Regulating shoot growth: hormonal control of a submergence-induced expansin gene transcript

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Abstract

Expansins are proteins that have the capacity to induce extension in isolated plant cell wall tissue in a pH dependent manner and are thought to be a major determinant for cell elongation. In the semi-aquatic species Rumex palustris, complete submergence induces enhanced petiole elongation, achieved by cell extension. This trait is functionally related to re-establishment of gas exchange between leaves and the air environment to relieve the flooding stress. Submergence of R. palustris results in higher endogenous ethylene concentrations, and the submergence/ethylene-induced elongation response is regulated by the down-stream hormones gibberellic acid (GA), abscisic acid (ABA) and auxin. Previous work has shown that the onset of the submergence-induced petiole elongation coincides closely with the up-regulation of an expansin transcript (RpEXP1), and expansin activity. This study shows that the submergence-induced up-regulation of RpEXP1 mRNA in R. palustris petioles is under direct control of ethylene. GA, ABA and auxin, do not act as down-stream hormones of ethylene for regulation of RpEXP1 transcript abundance, although a combined effect of submergence and 1-naphtalene acetic acid (1-NAA), a synthetic auxin, also resulted in enhanced RpEXP1 transcript levels.
Introduction

Plant cell extension is brought about by a combination of turgor pressure and loosening of the cell walls. Although turgor pressure is required for cell extension, elongation of plant cells is mainly regulated by modulation of cell wall properties (Cosgrove, 2000a; Kutschera, 2001). A prime candidate for modulating cell wall properties, and thus cell elongation, are expansins (Cosgrove, 2000a). Expansins were first described by McQueen-Mason et al. (1992) as proteins responsible for the acid-induced extension of etiolated cucumber hypocotyls. The current model of the mode of expansin action hypothesizes cleavage of cellulose-hemicellulose hydrogen bonds. This model is based on the lack of detectable cellulase activity, decreased activity when water is replaced by deuterated water (the latter having stronger hydrogen bonds) and activity on composites made of cellulose and xyloglucan polymers (McQueen-Mason and Cosgrove, 1994; Whitney et al., 2000).

Expression of expansins is correlated with various processes involving cell wall loosening, such as tissue softening in ripening fruits (Rose and Bennett, 1999), meristem development (Pien et al., 2001) and elongation growth (McQueen-Mason, 1995; Cho and Kende, 1997a, 1997b, 1997c). The role of expansin proteins involved in elongation growth is further strengthened by studies with anti-sense expansin constructs under the regulation of an expansin promoter in Arabidopsis (Cho and Cosgrove, 2000) and inducible sense and anti-sense expansin constructs in rice (Choi et al., 2003). Inducement of a sense OsEXP4 in rice resulted in enhanced coleoptile and mesocotyl elongation, while induction of an anti-sense OsEXP4 construct reduced the growth of these tissues (Choi et al., 2003). By contrast, constitutive over-expression or repression of expansins resulted in an absence of a phenotype (Schipper et al., 2002), or contradictory phenotypes, such as reduced growth in expansin over-expressing tomato plants (Rochange et al., 2001). These results are probably caused by redundancy in the expansin gene family in plants (see http://www.bio.psu.edu/expansins) and feedback mechanisms present in plants adjusting cell wall characteristics to counteract the effect of constitutive expansin over-expression (Rochange et al., 2001).

The river-plain species Rumex palustris shows a striking morphological response upon submergence, first the petioles bend upwards (hyponastic growth), followed by an enhanced elongation of the petioles (Banga et al., 1997; Cox et al., 2003). These traits restore air contact and gas exchange with the atmosphere and improve plant survival in flooding-prone environment. Submergence-induced elongation of R. palustris petioles is a good model to study tissue and cellular extension, since i) the elongation is achieved by cell elongation, without involvement of cell division (Voesenek et al., 1990), ii) the enhanced cellular extension is distributed equally over the entire petiole (Rijnders et al., 1996) and iii) the elongation response can be switched on and off in a reliable way by submergence and ethylene treatments, allowing comparative studies of elongation kinetics and cell extension mechanisms, like expansins (Chapter 1).
Submergence-induced petiole elongation of *R. palustris* is under control of various plant hormones. When submerged, the basal production of ethylene of *R. palustris* shoot tissue is maintained (Voesenek *et al.*, 1993; Banga *et al.*, 1996b), leading, together with the lower diffusion rate of ethylene in water than in air (Jackson, 1985), to ethylene concentrations of 1 µl l⁻¹ within one hour of submergence. This concentration is already saturating the petiole elongation response (Banga *et al.*, 1996a). Both submergence and ethylene exposure result in a rapid decline in abscisic acid (ABA) concentrations, preceding the start of petiole elongation. Moreover, ABA added to submerged plants severely inhibits the elongation response (Voesenek *et al.*, 2003a; Benschop *et al.*, unpublished results). Next to ABA, gibberellic acid (GA) also acts downstream of ethylene in the elongation response. Submergence of *R. palustris* results in increased GA concentrations, while submerged plants pre-treated with GA biosynthesis inhibitors result in retarded petiole elongation, which could be rescued by addition of GA₃ (Rijnders *et al.*, 1997). In addition to ABA and GA, auxins are also involved in the ethylene-driven submergence response of *R. palustris*. A rise in the native auxin indole-3-acetic acid (IAA) was observed upon 4 h of submergence (Cox *et al.*, unpublished results). Removal of the leafblade, in this way reducing internal IAA concentrations, inhibited petiole elongation and could be rescued by addition of the synthetic auxin 1-naphtolene acetic acid (1-NAA; Cox *et al.*, unpublished results).

Vriezen *et al.* (2000) showed that in petioles of submerged *R. palustris*, the transcription of an expansin gene, *RpEXP1*, was up-regulated. Next to the transcript concentrations, the amount of expansin proteins of the size group containing *RpEXP1*, and the acid-induced extension of petiole cell walls, as a reflection of the expansin activity in planta, increased upon submergence (Chapter 1). These results indicate that expansin action might be involved in the submergence-induced petiole elongation of *R. palustris*. Similar to the elongation response (Voesenek and Blom, 1989), *RpEXP1* mRNA expression is also induced by ethylene exposure (Vriezen *et al.*, 2000). However, since ethylene is the start of the signal transduction pathway leading to enhanced petiole elongation, with ABA, GA and auxin as downstream intermediates (Voesenek *et al.*, 2003a), it is not known whether ethylene has a direct effect on *RpEXP1* transcription or an indirect effect via a downstream hormone. This study evaluates the hormonal regulation of *RpEXP1* transcription in order to find the key regulating hormone for the expansin-driven cell elongating mechanism. Our results indicate that *RpEXP1* transcription is under direct control of ethylene and that none of the known downstream hormones of the signal transduction pathway for petiole elongation (ABA, GA or auxin) act as intermediates on *RpEXP1* expression, although a stimulatory effect was observed when both the submergence and 1-NAA stimuli were present.
Results

RpEXP1 transcription is up-regulated by submergence

The mRNA expression of five expansin genes (RpEXP1, 8, 10, 15 and 18) in R. palustris petioles in response to submergence was determined in a previous study (Chapter 1). Of these genes, only RpEXP1 transcription showed an up-regulation upon submergence. Transcript levels increase within 2 h and maintained at higher levels for at least another 24 h. Submergence-induced up-regulation of RpEXP1 was confirmed in the present study (Figure 1). When plants were de-submerged after 10 hr of submergence by lowering the water to soil level and thus relieving the submergence signal, RpEXP1 concentrations decreased to air control levels (Figure 1). These results show that RpEXP1 transcript abundance is strongly regulated by submergence. The time point t=6 (6 h of submergence) was chosen to study the hormonal regulation of RpEXP1 transcription.

RpEXP1 transcription is regulated by ethylene

Similar as has been found for submergence, application of 5 µl l⁻¹ external ethylene in air for 6 h, induced a significant rise in RpEXP1 transcript levels (P<0.05, Figure 2). mRNA concentrations of RpEXP8, 10 and 18 did not show any change upon ethylene treatment, while the amount of RpEXP15 mRNA significantly decreased upon treatment with ethylene (Figure 2). The ethylene sensing-inhibitor 1-MCP binds to multiple ethylene receptors (Hall et al., 2000) and in this way blocks ethylene binding to the receptor (Sisler et al., 1996). Pre-treatment of plants with 1 µl l⁻¹ 1-MCP, a concentration high enough to inhibit petiole elongation (Benschop et al., unpublished results) blocked the up-regulation of RpEXP1 in petioles of submerged plants (Figure 3), indicating that submergence-induced up-regulation of RpEXP1 requires ethylene action.

Figure 1. RpEXP1 expression in 3rd petioles of air-grown (open circles), submerged (filled squares), or de-submerged (open triangles) R. palustris plants, relative to air at t=0. During the de-submergence, after 10 h of submergence, water was lowered to soil level. mRNA concentrations were measured using real-time RT-PCR with 18S RNA as internal standard. Mean ± se, n=3. Grey horizontal bar indicates 8 h dark period.
ABA is not involved in submergence-induced RpEXP1 transcription

Since RpEXP1 is the only expansin gene that so far showed enhanced transcript concentrations in petioles of R. palustris upon submergence (Chapter 1) and ethylene treatment (Figure 2), the expression profile of this gene was further analysed for its response to other hormones acting downstream of ethylene in the submergence-induced elongation response of R. palustris. Next to a rapid rise in ethylene (Banga et al., 1996a), ABA concentrations also showed a fast decrease upon submergence and ethylene exposure (Voesenek et al., 2003a; Benschop et al., unpublished results). Plants submerged in water containing 10 µM ABA to keep internal ABA concentrations in petioles high, showed a reduced elongation response to submergence (Benschop et al., unpublished results). Based on these results we hypothesised that ABA acts as a negative regulator of RpEXP1 transcript abundance. However, when plants were submerged in water containing 10 µM ABA, no decline in RpEXP1 abundance was observed (Figure 4).
GA manipulations do not show an effect on *RpEXP1* transcription

Next to ABA, the submergence-induced petiole elongation is also regulated by GA (Rijnders *et al.*, 1997). Pre-treatment of plants with the GA-biosynthesis inhibitor paclobutrazol inhibits submergence-induced petiole elongation that could be rescued by addition of GA to the plants (Rijnders *et al.*, 1997). The amount of *RpEXP1* mRNA in petioles was not affected by pre-treatment with paclobutrazol (P=0.94, figure 5), while the pre-treated plants did show typical paclobutrazol-induced growth reduction and darkening of the leaves. GA treatment for 6 h also lacked an effect on the amount of *RpEXP1* transcript (P=0.45, Figure 5). Similarly, no effect of GA on *RpEXP1* mRNA levels was observed when GA was given 11 and 1 h before the start of the experiment (data not shown). Since the GA-induced elongation observed by Rijnders *et al.* (1997) could be explained by specific up-regulation of other expansin transcripts, the transcript levels of *RpEXP8*, 10, 15 and 18 were also determined. Similar to the amount of *RpEXP1* mRNA, the transcript abundances of these other expansin genes were not depressed by paclobutrazol pre-treatment, nor stimulated by GA addition (data not shown).
Paclobutrazol induces a GA$_3$ independent increase in expansin protein

GA and paclobutrazol did not affect the mRNA concentration of $RpEXP1$. We can not, however, exclude that GA affects the translation of $RpEXP1$ mRNA. Therefore, we also studied the regulation of expansins protein expression in response to paclobutrazol and GA treatments. When Western blots of $R. palustris$ crude cell wall extracts were incubated with LeEXP2 antibody, three major bands were observed of 24, 26 and 28 kD (Chapter 1). Only the 24 kD band, containing e.g. RpEXP1, increased upon submergence with the largest increase 13 h after submergence (Chapter 1). Figure 6 shows the relative intensity of the 24 kD expansin band in $R. palustris$ crude cell wall protein extracts. A submergence period of 13 h increased the amount of 24 kD expansin protein, which unexpectedly increased even further when the plants were pre-treated with paclobutrazol. When paclobutrazol pre-treated plants were flooded in a 10 µM GA$_3$ solution, no reversion of the paclobutrazol-induced increase was observed. Paclobutrazol nor GA$_3$ showed an effect on the amount of 26 kD or 28 kD expansin proteins (data not shown), indicating that the paclobutrazol-induced increase is specific for the 24 kD expansin and not an artefact of loading. The $RpEXP1$ mRNA profiles of the 13 h treated plants was similar to the results obtained after 6 h (Figure 6, data not shown).

$RpEXP1$ transcription is up-regulated by 1-NAA in submerged plants but not in air-grown plants

Auxin is required for submergence-induced petiole elongation (Cox et al., unpublished results). Removal of the leaf blade resulted in 36 to 62 % lower auxin concentrations in the petiole and a lack of submergence-induced petiole elongation for 8 h (Cox et al., unpublished results). The growth reduction could be restored by application of the synthetic auxin 1-NAA to the submergence-water (Cox et al., unpublished results). Detachment of the leaf blade from the petiole led to an overall significant reduction of $RpEXP1$ transcript abundance (P=0.005) and was most pronounced in air-grown plants (Figure 7). Addition of auxin to intact or leaf-
detached plants did not cause a change in \textit{RpEXP1} transcript abundance (Figure 7), while air-grown plants treated with 1-NAA did show typical 1-NAA-induced bending of the leaves (data not shown). These results indicate that, in petioles of air-grown plants, the inhibiting effect of leaf blade removal on \textit{RpEXP1} transcript abundance is not a specific auxin effect. Petioles of submerged plants showed a marked increase in \textit{RpEXP1} transcript levels (Figure 7). Addition of 1-NAA to the submergence water increased the transcript levels of \textit{RpEXP1} even further (Figure 7). Since auxin is known to stimulate ethylene production (Yang and Hoffman, 1984), we tested whether the auxin stimulation of \textit{RpEXP1} transcription was due to a stimulation of ethylene production by blocking the ethylene signalling with 1-MCP. Figure 8 shows that 1-MCP did lower the amount of \textit{RpEXP1} transcription, but that blocking the ethylene pathway with a 1-MCP pre-treatment could not block the whole stimulatory effect of the submergence-auxin interaction.

**Discussion**

\textit{R. palustris} petioles show enhanced elongation in response to submergence, ethylene exposure (Voesenek and Blom, 1989) and GA addition (Rijnders et al., 1997), while ABA addition results in slower submergence-induced elongation rates (Voesenek et al., 2003a). In a previous study we showed that \textit{RpEXP1} mRNA, amount of expansin proteins of the size group containing \textit{RpEXP1} and expansin activity levels rise in petioles upon submergence (Chapter 1). In the present paper, we studied the hormonal regulation of \textit{RpEXP1} transcript abundance in \textit{R. palustris} petioles in relation to submergence-induced elongation. This research led to a model covering the hormonal regulation of \textit{R. palustris} petiole elongation (Figure 9), revising a part of the models proposed by Peeters et al. (2002) and Voesenek et al. (2003a).

Ethylene, as the prime signal in the submergence response, enhanced, next to the petiole elongation rate, also the \textit{RpEXP1} transcript concentration in \textit{R. palustris} petioles (Figure 2). The regulation of \textit{RpEXP1} mRNA concentration by ethylene is further strengthened by inhibition of the submergence-induced increase in \textit{RpEXP1} transcript levels by the ethylene perception-inhibitor 1-MCP (Figure 3). The rachis of the fern \textit{Regnellidium diphyllum} also has the capacity to elongate strongly when
submerged or exposed to ethylene (Cookson and Osborne, 1978). Kim et al. (2000) showed that both treatments induced increased concentrations of the α-expansin gene RdEXP1 in this species. Transcription levels of a second α-expansin gene, RdEXP2, did not change upon submergence nor ethylene exposure, indicating that, like in R. palustris, submergence and ethylene induce the expression of a specific expansin gene. In R. acetosa, a species of the same genus as R. palustris, but not showing submergence-induced petiole elongation, ethylene exposure lead to decreased concentrations of RaEXP1 (Vriezen et al., 2000). These results indicate that ethylene exposure does not necessarily induce expansin transcript expression and that ethylene-induced transcript expression coincides positively with the growth response of the plant species to ethylene. Since ethylene is a prime submergence signal for many plant species (Voeseñek and Blom, 1999; Jackson and Ram, 2003) and results in a cascade of changing hormone concentrations and sensitivities (Kende et al., 1998, Voeseñek et al., 2003a), we studied whether the observed induction of RpEXP1 mRNA was directly regulated by ethylene, or via downstream hormones (Figure 9).

Submergence and ethylene exposure result in a rapid decline in ABA concentration in petioles of R. palustris (Benschop et al., unpublished results) and this decline in ABA might act as an intermediate between ethylene and expansin transcription. Keeping ABA concentrations high in submerged plants and in this way reducing the submergence-induced elongation (Benschop et al., unpublished results), however, did not result in an inhibition of RpEXP1 levels (Figure 4). We therefore conclude that ABA does not regulate submergence-induced elongation by inhibiting RpEXP1 transcription (Figure 9). Similar ABA-independency of expansin transcription in a cell elongation response was observed in drought stressed maize roots (Wu et al., 2001), where ABA is needed to maintain root growth under low water potentials. Drought stress induced higher expression of maize expansin mRNAs. However, ABA manipulations affecting root growth had, like in submerged R. palustris petioles, no effect on expansin transcript levels (Wu et al., 2001). The mechanism affected by ABA might be alkalisation of the apoplast (Balsevich et al., 1994), that would inhibit the activity of expansins, since these are most active at acidic pH (McQueen-Mason et al., 1992).

Since GA promotes petiole elongation in R. palustris (Rijnders et al., 1997) and GA is the key hormone in the submergence-induced internode elongation of deep water rice (Kende et al., 1998), inducing more transcripts of α- and â-expansins in rice (Lee and Kende, 2001; 2002), we expected an inhibiting effect of the GA biosynthesis-inhibitor paclobutrazol and a stimulatory effect of GA on RpEXP1 transcript levels. Pre-treatment of R. palustris plants with paclobutrazol, nor addition of GA3, had any effect on RpEXP1 transcript levels (Figure 5). Four other studied expansin genes (RpEXP8, 10, 15 and 18) also showed no increase in transcript concentrations upon treatment with GA. Next to the expansin transcript levels, the amount of expansin proteins also did not decrease upon paclobutrazol pre-treatment (Figure 6). These results indicate that in R. palustris, the stimulatory effect of GA on petiole elongation growth is not via a stimulation of the amount of expansin transcript or proteins (Figure 9).
Pre-treatment with the GA biosynthesis inhibitor paclobutrazol led to higher amounts of the 24 kD protein in submerged *R. palustris* petioles (Figure 6), while such an increase was not observed for two other expansin protein bands (26 kD and 28 kD, data not shown). The accumulation of a 24 kD expansin protein by paclobutrazol could be caused by higher translation rates, slower turnover of the proteins, or increased transcription of expansins other than RpEXP1 and RpEXP8 (expansins studied with real-time RT-PCR and belonging to the 24 kD size group). No distinction between these mechanisms could be made with the present data, nor whether the observed paclobutrazol-induced increase of the 24 kD expansin protein is caused by GA biosynthesis inhibition, or by an unknown action of paclobutrazol. As an alternative, paclobutrazol might also act indirectly via the reduced growth of plants that are treated with paclobutrazol 4 d in advance (Rijnders *et al.*, 1997), triggering a feed-back loop to maintain tissue elongation via more expansin proteins while the GA-regulated elongation mechanism is inhibited and which is not yet reversed after 13 h GA treatment. GA has shown to alter various cell wall properties, such as inducing reorientation of cellulose microfibril arrangement more traverse to the cell axis in Zea mays and in deep water rice (Mita and Katsumi, 1986; Sauter *et al.*, 1993), and increasing xyloglucan endotransglycosylation (XET) activity in cucumber hypocotyls (Potter and Fry, 1994). In peas, GA showed an effect on the yield threshold of the cell walls (Cosgrove and Sovonick-Dunford, 1989). Whether these mechanisms cause the GA-induced petiole elongation in *R. palustris* remains to be elucidated.

Submergence induced a rise in IAA concentration in the peripheral tissues of *R. palustris* (Cox *et al.*, unpublished results). A lower IAA concentration induced by cutting off the leaf blade resulted in a temporal reduction of submergence-induced petiole elongation rate for at least 8 h, and could be rescued by addition of 1-NAA (Cox *et al.*, unpublished results). The auxin dependency of the elongation response is not the result of a dependency of expansin expression on auxin, since no 1-
NAA-induced \textit{RpEXP1} transcription was observed after 6 h of treatment (Figure 7, Figure 9). However, in combination with submergence, auxin did show a stimulatory effect on \textit{RpEXP1} mRNA abundance. Auxin is known to enhance ethylene production (Yang and Hoffman, 1984). We therefore studied whether the observed interaction of submergence and 1-NAA, leading to enhanced \textit{RpEXP1} transcript abundance, involved ethylene. Pre-treatment of plants with the ethylene antagonist 1-MCP reduced the \textit{RpEXP1} transcript levels in petioles of plants submerged with 1-NAA (Figure 8), but not to levels of 1-MCP pre-treated submerged plants without 1-NAA (Figure 8). These results indicate an ethylene independent interaction between submergence and 1-NAA that has an additive effect on \textit{RpEXP1} transcript abundance. The nature of this interaction remains to be elucidated.

Submergence-induced petiole elongation coincides closely with the up-regulation of \textit{RpEXP1} (Chapter 1). The close correlation between \textit{RpEXP1} expression and \textit{R. palustris} petiole elongation did not hold for the data presented in this study. Hormonal manipulations affecting petiole elongation, such as GA addition (Rijnders \textit{et al.}, 1997), did not result in a concurrent change in \textit{RpEXP1} transcript abundance (Figure 5). These observations led us to the conclusion that hormones altering \textit{R. palustris} petiole elongation do so by acting on mechanisms modulating \textit{RpEXP1} action, downstream of \textit{RpEXP1} transcription, or by cell wall loosening mechanisms independent of expansin action (Figure 9).

\textit{RpEXP1} transcription is likely to be under direct control of ethylene, since none of the known downstream components of the submergence-induced petiole elongation signal transduction pathway (ABA, GA, or auxin) showed a regulatory effect on \textit{RpEXP1} transcript levels (Figure 9). We therefore expect to find an Ethylene Responsive Element (ERE) in the promoter sequence of \textit{RpEXP1}. EREs have been found in promoter sequences of the rice expansin genes \textit{OsEXP11, 15} and the \textit{â}-expansin \textit{OsEXPB4} (Lee \textit{et al.}, 2001). However, only \textit{OsEXP2} and \textit{OsEXP4}, genes lacking an ERE in their promotor sequence, showed an up-regulation upon submergence, both in the amount of transcript and protein (Cho and Kende, 1997a, 1997c), while ethylene is also the prime signal for submergence-induced stem elongation in deep water rice, (Métraux and Kende, 1983). The submergence-induced internode elongation in deep water rice is most likely regulated by the downstream hormone GA (Kende \textit{et al.}, 1998), since the whole response can be blocked by GA biosynthesis inhibitors and mimicked by GA addition. This also holds true for the expression of \textit{OsEXP2} and \textit{OsEXP4}, both of which contain GA-responsive elements in their promotor sequences (Lee \textit{et al.}, 2001).

This study indicates that submergence-induced up-regulation of \textit{RpEXP1} transcript abundance is under direct control of ethylene. By contrast, although GA (Rijnders \textit{et al.}, 1997), ABA (Benschop \textit{et al.}, unpublished results) and auxin (Cox \textit{et al.}, unpublished results) have been shown to modulate submergence-induced petiole elongation in \textit{R. palustris}, the present work shows that these hormones do not regulate expression of \textit{RpEXP1}, but might work on \textit{RpEXP1} activity or other cell wall modifying mechanisms (Figure 9).
Materials and methods

Plant material and growth conditions

Rumex palustris plants were grown in 80 ml pots as described in chapter 1, and were used when plants were 24 d old. Plants used for the submergence time series and ethylene experiment were 2 d older, this difference in age did not affect the transcription pattern of *RpEXP1* upon submergence (data not shown). Plants were submerged in glass containers with 7 l water that had been acclimatized to the growth room conditions over night. Only the 3rd petiole was harvested and each replicate for the mRNA and protein experiments consisted of 5 petioles from different individual plants, with the exception of the ethylene experiment, where 2 petioles per replicate were used.

Hormone treatments

Ethylene (Analytical grade, Hoekloos, Schiedam, The Netherlands) was applied in a flow-through system with 70% RH, 20°C and 200 µmol m$^{-2}$ s$^{-1}$ PPFD. Plants were treated with 5 µl l$^{-1}$ ethylene or with air without added ethylene. 1-Methylcyclopropene (1-MCP, Ethylbloc, Floralife inc., Walterboro, USA) was applied in 24 l chambers (desiccators) at 1 µl l$^{-1}$ for 1 h, prior to the submergence experiment. Non-1-MCP treated plants were put in similar desiccators for the same duration.

(±)-ABA (Acros, ‘s-Hertogenbosch, The Netherlands) was prepared as 25 mM stock in 96 % ethanol and diluted to 10 µM in water. Non-ABA treated submerged plants were exposed to the same ethanol concentrations in the water.

Paclobutrazol (Duchefa, Haarlem, The Netherlands) was prepared as 0.1 M stock in 96 % ethanol and diluted to 50 µM in water. 10 ml of 50 µM paclobutrazol was given to the soil of the plants 4 d before the experiment. Non-paclobutrazol treated plants were pre-treated with water with the same concentration of ethanol. GA$_3$ (Duchefa, Haarlem, The Netherlands) was prepared as 0.1 M stock in 96 % ethanol and diluted with water to the appropriate concentration. The shoots of GA-treated air-grown plants were dipped in 10 µM GA$_3$, 0.1 % Tween 20 solution and were fed 10 ml of 50 µM GA$_3$ to the soil. Submerged GA-treated plants were flooded in 10 µM GA$_3$. Non-GA-treated plants were treated with similar solutions with GA$_3$ omitted.

In some experiments, leaf-blades were cut off 30 min before the start of the experiment. 1-Naphtalene acetic acid (1-NAA; Duchefa, Haarlem, The Netherlands) was prepared as 1 M stock in 96 % ethanol and diluted to appropriate concentrations in water. Shoots of 1-NAA-treated air-grown plants were dipped in 10 µM 1-NAA, 0.1% Tween 20 solution and 10 ml 50 µM 1-NAA was added to the soil. Submerged 1-NAA-treated plants were flooded in 10 µM 1-NAA. Non-1-NAA-treated plants were treated with similar solutions with 1-NAA omitted.

RNA extraction and analysis

RNA was extracted from *R. palustris* 3rd petioles according to Kiefer *et al.* (2000) with modifications described in chapter 1. cDNA was synthesised from 1 µg total RNA with SuperScript III (Invitrogen, Breda, The Netherlands) according to the manufactures protocol, but with ¼ of the reverse transcriptase and 5 µM random hexamers for 1 h at 50°C and diluted 2.2 times before analysis. Real-time RT-PCR was performed (Heid *et al.*, 1996) as described in chapter 1.
Western blot analysis

Crude cell wall protein extracts were made according to Rochange et al. (2001) with modifications described in chapter 1. Samples were loaded on a 15 % SDS-PAGE relative to the cell wall dry weight to gain a physiological representation of the amount of expansin protein (Cosgrove and Li, 1993; Cho and Kende, 1997c; chapter 1). The proteins were blotted and detected as described in chapter 1. As primary antibody the LeEXP2, a gift from Dr. A.B. Bennett and Dr. A.L.T. Powell (University of California, Davis), was used in a 1:1500 dilution.

Statistical analysis

Univariate analysis of variance was performed on the data of the GA and 1-NAA experiment, using SPSS 10.0.7 for Windows (SPSS inc., Chicago, Illinois, USA). Statements about differences in the GA and 1-NAA experiments (Figures 5 and 7) are statistically valid.

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