene production was similarly modulated in the two varieties by ACC and AVG, ethylene production was measured 1, 3 and 7 days after adding the two chemicals (Fig. 4). In control conditions, ethylene production was significantly lower in FR13A than in Nipponbare after 3 days of culture. Along the time course, the addition of ACC significantly enhanced ethylene production in FR13A, whereas AVG inhibited it (Fig. 4a). The beneficial effect of ACC on FR13A regeneration performances is probably related to the increased production of ethylene, which is lower in this landrace than in Nipponbare. When testing the effects of ACC and AVG on ethylene production in Nipponbare, we observed an effect similar to the one observed for FR13A along all the time course (Fig. 4b and data not shown). In the case of Nipponbare, no effects related to ACC were observed on the performance indexes (Fig. 3), indicating that its regeneration does not depend on exogenous ethylene precursors supply but rather on ethylene endogenous production that is probably sufficient. Indeed, ethylene repression obtained by adding AVG had a negative effect on the regeneration performances of Nipponbare (Fig. 3).

We next investigated whether ACC and AVG affected the anaerobic gene responses. The presence or absence of the *SUB1A* gene in the two varieties could be a key determinant in the activation of the fermentative metabolism affecting tolerance to the in vitro hypoxic conditions.

The environment created by closed containers and water submergence may be considered similar since it results in oxygen deprivation and ethylene accumulation.

We analyzed *SUB1A* regulation together with the genes involved in the fermentation pathway (i.e. *PDC* and *ADH*) (Fig. 5). The induction of *SUB1A* by ethylene is the master regulator for the fermentation-related genes under low oxygen in *SUB1A*-varieties, a mechanism that enhances survival (Fukao et al. 2006). As expected, *SUB1A* was upregulated in FR13A under regeneration and its regulation was enhanced when ACC was added to the medium (Fig. 5). In vitro culture in closed containers suffers from the accumulation of ethylene (produced by plants or media), making the environment very different from that experienced by non-enclosed plants (Biddington 1992; Mensuali-Sodi et al. 1993).

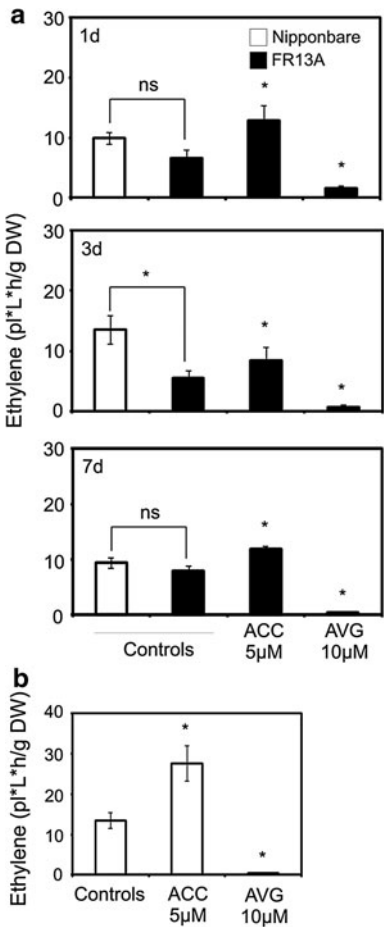

Fig. 4 Ethylene production by Nipponbare and FR13A after 28 days of calli growth, adding 5 μM ACC and 10 μM AVG to MS medium (controls). **a** FR13A ethylene production 1, 3 and 7 days after adding ACC and AVG; **b** Nipponbare ethylene production 3 days after adding ACC and AVG. Student's *t* test significance (* $p < 0{,}05$) was calculated for each variety by comparison between the treated samples and the respective controls. For the control samples, significance was calculated between the two varieties

ADH and *PDC* were similarly upregulated in both Nipponbare and FR13A calli under control conditions when compared to aerobic leaves and roots, suggesting that the calli were growing in a hypoxic environment (Fig. 5). In FR13A ACC induced, as expected, *SUB1A*, and *ADH* and *PDC* also showed a similar increase (Fig. 5). Moreover, AVG downregulated *SUB1A* and *PDC* genes in FR13A alone (Fig. 5). Nipponbare and FR13A behaved differently following ACC treatment probably because of the activation of *SUB1A* and downstream genes. Since *SUB1A* is also known to repress ethylene production itself (Fukao et al. 2006, Fig. 6), the significant differences observed in the ethylene level between FR13A and Nipponbare controls at 3 days of culture (Fig. 4), were possibly due to the activation of the *SUB1A* gene in FR13A (Fig. 5), which repressed basal ethylene production.

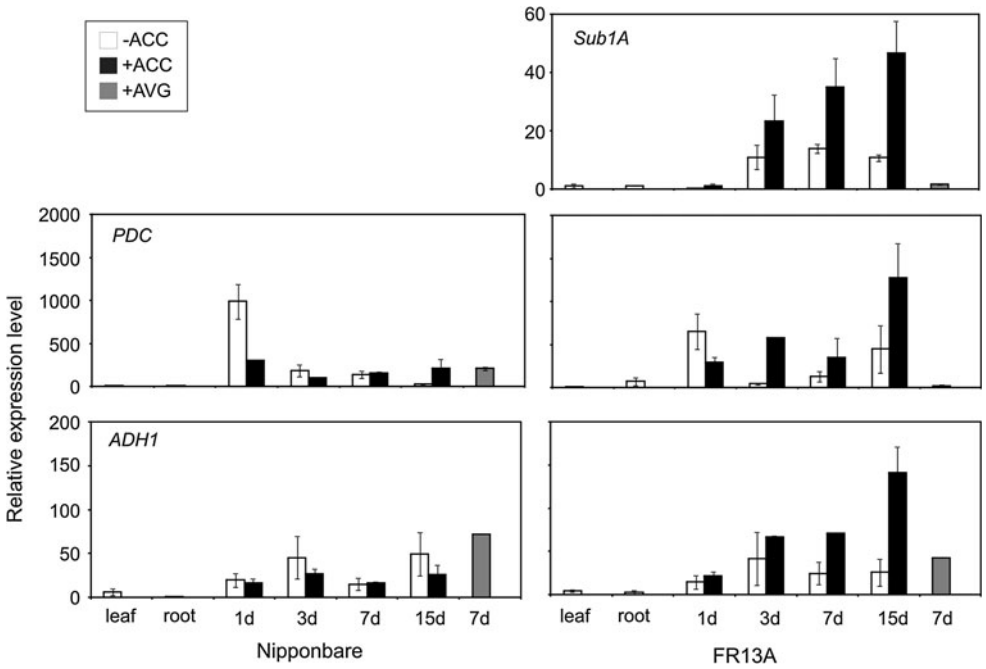

Fig. 5 Anaerobic gene expression in Nipponbare and FR13A after 1, 3, 7 and 15 days on regeneration medium without or with ACC (5 μM) and after 7 days with AVG (10 μM)

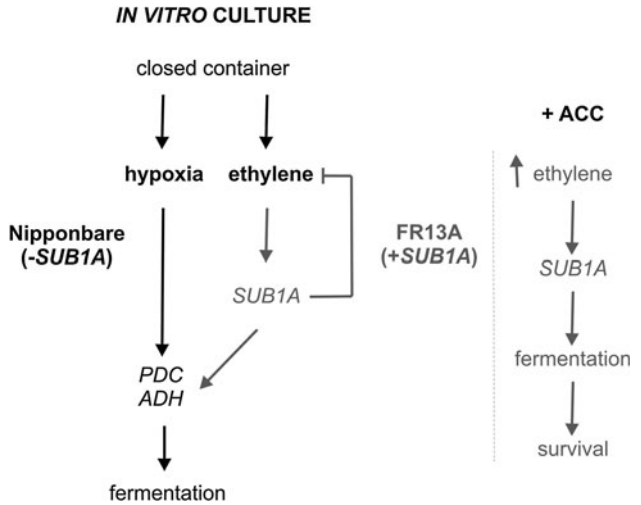

Fig. 6 The pathways activated under regeneration in rice FR13A and Nipponbare plants that do and do not harbour the *SUB1A* gene

Conclusions

The use of ethylene precursors or inhibitors in plant in vitro culture is known to have different consequences depending on the genotype used. The ethylene-responsive *SUB1A* gene, when present, profoundly affects rice survival in the hypoxic environment generated under flooding. In this paper, we have shown that the ACC-released ethylene is required for an efficient regeneration of the *SUB1A* landrace FR13A. In vitro growth is often associated with the generation of a hypoxic environment, and FR13A grown in vitro displays an ethylene-dependent activation of the genes involved in hypoxia tolerance. It is thus tempting to speculate that ethylene-dependent activation of *SUB1A* might be required for the efficient regeneration of rice varieties harbouring this gene, and further analyses are under way to evaluate this possibility. Our ACC-containing regeneration medium provides an easy method to obtain a better regeneration efficiency of the *SUB1A*-harbouring FR13A rice.

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