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RESEARCH PAPER

RP-ACS1, a flooding-induced 1-aminocyclopropane-1-carboxylate synthase gene of *Rumex palustris*, is involved in rhythmic ethylene production

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Abstract

Many semi-aquatic plants respond to flooding by elongating the shoot to reach the water surface. This response is initiated by accumulation of ethylene in the plant due to decreased gas-exchange and continued ethylene production during submergence. Ethylene biosynthesis is often limited by the availability of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, synthesized by ACC synthase. Here, is reported the cloning of a *Rumex palustris* cDNA corresponding to an ACC synthase gene (*RP-ACS1*), whose expression is induced by submergence in the long term but does not precede the observed short-term increase in ACS activity. Under aerated conditions, *RP-ACS1* messenger accumulation exhibited circadian rhythmicity with high levels in the dark phase and low levels in the light phase, similar to the oscillations in ethylene production under these conditions. ACC oxidase (*RP-ACO1*) messenger accumulation also showed a rhythmic pattern, but opposite to that of *RP-ACS1*, and closely resembled the ethylene oscillation found in *R. palustris* plants that were waterlogged. Together the results indicate that transcriptional regulation of *RP-ACS1* may directly control rhythmic ethylene production under aerated condition and suggest that post-transcriptional regulation is important in initial up-regulation of ACS activity upon submergence.

Key words: Ethylene, ACC synthase, submergence, *Rumex*, circadian rhythm.

Introduction

The gaseous plant hormone ethylene has been shown to influence many processes in plants such as germination, root-hair initiation, flower senescence, and fruit ripening. Furthermore, responses to a wide variety of stresses such as drought, wounding, pathogen attack, and waterlogging depend on ethylene and are initiated by an increase in ethylene biosynthesis (Jackson, 1985; Abeles *et al*., 1992). The ethylene biosynthetic pathway has been extensively studied in many plant species (Yang and Hoffman, 1984; Kende, 1993; Fluhr and Mattoo, 1996). The precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), is produced via conversion of *S*-adenosylmethionine (SAM) by ACC synthase, and ACC is converted to ethylene, CO_2, and HCN by ACC oxidase in an O_2-dependent process. In a number of plants ACC synthase and ACC oxidase proteins are encoded by gene families whose members are differentially regulated (Fluhr and Mattoo, 1996). Generally, the rate-limiting step in ethylene biosynthesis is the conversion of SAM to ACC (Yang and Hoffman, 1984), and an increase in ethylene production is often preceded by induction of ACC synthase genes (Rottmann *et al*., 1991; Botella *et al*., 1993; Oetiker

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et al., 1997), although post-transcriptional regulation of ACC synthase has also been observed in some cases (Spanu *et al*., 1994; Vogel *et al*., 1998). ACC oxidase acitvity can be limiting in ethylene biosynthesis in situations of high ethylene production or low oxygen concentration (English *et al*., 1995; Yamamoto *et al*., 1995; Barry *et al*., 1996; Lasserre *et al*., 1996; Vriezen *et al*., 1999).

Rumex palustris is a semi-aquatic plant that grows mainly in flooding-prone river areas, and serves as a model species for studying the physiology of flooding tolerance (Peeters *et al*., 2002; Voesenek *et al*., 2003*a*). Hyponastic growth and rapid elongation of the petiole cells are major processes which keep the foliage of *R. palustris* plants above the rising water surface (Voesenek *et al*., 1990*b*; Cox *et al*., 2003). Ethylene plays an important role in the induction and maintenance of this response, as it does in other semi-aquatic and amphibious species (Musgrave *et al*., 1972; Metraux and Kende, 1983; Blom *et al*., 1994). Furthermore, in *R. palustris*, enhanced rates of ethylene production are important for fast elongation to continue when shoot tips emerge from the floodwater (Voesenek *et al*., 2003*b*). It has been reported previously that, upon submergence, the conversion rate of ACC to ethylene decreases, although ACO transcript level and *in vitro* protein activity increase (Banga *et al*., 1996; Vriezen *et al*., 1999). The rate of conversion of SAM to ACC is temporarily induced by submergence, but the contributions of the *ACS* gene and the activity of the corresponding protein are unkown (Banga *et al*., 1996).

Under drained conditions, *R. palustris* produces ethylene at a low level. However, when the soil is saturated with water (waterlogging) ethylene biosynthesis is induced and the ethylene release shows a clear rhythm with peaks during the day (Voesenek *et al*., 1990*a*). Rhythmic ethylene production has been observed in various species (El-Beltagy *et al*., 1976; Rikin *et al*., 1984; Ievinsh and Kreicbergs, 1992; Kathiresan *et al*., 1996; Machácková *et al*., 1997; Beßler *et al*., 1998; Finlayson *et al*., 1998; Dziubinska *et al*., 2003; Yamasaki *et al*., 2003) and can have a circadian nature (Rikin *et al*., 1984; Finlayson *et al*., 1998; Dziubinska *et al*., 2003), or be under strict control of the light (Beßler *et al*., 1998). The conversion step at which rhythmic ethylene production is regulated also differs between species. As expected, in most species ACC synthesis seems to determine the formation rate of ethylene (Rikin *et al*., 1984; Machácková *et al*., 1997; Beßler *et al*., 1998; Yamasaki *et al*., 2003). However, fluctuations in ACC oxidase activity are responsible for generating the ethylene rhythm in an ethylene-overproducing cultivar of *Sorghum bicolor* (Finlayson *et al*., 1999) and may also be in *Stellaria longipes*, in which ACC oxidase activity oscillates in phase with ethylene production (Kathiresan *et al*., 1996).

To study the role of ACC synthase in ethylene production in *R. palustris* during submerged and non-submerged conditions, the most abundant *R. palustris* ACC synthase gene, *RP-ACS1*, was identified, and its expression compared with ACC synthase activity and ethylene production. Expression of *RP-ACS1* was induced by submergence, but the induction did not coincide with induction of ACS enzyme activity. Under aerated conditions *RP-ACS1* mRNA concentration fluctuated in a rhythm that was highly similar to the rhythm observed in ethylene production.

Materials and methods

Plant material and growth conditions

Achenes of *Rumex palustris* Sm. were collected from river areas near Millingen, The Netherlands. Germination and growing conditions were as described by Banga *et al*. (1996). All of the plants used for experiments were 26–30-d-old. Plants were grown under alternating light regimes (16 h light; PPFD 65 μmol m^{-2} s^{-1}; 8 h dark) at a constant temperature of 22 °C. For the experiments involving submergence, plants were kept under constant light and temperature, starting 24 h before submergence. Submergence took place in an open tank with 25 cm tap water at 22 °C. At several time points after submergence shoots or petioles and lamina of leaf 4, the youngest fully developed leaf, were cut and directly frozen in liquid nitrogen. Northern analysis showed that the expression patterns of the genes studied were comparable in leaves 3, 4, and 5 of 4-week-old *R. palustris* plants (data not shown). For the experiments under aerated conditions, the alternating light regime was continued for two full cycles and then the regime was changed to constant light, all at constant temperature.

Isolation of ACC synthase cDNA fragments

Primers TZ-1F$^{\alpha}$ and TZ-2R, which were based on conserved ACC synthase protein sequences, have been described before (Zarembinski and Theologis, 1993). Degenerated primers RP-SYN2 and RP-SYN3 were based on conserved DNA sequences of known ACC synthase sequences obtained from GenBank (RP-SYN2: 5′-CCCAKCRG-CYTCAATYTGYAC-3′; RP-SYN3: 5′-CCRAYTCKRAADCCW-GGBARSCCCAT-3′ using IUB codes). cDNA was isolated from an *R. palustris* cDNA Uni-ZAP XR library (Vriezen *et al*., 1997) using the mass excision protocol of the manufacturer (Stratagene). The isolated pBluescript phagemid was used for PCR reactions after phenol–chloroform extraction and ethanol precipitation. A 200 ng aliquot of this DNA was used for a PCR reaction in 100 μl PCR buffer with 1.25 mM $MgCl_2$, 200 pmol of each dNTP, 100 pmol of each primer RP-SYN2 and RP-SYN3, and 0.8 units of thermostable DNA polymerase (Goldstar, Eurogentec). Thirty cycles of 1.5 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C were performed, preceded by 4 min at 96 °C, and terminated with 10 min at 72 °C, in a Thermal Cycler (Perkin Elmer).

A second PCR reaction was performed with 200 ng of the pBluescript phagemid in 100 μl PCR buffer with 1.5 mM $MgCl_2$, 200 pmol of each dNTP, 100 pmol of each primer TZ-1F$^{\alpha}$ and TZ-2R, and 0.4 units of thermostable DNA polymerase. The amplification reaction consisted of five cycles of 1.5 min at 94 °C, 1 min at 37 °C, and 1 min at 72 °C, followed by 35 cycles of 1.5 min at 94 °C, 1 min at 45 °C, and 1 min at 72 °C, preceded by 4 min at 96 °C, and terminated with 10 min at 72 °C. A 5 μl aliquot of this reaction mixture was re-amplified under the same conditions for 35 cycles. After the DNA fragment size was verified, the PCR products (a 152 bp fragment with the TZ-1F$^{\alpha}$ and TZ-2R primers and a 660 bp fragment with the RP-SYN2 and RP-SYN3 primers) were cloned into the pCRII vector using a TA Cloning Kit (Invitrogen) and sequenced.

cDNA library screening

Using a *R. palustris* cDNA library (Vriezen *et al*., 1997) approximately 0.8×10^6 plaques were screened with a [α-^{32}P]dATP-labelled 660 bp ACC synthase PCR fragment according to the manufacturer's protocol (Statagene). Filters were prehybridized for 1 h and hybridized overnight at 65 °C with a solution containing 5×SSC, 5×Denhardt's reagent, 0.5% (w/v) SDS, and 100 μg ml^{-1} denatured, fragmented salmon sperm DNA. Membranes were then washed twice in 2×SSC plus 0.1% (w/v) SDS at 65 °C for 15 min each and twice in 0.2×SSC plus 0.1% (w/v) SDS for 15 min. The blots were exposed to film (X-Omat AR, Kodak) with two intensifying screens at −80 °C for 16–48 h. After three rounds of screenings 13 positive plaques were identified and 10 were sequenced and proved to be identical ACC synthase homologues (*RP-ACS1*; Genbank accession number AF038945).

DNA and RNA isolation and blot hybridization analysis

Genomic DNA was isolated according to Van Eldik *et al*. (1995). Southern blotting, DNA fixation, and hybridization were performed on a nylon membrane (Hybond-N, Amersham) according to the manufacturer's directions. The full-length *RP-ACS1* cDNA and the pcr7Rp fragment were labelled with [α-^{32}P]dATP by the random-priming method, and used as probes. Prehybridization, hybridization, and washing conditions were the same as described for the library screening.

For RNA gel blots, total RNA was isolated according to Van Eldik *et al*. (1995) and separated on a 1% (w/v) agarose gel containing 0.4 M formaldehyde and 0.1 μg ml^{-1} ethidium bromide. RNA was transferred to a nylon membrane (Hybond-N, Amersham) according to the manufacturer's directions. Prehybridization, hybridization, and washing conditions were the same as described for the library screening. Hybridizations were performed using the full-length cDNAs *RP-ACS1*, *RP-ACO1* (accession no. Y10034), *RP-CAB1* (accession no. AF165529), or *Rp-EXP1* (accession no. AF167360) as probes. The blots were exposed to film (X-Omat AR, Kodak) with two intensifying screens at −80 °C for 10–14 d (*RP-ACS1* and *Rp-EXP1*) or 1–2 d (*RP-ACO1* and *RP-CAB1*). For hybridization with the *RP-ACO1* probe, the blots used for *RP-ACS1* hybridization were stripped and rehybridized with labelled *RP-ACO1*, which was later repeated for *Rp-EXP1*. Finally, hybridization with a tobacco ribosomal cDNA (kindly provided by Dr K Weterings, University of Nijmegen) was performed to confirm equal loading of the membranes. The autoradiographs were scanned with a densitometer (Molecular Imager FX, Bio-Rad), and the signals were quantified (Molecular Analyst, Bio-Rad). The value for the messenger concentration is the density of the band on the autoradiograph corrected for the density of the 28S band. All analyses of messenger concentrations were done at least twice with different plants in different experiments to ensure that the observed patterns of the mRNA concentration were reproducible.

Analyses of in vitro *ACC synthase capacity*

Shoots were excised at the soil surface, weighed, frozen in liquid N_2 and subsequently stored at −80 °C. Prior to extraction, samples were ground in liquid N_2. Half a millilitre of extraction buffer (600 mM TES pH 8.5, 5 mM DTT, 10 μM pyridoxal-5-phosphate; pH adjusted using KOH) was added per gram fresh weight. The slurry was centrifuged at 20 000 *g* for 20 min at 4 °C and the supernatant dialysed overnight against two changes of buffer (10 mM TES pH 8.5, 10 μM pyridoxal-5-phosphate) at 4 °C. A minimum of 25 ml of incubation dialysis buffer was used per millilitre of supernatant. In the assay, 150 μl extraction buffer was added to 1 ml supernatant and 100 μl AdoMet solution (10 mM). The vial was closed air-tight with a septum and the reaction mixture was placed on an orbital shaker at 30 °C for 1 h. ACC was determined by the method of Lizada and Yang (1979) with internal standardization (ACC from Sigma). ACC was chemically converted into ethylene, which was measured with a gas chromatograph [Synspec GC 955-100 equipped with a photo ionization detector and a stainless-steel column (0.12×2.0 m) filled with Haysep R mesh 80/100 (temperature 105 °C; carrier gas N_2)]. This experiment was repeated with similar results. To test for possible differences in the recovery of ACC synthase activity from tissues isolated under control conditions (low expected activity) and after 2 h of de-submergence (high expected activity), equal masses of tissue homogenates were mixed and the ACC synthase activity was determined. The activity varied between 2% and 4% of the expected activity calculated for the mixed samples.

Ethylene measurements

For ethylene measurements, plants were transferred from a tray to a Petri dish (two plants per Petri dish) after gently removing the surplus of soil, which was not in contact with the roots. The Petri dishes were placed in cuvettes and the open cuvettes were kept in the growth chamber used for ethylene measurements for 2 d to acclimatize the plants. Some of the removed soil was put in a Petri dish to serve as a control in the measurements. Plants were kept under alternating light regimes (16 h light; PPFD 150 μmol m^{-2} s^{-1}; 8 h dark) and later in the experiment under continuous light, always at a constant temperature of 21 °C. Plants were watered well to prevent closure of the stomata in the light. Ethylene measurement was performed using a sensitive laser-based photoacoustic detector in combination with a gas flow-through system (flow of 2.5 l h^{-1}), as described before (Montero *et al*., 2003 and references therein). Measurements were done twice, with three cuvettes in each measurement (two with plants and one with soil); representative data are shown in the Results.

Results

Cloning of ACC synthase cDNAs from R. palustris

Partial cDNAs encoding ACC synthase were generated by PCR with degenerated primers on a cDNA library constructed from leaf RNA isolated from *R. palustris* plants submerged for 24 h. Two combinations of degenerated oligonucleotides were used for this reaction. The primer combinations TZ-1F$^{\alpha}$/TZ-2R and RP-SYN2/RP-SYN3 led to the synthesis of a 152 bp and a 660 bp fragment, respectively. Sequence analysis of several subclones of these fragments demonstrated that they were all 100% homologous in the overlapping sequences, indicating that they all derived from the same cDNA. The 660 bp fragment showed high homology to known ACC synthase sequences from other species and was subsequently used to isolate full-length clones from the same *R. palustris* cDNA library. Ten of the clones obtained proved to be identical in sequence, and the corresponding gene was designated *RP-ACS1*. The deduced RP-ACS1 protein consists of 489 amino acids and has a molecular weight of 55 kDa and a pI of 6.89. It contains all seven domains that are conserved in many ACC synthases (Dong *et al*., 1991; Theologis, 1992) and also the 11 amino acids conserved in various aminotransferases (Rottmann *et al*., 1991). The RP-ACS1 protein shares highest similarity (68%) with a functional ACC synthase from citrus (CS-ACS1; Wong *et al*., 2001).

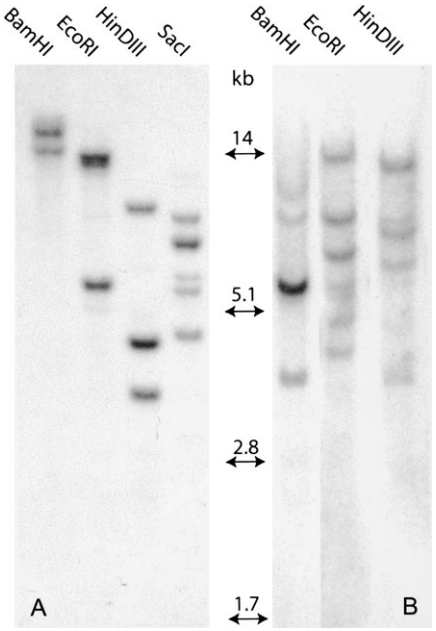

Fig. 1. DNA gel blot analysis of the ACC synthase gene family in the *R. palustris* genome. Ten micrograms of digested DNA was loaded per lane. Molecular length standards are indicated in the middle (kb). Probes were (A) the full-length *RP-ACS1* cDNA or (B) PCR-fragment pcr7Rp.

Furthermore, sequencing of PCR fragments pcr2Rp and pcr7Rp (accession nos AF041480 and AF041481) amplified from *R. palustris* genomic DNA with primer combination TZ-1F$^{\alpha}$/TZ-2R showed the existence of at least two additional ACC synthase genes. Southern analysis of genomic DNA using the full-length *RP-ACS1* cDNA and pcr7Rp as probes confirmed the presence of a multi-gene ACC synthase family (Fig. 1). However, PCR with the same TZ-1F$^{\alpha}$/TZ-2R primers on cDNA isolated from the cDNA library, and RNA gel-blot analyses of *R. palustris* RNA using the pcr2Rp or pcr7Rp fragment as a probe, did not produce evidence for the presence of messengers that could correspond to the other two genes, either under submerged or non-submerged conditions.

RP-ACS1 *expression upon submergence*

RP-ACS1 messenger could be detected in total shoots (five petioles + five leaf blades) and in the roots of young *R. palustris* plants that were submerged and kept under constant light and temperature. Figure 2A shows that the *RP-ACS1* mRNA level in the shoot remained practically constant during the 12 h of submergence, and started to accumulate to higher levels at 24 h that persisted up to 48 h. *RP-ACS1* was also expressed in the roots during this treatment, but no increase was observed after submergence. By contrast, direct measurement of ACC synthase activity in shoots of submerged and non-submerged plants showed that activity of the protein was strongly enhanced after 6 h of submergence, after which it declined again (Fig. 2B). The plants used in all these experiments were composed of five

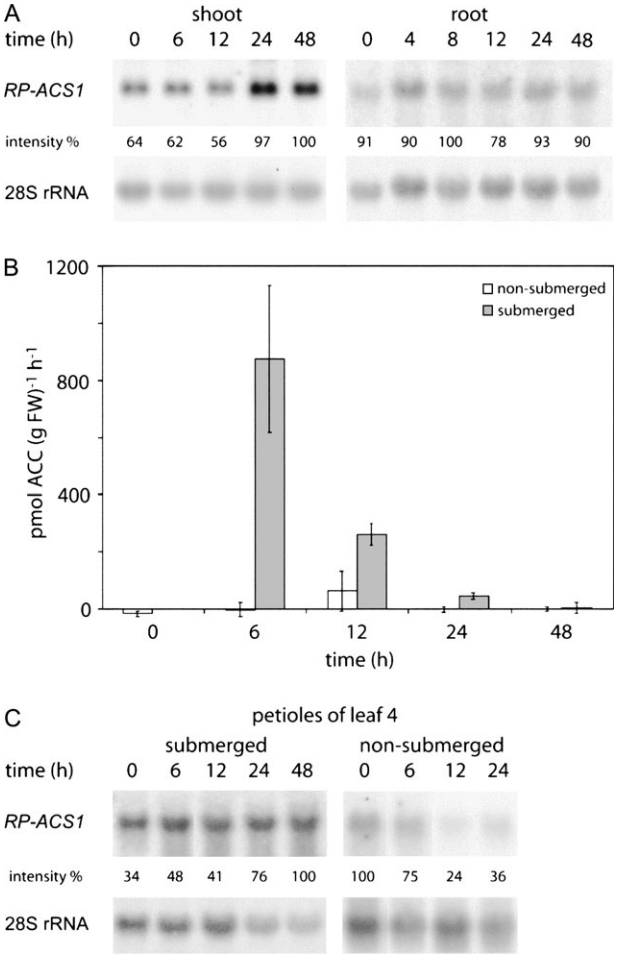

Fig. 2. Accumulation of *RP-ACS1* mRNA and ACS activity in *R. palustris* tissues upon submergence, under constant light. (A) *RP-ACS1* transcript level in shoot and root. (B) ACC synthase activity in shoots of submerged and non-submerged plants. (C) *RP-ACS1* transcript levels in petioles of leaf 4, the youngest fully grown leaf, of submerged and non-submerged plants. The RNA gel-blots were re-probed with 28S rRNA as a control for the loaded amount of total RNA (10 μg).

leaves, arranged in a rosette, of which the youngest (leaves 4 and 5) are the most responsive to ethylene (Voesenek *et al*., 1990*b*). To ensure that a local increase in *RP-ACS1* transcript level in responsive tissue had not been overlooked, due to dilution with non-responsive tissue, RNA from petioles of leaf 4 was analysed separately. Figure 2C also shows that, in leaf 4, *RP-ACS1* transcript level remained constant during the first 12 h after submergence. Surprisingly, however, analysis of *RP-ACS1* transcript accumulation in leaf 4 of non-submerged control plants showed that during 1 d the transcript level decreased significantly.

Further localization of the expression of *RP-ACS1* was performed by analysing total RNA of several segments of the petioles and lamina of leaf 4. Figure 3 shows that before submergence (t=0 h) the majority of the ACC synthase mRNA was localized in the petioles and was low, but detectable, in the lamina. After 24 h of submergence the

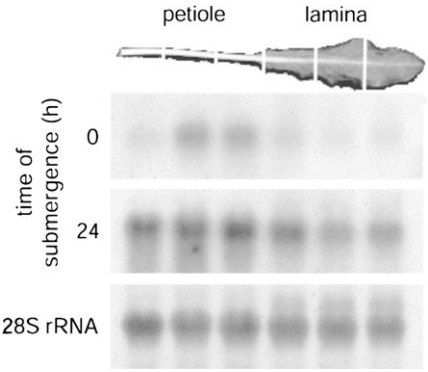

Fig. 3. Expression of *RP-ACS1* in petiole and lamina segments of submerged *R. palustris* plants (50 plants were sampled at each time point). The leaf at the top displays the segments of leaf 4 that were used for the RNA isolation and corresponds with the position of the lanes below the picture. The RNA gel-blot was re-probed with 28S rRNA as a control for the loaded amount of total RNA (10 μg, only reflected for t=24 h).

RP-ACS1 mRNA level had increased in both tissue types, but was still higher in the petioles.

Rhythmic messenger accumulation

Modulation of *RP-ACS1* mRNA levels in non-submerged plants (Fig. 2C) led us to analyse the expression pattern of the *R. palustris* ethylene biosynthetic genes in the shoot at several time points over 2 d with an 8 h dark phase and an additional 2.5 d with constant light (Fig. 4). The expression of chlorophyll *a*/*b*-binding protein (*CAB*) genes is known to show a strong circadian rhythm that persists under constant light (McClung, 2001). The messenger level of a *R. palustris CAB* gene (*RP-CAB1*; unpublished results) was used as an endogenous control of the circadian rhythm during these experiments. The first sample every day was taken 30 min before the end of the dark phase. The *RP-CAB1* messenger levels were still low at this time point but increased rapidly to a maximum in the middle of the light phase. The amplitude and the period of this rhythm were not affected by exposure to constant light for two nights (Fig. 4B). The messenger accumulation of *RP-ACS1* also showed a rhythmic pattern, but opposite to that of *RP-CAB1* (Fig. 4C). The highest levels were found when the plants were still in the dark, and the lowest levels were found at the middle of the light phase. Cycling of the ACC synthase transcript levels persisted for at least 2 d in the plants when subjected to continuous light, although the amplitude of the rhythm decreased strongly. The ACC oxidase (*RP-ACO1*, 1.3 kb; Vriezen *et al*., 1999) messenger accumulation displayed a rhythm with the peaks in the middle of the light phase as was found for the *RP-CAB1* mRNA accumulation. However, the amplitude of the rhythm was drastically reduced after the cycle under constant light, and no increase in messenger was seen after the second cycle (Fig. 4D; days 4 and 5).

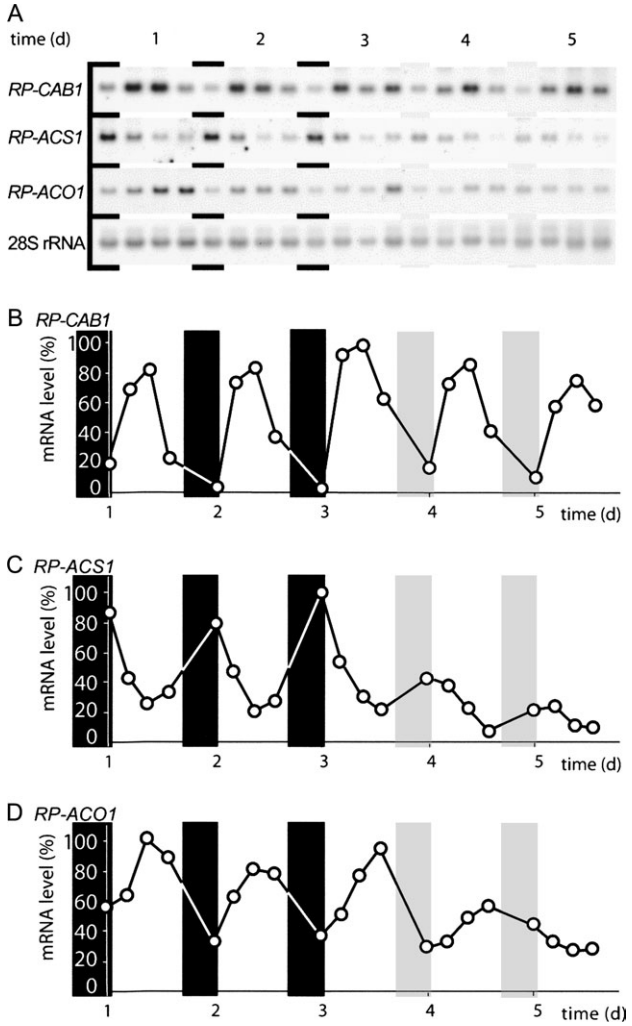

Fig. 4. The effect of photoperiodic regime on the mRNA concentration of *RP-CAB1* (A, B), *RP-ACS1* (A, C), and *RP-ACO1* (A, D) in shoots of *R. palustris* (five plants per sample) grown in drained soil. The black bars represent the dark phase and the grey bars represent the nights with the lights on (continuous light). The RNA gel-blot was re-probed with 28S rRNA as a control for the loaded amount of total RNA (10 μg).

Rhythmic ethylene production

Under non-submerged conditions, it is expected that ACC synthase activity is rate-limiting in ethylene production. To determine possible consequences of oscillations in *RP-ACS1* transcript levels, measurement of ACC synthase activity and ethylene production of *R. palustris* plants grown under the same light regime was attempted. However, under non-submerged conditions ACC synthase activity was too low for accurate measurement (see control plants in Fig. 2B). Ethylene production was analysed using laser-driven photoacoustic spectroscopy, a system able to measure ethylene in the pl l^{-1} area (Montero *et al*., 2003 and references therein). Figure 5A shows that during the light–dark cycles ethylene production was low in the light phase and increased in the dark phase. Under constant light conditions this rhythm persisted, with lower amplitude. It

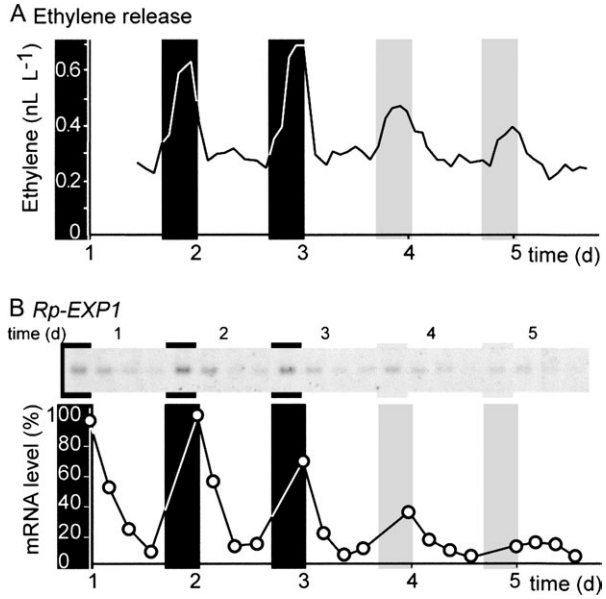

Fig. 5. Ethylene production (A) and *Rp-EXP1* mRNA levels (B) of *R. palustris* under a 16 h light/8 h dark photoperiod and under constant light, all at constant temperature. The black bars represent the dark phase and the grey bars represent the nights with the lights on (continuous light). Comparable patterns in ethylene release were obtained from a simultaneous measurement of plants in another cuvette and in two cuvettes from a fully independent replicate. (B) Steady-state mRNA levels from *R. palustris EXP1* (Vriezen *et al*., 2000). The full-length cDNA was hybridized to the same RNA gel-blot as shown in Fig. 4. The graph shows relative mRNA levels adjusted for the loaded amount of RNA as determined by 28S rRNA hybridization.

has been reported that under light conditions photosynthesis may inhibit ethylene production by decreasing the level of carbon dioxide in the cuvette (Kao and Yang, 1982). Carbon dioxide levels in the cuvette were indeed lower in the light, and supplementing the incoming air with extra carbon dioxide resulted in an increase in ethylene production (data not shown). Although this effect may have enhanced the difference between ethylene production in the light and in the dark, the persistence of the rhythm in constant light indicated that ethylene production did oscillate. *Rp-EXP1* encodes for an α-expansin gene that is regulated by ethylene (Vriezen *et al*., 2000) and is used here as a marker for ethylene response. Figure 5B shows that its mRNA accumulates with a comparable pattern as *RP-ACS1* mRNA (Fig. 4A, C), possibly following ethylene release (Fig. 5).

Discussion

ACC synthase genes are members of gene families and have been shown to be induced or inhibited in different plant tissues by stimuli such as auxin (Zarembinski and Theologis, 1994), wounding (Liu *et al*., 1993), submergence (Van der Straeten *et al*., 1997; Zarembinski and Theologis, 1997), and pollination (Bui and O'Neill, 1998). Also in *R. palustris*, multiple ACC synthase genes are present (Fig. 1). This suggests that the *R. palustris* ACC synthase genes may be differentially expressed and that more than one heterologous ACC synthase messenger may accumulate in a given tissue. However, using PCR with degenerated primers, it was possible to isolate only one ACC synthase cDNA (*RP-ACS1*) from leaves of *R. palustris* submerged for 24 h, although the same degenerated primers have been successfully used to amplify other ACC synthases from genomic DNA of *R. palustris* (pcr2Rp, pcr7Rp) and from *R. acetosa*, *Nicotiana tabacum*, *Ranunculus sceleratus* (data not shown), and rice (Zarembinski and Theologis, 1993). Because RNA gel blots probed with the ACC synthase gene fragments pcr2Rp and pcr7Rp also did not produce evidence for the presence of other messengers in *R. palustris* leaves under submerged or aerated conditions, it was assumed that *RP-ACS1* is most likely to be the gene responsible for the major part of ACC production in the *R. palustris* tissues used in the experiments.

The role of RP-ACS1 *during submergence*

It has been reported that ACC concentration increases in the roots and shoots of submerged *R. palustris* plants (Banga *et al*., 1996) and also in the intercalary meristem of rice plants (Cohen and Kende, 1987; Zarembinski and Theologis, 1997), with the maximum levels reached within the first hour of submergence. Detailed studies on conversion rates and on ACC oxidase gene expression and activity have shown that ACC accumulation in *R. palustris* is caused, in part, by the decreased activity of ACC oxidase during submergence (Banga *et al*., 1996; Vriezen *et al*., 1999). In addition, Banga *et al*. (1996) calculated that submergence caused a short-term increase in ACC synthesis and this could be confirmed by measurement of ACC synthase activity (Fig. 2B). The present results show that *RP-ACS1* transcript level in the root did not increase during 48 h of submergence and remained at a constant level for at least 12 h in the shoot (Fig. 2A, C). This discrepancy between *RP-ACS1* messenger concentration and ACC synthesis suggests that ACC synthase activity is regulated at a post-transcriptional level, as had already been observed in other species (Spanu *et al*., 1994; Vogel *et al*., 1998). The RP-ACS1 protein contains the conserved serine residue in the C-terminus, which is phosphorylated in tomato LE-ACS2 and thought to be involved in the regulation of ACS activity and turnover (Tatsuki and Mori, 2001; Chae *et al*., 2003; Wang *et al*., 2004). However, activity of other ACC synthases cannot be completely excluded as the genome sequence of *R. palustris* is largely unknown.

A study of the expression of *RP-ACS1* in petioles of plants grown under non-submerged, but otherwise comparable, conditions indicated that the *RP-ACS1* mRNA concentration was at a relatively high level at the beginning of the day and decreased during the subsequent hours (Figs 2C, 4C). So, although the *RP-ACS1* transcript level did not increase in the shoot during the first hours of submergence,

it was concluded that flooding maintains the messenger concentration at a discrete level and thus makes it possible for the ACC concentration to increase in the plant relative to the control.

Further analysis of the localization of the messenger during submergence showed that the ACC synthase transcript was most abundant in the petioles, the part of the plant that elongates most after submergence (Voesenek *et al*., 1990*a*), but that it also started to accumulate in the lamina after 24 h under water (Fig. 3). It has been found previously that ACC oxidase mRNA, and activity upon submergence, are also localized in the petioles (Vriezen *et al*., 1999), which indicates that ACC synthesis occurs mainly at the site of ethylene production. Although localized ethylene production does not seem to be useful during complete submergence, as ethylene accumulates in the whole plant (Banga *et al*., 1996), it is probably very useful to ensure high ethylene concentrations in the petioles when the plant is only partly submerged and the ethylene produced can diffuse away freely (Voesenek *et al*., 2003*b*). Comparable results were obtained by Zarembinski and Theologis (1997) who found within 12 h of partial submergence an induction of *OS-ACS1* mRNA in the cell elongation zone of deep-water rice internodes.

Rhythmic ethylene production and ethylene biosynthesis gene expression

In non-submerged, soil-grown *R. palustris* plants ethylene production showed a clear rhythm with the highest levels in the dark and the lowest level under light conditions (Fig. 4E). In many plants ACC synthase activity has been shown to be rate-limiting in ethylene production under non-stressed conditions (Yang and Hoffman, 1984; Kende, 1993), so the observation that ethylene production is correlated with *RP-ACS1* messenger levels in *R. palustris* plants suggests that ACC activity is regulated at the transcript level under these conditions. By contrast, under submerged and hypoxic conditions, or in cases of high ethylene production, ACC oxidase activity has been found to be rate-limiting (English *et al*., 1995; Yamamoto *et al*., 1995; Barry *et al*., 1996; Lasserre *et al*., 1996; Vriezen *et al*., 1999). Interestingly, waterlogged or hydroponnically grown *R. palustris* plants produce more ethylene, with a rhythm that is opposite to that found in soil-grown plants (Voesenek *et al*., 1990*a*, 1997), and closely resembles the fluctuations in *RP-ACO1* messenger levels as determined in this study. The function of rhythmic ethylene production in plants remains unclear. During submerged conditions, the accumulated ethylene induces growth of the petioles (Voesenek *et al*., 1990*b*). The low amount of ethylene produced in *R. palustris* under drained conditions may also have a slight growth-stimulating effect, as petiole growth during the dark phase seems to be slightly higher than during the light phase (Voesenek *et al*., 1997). Moreover, the mRNA level of a growth-associated *R. palustris* α-expansin gene, *Rp-EXP1*, also shows a circadian pattern comparable to ethylene release. This gene is known to be responsive to ethylene (Vriezen *et al*., 2000), suggesting that the plant responds to the rhythm of the basal ethylene production.

The messenger accumulation pattern of the ethylene biosynthetic genes showed that they were regulated by multiple signals. The (initial) persistence of the rhythms in *RP-ACS1* and *RP-ACO1* transcript levels under constant light (Fig. 4B), indicates an influence of the circadian clock on the mRNA levels. Unlike the *CAB* gene, however, the amplitude of the *RP-ACS1* and *RP-ACO1* rhythm decreased strongly when the dark phases were omitted. This means that, next to the circadian clock, a diurnal signal also influenced the messenger levels. The pathway that controls rhythmic expression of the ethylene biosynthetic genes in *R. palustris* and other species is still unknown, but may act via a sub-group of MYB-related transcription factors that were shown to be involved in the regulation of circadian gene expression in *Arabidopsis* (Schaffer *et al*., 1998; Wang and Tobin, 1998; Kuno *et al*., 2003).

Conclusion

The regulation of ethylene biosynthesis in *R. palustris* and other plants is complex because many genes encoding for ACC synthase and ACC oxidase and many other factors may influence ethylene release. In this report, the rhythmic patterns of ethylene synthesis, and of *RP-ACS1* and *RP-ACO1* mRNA accumulation, are shown in soil-grown, non-submerged *R. palustris*. Under these growth conditions, ACC synthesis is expected to be rate-limiting, and the pattern of *RP-ACS1* mRNA accumulation correlated with ethylene production. Submergence, which results in rapid changes of many physiologically important parameters, like the level of oxygen and carbon dioxide, induces ACC synthase activity in the short term, possibly via post-transcriptional or post-translational regulation. Increased ACC synthase activity, together with a decreased ACC oxidase activity, causes accumulation of ACC in the shoot during submergence.

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References

Abeles FB, Morgan PW, Saltveit Jr ME. 1992. *Ethylene in plant biology*, 2nd edn. San Diego, CA: Academic Press.

Banga M, Slaa EJ, Blom CWPM, Voesenek LACJ. 1996. Ethylene biosynthesis and accumulation under drained and submerged conditions. *Plant Physiology* **112,** 229–237.

Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D. 1996. Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *The Plant Journal* **9,** 525–535.

Beßler B, Schmitgen S, Kühnemann F, Gäbler R, Urban W. 1998. Light-dependent production of ethylene in *Tillandsia usneoides* L. *Planta* **205,** 140–144.

Blom CWPM, Voesenek LACJ, Banga M, Engelaar WMHG, Rijnders JHGM, Vandesteeg, HM, Visser EJW. 1994. Physiological ecology of riverside species: adaptive responses of plants to submergence. *Annals of Botany* **74,** 253–263.

Botella JR, Schlagnhaufer CD, Arteca JM, Arteca RN, Phillips AT. 1993. Identification of two new members of the 1-aminocyclopropane-1-carboxylate synthase-encoding multigene family in mung bean. *Gene* **123,** 249–253.

Bui AQ, O'Neill SD. 1998. Three 1-aminocylopropane-1-carboxylate synthase genes regulated by primary and secondary pollination signals in orchid flowers. *Plant Physiology* **116,** 419–428.

Chae HS, Faure F, Kieber JJ. 2003. The *eto1*, *eto2*, and *eto3* mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. *The Plant Cell* **15,** 545–559.

Cohen E, Kende H. 1987. *In vivo* 1-aminocyclopropane-1-carboxylate synthase activity in internodes of deepwater rice. *Plant Physiology* **84,** 282–286.

Cox MCH, Millenaar FF, De Jong van Berkel YEM, Peeters AJM, Voesenek LACJ. 2003. Plant movement. Submergence-induced petiole elongation in *Rumex palustris* depends on hyponastic growth. *Plant Physiology* **132,** 282–291.

Dong JG, Kim WT, Yip WK, Thompson GA, Li L, Bennett AB, Yang SF. 1991. Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. *Planta* **185,** 38–45.

Dziubinska H, Filek M, Koscielniak J, Trebacz K. 2003. Variation and action potentials evoked by thermal stimuli accompany enhancement of ethylene emission in distant non-stimulated leaves of *Vicia faba* minor seedlings. *Journal of Plant Physiology* **160,** 1203–1210.

El-Beltagy AS, Kapuya JA, Madkour MA, Hall MA. 1976. A possible endogenous rhythm in internal ethylene levels in the leaves of *Lycopersicon esculentum* Mill. *Plant Science Letters* **6,** 175–180.

English PJ, Lycett GW, Roberts JA, Jackson MB. 1995. Increased 1-aminocyclopropane-1-carboxylic acid oxidase activity in shoots of flooded tomato plants raises ethylene production to physiologically active levels. *Plant Physiology* **109,** 1435–1440.

Finlayson SA, Lee I-J, Morgan PW. 1998. Phytochrome B and the regulation of circadian ethylene production in sorghum. *Plant Physiology* **116,** 17–25.

Finlayson SA, Lee I-J, Mullet JE, Morgan PW. 1999. The mechanism of rhythmic ethylene production in sorghum. The role of phytochrome B and simulated shading. *Plant Physiology* **119,** 1083–1089.

Fluhr R, Mattoo AK. 1996. Ethylene – biosynthesis and perception. *Critical Reviews in Plant Sciences* **15,** 479–523.

Ievinsh G, Kreicbergs O. 1992. Endogenous rhythmicity of ethylene production in growing intact cereal seedlings. *Plant Physiology* **100,** 1389–1391.

Jackson MB. 1985. Ethylene and responses of plants to soil waterlogging and submergence. *Annual Review of Plant Physiology* **36,** 145–174.

Kao CH, Yang SF. 1982. Light inhibition of the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in leaves is mediated through carbon dioxide. *Planta* **155,** 261–266.

Kathiresan A, Reid DM, Chinnappa CC. 1996. Light- and temperature-entrained circadian regulation of activity and mRNA accumulation of 1-aminocyclopropane-1-carboxylic acid oxidase in *Stellaria longipes*. *Planta* **199,** 329–335.

Kende H. 1993. Ethylene biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **44,** 283–307.

Kuno N, Møller SG, Shinomura T, Xu XM, Chua NH, Furuya M. 2003. The novel MYB protein EARLY-PHYTOCHROME-RESPONSIVE1 is a component of a slave circadian oscillator in *Arabidopsis*. *The Plant Cell* **15,** 2476–2488.

Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balague C. 1996. Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). *Molecular and General Genetics* **251,** 81–90.

Liu D, Li N, Dube S, Kalinski A, Herman E, Mattoo AK. 1993. Molecular characterization of a rapidly and transiently wound-induced soybean (*Glycine max* L.) gene encoding 1-aminocyclopropane-1-carboxylate synthase. *Plant and Cell Physiology* **34,** 1151–1157.

Lizada MCC, Yang SF. 1979. A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. *Analytical Biochemistry.* **100,** 140–145.

Machácková I, Chauvaux N, Dewitte W, Onckelen H. 1997. Diurnal fluctuations in ethylene formation in *Chenopodium rubrum*. *Plant PhysioIogy* **113,** 981–985.

McClung CR. 2001. Circadian rhythms in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 139–162.

Metraux JP, Kende H. 1983. The role of ethylene in the growth response of submerged deep water rice. *Plant Physiology* **72,** 441–446.

Montero C, Cristescu SM, Jimenez JB, Orea JM, Hekkert STL, Harren FJM, Urena AG. 2003. trans-Resveratrol and grape disease resistance. A dynamical study by high-resolution laser-based techniques. *Plant Physiology* **131,** 129–138.

Musgrave A, Jackson MB, Ling E. 1972. *Callitriche* stem elongation is controlled by ethylene and gibberellin. *Nature* **238,** 93–96.

Oetiker JH, Olson DC, Shiu OY, Yang SF. 1997. Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*). *Plant Molecular Biology* **34,** 275–286.

Peeters AJM, Cox MCH, Benschop JJ, Vreeburg RAM, Bou J, Voesenek LACJ. 2002. Submergence research using *Rumex palustris* as a model: looking back and going forward. *Journal of Experimental Botany* **53,** 391–398.

Rikin A, Chalufz E, Anderson JD. 1984. Rhythmicity in ethylene production in cotton seedlings. *Plant Physiology* **75,** 493–495.

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campell AD, Theologis A. 1991. 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multi-gene family whose transcription is induced during fruit and floral senescence. *Journal of Molecular Biology* **222,** 937–961.

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre IA, Coupland G. 1998. The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* **93,** 1219–1229.

Spanu P, Grosskopf DG, Felix G, Boller T. 1994. The apparent turnover of 1-aminocyclopropane-1-carboxylate synthase in tomato cells is regulated by protein phosphorylation and dephosphorylation. *Plant Physiology* **106,** 529–535.

Tatsuki M, Mori H. 2001. Phosphorylation of tomato 1-aminocyclopropane-1-carboxylic acid synthase, LE-ACS2, at the C-terminal region. *Journal of Biological Chemistry* **276,** 28051–28057.

Theologis A. 1992. One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. *Cell* **70,** 181–184.

Van der Straeten D, Anuntalabhochai S, Van Caeneghem W, Zhou Z, Gielen J, Van Montagu M. 1997. Expression of three members of the ACC synthase gene family in deepwater rice by submergence, wounding and hormonal treatments. *Plant Science* **124,** 79–87.

Van Eldik GJ, Vriezen HW, Wingers M, Ruiter RK, Van Herpen MMA, Schrauwen JAM, Wullems GJ. 1995. A pistil-specific gene of *Solanum tuberosum* is predominantly expressed in the stylar cortex. *Sexual Plant Reproduction* **8,** 173–179.

Voesenek LACJ, Benschop JJ, Bou J, Cox MCH, Groeneveld HW, Millenaar FF, Vreeburg RAM, Peeters AJM. 2003*a*. Interactions between plant hormones regulate submergence-induced shoot elongation in the flooding-tolerant dicot *Rumex palustris*. *Annals of Botany* **91,** 205–211.

Voesenek LACJ, Harren FJM, Bögemann GM, Blom CWPM, Reuss J. 1990*a*. Ethylene production and petiole growth in *Rumex* plants induced by soil waterlogging. *Plant Physiology* **94,** 1071–1077.

Voesenek LACJ, Jackson MB, Toebes AHW, Huibers W, Vriezen WH, Colmer TD. 2003*b*. De-submergence-induced ethylene production in *Rumex palustris*: regulation and ecophysiological significance. *The Plant Journal* **33,** 341–352.

Voesenek LACJ, Perik PJM, Blom CWPM, Sassen MMA. 1990*b*. Petiole elongation in *Rumex* species during submergence and ethylene exposure: the relative contributions of cell division and cell expansion. *Journal of Plant Growth Regulators* **9,** 13–17.

Voesenek LACJ, Vriezen WH, Smekens M, Huitink F, Bögemann GM, Blom C. 1997. Ethylene sensitivity and response sensor expression in petioles of *Rumex* species at low O_2 and high CO_2 concentrations. *Plant Physiology* **114,** 1501–1509.

Vogel JP, Woeste KE, Theologis A, Kieber JJ. 1998. Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. *Proceedings of the National Academy of Sciences, USA* **95,** 4766–4771.

Vriezen WH, De Graaf B, Mariani C, Voesenek LACJ. 2000. Submergence induces expansin gene expression in flooding-tolerant *Rumex palustris* and not in flooding-intolerant *R. acetosa*. *Planta* **210,** 956–963.

Vriezen WH, Hulzink R, Mariani C, Voesenek LACJ. 1999. 1-Aminocyclopropane-1-carboxylate oxidase activity limits ethylene biosynthesis in *Rumex palustris* during submergence. *Plant Physiology* **121,** 189–195.

Vriezen WH, van Rijn CPE, Voesenek LACJ, Mariani C. 1997. A homolog of the *Arabidopsis thaliana ERS* gene is actively regulated in *Rumex palustris* upon flooding. *The Plant Journal* **11,** 1265–1271.

Wang KL, Yoshida H, Lurin C, Ecker JR. 2004. Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein. *Nature* **428,** 945–950.

Wang Z-Y, Tobin EM. 1998. Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* **93,** 1207–1217.

Wong WS, Li GG, Ning W, Xu ZF, Hsiao WLW, Zhang LY, Li N. 2001. Repression of chilling-induced ACC accumulation in transgenic citrus by over-production of antisense 1-aminocyclopropane-1-carboxylate synthase RNA. *Plant Science* **161,** 969–977.

Yamamoto M, Miki T, Ishiki Y, Fujinami K, Yanagisawa Y, Nakagawa H, Ogura N, Hirabayashi T, Sato T. 1995. The synthesis of ethylene in melon fruit during the early stage of ripening. *Plant and Cell Physiology* **36,** 591–596.

Yamasaki S, Fujii N, Takahashi H. 2003. Photoperiodic regulation of CS-ACS2, CS-ACS4 and CS-ERS gene expression contributes to the femaleness of cucumber flowers through diurnal ethylene production under short-day conditions. *Plant, Cell and Environment* **26,** 537–546.

Yang SF, Hoffman NE. 1984. Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology* **35,** 155–189.

Zarembinski T, Theologis A. 1993. Anaerobiosis and plant growth hormones induce two genes encoding 1-aminocyclopropane-1-carboxylate synthase in rice (*Oryza sativa* L.). *Molecular Biology of the Cell* **4,** 363–373.

Zarembinski TI, Theologis A. 1994. Ethylene biosynthesis and action: a case of conservation. *Plant Molecular Biology* **26,** 1579–1597.

Zarembinski TI, Theologis A. 1997. Expression characteristics of *OS-ACS1* and *OS-ACS2*, two members of the 1-aminocyclopropane-1-carboxylate family in rice (*Oryza sativa* L. cv. Habiganj Aman II) during partial submergence. *Plant Molecular Biology* **33,** 71–77.