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3 **The response to submergence of lowland rice cv Senia is mediated by gibberellins**

4 **but not by ethylene**

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Running title: Response of lowland rice to submergence

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Summary

We have examined the gibberellin (GA) and ethylene regulation of submergence-induced elongation in seedlings of the submergence tolerant lowland rice (*Oryza sativa* L.) cvs Senia and Bomba. Elongation was enhanced after germination to facilitate water escape and reach air. Submerged-induced elongation depends on GA because it was counteracted by paclobutrazol (an inhibitor of GA biosynthesis), an effect that was negated by GA_3. Moreover, in the cv Senia submergence increased the content of active GA_1 and its immediate precursors (GA_{53}, GA_{19} and GA_{20}) by enhancing expression of several GA biosynthesis genes (*OsGA20ox1* and *-2,* and *OsGA3ox2)*, but not by decreasing expression of several *OsGA2ox* (GA inactivating genes). Senia seedlings, in contrast with Bomba seedlings, did not elongate in response to ethylene or 1-aminocyclopropane-1-carboxylic-acid (ACC; an ethylene precursor) application, and submergence-induced elongation was not reduced in the presence of 1-methylcyclopropene (1-MCP; an ethylene perception inhibitor). Ethylene emanation was similar in Senia seedlings grown in air and in submerged-grown seedlings following de-submergence, while it increased in the case of Bomba. The expression of ethylene biosynthesis genes (*OsACS1*, *-2* and *-3*, and *OsACO1*) was not affected in Senia, but that of *OsACS5* was rapidly enhanced in Bomba upon submergence. Our results support the conclusion that submergence elongation enhancement of lowland rice is due to alteration of GA metabolism leading to an increase of active GA (GA_1) content. Interestingly, in the case of cv Senia, in contrast with cv Bomba, this is triggered through an ethylene-independent mechanism.

Keywords: Ethylene, Gibberellins, Lowland rice, *Oryza sativa*, Submergence

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic-acid; GA, gibberellin; GAox, GA oxidase; 1-MCP, 1-methylcyclopropene; PAC, paclobutrazol; WT, wild type

1 **Introduction**

2 Flooding is an environmental stress that can affect plant architecture and crop
3 production. Research on this topic has concentrated mostly on a few relatively flood-tolerant
4 species from *Oryza*, *Rumex* and *Echinochloa* genera (Jackson, 2008; Bailey-Serres and
5 Voesenek, 2008). It has been shown that many plants elongate to escape from the asphyxiation
6 produced by submergence (Voesenek and Blom, 1999), in a process involving different
7 hormones such ethylene, gibberellins (GA) and abscisic acid (Musgrave et al., 1972; Rijnders et
8 al., 1997; Kende et al., 1998; Benschop et al., 2006; Jackson 2008).

9 Rice (*Oryza sativa* L.) is a semiaquatic species with increased shoot elongation when
10 the plant is totally or partially submerged. According to submergence habit, two main ecotypes
11 can be distinguished: deepwater rice, which is cultivated in habitats with occasional floods
12 during which internodes elongate dramatically to escape from flooding (Kende et al., 1998), and
13 lowland rice, which germinates usually under submergence and whose coleoptile and young
14 leaves growth is normally enhanced under these conditions (Jackson et al., 1987). An exception
15 is the submergence tolerant lowland rice varieties, where shoot elongation is restricted under
16 submergence to conserve energy reserves and reduce carbohydrate consumption to enable
17 development restarting upon eventual de-submergence (Ismail et al., 2009; Kawano et al.,
18 2009). This tolerance to submergence is conferred by *Sub1A*, a gene encoding an ethylene
19 responsive factor that increases the levels of GA signalling repressors SLR1 and SLRL1 thus
20 reducing GA-inducible expression under submergence (Setter and Laureles, 1996; Perata and
21 Voesenek, 2007; Fukao and Bailey-Serres, 2008b). In contrast, the increased elongation
22 capacity of normal lowland rice is certainly an advantage in the case of germination under
23 shallow layers of water (about 10-15 cm) only during their early growth stages, as occurs in
24 most Mediterranean rice growing countries, to help reaching contact with air and start active
25 photosynthesis as soon as possible.

26 During the last twenty years, much progress has been made on the submergence
27 response of deepwater rice internodes, and a model explaining this growth induction has been
28 proposed. According to this model, the response of rice internodes to flooding would be

1 mediated by at least three interacting hormones, namely ethylene, abscisic acid (ABA) and
2 gibberellins (GA) (Kende et al., 1998; Van Der Straeten et al., 2001; Vriezen et al. 2003). The
3 increase of ethylene concentration would promote rice internode elongation mediated by an
4 increase of GA response and concentration. However, in contrast to deepwater rice, much less is
5 known on lowland rice elongation upon water submergence. In young lowland rice seedlings,
6 coleoptile and leaf sheath elongation is stimulated by ethylene and GA (Raskin and Kende,
7 1983; Furakawa et al., 1997; Van Der Straeten et al., 2001; Fukao and Bailey-Serres, 2008a)
8 application, indicating that these hormones may be involved in their submergence-induced
9 elongation. It has been suggested that ethylene stimulates leaf elongation (at least under air-
10 grown conditions) by increasing GA_1 responsiveness and turnover (Furukawa et al., 1997).
11 Lowland 9-d-old rice seedlings (cv IR36) respond to submergence with a temporal burst of
12 growth, and that sustained growth is correlated with induction of *OsACS5*, although no change
13 in GA content (GA_{19} and GA_{20}) was observed (Van Der Straeten et al., 2001).
14 In this report we have investigated the role of GAs and ethylene in the response of
15 lowland rice seedlings to submergence early after germination using Senia and Bomba, two
16 non-tolerant cultivars of rice to submergence, largely grown in the Comunidad Valenciana,
17 Spain, for their excellent organoleptic properties (mainly tenderness, reduced stickiness, and
18 large capacity of flavour incorporation). We found that submergence-induced elongation
19 depended on GA in both cases, through alteration of GA metabolism leading to an increase of
20 GA_1 content. But, interestingly, while this elongation also depends on ethylene in Bomba, it
21 does not in Senia. This suggests that submergence-induced elongation mediated by GA in rice
22 may be triggered, at least in some cases, through an ethylene independent mechanism.
23
24 **Materials and methods**
25 **Plant material and growth conditions**
26 Seeds of rice [*Oryza sativa* L. var. japonica cvs Senia, short, and Bomba, tall;
27 COPSEMAR, Sueca, Spain] sterilised for 5 min in 70% ethanol followed by 45 min in 2.5%
28 sodium hypochlorite containing 0.05 % Tween 20 were placed in petri dishes, covered with

distilled water and kept at 27.C in the dark for 3 d. Germinated seeds were placed in 1 L glass vessels (8 seeds per vessel) containing 40 mL of 0.35 mM MES pH 5.8 medium solidified with 0.45% (w/v) industrial agar (Pronadisa, Alcobendas, Madrid), covered with loose transparent lids and kept in a growth chamber at 25.C under16 h light (40 ømol m^{-2} s^{-1})/ 8 h darkness. Four days later (time 0) 40 ml of 0.35 mM MES pH 5.8 solution (just covering the base of the seedlings) were added to control vessels to avoid desiccation. For submergence experiments, 800 mL of that solution (14 cm deep water layer) were added to the vessels. At this time, the first leaf had already emerged from the coleoptile and some roots had grown within the solidified medium.

Material for RNA and GA analysis was taken 2 h after starting the light period to avoid possible effects of circadian rhythm on transcript and hormone content, frozen in liquid N_2 and stored at -80.C until analysis.

The experiments were repeated three times and each replicate consisted of material from at least 15 seedlings.

Plant growth substances and hypoxia application

GA_3 (1 øM) (Duchefa, http://www.duchefa.com), Paclobutrazol (10 øM) (PAC; a GA synthesis inhibitor) (Duchefa) and 1-aminocyclopropane-1-carboxylic-acid (50 øM) (ACC; an ethylene precursor) (Sigma-Aldrich, http://www.sigma-aldrich.com) were applied in the 0.35 mM MES pH 5.8 solution. Final concentrations were calculated considering the total volume of solution plus solidified medium in the vessel.

Ethylene was applied to the seedlings at a final concentration of 10 øL L^{-1} by placing the vessels, with lids removed, in an airtight transparent plastic box (125 L), and introducing in the box with a syringe an aliquot from a concentrated ethylene stock. The box was ventilated twice a day and fresh ethylene applied again. We checked by gas chromatography analysis that, using these conditions, ethylene concentration in the box remained essentially constant during the experiments. Control seedlings were grown in vessels placed in a similar plastic box but without applied ethylene.

1-Methylcyclopropene (1-MCP; a gaseous ethylene perception inhibitor) was released from Ethylbloc (Floralife, Walterboro, SC, USA) [0.14% (w/w), 1-MCP] by stirring a water solution of the compound in an airtight flask at 40°C for 12 min. 1-MCP was then collected from the headspace with a syringe and introduced in the 125 L airtight transparent plastic box containing the 4-d-old seedlings (time 0), at final 10 μL. L^{-1} concentration for 6-h treatment. The submergence and hormone treatments were started after that time.

RNA isolation

RNA was isolated using TRIZOL®Reagent according to manufacturer instructions (Invitrogen™ Life technologies, Barcelona, Spain).

Northern-blot analysis

Transcript levels of GA metabolism genes were determined by Northern-blot analysis. Thirty micrograms of total RNA were separated by electrophoresis on 1% (w/v) agarose gel containing 8% (v/v) formaldehyde and blotted to Hybond N^+ membrane (Amersham) in 20X SSC by capillary transfer.

DNA for Northern-blot analysis was produced for each of the following genes of GA metabolism by PCR amplification using rice cDNA from Senia designed from published sequences of *OsGA20ox1* (U50333), *OsGA20ox2* (AY114310) and *OsGA3ox2* (AB056519), and those of rice BAC clones from Monsanto for *OsGA2ox1* (OSM16355), *OsGA2ox2* (OSM13691) and *OsGA2ox3* (OSM126157), using the specific primers described in Supplementary Table 1. DNA probes were labelled with [^{32}P]dCTP using the Ready-To-Go Labelling Beads kit (Amersham Biosciences). After hybridization at 42.C for 1 h in 0.25 M KH_2PO_4/K_2HPO_4 buffer containing 7% SDS, 1 M EDTA, 10% PEG 6000, 0.25 M NaCl, 40% formamide and 0.2 mg/ ml salmon sperm, the filters were washed with 1x SSC, 0.1% SDS at 55°C-60°C then with 0.1X SSC, 0.1% SDS at 60.C for 10-15 min and exposed for autoradiography (Kodak X-OMAT LS, Amersham Biosciences). Prior to any subsequent hybridization, the filters were stripped with hot water containing 0.1% SDS, as recommended by the manufacturer. Equal RNA loading was confirmed by ethidium bromide staining of ribosomal RNAs. mRNA signals in hybridized filters were scanned and quantified by

Phosphorimager analysis using ImageReader and ImageGauge software (Fuji, Tokyo). For quantitative RNA analysis, ratios of gene signals to actin ethidium bromide signals [obtained by RT-PCR as described later, scanned and quantified using the GeneTools (SynGene, Frederick, MD, USA) program] were used. The experiment was repeated using three independent biological replicates with similar results, and relative contents from a representative experiment are given in Results.

RT-PCR analysis

Transcript levels of ethylene metabolism genes (*OsACS1*, *OsACS2*, *OsACS3*, *OsACS4*, *OsACS5*, *OsACO1* and *OsACO2*) were determined by semiquantitative RT-PCR analysis. First-strand cDNA synthesis was carried out using 3 øg of total RNA and a first-strand cDNA synthesis kit (Amersham Biosciences). PCR was performed in 50 øL total volume solution containing 1 øL aliquot of the cDNA reaction, 0.2 øM gene-specific primers (see Supplementary Table 1), 0.2 mM dNTPs, 5% DMSO, 2.5 mM $MgCl_2$, 2.6 units of Expand High Fidelity Taq DNA polymerase (Roche), and 10X reaction buffer. The primers were located at different consecutive exons of each gene to prevent that the PCR products might be produced from contaminating genomic DNA. Thermocycling conditions were: 5 min at 95.C followed by 15 cycles of 95.C/ 45 s, 60-64.C/ 45 s and 72.C/ 45-60 sec, 5-15 cycles of 95.C/ 45 s, 60-64.C/ 45 s and 72.C/ 60-75 sec, and a final extension of 7 min at 72.C. For semiquantitative RNA analysis, ratios of gene to actin ethidium bromide signals were scanned and quantified using the program GeneTools (SynGene). The experiment was repeated using three independent biological replicates with similar results, and relative contents from a representative experiment are given in Results.

Determination of ethylene emanation

Germinated seedlings were placed in 20 mL glass vials (30 seedlings per vial) containing 3 mL of 0.35 mM MES pH 5.8 and 0.45 % agar and cultured at 25 .C in a growth chamber under the temperature and light conditions described before. After four days, half of the vials (five replicates per treatment) were filled up with water and the other half left as control. After 24 h of submergence the water was removed, all the vials sealed with rubber caps

1 (Sigma-Aldrich) and maintained under light. Ethylene emanation was determined taking 1 mL
2 aliquots from the sealed vials with an air-tight syringe after 1, 2, 3, 4 and 6 h incubation to
3 check for linear response of accumulated ethylene, thus discarding possible early post
4 submergence stress ethylene production. The vials were vented between samplings to prevent
5 CO_2 and O_2 accumulation. Ethylene was determined using a TRB-1 TRACER column (60 m
6 long x 0.56 mm internal diameter) at 60.C, connected to a flame ionization detector in a 4890
7 Hewlett-Packard gas chromatograph, and equipped with a 3395 Hewlett-Packard integrator
8 (Palo Alto, CA, USA). Ethylene quantification was carried out by measuring the area of the
9 peak with the same retention time as pure ethylene (0.98 min) and using an ethylene standard
10 curve (0.05 to 5 nl per injection).

11 **Quantification of gibberellins**

12 Fresh material aliquots (100 mg) ground in a mortar with liquid N_2 were extracted in
13 0.5 ml of 80% methanol overnight at 4.C in the presence of internal GA standards (300 pg each
14 of $[^2H_2]GA_1$, GA_8, GA_{19}, GA_{20}, GA_{29}, and GA_{53}, purchased from Prof L. Mander, Australian
15 National University, http://www.anu.edu.au). The mixture was centrifuged for 15 min at 12000
16 g at 4.C, and the pellet washed with 0.5 ml of 80% methanol and centrifuged again. Combined
17 methanolic extracts were taken to dryness under vacuum, dissolved in 50 øL of methanol plus
18 450 L of H_2O pH 8.0 and applied to a pre-equilibrated (5 mL of methanol plus 10 mL of H_2O
19 pH 8.0) Bond Elut SS-SAX column (500 mg; Varian, Middelburg, The Netherlands). The
20 column was washed with 5 mL of H_2O pH 8.0 and GAs were eluted with 4 mL of 0.2 M formic
21 acid. The effluent was passed through a pre-equilibrated (5 mL of methanol and 10 mL of H_2O
22 pH 3.0) Bond Elut C_{18} column (500 mg; Varian, Middelburg, The Netherlands), washed with 5
23 mL H_2O pH 3.0 and GAs eluted with 5 ml 80% methanol before drying in vacuum. The
24 residues were dissolved in 0.5 mL methanol and methylated with ethereal diazomethane before
25 HPLC separation. Methylated dried samples were dissolved in 0.5 ml 10% methanol, injected
26 onto a 4 øm Nova-Pak® C_{18} column (3.9 mm x 150 mm, Waters, Barcelona, Spain) and eluted
27 with a linear gradient of 10% to 100% methanol containing 50 øL L^{-1} acetic acid over 40 min at
28 1 mL min^{-1}. Fractions were grouped according to expected GA retention times and taken to

dryness in vacuo. Dried samples were silylated and used for GA quantification by GC-MS as described by Peng et al. (1999).

Statistical analysis

Statistical analysis of data was carried out with the Student´s *t* test and multiple-range test using the JMP statistical package.

Results

Growth induction of lowland rice by submergence

To characterize the response of lowland rice to submergence, the growth of different organs of cv Senia seedlings (4-d-old at the time of starting the experiment) in air or submerged was determined during 8 d. The first leaf and the second leaf blade did not elongate between d2 and d8, and their lengths were similar in non-submerged and submerged seedlings (Figs 1A and 1B). By contrast, the second leaf sheath (Fig. 1C) and the entire third leaf (Fig. 1D) elongated during that period, much more in submerged that in non-submerged seedlings. This could be detected from 2 d after submergence in the first case, and from 4 d in the second case, with a maximum after 6 d of starting submergence. Similar results were obtained with cv Bomba seedlings (results not presented; see Figs 2B and 5B and D for response to submergence of the second leaf sheath). Therefore, to investigate the mechanism of elongation induced by early submergence in rice the response of the second-oldest leaf sheath was selected. A picture of rice seedlings after 4 d under non-submergence and submergence conditions is presented in Fig. 1E.

Role of gibberellins on submerged induced elongation

The response to 10 øM GA_3 and 1 øM PAC (an inhibitor of GA biosynthesis) of seedlings grown in air or under water for 4 d was investigated. As seen in Fig. 2, GA_3 induced sheath elongation of seedlings of both cultivars grown in air or submerged. PAC inhibited elongation, and this was fully reversed by GA_3 in the case of Senia, and partially in Bomba. Interestingly, application of GA_3 to PAC treated seedlings produced about double elongation in

1 submerged than in air grown seedlings (48.5 vs 25.5 mm in Senia, and 28.1 vs 15.2 mm in
2 Bomba), suggesting that GA responsiveness increased under submergence.

3 To determine whether submergence altered GA content, the levels of GAs from the
4 early-13-hydroxylation pathway, the main GA biosynthesis pathway in rice (Kobayashi et al.
5 1989, 2000), were determined in Senia seedlings after 4 d of submergence (Fig. 3). The material
6 used for GA quantification was composed mainly of organs responding to submergence
7 (second- and third-oldest leaves; see Fig. 1); the inclusion of the minor first-leaf and the second-
8 oldest leaf blade (which did not respond to submergence) should have no significant or only a
9 diluting effect. The content of GA_1 (the active GA in rice) in submerged seedlings was about
10 three times higher than in air-grown seedlings, but that of GA_8 (a GA_1 metabolite) was not
11 significantly different to control seedlings. The concentration of GA_{53} and GA_{19}, two GA_1
12 precursors, also increased (between three and four times) during submergence, whereas that of
13 GA_{20} (the immediate GA_1 precursor) was not altered. The concentration of GA_{29}, a GA_{20}
14 metabolite did not vary either.

15 Relative transcript contents of genes encoding enzymes of GA biosynthesis
16 (*OsGA20ox1* and *-2* and *OsGA3ox1* and *-2*) and catabolism *(OsGA2ox1, -2* and *-3)* were
17 determined by Northern blot analysis in Senia seedlings after 1 h to 4 d of submergence to know
18 whether the effect of submergence on GA levels was the result of a change in expression of
19 those metabolic genes. As shown in Fig. 4, an increase of mRNA levels of *OsGA20ox1* and
20 *OsGA20ox2* was observed, as early as 2 h after starting submergence in the second case (the low
21 transcript levels of *OsGA20ox2* prevented their accurate quantification), and between 4 h and 2
22 d in the first case (with a maximum at 1 d of submergence). Relative content of *OsGA3ox2*
23 transcripts was higher at 2 h and 4 h after submergence (Fig. 4). A transient increase of
24 *OsGA2ox3* transcripts was also found early after starting submergence (between 1 h and 2 h),
25 but it could not be accurately quantified due to relatively low transcript levels (Fig. 4).
26 Expression of *OsGA3ox1*, *OsGA2ox1* and *OsGA2ox2* could not be detected by Northern
27 analysis.

28 **Role of ethylene on induction of elongation by submergence**

When ethylene (10 øL L^{-1}) was applied in the atmosphere it had no effect on sheath elongation of Senia seedlings (Fig. 5A), while those of Bomba elongated significantly more than control, although less than submerged seedlings (Fig. 5B). The effect of ethylene was also analyzed by applying ACC (50 øM) in the medium of air-grown or submerged seedlings. ACC (a precursor of ethylene biosynthesis) had also no effect in Senia -(Fig. 5C), but enhanced elongation of air-grown Bomba seedlings (in this case as much as submergence), but not on submergence-induced seedlings (Fig. 5D). The larger efficiency of ACC compared to ethylene application on elongation of Bomba seedlings is not easy to explain, but it could be due to a supra- or suboptimal dose of applied ethylene. Importantly, the application of 1-MCP (10 øL L^{-1}) (an inhibitor of ethylene action) did not alter sheath elongation of Senia seedlings grown either in air or submerged, in the absence or presence of ACC (Fig. 5C). By contrast, in Bomba, 1-MCP negated the effect of ACC in air-grown seedlings, and abolished almost completely submergence-induced elongation (Fig. 5D). 1-MCP did not affect shoot elongation of air-grown seedlings (Fig. 5D).

To get further insight on the possible effect of ethylene, ethylene emanation (nL g FW^{-1} h^{-1}) was estimated in air grown and submerged rice seedlings by gas chromatography. In Senia, the rate of production of a compound with the same retention time as ethylene was similar in seedlings grown in air and submerged (12-14 nL g FW^{-1} h^{-1}) (Fig. 5E). This means that accumulation of ACC upon submergence and production of ethylene due to de-submergence stress, as described by Voesenek et al. (2003), may not occur or not been detected under our conditions. The amount of ethylene produced by air-grown Bomba seedlings was about half that Senia, and after one day of submergence it was twice that of non-submerged seedlings (Fig. 5E).

We also investigated, by semiquantitative RT-PCR, the effect of submergence on the expression of genes encoding the ethylene biosynthetic enzymes ACC synthases (*OsACS1*, *-2* and *-5*) and ACC oxidases (*OsACO1* and *-2*). In Senia, *OsACS* transcript levels did not increase (*OsACS2*) or very little *(OsACS1* and *-5*; relative contents in submerged seedlings were always ª twice the controls) (Fig. 6A). A transient decrease in *OsACS2* mRNA content was observed

during the first two hours of submergence. *OsACO1* transcript content remained essentially unaltered during the first 8 h of submergence and decreased afterwards (Fig. 6A). Expression of *OsACO2* was not detected either in control or submerged seedlings (data not presented). In Bomba, the effect of submergence on *OsACS1* and *-2* expression was similar to that described before for Senia, except for a transient increase of *OsACS1* transcripts at 1 d (compare Figs. 6A and 6B). In contrast, increase of *OsACS5* transcript content was much higher in submerged Bomba than in Senia seedlings at all times between 1 h and 4 d of submergence (with a maximum of about five times at 2 h) (Fig. 6B). *OsACO1* transcripts decreased slightly from 4 h after starting submergence in Bomba, earlier than in Senia (from 1 d in this case) (Fig. 6B). *OsACO2* expression was relatively higher in Bomba than in Senia, but we did not find any effect of submergence (Fig. 6B).

Discussion

Submergence induces GA-dependent elongation and increases GA_1 content by altering GA metabolism

We found that 4-d-old rice seedlings of Senia and Bomba, two short lowland cultivars var. japonica, grown under water displayed higher elongation of their second and third leaves (Figs 1, 2 and 5). allowing the young plants to escape from submergence early after germination in shallow waters. GA_3 enhanced and PAC (an inhibitor of GA biosynthesis) application negated sheath elongation in seedlings grown either in air or submerged (Fig. 2), suggesting that the elongation depends on GA under both culture conditions. Application of GA and PAC has also been shown previously to alter leaf elongation of young lowland rice seedlings grown in air and submerged (Setter and Laureles, 1996; Furukawa et al., 1997; Van Der Straeten et al., 2001). Our hypothesis was further confirmed by showing that GA_1 concentration, the dominant bioactive GA in vegetative organs of rice (Kobayashi et al., 1989) increased significantly in the aerial part of submerged Senia seedlings (up to five times after four days) as well as its precursors GA_{53} and GA_{19}, and its metabolite GA_8 (Fig. 3). Surprisingly, in contrast to our results, GA_{19} and GA_{20} levels (GA_1 concentration was not determined) were not altered upon

submergence in lowland IR36 seedlings (Van Der Straeten et al., 2001). This lack of effect may be due to the older seedlings (9-d-old seedlings vs barely germinated seeds in this work), or to the var. indica rather than japonica used in this work. Increase of GA_1 content in petioles of flooding-tolerant *Rumex palustris*, but not in flooding-intolerant *Rumex acetosa* has also been observed (Rijnders et al., 1997).

The higher levels of *OsGA20ox1*, *OsGA20ox2* and *OsGA3ox2* mRNA during submergence of Senia seedlings (Fig. 4) suggest that GA_1 accumulation was probably the result of enhanced expression of those GA biosynthesis genes. On the other hand, since the expression of *GA2ox3* (the main *GA2ox* gene in rice) apparently did not decrease, this indicates that reduction of GA catabolism may not be involved in the increase of active GA upon submergence. However, although GA 2-oxidase activity is the main GA inactivation pathway, the possibility that other kinds of GA inactivating enzymes might contribute to GA_1 content modification (Yamaguchi, 2008) can not be excluded. Our results agree with previous reports showing that *OsGA20ox1*, *OsGA20ox2* and *OsGA3ox2* are involved in vegetative development of rice (Itoh et al., 2001; Oikawa et al., 2004). As expected, *OsGA3ox1* mRNA was not detected in rice seedlings probably because its expression is limited to flowers and epithelium (Kaneko et al., 2003). This excludes the implication of *OsGA3ox1* in submergence rice response. On the other hand, the higher response to GA_3 of submerged compared to air-grown seedlings in the presence of PAC (Fig. 2) indicates that, in addition to increasing active GA concentration, submergence also increases GA response, as occurs in deepwater rice internodes (Raskin and Kende, 1984).

Interestingly, a transient increase of *OsGA2ox3* mRNA accumulation was observed during early submergence (first two hours), followed by a drop to control levels (Fig. 4). Two other *OsGA2ox* (*OsGA2ox1* and *-2*) presented the same transient mRNA accumulation pattern (results not shown). The accumulation of *OsGA2ox* mRNA during early submergence could be due to a general stress response leading to temporary decrease of growth, later counteracted by the positive elongation response induced by submergence.

Submergence-induced elongation in Senia does not depend on ethylene

It is currently accepted that ethylene accumulated as a result of both physical entrapment and enhanced synthesis is the signal triggering the transduction pathway leading to elongation under submergence (Jackson, 2008). Induction of elongation under submergence driven by ethylene has been observed in species from genera such Callitriche, Ranunculus and Rumex (Voesenek and Blom, 1999), in deep-water rice (Kende et al., 1998; Van der Straeten et al., 2001; Vriezen et al., 2003) and in diverse lowland rice cultivars (Jackson et al., 1987; Furukawa et al., 1997; Van Der Straeten et al., 2001). In the tall lowland rice cultivar Bomba the submergence-induced elongation was also mediated by ethylene because: a) the application of ACC or ethylene to non-submerged seedlings induced sheath elongation (in the case of ACC to a value similar to that obtained by submergence); b) purported ethylene emanation capacity increased more than twice upon submergence; and c) the effect of submergence was almost completely negated by 1-MCP (an inhibitor of ethylene action) (Figs 5B, 5D and 5E). The application of ACC to submerged seedlings did not produce additional elongation (Fig. 5D), probably because the concentration of ethylene in those seedlings was saturating under flooding conditions. The problems raised in the determination of ethylene production during submergence have been investigated in Rumex (Voesenek et al., 2003, and references herein). In our case, our determination conditions discarded trapped ethylene, as well as the possibility of ethylene produced from previously accumulated ACC or de-submergence stress. Although levels of gene expression are not necessarily correlated with protein level or activity, it is of interest that the enhanced ethylene emanation in Bomba upon submergence was associated with a rapid (within 1 h) increase of *OsACS5* transcript levels (up to five-fold after 2 h). A temporary increase of *OsACS1* transcript level (up to three fold) after long-term (24 h) submergence was also observed (Fig. 5B). De novo biosynthesis of ethylene in submerged IR36 seedlings is also associated with induction of *OsACS5* within 1 h of submergence, localized in vascular bundles of leaf sheaths and young stems (Van Der Straeten et al. 2001, Zhou et al. 2002), while increased expression of *OsACS1* occurs only after long time submergence (between 12 h and 2 d). All these results suggest that in Bomba *OsACS5* and *OsACS1* play roles in ethylene biosynthesis associated with early and sustained submergence, respectively.

1 By contrast, the following evidence suggests that enhancement of seedling elongation
2 under submergence in the cv Senia is not mediated by ethylene: a) air-grown seedlings did not
3 respond to ethylene and ACC; and b) elongation of air-grown and submerged seedlings was not
4 reduced by 1-MCP application (Figs 5A and C). Also, purported ethylene emanation determined
5 in submerged plants following de-submergence was similar to ethylene emanation in seedlings
6 maintained in air (Fig. 5E). Moreover, the expression of diverse genes encoding ethylene
7 biosynthesis enzymes (*OsACS1*, *-2* and *-3* and *OsACO1*) did not increase in submerged
8 seedlings (Fig. 6A). Only *OsACS5* expression increased, although for a short time (between 1
9 and 2 h of submergence) and much less than in Bomba (always less than twice the level in
10 control). It could be argued that ethylene is produced at an optimal level in Senia and that this is
11 the reason why applied ethylene had no effect on elongation. But this possibility is not
12 supported by the fact that 1-MCP did not reduce elongation of air-grown or submerged
13 seedlings.
14 No reports are found in the literature on expression of genes encoding ACC oxidases in
15 lowland rice. We found that *OsACO1* transcript levels decreased after 8 h (cv Bomba) and 1 d
16 (cv Senia) of submergence, while those of *OsACO2*, the second gene encoding ACO in rice, did
17 not vary significantly upon submergence in Bomba (Fig. 6) and were not detected in Senia.
18 Therefore, ethylene synthesis in lowland rice seedlings upon submergence does not seem to be
19 regulated by enhanced expression of *ACO* genes.
20 In conclusion, we show in this work that submerged-induced seedling elongation of the
21 lowland rice cvs Senia and Bomba is due to enhanced GA biosynthesis leading, at least in the
22 case of Senia, to higher GA_1 concentration as a result of increased expression of the GA
23 biosynthesis genes *OsGA20ox1*, *OsGA20ox2* and *OsGA3ox2*. But, contrary to cv Bomba, in cv
24 Senia this effect does not seem to be mediated by ethylene. In *Potamogeton pectinatus*, another
25 monocotyledon, submergence-induced response does not depend on ethylene (Voesenek and
26 Blom, 1999) but is driven by the increase in acidity produced by CO_2 accumulation (Summers
27 and Jackson, 1996). It is thus tempting to speculate that an increase in acidity produced by CO_2
28 accumulation might be the ethylene-independent signal triggering GA biosynthesis in the cv

Senia. Hypoxia (3% O_2) enhanced sheath elongation of both Senia and Bomba seedlings (data not presented). However, although we do not know the actual O_2 levels in submerged seedlings, it is highly unlikely that this might be the initiating factor, because those seedlings should be photosynthetically active under water and accumulate oxygen. In any case, regardless of the molecular mechanism responsible of GA metabolism alteration, our results indicate that submergence-induced elongation in lowland rice may not necessarily be mediated by ethylene.

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1 **Figure legends**

2 **Figure 1.** Growth response of 4-d-old lowland rice cv Senia to submergence. Seedlings were 3 submerged at time 0, or maintained in air for 8 d and the length of different leaves (numbered 4 from the base) was measured every 2 d. (A) First-oldest leaf; (B) Blade of second-oldest leaf; 5 (C) Sheath of second-oldest leaf; (D) Third-oldest leaf; (E) Picture of representative seedlings 6 grown for 4 d in air or under water. The arrows indicate the junction of insertion between blade 7 and sheath of the second-oldest blade. At each point, data are mean values µ SD from at least 15 8 seedlings. In C and D, the differences between submerged and control data were at all times 9 significantly different ($P<0.05$).

10 **Figure 2.** Effect of GA_3 and PAC application on elongation of the second-oldest leaf sheath of 11 Senia (A) and Bomba (B) seedlings grown in air or submerged. The treatments (start of 12 submergence and application of 10 øM GA_3 and/or 1 øM PAC) were carried out on 4-d-old 13 seedlings, and sheath length was measured 4 d later. Data are mean values µ SD from at least 15 14 seedlings. At each experiment, values with different letters were significantly different 15 ($P<0.05$). C, control; PAC, paclobutrazol. At each experiment, values marked with different 16 letters were significantly different ($P<0.05$).

17 **Figure 3.** Concentration (pg mg FW^{-1}) of GAs in air-grown and submerged Senia seedlings. 18 Entire aerial part was collected for GA analysis from seedlings after 4 d of submergence. Data 19 are mean values µ SD from four biologically independent determinations. Asterisks note values 20 significantly different to control ($P<0.05$).

21 **Figure 4.** Time-course effect of submergence on transcript levels of *OsGA20ox1*, *OsGA20ox2*, 22 *OsGA3ox2* and *OsGA2ox3* in Senia seedlings. Total RNA was isolated from seedlings 23 submerged or not for 1 h, 2 h, 4 h, 8 h, 1 d, 2 d and 4 d and analyzed by Northern blot (30 øg 24 RNA per lane). Transcript levels were estimated as described in Materials and methods using 25 RNA from three independent biological samples, with similar results. Numbers below 26 submergence lanes are relative transcript levels compared to the corresponding control time 27 seedlings grown in air, from a representative experiment (upper panel). In the case of *Os20ox2* 28 and *Os2ox3* their relatively low transcript levels prevented accurate quantification. In the lower

panels, transcript level in the control sample for each sampling time was set to 1, and level in the submerged sample was calculated relative to the corresponding control value.

Figure 5. Ethylene and elongation of Senia (A, C, E) and Bomba (B, D, E) seedlings under submergence. (A, B) Time-course of sheath elongation in control and in response to submergence and ethylene (10 øL. L^{-1}). Treatments were started at day 0 (4-d-old seedlings) and maintained for the duration of the experiment (8 days). At each point, data are mean values µ SD (n º 15). (C, D) Effect of ACC (50 øM) and 1-MCP (10 ppm) on elongation of seedlings grown in air or submerged. Treatments were carried out on 4-d-old seedlings (day 0) and length of the second-oldest leaf sheath was determined 4 d later. Data are mean values µ SD (n º 15). (E) Ethylene emanation (nl g FW^{-1} h^{-1}) from submerged and non-submerged Senia and Bomba seedlings. 4-d-old seedlings were non-submerged or submerged during 1 d and the water removed before quantification of ethylene emanation. Data are mean values µ SD (n = 5, 30 seedlings per replicate). At A, B and E asterisks indicate values significantly different to control (P<0.05). At C and D, at each experiment, values with different letters were significantly different (P<0.05). C, control; S, submerged; E, ethylene. Asterisks note values significantly different to control (P<0.05)

Figure 6. Time-course of the effect of submergence on transcript levels of *OsACS1*, *OsACS2*, *OsACS5* and *OsACO1* in Senia (A) and Bomba (B) seedlings. In the later case, *OsACO2* transcripts were also determined. Transcript levels were estimated by semiquantitative RT-PCR analysis, as described in Materials and methods. Numbers below submergence lanes are relative transcript levels compared to the corresponding control time seedlings grown in air, from a representative experiment (upper panel). See legend of Fig. 4 for further details. In the lower panels, transcript level in the control sample for each sampling time was set to 1, and level in the submerged sample was calculated relative to the corresponding control value.

1 **Supplementary Table 1.** Primer sequences used to produce DNA probes for Northern-blot

2 analyses and for semiquantitative RT-PCR

3

	Sense (5´- 3´)	Antisense (5´- 3´)
OsGA20ox1 **(U50333)**	**GATCCCGTCGCAGTTCATAT**	**TCATCTCCAGCCGATGAGTA**
OsGA20ox2 **(AY114310)**	**CAACTCACTCCCGCTCAACACAGC**	**CTTCATCTCCTCGCAGACTTCT**
OsGA3ox2 **(AB056519)**	**CACTTGAAGAACCCGCTCTG**	**GTGCCCGACCAGAAGGAAC**
OsGA2ox1 **(OSM 16355)**	**ACGATAGCGACGGTGGACAT**	**TATGCTTTTCCCTCACTGGC**
OsGA2ox2 **(OSM 13691)**	**GAGCTCGAGCAGATAGCCC**	**AAATCTGCAGAGCCTGTCGT**
OsGA2ox3 **(OSM 126157)**	**TCTTCAGGGCCGTCAACC**	**GCGTGTACATGGTCCTCT**
OsACS1	**TGGTCGCCGAGGAGAAGCCGCAG**	**GTGGCGGAGTCGACGAACGACC**
OsACS2	**GCATCGACCTTCTCTCGACGAAAGC**	**ATGGGCTGGGCTGAGGTGGTGG**
OsACS3	**ATGGCGATGTCGGCGGCTCACGGC**	**AGCAGCCCAGTCTATGGAGTGC**
OsACS4	**CTGACGGCTGCGCCGGCGCCTCG**	**TTCCCAATTGTTGCTTTGCACCAC**
OsACS5	**CCTCACCTTCATCCTCGCCGACCCC**	**GCTGGCAGCTAGCTTGTTGCTTTGTTCC**
OsACO1	**ATGGCACCGACTTCGACGTTCCC**	**CCTGATGTCGTCGTCGAGGT**
OsACO2	**GATCAACATGGAGAACCTGGA**	**AGGTGCTCTCCCAGTCGAT**

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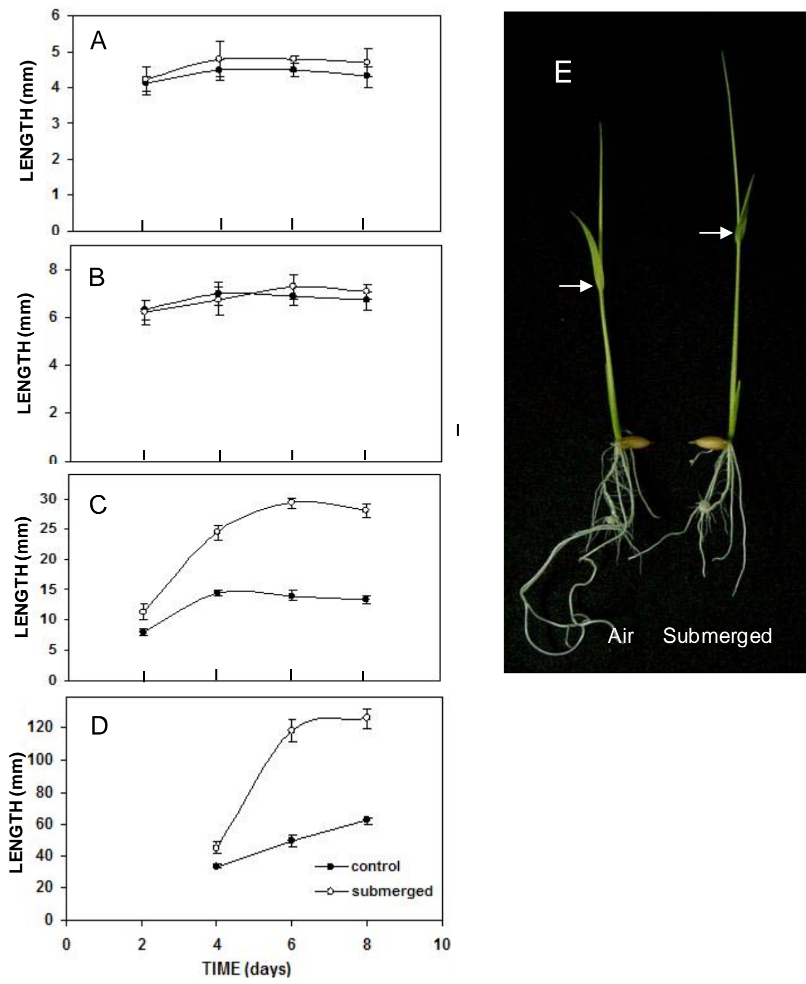
2 Figure 1



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1 Figure 2

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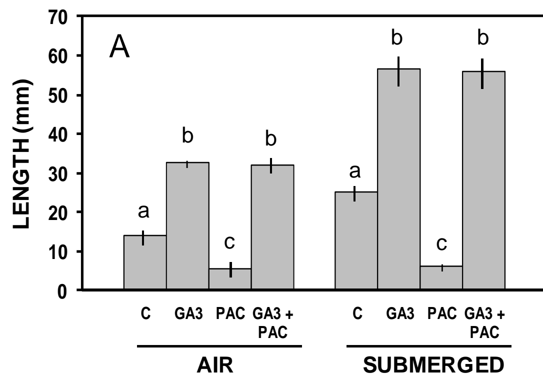

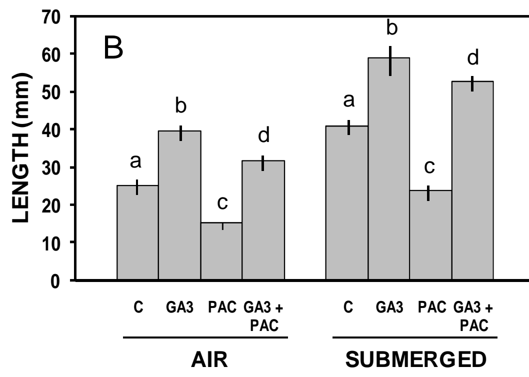

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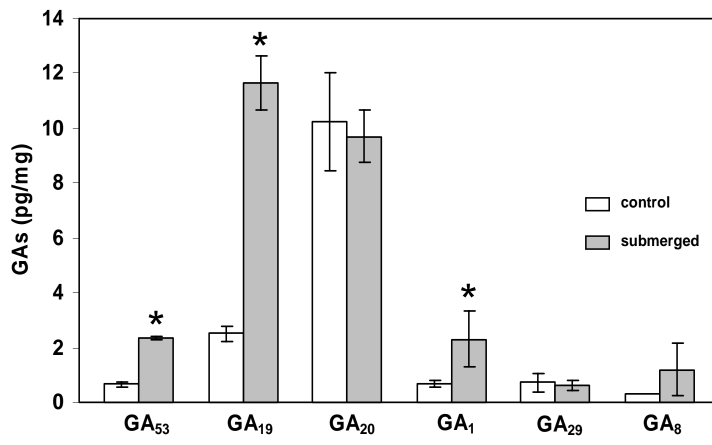

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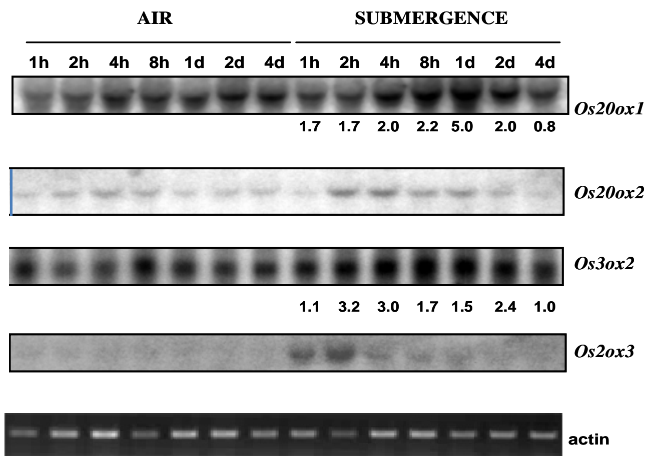

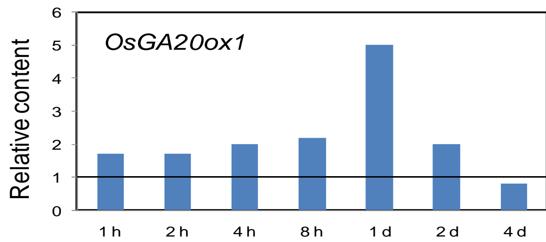

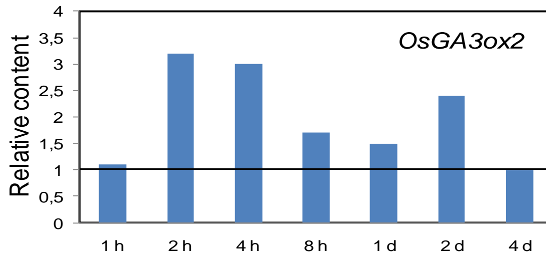

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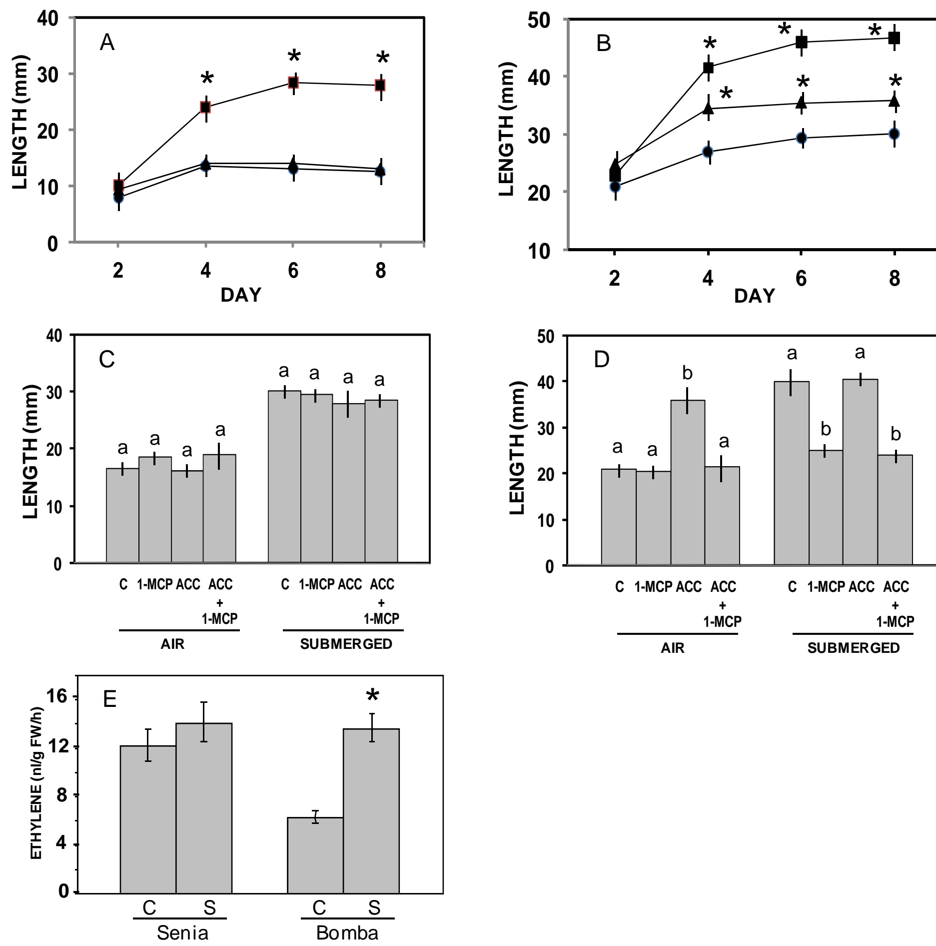

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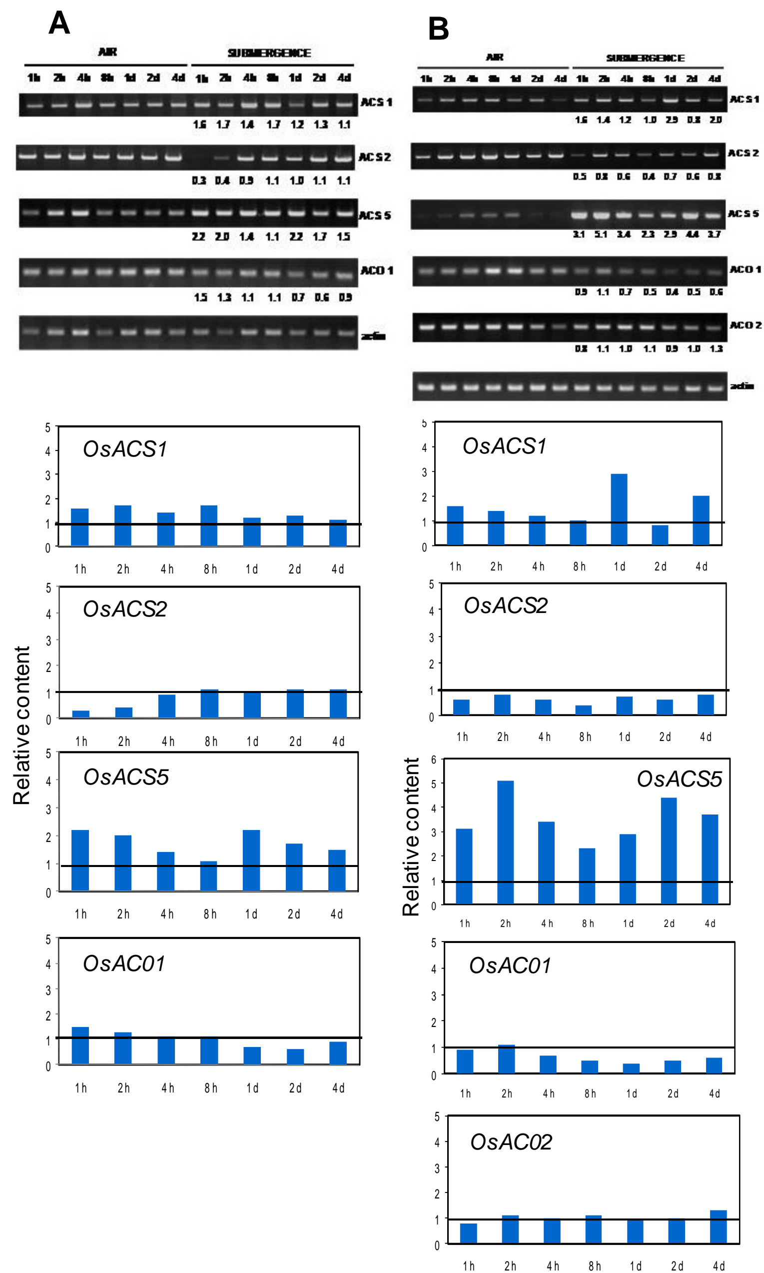

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