A Comparative Molecular-Physiological Study of Submergence Response in Lowland and Deepwater Rice[1]

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Survival of rice (*Oryza sativa*) upon an extreme rise of the water level depends on rapid stem elongation, which is mediated by ethylene. A genomic clone (*OS-ACS5*) encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which catalyzes a regulatory step in ethylene biosynthesis, has been isolated from cv IR36, a lowland rice variety. Expression was induced upon short- and long-term submergence in cv IR36 and in cv Plai Ngam, a Thai deepwater rice variety. Under hypoxic conditions, abscisic acid and gibberellin had a reciprocal opposite effect on the activity of *OS-ACS5*. Gibberellin up-regulated and abscisic acid down-regulated *OS-ACS5* mRNA accumulation. Growth experiments indicated that lowland rice responded to submergence with a burst of growth early on, but lacked the ability to sustain elongation growth. Sustained growth, characteristic for deepwater rice, was correlated with a prolonged induction of *OS-ACS5*. In addition, a more pronounced capacity to convert ACC to ethylene, a limited ACC conjugation, and a high level of endogenous gibberellin$_{20}$ were characteristic for the deepwater variety. An elevated level of *OS-ACS5* messenger was found in cv IR36 plants treated with exogenous ACC. This observation was concomitant with an increase in the capacity of converting ACC to ethylene and in elongation growth, and resulted in prolonged survival. In conclusion, *OS-ACS5* is involved in the rapid elongation growth of deepwater rice by contributing to the initial and long-term increase in ethylene levels. Our data also suggest that ACC limits survival of submerged lowland rice seedlings.

Rice (*Oryza sativa*) is a semiaquatic plant adapted to survive submergence for a certain period of time. Rice is classified into three ecotypes according to its water requirement and tolerance: highland, lowland (irrigated or rain-fed), and deepwater rice (Takahashi, 1984). Deepwater rice grows in tidal swamps and in rain-fed areas of Southeast Asia where fields can be covered with water levels of more than 1 m during the monsoon season (Catling, 1985). Sustained stem elongation ensures its survival upon submergence. In extreme cases a total height of 7 m has been recorded (Vergara et al., 1976). The latter type is called floating rice because leaves and stems float on the surface when water levels recede.

Rapid elongation in response to submergence is triggered by oxygen deficiency and is controlled by ethylene (Kende et al., 1998). Ethylene accumulates as a result of physical entrapment. In addition, the subatmospheric oxygen level leads to an increase in de novo ethylene synthesis (approximately 8-fold) through the activation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS; Métraux and Kende, 1983; Cohen and Kende, 1987). ACS is encoded by a multigene family that consists of at least five members in rice (Zarembinski and Theologis, 1993, 1997; Van Der Straeten et al., 1997). *OS-ACS1* is the only rice *ACS* gene that has been implicated in the submergence response thus far (Zarembinski and Theologis, 1997). Furthermore, the elevated level of ethylene in submerged plants promotes elongation growth through a modulation of the balance in gibberellin (GA) and abscisic acid (ABA; Hoffmann-Benning and Kende, 1992; Azuma et al., 1995). The increase in GA concentration and responsiveness stimulates cell division and elongation activities (van der Knaap and Kende, 1995; van der Knaap et al., 1997; Lorbiecke and Sauter, 1998). In addition, expansins appear to play an important role in mediating rapid stem elongation (Cho and Kende, 1997, 1998).

The studies mentioned above were focused on the growth of internodes of rice plants aged between 3 and 13 weeks. As a consequence, very little is known on the submergence response of seedlings. Yet in those cases where seedlings live under fully submerged conditions for several days, yield losses are considerable. Submergence is indeed a major constraint to rice production (Widawsky and O'Toole, 1990; Herdt, 1991). Low-yielding deepwater and floating rice varieties are currently cultivated in those

[1] This research was supported by the European Union (grant no. International Scientific Cooperation China CI1*–CT93–0082) and by the Geconcerteerde Overlegde Acties (grant no. GOA 96016). D.V.D.S. and H.A.V.O. are Research Directors of the Fund for Scientific Research (Flanders).

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areas where water depths remain above 50 cm for several months. Submergence tolerance is also required in flash-flooded rain-fed areas where lowland varieties are grown. Biochemical and genetic characteristics of adaptation to submergence have been studied in detail (Nandi et al., 1997; Setter et al., 1997; Vartapetian and Jackson, 1997; Mackill et al., 1999). Significant progress was also made on the molecular characterization of responses to hypoxic and anoxic stress (Chang et al., 2000; Dennis et al., 2000). Despite all these advances, the mechanism that senses the change in oxygen availability and translates the message into the adaptive response remains largely unknown.

High-nutritional and high-yielding lowland cultivars could be adapted by genetic engineering, allowing them to grow in flooded areas or tidal swamps. As a first step toward this goal we have studied an *ACS* gene (*OS-ACS5*) that is induced by submergence in lowland and in deepwater rice. By correlating growth with expression patterns for submergence-induced ACS, in vitro ACC oxidation capacity, and levels of ACC, ethylene, ABA, and GA, we have tried to underpin the molecular and biochemical alterations that contribute to the difference in flooding tolerance. Although not being limited to the response of seedlings, our study pays particular attention to the reaction in early development.

RESULTS

Isolation and Characterization of *OS-ACS5*, a Genomic ACS Clone from Lowland Rice (cv IR36)

A genomic library of cv IR36 was screened at low stringency with ACS cDNAs from deepwater rice (cv Plai Ngam; Van Der Straeten et al., 1997). Two positive clones overlapped each other by restriction mapping and by DNA sequence analysis. The nucleotide sequence of a 4,328-bp stretch was determined (accession no. X97066). Comparison of the deduced polypeptide to a peptide sequence of 34 amino acids in five different rice ACS isoforms (Zarembinski and Theologis, 1993) and of a 102-bp DNA sequence (accession no. U65704) allowed us to identify the gene as *OS-ACS5*. This gene includes an open reading frame interrupted by two introns and flanked by a 1.7-kb 5′-untranslated region and a 1.0-kb 3′-untranslated sequence. A comparison with *OS-ACS1*, which has four exons and three introns (Zarembinski and Theologis, 1993), revealed that the equivalent of the first intron was lost in *OS-ACS5*. A similar structure was found in potato (Destéfano-Beltrán et al., 1995). The deduced polypeptide consists of 461 amino acids and has a calculated molecular mass of 49.9 kD and a pI of 7.28. Sequence alignment of *OS-ACS5* revealed significant similarity with other *ACS*. Most notably, an amino acid identity of 61% was found with an orchid *ACS* gene (accession no. L07882) and 56% with *OS-ACS1* from cv IR36 (Zarembinski and Theologis, 1993).

A comparison of the *OS-ACS5* promoter with that of *LE-ACS3*, which is responsive to submergence in tomato (Olson et al., 1995), did not reveal any significant similarity. Because rhythmic ethylene production had been demonstrated in several species (Kathiresan et al., 1996; Macháčková et al., 1997; Finlayson et al., 1998), we searched for homology with a circadian clock-regulated element (Carré and Kay, 1995) within the *OS-ACS5* promoter. However, no significant similarity was found. After PCR amplification and sequencing of a 413-bp fragment of the *OS-ACS5* promoter in cv Plai Ngam (see "Materials and Methods"), an interesting feature became apparent. Even though both promoter fragments were essentially identical, the promoter of the lowland variety lacked 38 bp between −258 and −295 upstream of the start codon in deepwater rice (Fig. 1).

Genomic DNA Blot of *OS-ACS5* for Lowland and Deepwater Rice

To reveal the existence of a closely related member in the cv IR36 *ACS* gene family, a genomic DNA gel blot was performed under very stringent conditions. Genomic DNA from cv IR36 and cv Plai Ngam was digested with *Eco*RI, *Bgl*II, *Xho*I, and *Eco*RI/*Pst*I. Single bands hybridized upon digestion with *Xho*I, *Eco*RI/*Pst*I, and *Eco*RI, with sizes corresponding to those predicted from the sequence (data not shown). The digestion with *Bgl*II yielded two predicted bands of 4.3 and 2.9 kb. The intensity of the bands correlated to a single-copy gene according to a copy number reconstruction analysis. Deepwater rice had a pattern identical to that of cv IR36 in all lanes, supporting the existence of an *OS-ACS5* ortholog in these plants.

Developmental and Stress-Regulated Expression of *OS-ACS5*

Figure 2A shows the expression of *OS-ACS5* at different stages of vegetative growth in cv IR36. A significant stimulation was found when plants at any age were submerged for 4 h. However, a peak in mRNA accumulation occurred in 5-week-old submerged plants. To determine whether *OS-ACS5* plays a role in the initial growth-promoting increase in ethylene synthesis, short-term submergence induction was investigated and compared with *OS-ACS1*, which is induced within 12 h of submergence and by low oxygen treatment (Zarembinski and Theologis, 1997). Figure 2B shows an RNA gel blot of 9-d-old seedlings submerged for 0.5, 1, and 2 h. In cv IR36, a peak in *OS-ACS5* messenger accumulation occurred after 1 h of treatment, whereas in cv Plai Ngam, equally high levels were observed after 1 and 2 h of submergence (on average a 3-fold

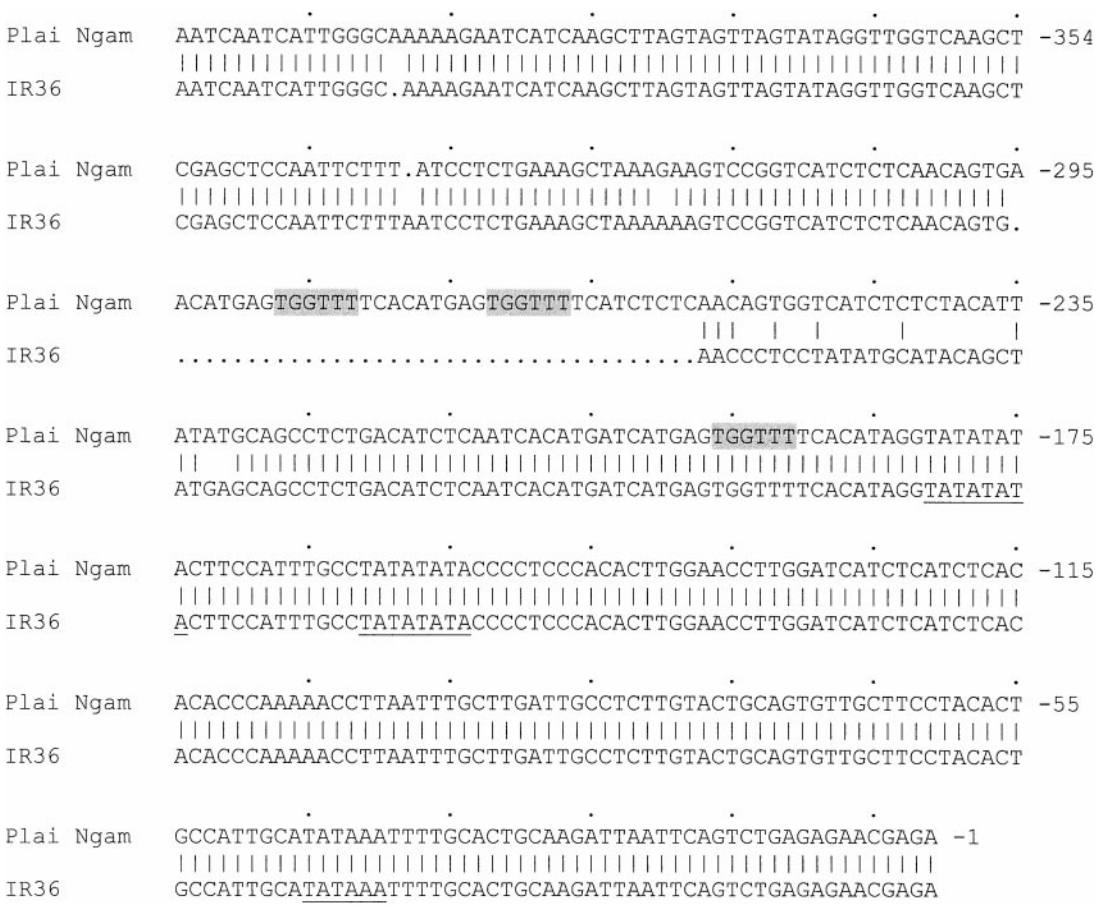


Figure 1. Alignment of promoter sequences of *OS-ACS5* in cv Plai Ngam and cv IR36. The alignment was made using the Genetics Computer Group program (version 10.0; Madison, WI). The region between −295 and −230 is highly divergent and covers a gap of 38 bp in cv IR36. Shaded boxes correspond to GT-like motifs typical for anaerobic response elements. Putative TATA boxes are underlined. Nucleotide numbering is relative to the start codon in cv Plai Ngam.

induction). In contrast, *OS-ACS1* mRNA was repressed in both varieties and accumulated to a level approximately 10-fold lower than that of *OS-ACS5* in the same tissues.

When plants were treated with 10 μM cycloheximide for 4 h followed by 6 h of incubation without the translation inhibitor, a 3-fold induction of *OS-ACS5* mRNA was observed (data not shown). An increase of the steady-state mRNA level upon cycloheximide treatment was also demonstrated for the *OS-ACS1*, *OS-ACS2*, and *OS-ACS3* genes (Zarembinski and Theologis, 1993). Wounding had no effect on the *OS-ACS5* steady-state level in cv Plai Ngam or in cv IR36 (data not shown).

Comparison of the *OS-ACS5* mRNA Level and Growth in cv IR36 and cv Plai Ngam

Figure 3 presents the biometric growth data of 9-d-old (Fig. 3B) and 9-week-old (Fig. 3A) cv Plai Ngam or cv IR36 exposed to plant hormones (GA, ABA, and ethylene) in air or in a low-oxygen atmosphere for 2 d. Hypoxic treatment (mimicking the effect of submergence), as well as exposure to GA and ethylene all stimulated growth. A growth increase of 2- to 3-fold was observed upon exposure to GA in air. In contrast, supplementing ABA suppressed the growth by a factor of 2 to 4 (with the exception of 9-d-old cv IR36, where growth remained at control level). This suppression was not released by combination with a hypoxic treatment.

The steady-state mRNA level of *OS-ACS5* was assessed from the same samples (Fig. 3). Up- and down-regulation of expression was correlated with a positive or negative effect on growth, although this was less pronounced in hypoxia-treated seedlings. In 9-week-old cv Plai Ngam plants subjected to a hypoxic atmosphere, *OS-ACS5* mRNA was 5-fold induced. In air, GA and ethylene increased the steady-state level of transcription 2- to 3-fold, whereas in hypoxia, an 8-fold stimulation was noticed. ABA reduced the amount of mRNA to roughly 20% of the control level in air and low oxygen (Fig. 3A, top). This effect of ABA was also observed in 9-week-old cv IR36 plants (Fig. 3A, bottom), albeit only at subambient oxygen concen-

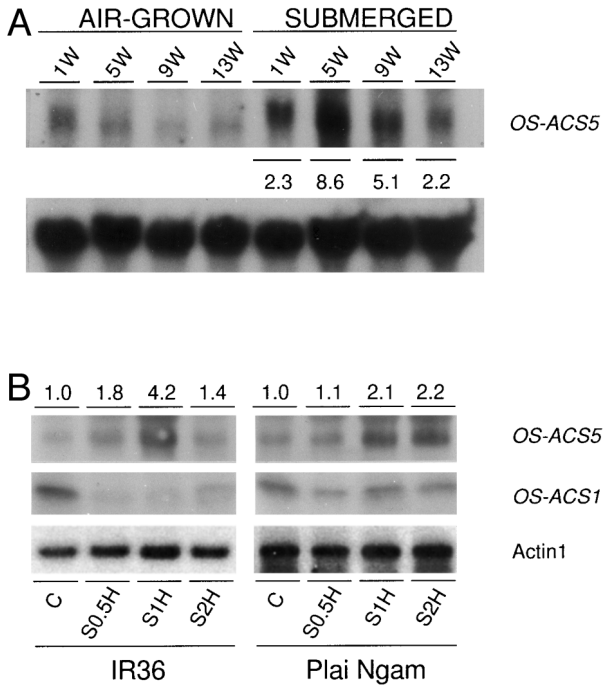


Figure 2. RNA gel-blot analysis of the expression of *OS-ACS5* and *OS-ACS1*. A, Transcript levels of *OS-ACS5* in cv IR36 at different developmental stages and upon 4 h of submergence. Total RNA (10 μg) was prepared from air-grown or submerged plants. Age of plants is indicated in weeks (W). Induction-fold was based on a densitometric scan, normalized to the values for 18S rRNA. B, Transcript levels of *OS-ACS5* and *OS-ACS1* upon short-term submergence. Poly(A)$^+$ (0.5 μg) was loaded on gel. Filters were reprobed with actin 1 to allow normalization of induction. Exposures were on phosphor-imager and lasted for 24 h and 10 d for *OS-ACS5* and *OS-ACS1*, respectively. Underlined numbers indicate relative message levels.

tration. The influence of GA was similar to that found in cv Plai Ngam. However, the combined treatment with hypoxia and ethylene did not result in an equal stimulation of the mRNA level when compared with cv Plai Ngam.

The difference in growth response to ABA between these two cultivars was more obvious in young rice seedlings (Fig. 3B). In cv Plai Ngam, addition of ABA suppressed the elongation in air by 70%. This reduction was even more severe in low oxygen (about 8-fold). In contrast, cv IR36 seedlings were more tolerant to ABA. No significant reduction in growth could be observed in air and only 40% in hypoxia. Nevertheless, a suppression of *OS-ACS5* mRNA upon ABA treatment was noticed in cv Plai Ngam and in cv IR36.

Deepwater and Lowland Rice Seedlings Respond Differently to Sustained Submergence

To mimic the natural growth situation in deepwater areas, a sustained submergence of 2 weeks was conducted on both cultivars at the age of 9 d. Figure

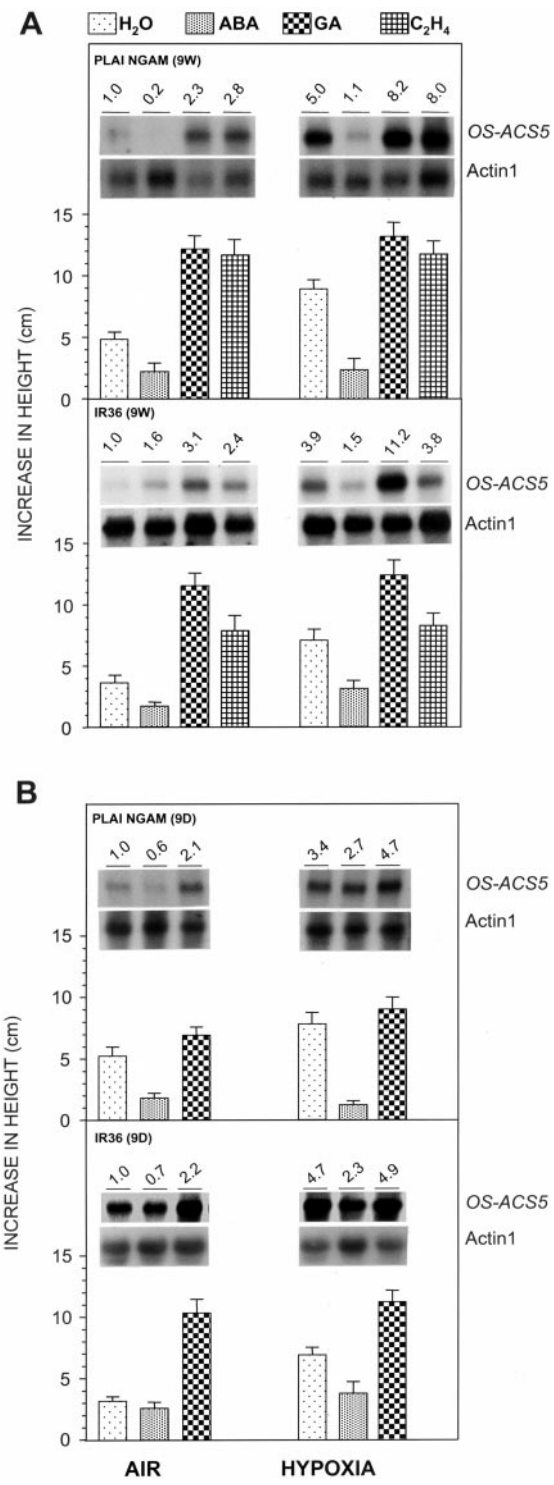


Figure 3. Morphometric analysis and RNA gel blots indicating the influence of hypoxia, hormones, and their combinations on rice growth and on the expression of *OS-ACS5* (48 h treatment). A, Nine-week-old plants (cv Plai Ngam, top; cv IR36, bottom); B, Nine-day-old seedlings (cv Plai Ngam, top; cv IR36, bottom). Poly(A)$^+$ RNA (0.5 μg) was prepared from all samples. Quantification was by scanning in a phosphor-imager. Blots were reprobed with a rice actin 1 cDNA to allow proper normalization. Underlined numbers indicate relative message levels. Biometric experiments were repeated three times (mean values ± SD are presented).

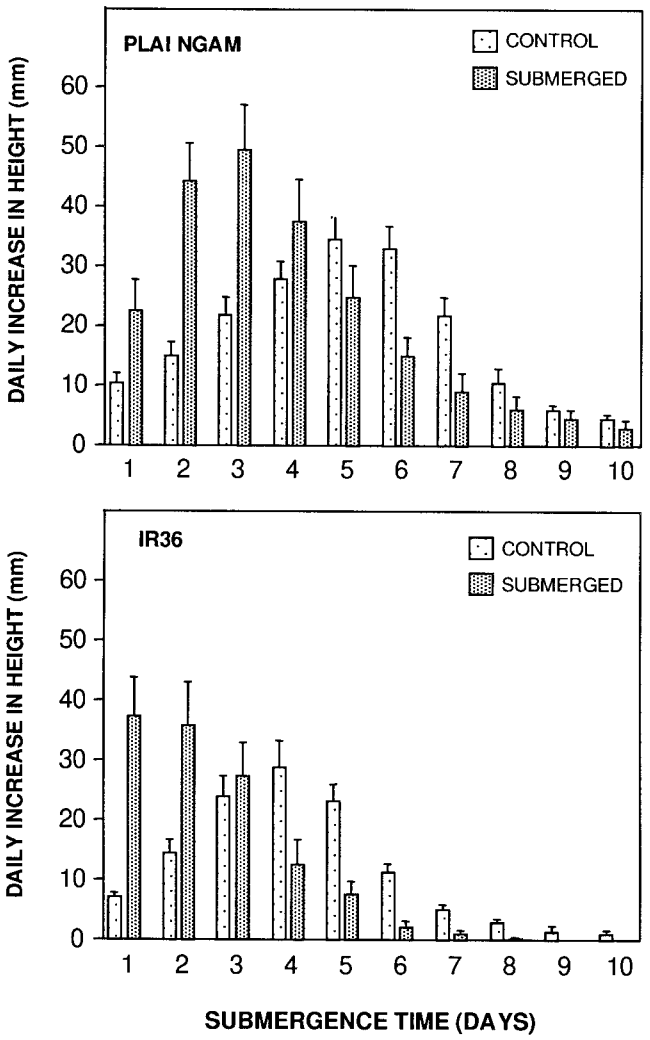


Figure 4. Growth of 9-d-old rice seedlings upon sustained submergence. Seedlings of cv Plai Ngam (top) or cv IR36 (bottom) were submerged for 10 d. Growth was measured daily. Data are mean values ± SD from 20 plants.

4 shows a record of the daily increase in height during the first 10 d of submergence. When grown in air, the elongation rate increased gradually, reaching a peak at d 4 or 5 in cv IR36 or in cv Plai Ngam, respectively. However, when submerged, cv IR36 had the most pronounced elongation on d 1. After 3 d, growth was strongly reduced and almost ceased after d 5 (Fig. 4, bottom). In contrast, the growth rate of cv Plai Ngam increased until d 3. A significant growth was observed all along the recording period (Fig. 4, top). The two varieties showed a different phenotypic appearance during the course of the experiment. The leaves of cv IR36 were already turning yellowish after 2 d of submergence and the plants were entirely chlorotic within a week. In contrast, cv Plai Ngam kept growing even after 10 d of submergence, although leaves turned light green or yellowish around d 7.

Exogenous ACC Treatment Results in a Constant Up-Regulation of *OS-ACS5* in Lowland and Deepwater Rice upon Long-Term Submergence and Correlates with an Increased Elongation Growth

Figure 5 (A and B, insets) shows RNA gel blots of tissues submerged for 2, 7, and 14 d in the absence or presence of 20 μM ACC, probed with *OS-ACS5* and *OS-ACS1*. In cv Plai Ngam and in cv IR36, a sustained high level of *OS-ACS5* mRNA was observed when ACC was added. In the lowland variety the average induction was approximately 3-fold; in the deepwater cultivar it was at least 6-fold. In both cases the enhanced accumulation of *OS-ACS5* was correlated with an increase in length. Ten days after submergence in the presence of ACC, the average additional

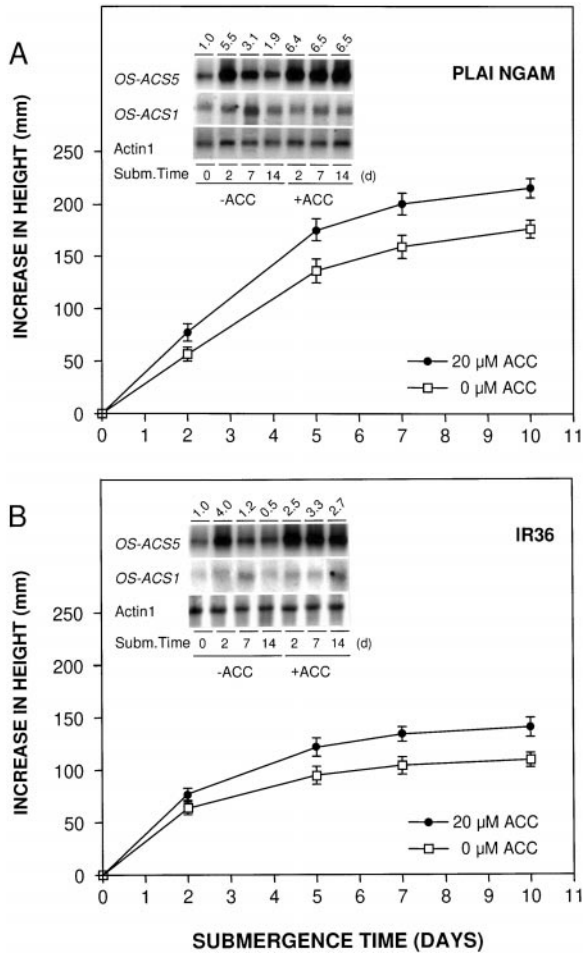


Figure 5. Influence of exogenous ACC on growth and ACS expression in submerged rice seedlings. Nine-day-old seedlings of cv Plai Ngam (A) and cv IR36 (B) were submerged for 2 weeks with or without 20 μM ACC. Height was measured at d 0, 2, 5, 7, and 10. Twenty seedlings were used per sample; data are mean values ± SD At indicated days after submergence, part of each sample was used for RNA extraction. RNA gel blots are shown as insets. Poly(A)$^+$ RNA (0.5 μg) was used for each sample. Exposure times are 24 h and 10 d for *OS-ACS5* and for *OS-ACS1*, respectively. Blots were reprobed with rice actin 1 cDNA for normalization. Underlined numbers indicate relative message levels.

increase in height was 33% for cv IR36 and 23% for cv Plai Ngam, compared with the values without exogenous ACC.

Furthermore, in the absence of ACC, *OS-ACS1* was significantly induced between 2 and 7 d in cv Plai Ngam, but not in cv IR36. However, it should be noted that the actual accumulation was again approximately an order of magnitude below that for *OS-ACS5* (exposure for *OS-ACS1* was 10-fold longer than for *OS-ACS5*). In contrast, upon addition of ACC, *OS-ACS1* mRNA levels remained at control level in cv Plai Ngam over the entire period of submergence, whereas in cv IR36 an induction was seen after 14 d.

Exogenous ACC Treatment Extends the Life Span of Submerged Lowland Rice

When seedlings were subjected to exogenous ACC, a delay in chlorosis was remarked in cv IR36. Therefore, chlorophyll levels were measured in both varieties after 2, 7, and 14 d of submergence in the presence or absence of 20 μM ACC. Figure 6 shows the chlorophyll levels relative to the level prior to submergence. No statistically significant differences were observed for cv Plai Ngam. Final levels remained close to 50% of the initial value. In contrast, cv IR36 plants displayed a clear retention of chlorophyll. After 14 d of submergence, the level had dropped to only 30%, against 14% in non-treated controls. As a consequence, plants looked healthier when 20 μM of ACC was supplied.

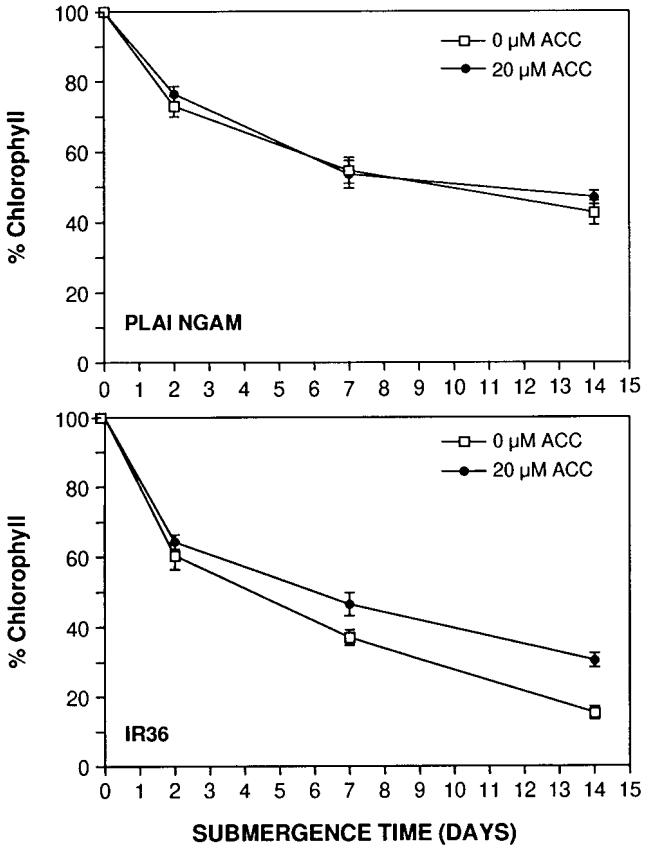


Figure 6. Delay in decrease of chlorophyll content upon sustained submergence of cv IR36 seedlings by exogenous ACC. Data points are expressed as a percentage of the total chlorophyll contents of 9-d-old air-grown seedlings. Data show average values ± SD from four independent repeats.

Submergence-Induced ACC Oxidase (ACO) Activity and Ethylene Emanation in Rice Seedlings

To assess the capacity of rice plants to convert the ethylene precursor ACC, in vitro ACO activity was measured (Vriezen et al., 1999; Fig. 7, A and B). Air-grown seedlings did not show significant differences in ACO activity over 7 d. However, a clear difference occurred upon submergence. After 2 d of treatment, a 6-fold induction of ACO activity was observed in deepwater rice. High levels were maintained until 14 d of submergence. In contrast, the induction was limited to 2.5-fold in cv IR36 after 2 d. No further increase was noticed upon prolonged submergence. When plants were treated with 20 μM ACC during submergence, the in vitro ACO activity after 7 d was 5-fold higher than that of air-grown seedlings and remained higher than the untreated controls after 14 d. In cv Plai Ngam, exogenous ACC did not yield a noteworthy effect, except for a slight decrease after 2 d.

Likewise, ethylene emanation most significantly increased in seedlings after 2 d of submergence (Fig. 7, C and D). Inductions were more elevated for cv Plai Ngam (3-fold), whereas a 2-fold increase was observed in cv IR36. When 20 μM ACC was administered during submergence, a 40% to 50% increase in ethylene release was found in the two cultivars.

ACC and Conjugated ACC Contents in Air-Grown and Submerged Lowland and Deepwater Rice

To verify the physiological relevance of the expression of the *OS-ACS5* gene in submerged rice, the levels of free ACC and conjugated ACC were measured (Martin et al., 1995) in the two varieties. As seen in Figure 8, the levels of endogenous ACC (Fig. 8A) and conjugated ACC (Fig. 8B) in air-grown plants of cv IR36 and cv Plai Ngam did not vary significantly over a period of 2 weeks; nor was there a remarkable difference between varieties. However, alterations were observed upon submergence. Although maximal levels of ACC occurring after 1 week of submergence were comparable in both varieties, the rate of accumulation was faster in cv IR36. Likewise, after 2 weeks of submergence, the decline of ACC contents to control levels was more rapid in cv IR36. It is interesting that this differential pattern of ACC accumulation and decline correlated more or

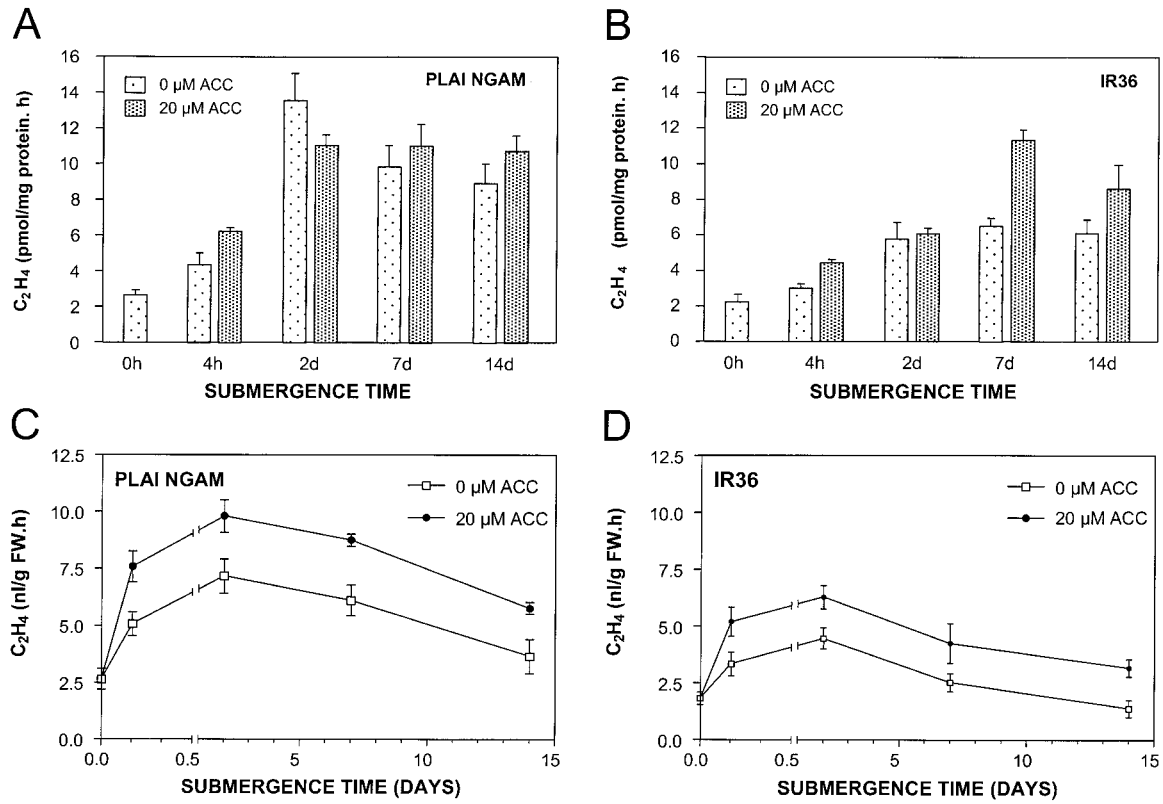


Figure 7. ACO activity and ethylene emanation from submerged rice seedlings. Seedlings were submerged in the absence or in the presence of 20 μM ACC for the time indicated. The mean value and SD were based on four independent experiments. A, In vitro ACO activity upon submergence of cv Plai Ngam seedlings. B, In vitro ACO activity upon submergence of cv IR36 seedlings. C, Ethylene emanation from submerged cv Plai Ngam seedlings. D, Ethylene emanation from submerged cv IR36 seedlings.

less with the difference in growth rate between the two lines as shown in Figure 4.

In addition, a significant difference was observed in the levels of conjugated ACC at each time point of submergence. Formation of ACC conjugates was always higher in cv IR36 than in cv Plai Ngam. Even after 2 weeks of submergence, the levels of conjugated ACC were still 65% higher in cv IR36.

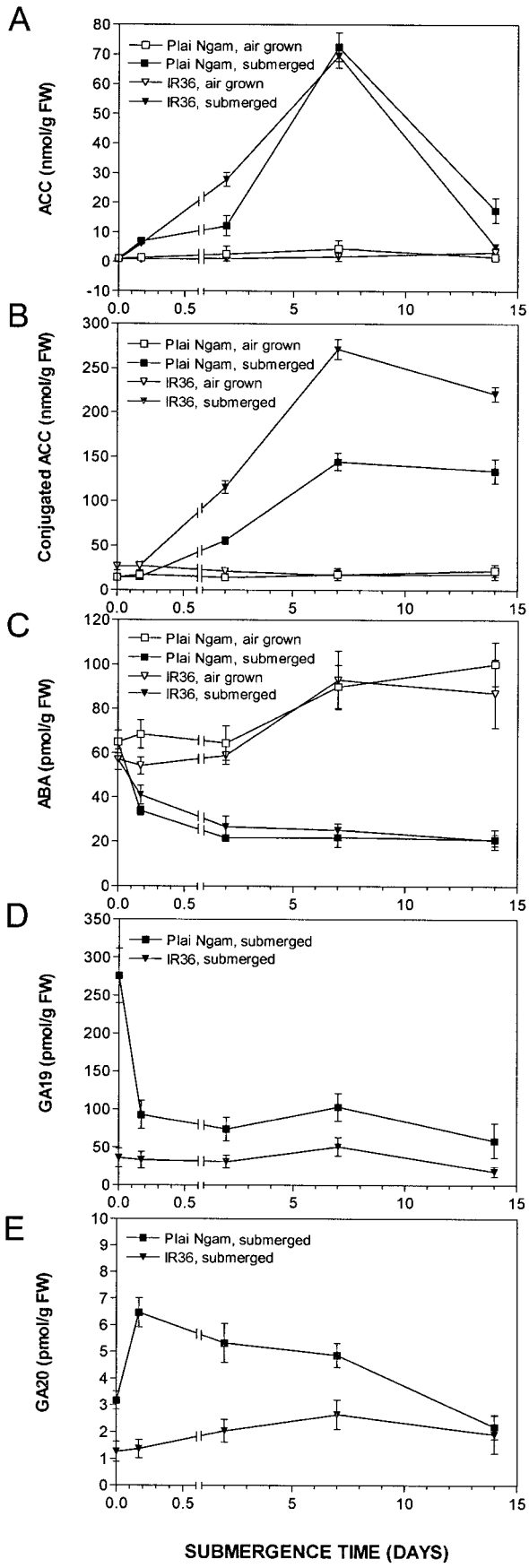
ABA and GA Contents in Air-Grown and Submerged Lowland and Deepwater Rice

The concentration of endogenous ABA and GA and the balance between these hormones are determining factors in submergence-stimulated elongation growth of adult rice (Hoffmann-Benning and Kende, 1992). To investigate whether similar changes occur in seedlings, the internal contents of ABA and GA were measured upon submergence. As shown in Figure 8C, the endogenous level of ABA in 9-d-old cv Plai Ngam was reduced to 45% of the control after 4 h of treatment. Cultivar IR36 reacted more slowly. Later on, the ABA levels in both varieties remained low at approximately 25% of the value in air-grown seedlings. On the other hand, GA_{19} and GA_{20} contents were significantly higher in deepwater rice. A decrease of GA_{19} (which is the precursor of GA_{20}) after 4 h of submergence correlated with a concomitant increase in GA_{20}. Cultivar IR36 showed a slight increase in GA_{20} until 7 d after submergence, but peak levels were 2- to 3-fold lower than the maximal concentrations in cv Plai Ngam.

DISCUSSION

In view of transferring the "floating trait" from low-yielding deepwater rice varieties to the high-yielding lowland cultivars, a comparison of the molecular aspects of ethylene biosynthesis in both types would be valuable. Here we have correlated the expression of two submergence-responsive *ACS* genes with growth patterns and with in vitro ACC oxidation, levels of ACC, ethylene, ABA, and GA. All traits were monitored in lowland and in deepwater rice seedlings. A limited set of experiments was also conducted on growing stem segments of adult plants. Basic similarities were found in the submergence response of seedlings and adult tissues at the molecular level, supporting previous physiological studies (Jackson and Pearce, 1991).

In contrast with *OS-ACS1*, an *ACS* gene that had previously been reported to contribute to longer-



term ethylene production upon submergence (Zarembinski and Theologis, 1997), *OS-ACS5* was induced by short-term treatment (1 h) in both cultivars (Fig. 2). A strong accumulation of *OS-ACS5* mRNA was also noted after 2 d of submergence for cv IR36 and cv Plai Ngam (Fig. 5). Therefore, in seedlings *OS-ACS5* may contribute to an initial, growth-promoting increase in ethylene synthesis and to the longer-term ethylene production. Thus, accumulation of ethylene by limited gas diffusion and an enhanced ethylene production may lead to the hormone level triggering the burst of growth in the early phase. *OS-ACS5* mRNA invariably accumulated to a much higher level than that of *OS-ACS1*. However, as nothing is known about the translation efficiency or the specific enzyme activities in vivo, *OS-ACS1* might also significantly contribute to the C_2H_4 production after long-term submergence. Given the fact that the *ACO* gene expression is enhanced upon submergence in deepwater rice (Mekhedov and Kende, 1996), submergence-induced ethylene production can be controlled at the synthase and at the oxidase levels. Confinement of expression of *OS-ACS* and *OS-ACO* genes to restricted cell types remains to be analyzed.

By exposing the plants to subambient oxygen concentrations close to those found in internodal lacunae of submerged deepwater rice (Stünzi and Kende, 1989), it was shown that the induction of *OS-ACS5* upon submergence may have resulted from oxygen deficiency. A homology search for an anaerobic response element (Olive et al., 1991; Walker et al., 1987) did not reveal the presence of a complete anaerobic response element. Yet, three consensus Myb-binding sites (T/C)(T/C) GGTTT were found in the cv IR36 promoter at −702, −966, and −1,326 bp upstream of the start codon (Fig. 1). In addition, three incomplete Myb-binding sites were present in the promoter fragment isolated from cv Plai Ngam. Two of these were located in the 38-bp region deleted in the cv IR36 promoter. It remains to be proven whether these sequences are involved in the differential response of these varieties to submergence.

The effects of ABA and GA on *OS-ACS5* gene expression were generally accompanied by a down- and up-regulation of growth, respectively (Fig. 3). These data are in accordance with the view that rapid elongation growth results from an altered balance between GA and ABA (Hoffmann-Benning and Kende, 1992). A positive feedback by GA and a negative effect of ABA could modulate *OS-ACS5* messenger accumulation. This hormonal feedback regu-

Figure 8. Endogenous hormone levels in air-grown and in submerged rice seedlings. A, Endogenous levels of ACC. B, Endogenous levels of conjugated ACC. C, Endogenous levels of ABA. D, Endogenous levels of GA_{19}. E, Endogenous levels of GA_{20}. Data represent mean values ± SD from two independent experiments, each with duplications. Samples were taken after 4 h, 2 d, 1 week, and 2 weeks of submergence. Air-grown rice of the same age was used as a control.

lation seemed less pronounced in cv IR36 seedlings, even though a stimulation/inhibition of growth was observed (Fig. 3). Moreover, a decrease in ABA and an increase in GA levels were measured in seedlings of both cultivars (Fig. 8). Auxins have been implicated in rice seedling growth, as well (Ishizawa and Esashi, 1983; Breviario et al., 1992). However, preliminary measurements of auxin did not reveal significant alterations in either of the cultivars tested upon submergence (data not shown).

Keith et al. (1986) suggested that the dissimilarity between non-deepwater and deepwater cultivars is based on the difference in duration of submergence that the plants can survive. The existence of a developmental control of GA-biosynthetic activity has been proposed, limiting the elongation capability of lowland rice at an early stage. Our results support, at least in part, this hypothesis. Although the growth response of cv IR36 and cv Plai Ngam seedlings and adult plants was quite similar upon short-term submergence, a marked difference arose from long-term submergence of seedlings (Figs. 4–6). In contrast to the lowland cultivar, deepwater rice had a boost of *OS-ACS5* and *OS-ACS1* accumulation, correlated with sustained growth. It is remarkable that when cv IR36 plants were treated with 20 μM ACC while being submerged, significantly higher levels were found for *OS-ACS5* together with a growth increase (Fig. 5B). This growth was also correlated with a higher retention of chlorophyll (Fig. 6B). Treatment of lowland rice with exogenous ACC also resulted in higher in vitro ACO activities, reaching values similar to those in deepwater rice after long-term submergence (Fig. 7). The effect of ACC on ACO activity is most certainly an ethylene-mediated induction, as shown previously in *Rumex palustris* (Vriezen et al., 1999). Ethylene emanation from ACC-treated lowland rice was also comparable with that from deepwater rice without exogenous ACC. Together, these results imply that ACC supply is a limiting factor for growth and survival of submerged lowland rice. Furthermore, a significant increase in ethylene emanation was measured in deepwater rice, all along the submergence treatment (Fig. 7C). Ethylene measurements, when submergence treatment terminated, strongly correlated with submergence tolerance in rice and in *Rumex* sp. (Khan et al., 1987; Vriezen, 2000). The current study indicates that in rice, the duration of submergence also enhances the subsequent ethylene release, suggesting a higher production during the submergence phase.

Additional differences between the two cultivars reside at later steps in the pathway (Figs. 7 and 8). The higher level of ACC conjugates in the lowland cultivar could result from the accumulation of ACC, whereas ACO activities are lower than in deepwater rice. A difference in ACC transport may exist, as well. It also remains to be seen whether low oxygen conditions differentially affect the catalytic activity of ACO in the two types. Regulation at the level of ethylene perception can play a role, but the receptors involved have yet to be identified.

In conclusion, survival of rice under sustained submergence is modulated at the level of the signaling hormone (ethylene) and the hormone that controls the elongation response (GA). Whether an enhanced ethylene level results in a prolonged survival due to a faster short-term response or as a result of an improved long-term adaptation related to a more optimal maintenance of carbohydrate and energy supplies and a stimulation of aerenchyma formation remains to be investigated.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Rice (*Oryza sativa* cv IR36 [lowland rice] and cv Plai Ngam [deepwater rice]) seeds were soaked in distilled water and kept at 24°C in the dark for 2 d. Imbibed seeds were germinated in the dark on vermiculite impregnated with one-half-strength Hoagland solution (Hoagland and Arnon, 1938). Seedlings were kept in a growth chamber at 28°C, 70% relative humidity, and under a photoperiod of 16 h of light/8 h of darkness, with a light intensity of 100 μmol m^{-2} s^{-1}. When older plants were needed, seedlings were transferred on the 10th d after germination and were cultured hydroponically in one-half-strength Hoagland solution under the same conditions.

Submergence, Hormone, Wounding, Cycloheximide, and Low-Oxygen Treatments

For submergence experiments, 12 rice plants were held upright using a perforated plastic plate in a 2-L glass cylinder filled with one-half-strength Hoagland solution. The solution was refreshed every 2nd d. Plants were grown under the conditions mentioned above. For gene expression analysis, submergence was sustained for 0.5 to 4 h on plants with indicated age (Fig. 2), whereas for comparison of growth data and *OS-ACS* inductions, as well as the biochemical measurements, 9-d-old seedlings were kept submerged for a period of up to 2 weeks (Figs. 4–8). For RNA gel blots on adult tissues (Fig. 2), entire stems were extracted, with the exception of 13-week-old plants, in which the oldest part (approximately 5 cm) was excluded.

Hormonal treatments were on plants of indicated age. Of each growth regulator (purchased from Sigma, St. Louis), 50 μM were administered by root absorption in one-half-strength Hoagland solution for 2 d. For mechanical wounding, plants were cut into 1- to 2-mm pieces with scissors. The cut tissue was kept in a covered Petri dish on a wet paper (Whatman, Clifton, NJ) for 5 h. The uptake of 10 μM cycloheximide was by root absorption for 4 h, followed by 6 h in one-half-strength Hoagland solution without the translation inhibitor. For a comparison of hormonal effects in normal and in low-oxygen conditions, 20 9-d-old seedlings or 12 stem pieces of 9-week-old plants (16 cm, plus 2 cm of roots) were placed vertically in a 100-mL beaker

filled with 40 mL one-half-strength Hoagland solution supplemented with 50 μM of the indicated growth regulators. The beaker was placed inside a 4-L glass jar through which hydrocarbon-free air or a hypoxic atmosphere (6% O_2, 3% CO_2, and 91% N_2) was flushed at a flux rate of 250 mL min^{-1}. When ethylene was used, 100 μL L^{-1} ethylene was diluted with ethylene-free air or with the low-oxygen gas mixture to a concentration of 1 μL L^{-1}. Elongation growth of 9-week-old plants was monitored by the total length increase. RNA was prepared of stem tissue only (12 pieces per sample). In seedlings, the elongation growth was monitored by the height of the second leaf.

Experiments were repeated three to four times. Samples for RNA gel blots were consistently taken at the same time of the day (i.e. 7 h after initiation of the light period) to exclude possible influences of circadian rhythms.

Isolation and Sequencing of the *OS-ACS5* Gene

A cv IR36 genomic library in EMBL-3 Sp6/T7 was used (Clontech, Palo Alto, CA). Screening was on Hybond N^+ (Amersham Pharmacia Biotech, Little Chalfont, UK) with a radiolabeled equimolar mixture of three partial cDNA fragments from deepwater rice *ACS* genes (Van Der Straeten et al., 1997) in a buffer with 6 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 1% (w/v) SDS, 5 × Denhardt's solution, and 100 mg L^{-1} denatured calf thymus DNA (Maniatis et al., 1982), and at low stringency (50°C, washed twice with 2 × SSC). Inserts of positive λ-clones were subcloned in pBluescript KS II as an *Eco*RI/*Xho*I fragment (Stratagene, La Jolla, CA). Double-stranded DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) with an automated 373A DNA sequencer (Applied Biosystems, Foster City, CA). The entire sequence was submitted to GenBank under accession number X97066.

PCR Amplification of an *OS-ACS5* Promoter Fragment of cv Plai Ngam and a Carboxyl-Terminal Fragment of *OS-ACS1*

A PCR fragment of the *OS-ACS5* promoter of cv Plai Ngam was generated from genomic DNA. Different sets of primers were designed based on the sequence of *OS-ACS5* in cv IR36. PCR amplification was performed with a *Pyrococcus woesci* proofreading DNA polymerase (Roche Diagnostics, Brussels). The reactions (30 cycles each) were performed on a thermocycler OmniGene TR3 CM220 (Hybaid Ltd., Ashford, UK). Genomic DNA from cv IR36 was used as a control. One pair of primers (Z29 and Z35) produced the expected 0.4-kb fragment from cv Plai Ngam. The primers (with melting temperatures) used were 5′-GAGAAAGAGAGAGAGAGTGAGC-3′ (55.9°C) and 5′-CAACAGCTTGCCACTCATGATGTTCTC-3′ (61.9°C) for Z29 and Z35, respectively. The sequences of two independently amplified DNA fragments were determined with an automated 377 DNA sequencer (Applied Biosystems) using a Bigdye Terminator Sequencing Kit (Applied Biosystems).

Using primers Z50 and Z52 on genomic DNA, an *OS-ACS1* fragment of 330 bp was amplified from cv Plai Ngam and from cv IR36. Both sequences were identical to the 990- to 1,319-bp fragment of the *OS-ACS1* cDNA (accession no. M96672; Zarembinski and Theologis, 1993). The homology of this fragment to *OS-ACS5* is 64%. The fragment was subcloned into pBluescript KS II. The sequences of the primers (with Tm) were 5′-TCGGCGACAGGGACTTCAC-3′ (55.7°C) and 5′-ACCTGAGGCGCTGCATGG-3′ (56.8°C) for Z50 and Z53, respectively. The Tm was calculated by the primer analysis software Oligo 4.0 (Rychlik and Rhoads, 1989).

Genomic DNA Gel Blot

Genomic DNA was prepared according to Shure et al. (1983). Three micrograms of DNA was digested with the appropriate restriction enzyme in a buffer provided by the manufacturer (Amersham Pharmacia Biotech). After separation on 0.8% (w/v) agarose, DNA was blotted on a Hybond N^+ membrane (Amersham Pharmacia Biotech). The probe (a 1.7-kb *Xho*I/*Eco*RI fragment from the genomic clone *OS-ACS5* covering most of the coding region) was labeled by random priming (T7 Quickprime Kit, Amersham Pharmacia Biotech). Hybridization was performed at 65°C for high-stringency or at 50°C for low-stringency conditions, respectively, and was essentially as described by Church and Gilbert (1984).

RNA Analysis

Total RNA from rice was prepared by the method of Jones et al. (1985). RNase-free DNase (Roche Diagnostics) treatment was performed to remove traces of genomic DNA. For preparation of poly(A)$^+$ mRNA, approximately 200 μg of total RNA was passed through an oligo(dT)-cellulose column according to the manufacturer's protocol [Poly(A) Quick Kit; Stratagene].

Ten micrograms of total RNA or 0.5 μg of mRNA was separated on a 1.2% (w/v) agarose gel containing 8% (v/v) formaldehyde. RNA was blotted on Hybond N^+ (Amersham Pharmacia Biotech) in 20× SSC. A riboprobe was prepared by T7 RNA polymerase transcription (Riboprobe Gemini II, Promega, Madison, WI). To reach the highest specificity, the 0.5-kb carboxyl-terminal part of the *OS-ACS5*-coding region was used (2,795–3,364 bp in X97066). This region is highly divergent among the *ACS* gene family members. A fragment of the same region was used to compare the expression with that of *OS-ACS1*. Because the average incorporation of radioactivity in probes was above 80% and the length of the *OS-ACS1* template was more than one-half that of *OS-ACS5* (330 versus 570 bp), it can be concluded that the *OS-ACS5* probe had a specific activity (on a molar basis) at most twice as high as that of the *OS-ACS1* probe. Therefore, *OS-ACS5* signals that were more than 10-fold higher in intensity compared with *OS-ACS1* represented an mRNA accumulation of at most an order of magnitude difference.

Prehybridization and hybridization were carried out at 45°C in a buffer containing 50% (v/v) formamide, 3× SSC, 5 × Denhardt's solution, 0.5% (w/v) SDS, and 100 μg mL^{-1} of salmon sperm DNA. Filters were washed in 0.1 × SSC plus 0.1% (w/v) SDS at the same temperature. To check for equal loading, filters were reprobed with an *Nsi*I/*Eco*RV fragment from a rice 17S rDNA (Takaiwa et al., 1984) for total RNA blots or with an *Eco*RI/*Sac*I fragment from rice actin 1 cDNA (McElroy et al., 1990) for mRNA blots. Filters were exposed to film (XAR, Kodak, Rochester, NY) or to a phosphor-Imager screen (445 S, Amersham Pharmacia Biotech) for quantifications. In the cases mentioned, quantifications were based on densitometric scanning (Ultroscan Laser Densitometer 2202, LKB, Bomma, Sweden). Blots were performed at least three times with samples from replicate tissues to ensure trustworthy interpretation of mRNA inductions. Although not reproducible in absolute terms, the trends of mRNA induction were similar. Typical experiments are shown.

Determination of Internal Contents of ACC, Conjugated ACC, ABA, GA, and Indole-3-Acetic Acid (IAA)

Twenty 9-d-old seedlings were submerged for 4 h, 2 d, 1 week, and 2 weeks. The aerial part was frozen in liquid nitrogen and ground to a fine powder. Approximately 0.5 g of fresh weight was used for each sample. Air-grown plants of the same age were used as a control. Experiments were repeated twice, each with duplicates.

ABA, ACC, GA, and IAA were analyzed by a combined solid-phase extraction procedure based on Prinsen et al. (1991) and Chauvaux et al. (1997). For isotope dilution purposes, ^{18}O-ABA (100 ng; prepared according to Gray et al., 1974), d_4-ACC (100 ng, 1-amino-, 2, 3, 3-$^2H_4$-cyclopropane-1-carboxylic acid; Sigma), 17,17-d_2-GA_1 and 17,17-d_2-GA_3 (25 ng each, provided by Dr. L. Mander, Research School of Chemistry, Australian National University, Canberra, Australia), and $^{13}C_6$-IAA (100 ng; Cambridge Isotope Laboratories, Andover, MA) were used. After methylation, methylated ABA was analyzed by gas chromatography-mass spectrometry (GC-MS) using negative chemical ionization (NH_4; HP 5890 series II coupled to a quadruple mass spectrophotometer [Trio 2000, Micromass, Manchester, UK]; column: 15 m BD-XLB, 0.25 mm i.d., 0.25-μm film diameter [J&W Scientific, Folsom, CA], gas phase He, 120°C to 240°C; 15° min^{-1}; $T_{inj.}$, 250°C). Diagnostic ions were 278 (Me-ABA) and 280 (Me-$^{18}O_1$-ABA; Gray et al., 1974). IAA was measured as pentafluorobenzyl (PFB)-IAA after derivatization with PFB bromide (Netting and Milborrow, 1988) by GC-MS CI$^-$. GC and column specifications were as described for ABA. Diagnostic ions were 174 (PFB-IAA) and 180 (^{13}C-PFB-IAA). GA_1 and GA_3 were analyzed after methylation and trimethyl silylation by GC-MS (Moritz and Monteiro, 1994). GC and column specifications were 150°C to 200°C at 20°C min^{-1}, 200°C to 260°C at 3°C min^{-1}, and 260°C to 300°C at 20°C min^{-1}. In this case, diagnostic ions were 506 (GA_1), 504 (GA_3), 508 (d_2-GA_1), and 506 (d_2-GA_3; Hasan et al., 1994). After phenylthiohydantoin (PTH) derivatization (Pilet and Saugy, 1985) and HPLC, PTH-ACC was analyzed by liquid chromatography-MS/MS ES$^+$ (Micromass Quattro II, C8 Lichrosphere 60 RP Select B [Merck-Schuchardt, Hohenbrunn bei Munich, Germany], 5 μm, 125 mm × 4 mm, MeOH/NH_4OAc; 50/50 (v/v), 800 μL min^{-1}, post-column split 1/20; source T 80°C, collision energy 20 eV, cone 30 V, P_{AR} 5 × 10^{-3} mbar). The diagnostic ions for multiple reaction monitoring were 218→98 (PTH-ACC) and 223→102 (d_4-PTH-ACC; Chauvaux et al., 1997). ACC conjugates were analyzed as PTH-ACC after dry acid hydrolysis (Cohen et al., 1986).

Determination of ACO Activity and Ethylene Production

In vitro ACO measurements were essentially performed according to Mekhedov and Kende (1996). Upon harvest plants were rinsed with distilled water and blotted dry. The aerial part was frozen in liquid nitrogen and ground to a fine powder. One gram of tissue was extracted with 3 mL of extraction buffer (100 mM Tris-HCl, pH 7.2, 30 mM Na-ascorbate, and 10% [w/v] glycerol) for 20 min at 4°C. The suspension was centrifuged at 5,000*g* for 15 min. The supernatant was recovered and further centrifuged at 12,000*g* for 20 min. The protein content in the supernatant was determined using a protein assay reagent (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. An aliquot containing 1 mg of protein was added to an 8-mL glass vial. This mixture was brought to a total volume of 2 mL of extraction buffer containing a final concentration of 4 mM ACC and 50 μM $FeSO_4$. The vial was sealed and incubated in the dark at 30°C for 1 h while being gently shaken. One milliliter of headspace was withdrawn and injected into a gas chromatographer (IGC-120DFL, Intersmat, Pavillons sous Bois, France). Data were generated from four independent experiments, each with duplicates.

To measure ethylene emanation entire seedlings (approximately 0.5 g fresh weight) were quickly blotted dry, weighed, and transferred to a 20-mL glass vial with a rubber cap. During these manipulations (a few minutes) ethylene trapped during submergence treatment could escape, allowing assessment of ethylene production. However, it should be noted that although allowing a qualitative comparison between treatments, the values measured cannot reflect absolute quantitative differences. A small piece of wet 3MM (Whatman) paper was added to keep the atmosphere moist. After 2 h, 1 mL of headspace was withdrawn and injected into a gas chromatographer Di200 (Delsi, Suresnes, France). A linear response was found up to 4 h of incubation. The calculation of ethylene production was based on a standard curve obtained from injections of a gas mixture containing 100 μL L^{-1} of ethylene (Air Liquide, Liège, Belgium). Experiments were repeated twice, each with two determinations.

Chlorophyll Determinations

Total chlorophyll ($a + b$) was measured according to Porra et al. (1989). Submerged seedlings were ground in liquid N_2. Of each sample, 100 mg was extracted with 8 mL of a mixture containing 80% (v/v) acetone and 20% (v/v) 2.5 mM Na-phosphate at pH 7.8 for 10 min in the dark while being gently shaken. Samples were centrifuged at 5,000*g* for 10 min. The chlorophyll content of the supernatant was spectrophotometrically measured at 663 nm and normalized on a fresh-weight basis. Data were generated from four repeats and expressed as a percentage of the values at the start of the experiment.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Jan Gielen and Wilson Ardiles for DNA sequencing, Gurdev Khush (International Rice Research Institute, Manila, Philippines) and Somboon Anuntalabhochai (Chiang Mai University, Chiang Mai, Thailand) for providing rice seeds (cv IR36 and cv Plai Ngam, respectively), Fumio Takaiwa (National Institute of Agrobiological Resources, Yatabe Ibaraki, Japan) for the rice 17s rDNA, and Ray Wu (Cornell University, Ithaca, NY) for the rice actin 1 cDNA. The authors thank Wim Vriezen (Ghent University, Belgium) for critical reading of the manuscript, Martine De Cock for lay-out, and Karel Spruyt, Rebecca Verbanck, and Christiane Germonprez for art work.

Received July 12, 2000; returned for revision September 1, 2000; accepted October 29, 2000.

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