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# Ethylene regulates fast apoplastic acidification and expansin A transcription during submergence-induced petiole elongation in *Rumex palustris*

**Robert A.M. Vreeburg[1,†], Joris J. Benschop[1,‡], Anton J.M. Peeters[1], Timothy D. Colmer[2], Ankie H.M. Ammerlaan[1], Marten Staal[3], Theo M. Elzenga[3], Raymond H.J. Staals[1], Catherine P. Darley[4], Simon J. McQueen-Mason[4] and Laurentius A.C.J. Voesenek[1,*]**

[1]*Plant Ecophysiology, Faculty of Biology, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands,*
[2]*School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia,*
[3]*Department of Plant Biology, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands, and*
[4]*Biology Department, University of York, PO Box 373, York, YO10 4 YW, UK*

Received 11 March 2005; revised 25 May 2005; accepted 27 May 2005.
[*]For correspondence (fax +31 302 518366; e-mail l.a.c.j.voesenek@bio.uu.nl).
[†]Present address: Edinburgh Cell Wall Group, Institute of Molecular Plant Science, University of Edinburgh, Daniel Rutherford Building, The Kings Buildings, Edinburgh EH9 3JH, UK.
[‡]Present address: Molecular Genetics, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

**Summary**

**The semi-aquatic dicot *Rumex palustris* responds to complete submergence by enhanced elongation of young petioles. This elongation of petiole cells brings leaf blades above the water surface, thus reinstating gas exchange with the atmosphere and increasing survival in flood-prone environments. We already know that an enhanced internal level of the gaseous hormone ethylene is the primary signal for underwater escape in *R. palustris*. Further downstream, concentration changes in abscisic acid (ABA), gibberellin (GA) and auxin are required to gain fast cell elongation under water. A prerequisite for cell elongation in general is cell wall loosening mediated by proteins such as expansins. Expansin genes might, therefore, be important target genes in submergence-induced and plant hormone-mediated petiole elongation. To test this hypothesis we have studied the identity, kinetics and regulation of expansin A mRNA abundance and protein activity, as well as examined pH changes in cell walls associated with this adaptive growth. We found a novel role of ethylene in triggering two processes affecting cell wall loosening during submergence-induced petiole elongation. First, ethylene was shown to promote fast net $H^+$ extrusion, leading to apoplastic acidification. Secondly, ethylene upregulates one expansin A gene (*RpEXPA1*), as measured with real-time RT-PCR, out of a group of 13 *R. palustris* expansin A genes tested. Furthermore, a significant accumulation of expansin proteins belonging to the same size class as RpEXPA1, as well as a strong increase in expansin activity, were apparent within 4–6 h of submergence. Regulation of *RpEXPA1* transcript levels depends on ethylene action and not on GA and ABA, demonstrating that ethylene evokes at least three, parallel operating pathways that, when integrated at the whole petiole level, lead to coordinated underwater elongation. The first pathway involves ethylene-modulated changes in ABA and GA, these acting on as yet unknown downstream components, whereas the second and third routes encompass ethylene-induced apoplastic acidification and ethylene-induced *RpEXPA1* upregulation.**

**Keywords: ethylene, expansins, apoplastic acidification, elongation growth, *Rumex*.**

## Introduction

Cell elongation requires loosening of cell walls, uptake of water and synthesis of cell wall polysaccharides (Cosgrove, 1999). Cell wall loosening is mediated by proteins, among which a key role is designated to expansins (McQueen-Mason *et al.*, 1992). Four sequence-related expansin families are currently distinguished in plants: EXPANSIN A (EXPA),

 

EXPB, EXPANSIN-LIKE A (EXLA) and EXLB (Kende *et al.*, 2004). Expansin activity has a pH optimum between 3.5 and 4.5 (McQueen-Mason *et al.*, 1992) and is hypothesized to break hydrogen bonds between hemicelluloses and cellulose microfibrils (McQueen-Mason and Cosgrove, 1994). In addition to expansins, cell walls contain a range of other enzymes that can influence wall extensibility by modifying the structure and interactions between wall polymers (Cosgrove, 2000).

The mRNA levels of expansin genes, and expansin protein levels, are strongly correlated with growth and development of root and shoot organs (Cho and Kende, 1998; Colmer *et al.*, 2004; Lee *et al.*, 2003; Wu *et al.*, 1996). The functionality of expansins in growth and development was demonstrated with transgenic approaches in several plant species and organs. Antisense plants showed smaller and shorter phenotypes (Cho and Cosgrove, 2000; Choi *et al.*, 2003; Zenoni *et al.*, 2004), whereas transgenics with induced expansin overexpression were larger (Choi *et al.*, 2003). Expansin mRNA levels and protein activity is not only controlled by developmental cues, but also by various environmental signals, such as drought (Jones and McQueen-Mason, 2004; Wu *et al.*, 2001), flooding (Cho and Kende, 1997a; Colmer *et al.*, 2004; Huang *et al.*, 2000; Kim *et al.*, 2000; Lee and Kende, 2001; Vriezen *et al.*, 2000) and gravity (Zhang and Hasenstein, 2000).

The direction and rate of plant cell expansion is controlled by several plant hormones. Often, these phytohormones mediate between environmental or developmental signals and target genes modifying cell wall properties, such as expansins. The plant hormones auxin (IAA; Catalá *et al.*, 2000; Hutchison *et al.*, 1999), gibberellin (GA; Cho and Kende, 1997b; Lee and Kende, 2001), cytokinin (O'Malley and Lynn, 2000; Wrobel and Yoder, 2001) and ethylene (Kim *et al.*, 2000; Vriezen *et al.*, 2000) regulate transcription of various expansin genes in several organs and species.

A spectacular growth response, correlated with the upregulation of expansins, is submergence-induced shoot elongation in some semi-aquatic plants (Kim *et al.*, 2000; Lee and Kende, 2001; Peeters *et al.*, 2002). This growth response brings shoot parts to the water surface. For the semi-aquatic dicot *Rumex palustris*, we demonstrated that expansin mRNA increased in petioles upon submergence and decreased again following de-submergence. However, resolution of these studies was restricted by use of a non-specific probe in Northern analyses (Vriezen *et al.*, 2000). Fast underwater elongation in *R. palustris* involves cell elongation only and requires the action of at least four plant hormones (Cox *et al.*, 2004; Voesenek *et al.*, 1990, 2003). The presumed primary signal is ethylene that accumulates in submerged tissues within 1 h of submergence (Banga *et al.*, 1996). Entrapped ethylene is responsible for a very fast downregulation of endogenous abscisic acid (ABA) levels (Benschop, 2004) and an increase in $GA_1$ levels (Benschop, 2004; Rijnders *et al.*, 1997). The fourth hormone that responds to the submergence signal is IAA, with an increase in lateral petiole regions upon submergence (Cox *et al.*, 2004).

The *Rumex* model system, with already detailed knowledge of hormonal interactions regulating submergence-induced petiole elongation, offers the unique opportunity to evaluate which components of this regulatory network control expansins, on the mRNA and protein level, and whether submergence induces changes in apoplastic pH. Furthermore, the involvement in this network of several plant hormones in combination with expansins as target genes allows us to establish whether this underwater response is controlled by a linear signal transduction cascade as suggested for underwater elongation of internodes of deep water rice (Kende *et al.*, 1998) or whether more parallel, simultaneously operating pathways are involved.

We suggest that expansin genes are downstream targets of the submergence-induced signal transduction pathway in *R. palustris*, ultimately leading to underwater escape. The aims of the present study were to (i) study submergence-induced apoplastic acidification and its hormonal regulation and (ii) elucidate the identity, kinetics and hormonal regulation of expansin activity, at both the mRNA and protein level, as related to submergence-induced petiole elongation in *R. palustris*.

## Results

### *Submergence induces fast petiole elongation*

High-resolution kinetic analyses of petiole elongation rates were obtained using linear variable displacement transducers. In both control and submerged plants, elongation rates of the second youngest petiole varied in time (Figure 1a). During an experiment of 30 h, there were two phases of enhanced elongation in submerged plants. The first phase of increased petiole elongation commenced approximately 4 h after submergence, and the second occurred 14 h later. These periods of faster elongation, interrupted by phases in which elongation rates did not differ from controls, reoccurred diurnally in experiments conducted over several days (Figure 1b), ultimately resulting in significantly longer petioles in submerged plants.

### *Cell wall acidification and increased extensibility accompany submergence-induced petiole elongation*

Acidic conditions have long been known to enhance cell elongation (Rayle and Cleland, 1992). This 'acid growth' response can be largely attributed to the acidic pH optimum for expansin activity (McQueen-Mason *et al.*, 1992). Therefore, a series of experiments were conducted to assess

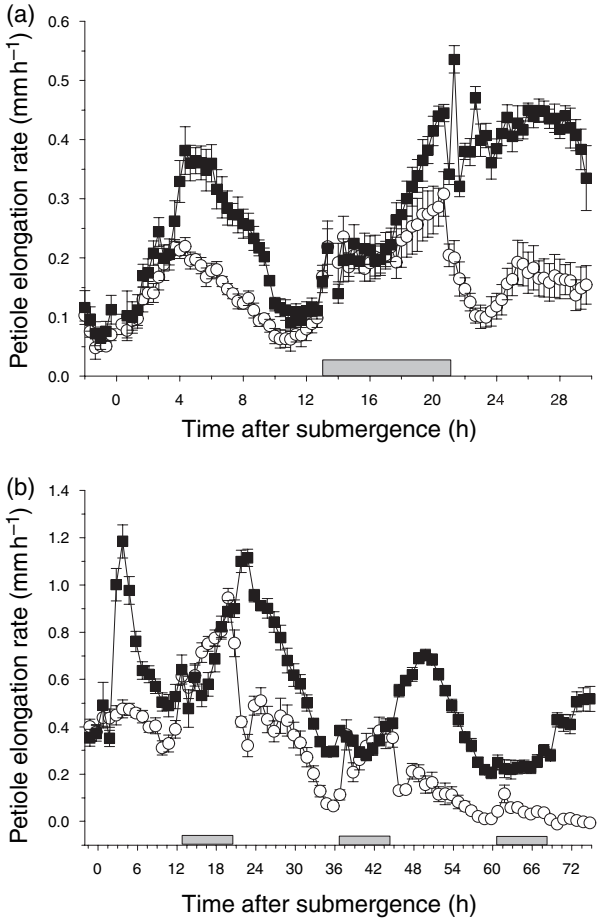


**Figure 1.** Elongation rates of the third petiole (second youngest on the plant) of *Rumex palustris* during a 30-h experiment (a) and a 72-h experiment (b). Plants were exposed to air (open circles) or complete submergence at $t = 0$ (filled squares, mean ± SE, $n = 6$). Growth rates were calculated every 20 min from length data obtained with a transducer set-up. Grey horizontal bar indicates 8 h dark period.

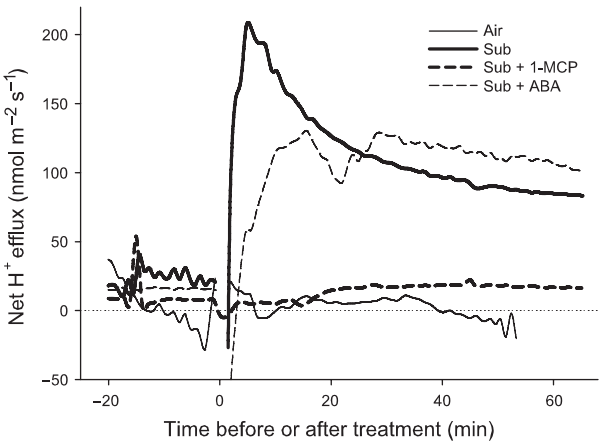


**Figure 2.** Typical traces of net proton efflux per square meter of petiole surface per second at the abaxial side of the third petiole in submerged (sub) and air-grown *Rumex palustris*. Thin solid trace: plant remained in air. Thick solid trace: plant submerged at $t = 0$. Thin dashed trace: petiole treated with 20 μM of ABA at $t = -2.5$ h and submerged at $t = 0$ in water containing 20 μM of ABA and 1 mM of KCl. Thick dashed trace: plants pre-treated for 40 min with 1-MCP and submerged at $t = 0$. All experiments were repeated at least three times with similar results.

whether: (i) net $H^+$ efflux and apoplastic pH are modified by submergence, ethylene or ABA, (ii) *in vivo* manipulations of pH affect petiole elongation, and (iii) *in vitro* acid-induced extensibility of petiole cell walls changes following submergence.

To assess the possibility that submergence leads to cell wall acidification, we first measured changes in pH at the petiole surface using a small pH electrode. During 4 h of submergence, pH decreased from an initial value of 5.9 ± 0.2 to 5.0 ± 0.2 ($n = 6$). This lower pH was still detectable in experiments lasting longer than 10 h of submergence (data not shown). Apoplastic acidification was detectable within 20 min of submergence, and this fast response prompted us to test whether ethylene or ABA, two hormones showing changes in concentration upon submergence within this time frame (Benschop, 2004; Voesenek *et al.*, 2003), influenced the rate of acidification. ABA at 20 μM, a concentration that inhibits submergence-induced petiole elongation by approximately 80% (Benschop, 2004), had no effect on submergence-induced acidification of the apoplast. By contrast, acidification upon submergence was inhibited in plants pre-treated with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception (data not shown).

To validate these surface pH measurements further, the Microelectrode Ion Flux Estimation (MIFE$^{TM}$) technique (Newman, 2001) was used to determine the net fluxes of $H^+$ from petioles. These experiments showed a high net $H^+$ efflux from petioles that commenced rapidly upon submergence, and remained high for the duration of the experiments (1 h) (Figure 2). The net $H^+$ efflux was prevented in plants pre-treated with 1-MCP, but was not influenced in plants submerged in a solution containing 20 μM of ABA. Taken together, the surface electrode and MIFE data suggest a novel regulatory role for ethylene in submergence-induced acidification in the petiole apoplast of *R. palustris*.

The importance of apoplast acidification on petiole elongation was tested *in vivo* by injections into petioles of solutions buffered at pH 4.0 or 6.0, and measuring subsequent elongation rates using linear variable displacement transducers. In non-submerged plants, injection of the pH 6.0 buffer did not change the rate of petiole elongation, whereas, pH 4.0 buffer invoked a rapid enhancement of elongation (Figure 3a). The enhanced elongation was only temporary, but re-occurred when a second injection with pH 4.0 buffer was performed (Figure 3a). In plants already submerged for 4 h, petiole elongation rates were, as expected, substantially higher than in air-exposed plants (Figure 3b). In these submerged plants, injection of pH 4.0 buffer hardly affected petiole elongation, whereas injection of the

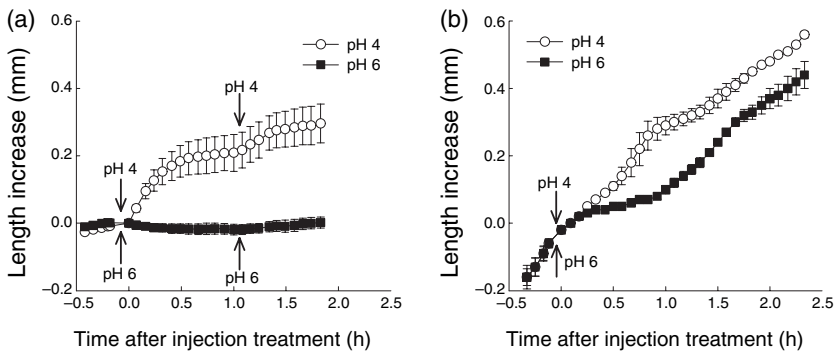


**Figure 3.** Increase in length of the third petiole of *Rumex palustris* injected with 50 mM of succinate buffer of pH 4 (open circles) or pH 6 (filled squares). Plants were growing in air (a) or submerged (b) at $t = -4$ h. In (a), injection with pH 6 is the control treatment; in (b) injection with pH 4 is the control treatment. Arrows indicate time of injection ($n = 2$–4; mean ± SE).

more neutral buffer (pH 6.0) transiently inhibited elongation (Figure 3b). These manipulations of apoplast pH using buffers, together with additional experiments in which 10 μM of fusicoccin was applied to petioles of air-exposed plants resulting in faster elongation (data not shown), suggest that acidification of the apoplast is a component of the mechanism leading to enhanced petiole elongation in *R. palustris*.

The magnitude of *in vitro* acid-induced extension (AIE) often reflects the growth rate of plant tissues and is probably the result of the action of primary wall loosening factors such as expansins and of secondary wall loosening factors like enzymes that modify the underlying structure of the wall (Cosgrove, 2000; McQueen-Mason, 1995). We used the *in vitro* AIE assay to gain insights into changes in extensibility upon submergence. It is assumed that the AIE of frozen, abraded and thawed tissue reflects the *in planta* activity of expansins. In air-exposed plants, AIE of the petioles generally declined with time (Figure 4). In submerged plants, AIE increased after 4 h, and at 6–7 h had reached a new, much higher level. The higher AIE was maintained for the duration of the experiment (28 h). The higher AIE of petioles from submerged plants suggests that more expansin protein was synthesized, and/or activity per unit protein increased (other than via pH changes), and/or the walls became more susceptible to expansin action. These three possibilities were examined in the series of experiments described below.

### *Submergence increases mRNA concentration of RpEXPA1, leading to enhanced levels of expansin protein in petioles*

To date, 19 expansin A genes have been cloned from *R. palustris* (Colmer *et al.*, 2004; Vriezen *et al.*, 2000). The mRNA levels of 13 were monitored using real-time RT-PCR.

Upon submergence, *RpEXPA1* mRNA levels were upregulated (Figure 5). The mRNA abundance of the other 12 expansins studied (*RpEXPA2*, *3*, *5*, *6*, *7*, *8*, *9*, *10*, *15*, *17*, *18* and *19*) were either not affected or downregulated by submergence (data not shown).

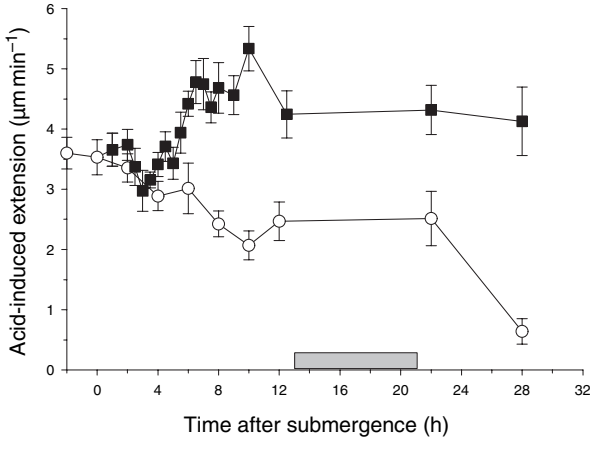


**Figure 4.** *In vitro* AIE of segments from the third petiole of *Rumex palustris* grown in air (open circles) or harvested at the indicated time after plant submergence (filled squares)(mean ± SE, $n = 10$). The AIE was measured in an extensometer with a constant load of 30 g, and is calculated as the extension rate in a 10-min interval after a pH change from 6.8 to 4.5, minus the extension rate in a 10-min interval before the pH change. Grey horizontal bar indicates 8 h dark period.

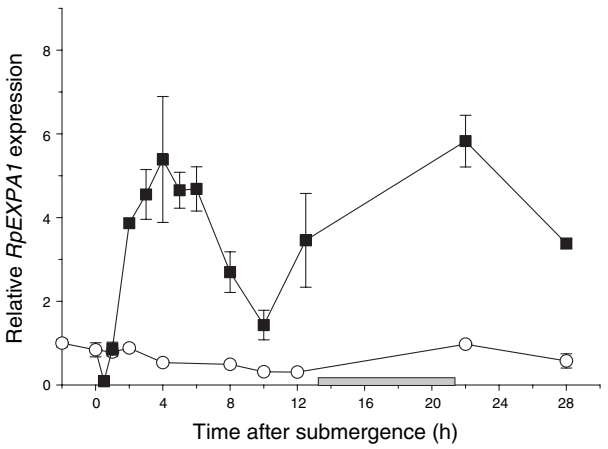


**Figure 5.** Relative transcript abundance of the *Rumex palustris* expansin A1 gene (*RpEXPA1*) in the third petiole of air-grown (open circles) or submerged (filled squares) plants (mean ± SE, $n = 3$). Values are measured with real-time RT-PCR with 18S as internal standard. Grey horizontal bar indicates 8 h dark period.

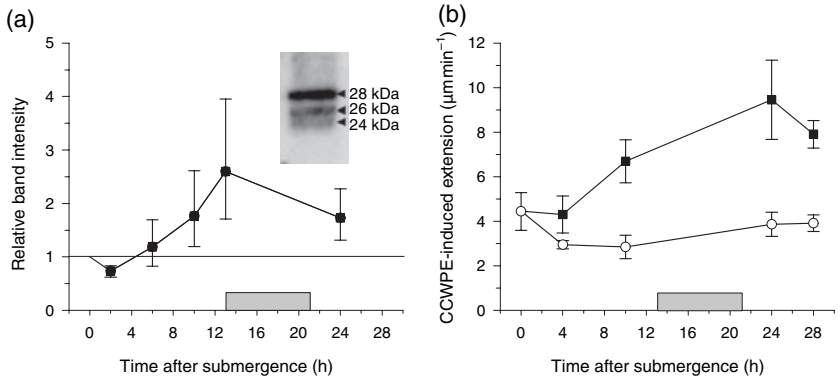


**Figure 6.** (a) Band intensity of a 24-kDa protein detected with LeEXP2 antibody in crude cell wall protein extracts (CCWPE) of the third petiole of submerged *Rumex palustris* petioles relative to the band intensities of air-grown plants (mean ± SE, $n = 4$). The control level is indicated by the horizontal line in the graph. Band intensities of submerged and air-grown samples were compared with each other on the same blot, making it possible to compare different blots with each other. The CCWPE was loaded on gel relative to the cell wall DW of the petioles used for the extraction. Grey horizontal bar indicates 8 h dark period. Inset: example of Western blot showing three expansin bands (24, 26 and 28 kDa) from CCWPE of *R. palustris* petiole tissue.
(b) Acid-induced extension of *Acetobacter xylinus*-derived cellulose/xyloglucan composite strips (2 × 12 mm) exposed to CCWPE of the third petiole of air-grown (open circles) or submerged (filled squares) *R. palustris*. The AIE was measured in an extensometer with a constant load of 20 g and the data were calculated as described in Figure 4 (mean ± SE, $n = 10$). Grey horizontal bar indicates 8 h dark period.

Following submergence, *RpEXPA1* transcript abundance had increased fourfold between 1 and 2 h (Figure 5). At the maximum, *RpEXPA1* transcript levels had increased sixfold, when compared with initial values (at the start of submergence). When expressed relative to air-exposed plants, the maximal increase in mRNA level was ninefold (4 h of submergence). The increase in *RpEXPA1* transcript abundance showed two distinct phases, similar in pattern to the diurnal changes in petiole elongation following submergence shown in Figure 1(a).

A phylogenetic comparison of the expansin A1 from *R. palustris* with putative orthologues in rice, Arabidopsis and *Regnellidium diphyllum* revealed a high similarity of *RpEXPA1* to *AtEXPA8* and *AtEXPA2* (data not shown). Less similarity was found with *OsEXPA1*, *2*, *3*, *4* and *10* and *RdEXPA1*. This *Regnellidium* gene and the five rice genes are associated with flooding-induced growth responses (Cho and Kende, 1997b; Kim *et al.*, 2000).

In order to evaluate the net result of increase in *RpEXPA1*, and decrease in several other *RpEXPA* genes (data not shown), on expansin protein abundance during submergence, Western blot analyses were performed using crude cell wall extracts. Based on predicted protein sizes of the nine available full-length *R. palustris* expansin cDNAs, proteins in the size range of 24.1–27.2 kDa were expected (signal peptides excluded). Three well-resolved bands were present at 24, 26 and 28 kDa on the Western blots using a polyclonal antibody raised against LeEXP2 (Figure 6a inset). The predicted protein sizes for the various cDNAs were assigned to one of three size categories (24.1–24.6, 25.7, 27.2 kDa), as shown in Table 1.

Time-series analysis showed that the intensity of the 26 and 28 kDa bands did not change, or decreased slightly, during 24 h of submergence (data not shown), a result consistent with the lack of submergence-induced upregulation of *RpEXPA10* and *RpEXPA18*. By contrast, the intensity of the 24 kDa band, that presumably contains, among others, the RpEXPA1 protein, increased from 6 h of submergence onwards (Figure 6a). However, in contrast to the observed pattern in *RpEXPA1* mRNA levels, no diurnal variation was observed in RpEXPA1 protein levels.

In addition to the Western blotting experiments to assess expansin protein abundance, we also tested expansin activity in crude protein extracts from the cell wall fraction of *R. palustris*, using *Acetobacter*-derived cellulose/xyloglucan composites. These composites have structural features in common with dicot cell walls, and provide a highly sensitive and uniform material for conducting assays of expansin activity (Whitney *et al.*, 2000). Expansin activity in submerged petioles increased relative to air-grown petioles from 4 h onwards and remained elevated for the duration of the experiment (Figure 6b).

**Table 1** Grouping of the full-length clones of *Rumex palustris* expansin A genes in three size groups. Protein sizes were calculated *in silico* without signal peptide (predicted using Signal P; Nielsen *et al.*, 1997) using DNA Star. Genes studied by real-time RT-PCR are indicated in bold

| Size range | | |
|---|---|---|
| **24.1–24.6 kDa** | **25.7 kDa** | **27.2 kDa** |
| **RpEXPA1** | **RpEXPA10** | **RpEXPA18** |
| **RpEXPA7** | RpEXPA11 | |
| **RpEXPA8** | RpEXPA12 | |
| RpEXPA13 | | |
| RpEXPA14 | | |

The final possible explanation for the increased AIE in submerged petioles (Figure 4) might be changes in cell wall susceptibility towards expansins. To examine this, heat-inactivated petiole segments of *R. palustris* were assayed for extension in response to the addition of equal amounts of celery crude cell wall protein extracts. These experiments showed no difference between submerged and air-grown petioles, when tested after 6, 10 and 24 h of treatment (data not shown).

In summary, increased *in vitro* acid-induced extensibility of petioles following submergence occurs concomitantly with an increase of expansin protein(s) of approximately 24 kDa, a size consistent with that predicted for RpEXPA1, the only expansin of the 13 studied that showed increased transcription following submergence. The increased level of expansin protein is consistent with increased activity (*Acetobacter* composite assay), and both were preceded by an increased in *RpEXPA1*.

### *Submergence-induced responses can be reversed by de-submergence*

To gain further insight into the regulation of expansins in relation to petiole elongation, we withdrew the submergence signal after 10 h by de-submerging plants to soil level, and a comprehensive set of measurements were taken after 14 h (this is 24 h after start of the experiment). Upon de-submergence, the petiole elongation rate returned to that of air-grown plants (Table 2). *RpEXPA1* transcript levels, which had risen during submergence (Figure 5), decreased following de-submergence to the values in air-grown plants (Table 2). Following de-submergence, both AIE and the amount of approximately 24 kDa expansin protein(s), returned to values close to those of air-grown plants. Although extractable expansin activity (*Acetobacter* composite assay) also declined, it lagged behind the more rapid decreases in the other parameters (Table 2).

The reversibility upon de-submergence of *RpEXPA1* concentration and protein activity as described above, together with the decline in the rate of elongation, shows that expansin activity correlates with petiole elongation, in response to submergence signals. The issue now remaining to be resolved is which of the four hormones known to be involved in submergence-induced petiole elongation (Cox *et al.*, 2004; Voesenek *et al.*, 2003), regulate *RpEXPA1* mRNA levels in *R. palustris*.

### *RpEXPA1 mRNA abundance is regulated by ethylene and auxin*

Previous work showed that expansin mRNA concentrations in *R. palustris* are upregulated by ethylene (Vriezen *et al.*, 2000). However, due to the method used it is not known which of the expansin genes are upregulated, and whether ethylene acts directly on the transcription, or indirectly via other hormones. The influences of ethylene, ABA, GA and 1-naphthalene acetic acid (1-NAA) on mRNA abundance of *RpEXPA1* were assessed after 6 h of submergence, as our kinetic analysis revealed high concentrations of this transcript (Figure 5) with petiole elongation occurring at maximum speed at this time (Figure 1).

Upon ethylene exposure (5 $\mu$l $l^{-1}$) *RpEXPA1* concentrations increased, *RpEXPA15* mRNA abundance was reduced, and no significant changes occurred for the other expansin genes studied (Figure 7a). In order to identify ethylene as the signal acting in submerged petioles, we used 1-MCP to inhibit ethylene perception. Pre-treatment with 1-MCP abolished the submergence-induced upregulation of *RpEXPA1* (Figure 7b).

Abscisic acid concentrations are reduced very quickly in petioles upon submergence (Benschop, 2004). We tested whether this decline in ABA is a signal for *RpEXPA1* upregulation by preventing the usual decline in ABA by external ABA addition to plants while submerged. Addition of ABA to submerged plants did not prevent the submergence-induced increase in *RpEXPA1* mRNA levels, indicating that a decline in ABA is not required for submergence-induced *RpEXPA1* upregulation (Figure 7c).

The possible role of GA in *RpEXPA1* expression was tested as this phytohormone is required for submergence-induced petiole elongation (Rijnders *et al.*, 1997) and has been shown to regulate expansins A and B in deepwater rice (Cho and Kende, 1997b; Lee and Kende, 2001). Submergence-induced upregulation of *RpEXPA1* is independent of GA action, as the GA biosynthesis inhibitor paclobutrazol was unable to reduce *RpEXPA1* mRNA abundance under water. Further support came from the observation that

| | Air-grown | Submerged | De-submerged |
|---|---|---|---|
| Growth rate (mm $h^{-1}$) | 0.10 ± 0.02 | 0.20 ± 0.01 | 0.11 ± 0.02 |
| *RpEXPA1* transcript abundance (rel.[a]) | 0.57 ± 0.17 | 3.38 ± 0.12 | 1.06 ± 0.11 |
| AIE ($\mu$m $min^{-1}$) | 0.64 ± 0.21 | 4.13 ± 0.57 | 1.07 ± 0.20 |
| 24 kDa protein levels (rel.[a]) | 1 | 1.73 ± 0.55 | 1.19 ± 0.25 |
| Extractable expansin activity ($\mu$m $min^{-1}$) | 3.92 ± 0.37 | 7.91 ± 0.62 | 5.50 ± 0.60 |

[a]Given value is a unit-less ratio.

**Table 2** Effects of de-submergence on the submergence-induced changes in growth, *RpEXPA1* expression, AIE, expansin protein level and expansin activity ($n = 3$–10; ±SE). Plants were submerged for 10 h, followed by at least 14 h of lowered water levels in which the shoots were re-exposed to air

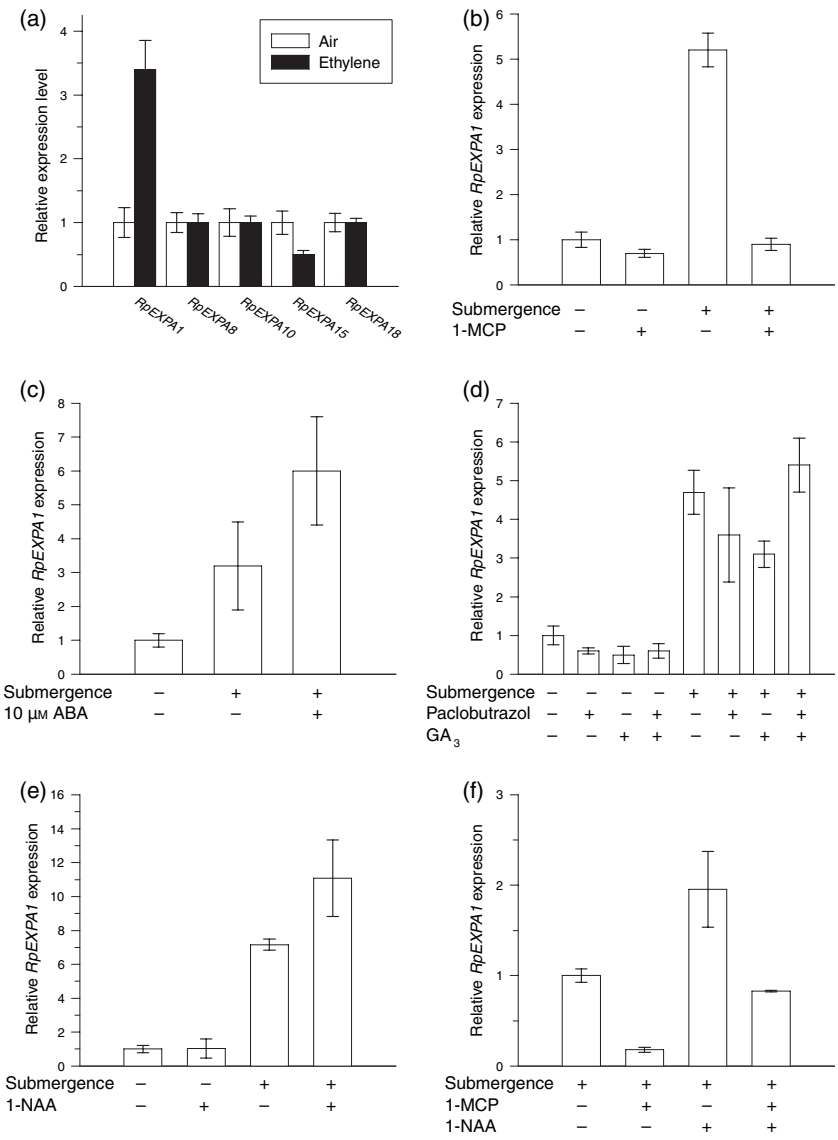


**Figure 7.** Relative transcript abundance of the *Rumex palustris* expansin A1 gene (*RpEXPA1*) after 6 h in the third petiole of plants treated with plant hormones and hormone inhibitors. The following treatments were applied: (a) transcription of *RpEXPA1*, *8*, *10*, *15* and *18* in petioles exposed to 5 μl $l^{-1}$ ethylene and air controls, (b) petioles submerged and pre-treated with the ethylene action inhibitor 1-MCP (1 μl $l^{-1}$ for 1 h), (c) petioles exposed to 10 μM of ABA, (d) petioles exposed to $GA_3$ (10 μM) and pre-treated with the GA biosynthesis inhibitor paclobutrazol (50 μM 4 days before start of experiment), (e) petioles exposed to 1-NAA (10 μM) and (f) petioles exposed to 1-NAA (10 μM) and pre-treated with 1-MCP (1 μl $l^{-1}$ for 1 h) (mean ± SE, $n$ = 2–4).

externally added $GA_3$ could not promote transcription in air-grown plants (Figure 7d).

Auxin plays a role in submergence-induced hyponasty and petiole elongation of *R. palustris* (Cox *et al.*, 2004). Treatment of air-grown petioles with 1-NAA did not affect the *RpEXPA1* mRNA concentration (Figure 7e). By contrast, a promoting effect of 1-NAA on *RpEXPA1* levels was observed in submerged plants. As auxin can stimulate ethylene production (Yang and Hoffman, 1984), we tested whether 1-NAA stimulation occurs via ethylene signalling, by blocking ethylene perception with 1-MCP. In this experiment, 1-NAA again caused further upregulation of *RpEXPA1* above that caused by submergence alone (Figure 7f). When ethylene action was abolished by 1-MCP, a substantial 1-NAA effect remained with *RpEXPA1* reaching values of non-treated submerged plants, which is much higher than submerged 1-MCP-treated plants without 1-NAA (Figure 7f). These results suggest that the effect of 1-NAA on *RpEXPA1* concentration in submerged petioles is independent of ethylene action.

In summary, the series of experiments on hormonal regulation show that ethylene, but not ABA and GA, can induce upregulation of *RpEXPA1* transcript abundance during submergence, and that auxin can enhance *RpEXPA1* mRNA to higher levels in submerged petioles in an ethylene-independent manner.

## Discussion

Submergence-induced petiole elongation enables *R. palustris* to emerge from flooded environments. This acclimation increases survival in environments characterized by shallow

and prolonged flooding (Voesenek *et al.*, 2004). The objective of the present work was to study submergence-induced apoplastic acidification and to elucidate the role of expansins, and their regulation by hormones, in bringing about underwater elongation.

Fast underwater petiole elongation commenced 4 h after submergence. This enhanced elongation matched fairly well with the kinetics of increased AIE of petioles, expansin protein abundance and activity if we take into account that the various methods applied differ in resolution (e.g. measuring elongation is much more sensitive than estimating protein abundance). The responses at the protein level were preceded by a specific increase in the transcript abundance for *RpEXPA1*, within 2 h of submergence. An even faster response was the rapid stimulation of net $H^+$ efflux from petiole cells, probably inducing a gradual acidification of the apoplast. Thus, our data suggest that submergence induces two major processes in petioles of *R. palustris*: (i) production of more expansin and (ii) apoplast acidification. These processes are both causally linked to elevated ethylene, the primary signal of submergence (Voesenek *et al.*, 2004).

### *Increased petiole elongation is preceded by net $H^+$ efflux that is under ethylene control*

Although $H^+$ extrusion commenced within minutes of submergence (Figure 2), petiole elongation rates did not increase until 4 h of flooding. This suggests that acidification for at least 4 h of submergence is required to enhance petiole elongation. The promoting effect of low apoplastic pH was demonstrated by manipulations of the endogenous pH that resulted in fast changes in petiole elongation (Figure 3a,b).

The possible roles of ABA and ethylene in triggering $H^+$ extrusion during submergence were tested. Based on earlier observations that ABA inhibits apoplastic acidification in guard cells (Goh *et al.*, 1996; Roelfsema *et al.*, 1998) and that it promoted extracellular alkalization in plant cells from other species (Balsevich *et al.*, 1994; Felle and Hanstein, 2002), we expected an influence of ABA on $H^+$ extrusion. Even though endogenous ABA levels decline rapidly in submerged petioles (Benschop, 2004), ABA levels did not appear to affect net $H^+$ extrusion (Figure 2). However, inhibition of ethylene perception abolished net $H^+$ extrusion (Figure 2), implicating ethylene as a controlling agent in apoplast acidification. This result suggests a novel role for ethylene in regulating apoplastic pH and it indicates that the ethylene signal may accumulate almost immediately upon submergence.

### *Expansin protein abundance and activity not always match elongation growth*

The submergence-induced increase in expansin protein(s) of the size class containing RpEXPA1 (Figure 6a), and expansin activity (Figure 6b), closely matched the initiation of fast underwater petiole elongation (Figure 1a) despite the serious differences in resolution of the techniques used. These findings are in agreement with responses of other species (Cho and Cosgrove, 2000; Cho and Kende, 1997c; Choi *et al.*, 2003; Huang *et al.*, 2000). However, the high temporal resolution of our experiments resulted in a novel observation: there are periods with high and low correlations between elongation and expansin activity existing in one system. There were periods during which petiole elongation rates in submerged plants were significantly faster than air-grown plants, but there was also a phase with equal rates (between 11 and 18 h of submergence; Figure 1a). The period in which submerged and air-grown petioles had equal elongation rates occurred despite significantly higher values in AIE, expansin protein abundance and *in vitro* expansin activity in the submerged plants (Figures 4 and 6a,b). This period with low correlation suggests that factor(s) other than expansin protein abundance must limit the *in vivo* rate of petiole elongation. Several processes act in concert to enable cell elongation (e.g. pH, various cell wall proteins and hydroxyl radicals; Cosgrove, 1999), and elucidation of which of these limits submergence-induced petiole elongation during the slower phase will be a priority for future work. As suggested by Caderas *et al.* (2000) a lack of correlation between expansin protein and growth may, in principle, also be related to a decrease in susceptibility of the cell walls to expansins. However, no differences in expansin susceptibility were found between submerged and air-grown petioles (data not shown), so susceptibility variation cannot explain the temporary lack of correlation between expansin protein and elongation rate.

### *Ethylene triggers the specific increase of the expansin RpEXPA1 transcript*

During submergence, a remarkable similarity was observed between the kinetics of *RpEXPA1* transcript abundance and petiole elongation (Figures 1a and 5). A similar transcription pattern also occurred for two other *R. palustris* genes, *Rp-ACO1* (Y10034; Vriezen *et al.*, 1999) and *Rp-ERS1* (U63291; Vriezen *et al.*, 1997). These genes encode for an enzyme in ethylene biosynthesis and for an ethylene receptor protein respectively. The similarity in kinetics of the mRNA abundance of these genes and petiole elongation in submerged *R. palustris* implicates a common regulatory system. Ethylene promotes the transcription of all three genes in *R. palustris* (*RpEXPA1*, present study; *Rp-ACO1*, Vriezen *et al.*, 1999; *Rp-ERS1*, Vriezen *et al.*, 1997). In addition, ERS and ACO orthologues in other species are also transcriptionally regulated by ethylene (Ciardi and Klee, 2001; Mekhedov and Kende, 1996; Mita *et al.*, 2002; Peck and Kende, 1995). Therefore, we suggest that ethylene is the common regulator controlling transcription of these three

genes and underwater elongation; either directly, or indirectly via an unknown signal transduction component(s). The similar transcription profiles of these three genes and petiole elongation could be explained by diurnal fluctuations in either: (i) endogenous ethylene concentrations, or (ii) sensitivity to ethylene. It seems unlikely that endogenous concentrations of ethylene fluctuate, to any great extent, during submergence (Banga *et al.*, 1996), as similar diurnal patterns of petiole elongation rates were also observed for plants treated with growth-saturating ethylene concentrations (data not shown). We therefore hypothesize that changes in ethylene sensitivity might explain the diurnal cycles in gene transcription and elongation.

Ethylene specifically triggers *RpEXPA1* transcription in submerged petioles of *R. palustris* (Figure 7a). The semi-aquatic fern *R. diphyllum* also responds to submergence by faster ethylene-mediated shoot extension (Kim *et al.*, 2000). Moreover, both submergence and ethylene treatment induced upregulation of an expansin A gene, *RdEXPA1* (AF202120) in rachises, the elongating organ of this species. Other expansin A genes are known to be regulated by ethylene in ripening tomato fruits (*LeEXPA1*; Rose *et al.*, 1997) and two during environmentally induced root hair initiation in Arabidopsis (*AtEXPA7* and *AtEXPA18*; Cho and Cosgrove, 2002).

In contrast to ethylene being the regulator of submergence-induced expansin mRNA level in *R. palustris* (present study) and *R. diphyllum* (Kim *et al.*, 2000), GA is considered to be the hormone that upregulates specific expansins A and B in deepwater rice (Lee *et al.*, 2001). The role of GA in internode extension in deepwater rice is further strengthened by the existence of GA-responsive elements in those expansin genes upregulated by submergence (Lee *et al.*, 2001). Interestingly, *RpEXPA1* concentration is GA independent (Figure 7d), although GA is required to sustain fast petiole elongation in *R. palustris* (Rijnders *et al.*, 1997). The need for GA in underwater petiole elongation, which is independent of *RpEXPA1* transcription, supports the hypothesis that ethylene stimulates elongation via several parallel transduction pathways and not via a linear transduction chain (Voesenek *et al.*, 2003). These results suggest that internode elongation in deepwater rice differs significantly from petiole elongation in *Rumex* in the way in which submergence-induced expansin transcription is regulated.

Independently of ethylene, auxin promoted *RpEXPA1* abundance in submerged petioles (Figure 7e,f). Auxin did not influence mRNA concentration in air-grown plants. Together, these results show an interaction between submergence and auxin. The nature of this interaction is not known. At least one other expansin A gene is also known to be upregulated by auxin, namely *LeEXPA2* (AF096776), the transcription of which occurs in the growing regions of tomato hypocotyls (Catalá *et al.*, 2000). Further evidence for a putative role of auxin in regulation of expansin genes is the existence of an auxin-response element in one expansin A gene in rice (Lee *et al.*, 2001).

### *Ethylene modulates three parallel pathways essential for submergence-induced petiole elongation*

The primary aim of the present study was to further clarify the regulatory network underlying submergence-induced petiole elongation in *R. palustris*. Our starting point was the existing knowledge of the hormonal regulation of submergence-induced petiole elongation in *R. palustris* (Peeters *et al.*, 2002). The present study extends this previous work with data on apoplastic acidification and its hormonal regulation and on a potential target gene encoding a cell wall-loosening protein (RpEXPA1). Upon submergence, ethylene accumulates rapidly in petiole tissues and evokes at least three parallel processes that correlate with fast underwater elongation. The first, apparent within minutes, is a stimulation of net $H^+$ efflux, leading to gradual cell wall acidification. The second, apparent within 2 h, is a transcriptional upregulation of *RpEXPA1*, followed by increased protein abundance and activity from 4–6 h onwards. Cell wall acidification presumably enhances the *in vivo* activity of the increasingly abundant RpEXPA1. The third process, also initiated by ethylene in petioles of *R. palustris*, is a cascade leading to increased concentrations of GA and auxin, and decreased levels of ABA (Benschop, 2004; Cox *et al.*, 2004; Voesenek *et al.*, 2003).

Our results suggest that under water petiole elongation in *R. palustris* is regulated via an ethylene-driven signalling network composed of parallel operating transduction pathways that result in coordinated elongation at the level of the whole petiole (Figure 8). The *Rumex* elongation system differs from the linear transduction cascade described for submergence-induced internode elongation in deepwater rice (Kende *et al.*, 1998).

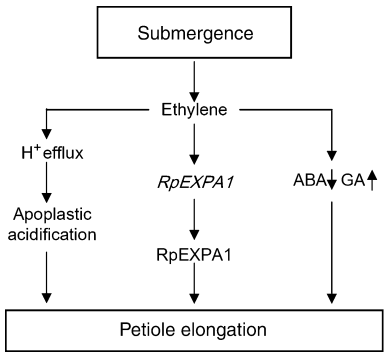


**Figure 8.** Signal transduction network for enhanced petiole elongation in *Rumex palustris* upon complete submergence. Accumulated ethylene triggers at least three parallel processes that, when integrated, stimulate cell elongation in petioles.

## Experimental procedures

### *Plant material*

*Rumex palustris* seeds were sown on black polyethylene beads (Elf Atochem, Marseille, France) floating on tap water in a transparent container. The container was placed in a growth cabinet at 12 h light, 20°C, 70 μmol $m^{-2}$ $sec^{-1}$ photosynthetic photon flux density and 12 h dark, 10°C. After 9 days, seedlings were potted in a mixture of 2:1 (v/v) potting soil and sand, with one seedling each in an 80-ml plastic pot. The potting mixture also contained 0.14 mg MgOCaO per pot. Prior to planting, each pot received 20 ml of nutrient solution containing: 7.5 mM $(NH_4)_2SO_4$, 15 mM of $KH_2PO_4$, 15 mM of $KNO_3$, 4.3 μM of $MnSO_4$, 1.8 μM of $ZnSO_4$, 0.32 μM of $CuSO_4$, 43 μM of $H_3BO_3$, 0.53 μM of $Na_2MoO_4$ and 86 μM of Fe-EDTA. The pots were placed in a growth chamber (16/8 h light/dark, 20°C, 70% relative humidity and 200 μmol $m^{-2}$ $sec^{-1}$ photosynthetic photon flux density) for 15 days. For the first 2 days following potting, the pots were kept in trays covered with glass, after which the pots were placed on an irrigation mat. The mat was automatically watered with tap water to saturation twice a day, after which water was drained. Plants used in all experiments were 24 days old.

The 24-day-old plants had an expanding third leaf and a newly emerging fourth leaf. Submergence was imposed by transferring pots into a series of 50 l tanks, half of which were then filled with tap water at 20°C and the other half were left containing air. Air-grown control plants were watered at the time of submergence and before the dark period. In all experiments, submergence was imposed at 10 AM. For de-submergence treatments, the water was siphoned out of the tanks to soil level.

### *Petiole elongation*

Petiole elongation was measured using linear variable displacement transducers as described in Voesenek *et al.* (2003), but with clamps designed to measure petiole elongation only. A net pulling weight of 5 g was used. This weight in itself did not affect the elongation rate of the petioles (data not shown). Elongation rates were calculated as the slope of lines fitted through data collected during 20 min intervals (180 data points).

### *Acid-induced extension, apoplastic pH measurements, net $H^+$ fluxes and pH manipulations*

Acid-induced extension of segments of the third petiole was measured in a custom-built constant load extensometer, modified from Cosgrove (1989), with a pulling force of 30 g. Petiole segments of 6 mm length were harvested (10 per time point per treatment) and stored frozen at –80°C. The frozen segments were abraded with carborundum slurry, thawed and pressed between two glass slides. After clamping in the extensometer the petiole segments were first incubated in 50 mM of HEPES, pH 6.8, for 30 min, after which the buffer was changed to 50 mM of sodium acetate, pH 4.5. An AIE was calculated by fitting a line through a 10-min interval starting 1 min after extension was enhanced, minus the slope of a line fitted through a 10-min interval starting 11 min before this enhanced rate.

Apoplastic pH was measured with a small flat surface pH electrode (tip size 1.5 mm, MI-406; Microelectrodes Inc., Bedford, NH, USA) in a custom-made cuvette in which the third petiole was bathed in 1 mM of KCl, while for air-grown controls the root, leaf blade and other leaves were not in solution whereas for submerged plants the roots and shoots were also submerged in 1 mM of KCl. The tip of a reference electrode (MI-401; Microelectrodes Inc.) was also positioned in the bath solution. The pH was measured at the abaxial side of petioles abraded with carborundum powder just prior to each experiment.

In some experiments, plants were pre-treated for 40 min with the ethylene perception inhibitor 1-MCP at 3 μl $l^{-1}$ (Ethylbloc; Floralife Inc., Walterboro, SC, USA). This pre-treatment completely blocked ethylene-induced petiole elongation in *R. palustris* (data not shown). In other experiments, some plants were submerged in 1 mM of KCl in tap water also containing 20 μM of ABA (dissolved in 0.1% ethanol; Acros, 's-Hertogenbosch, the Netherlands). All experiments were conducted at 20°C and 60 μmol $m^{-2}$ $sec^{-1}$ photosynthetic photon flux density.

Net fluxes of $H^+$ from petioles were measured using $H^+$-selective microelectrodes with the MIFE™ technique (Newman, 2001; Shabala *et al.*, 1997). Microelectrodes were pulled from borosilicate glass capillaries (GC150-10; Harvard Apparatus Ltd, Edenbridge, UK), silanized with tributylchlorosilane (Fluka 90974, Zwijndrecht, the Netherlands), back-filled with 15 mM of NaCl plus 40 mM of $KH_2PO_4$ and front-filled with Hydrogen Ionophore II (Cocktail A; Fluka 95297). The average response of the electrodes was 56.7 ± 1.0 mV per decade (i.e. pH unit) (pH range 5.1–7.8).

An intact plant was mounted in front of a horizontal microscope (Nikon, Badhoevedorp, the Netherlands). The third leaf was fixed watertight at the base of the petiole in a horizontal plastic container. The leaf blade was immobilized to the side of the container using Terostat. The petiole was gently rubbed with carborundum powder to remove the cuticle and submerged with 1 mM of KCl. For non-submerged controls, the roots, leaf blade and all other leaves were in air during the measurements. The $H^+$-selective microelectrode was mounted vertically in a holder (MMT-5; Narishige, Tokyo, Japan) on a three-way piezo-controlled micromanipulator (PCT; Luigs & Neumann, Ratingen, Germany) driven by a computer-controlled motor (MO61-CE08; Superior Electric, Bristol, CT, USA). The electrode was positioned 20 μm from the abaxial surface of the petiole. During measurements the distance between the surface of the petiole and the electrode was changed from 20 to 60 μm at a frequency of 0.1 Hz. Measurements were performed in the dark at 20°C. The experimental solution was boiled prior to use and during the experiments $CO_2$-free air was blown over the solution to minimize the effect of changing $CO_2$ concentrations on the pH of the experimental solution. The chemical activity of $H^+$ in solution was continuously recorded at the two distances from the petiole and from these data net $H^+$ fluxes were calculated according to Newman (2001).

Physiological activity of the tissue was checked after every experiment by following the extracellular pH change due to photosynthetic uptake of $CO_2$ induced by illumination of the tissue with 300 μmol $m^{-2}$ $sec^{-1}$ white light from a slide projector (projection lamp type 7748XHP; Philips, Eindhoven, the Netherlands) equipped with a fibreoptic cable.

In case of ABA treatment, the experimental solution contained 20 μM of ABA (stock solution 25 mM in ethanol). Pre-treatment with 1-MCP was performed as described below under the section Hormone treatments.

Apoplastic pH in petioles of *R. palustris* was manipulated and responses of elongation assessed. Two types of experiments were conducted: (i) Acidification was induced by applying fusicoccin (Sigma-Aldrich, Zwijndrecht, the Netherlands) as 10 μM in a 0.1% Tween 20 solution, to abraded petioles. Control petioles were also abraded and treated with a 0.1% Tween 20 solution. (ii) A succinate buffer (50 mM) with pH 4 or 6 was injected (7 μl) using a 10-μl syringe with a conical needle tip. The buffer was injected in air-grown petioles, or to petioles of plants that were submerged for 4 h.

Petiole elongation was measured with the transducers described above.

### *RNA isolation and cDNA synthesis*

RNA for real-time RT-PCR analysis from the various plant materials was isolated according to Kiefer *et al.* (2000) using Nucleon Phytopure DNA extraction resin (Amersham Biosciences, Roosendaal, the Netherlands). Residual genomic DNA was broken down after several treatments with RNase-free DNaseI (Amersham Biosciences). First-strand cDNA synthesis used approximately 1 μg of total RNA, random hexamer nucleotides (Roche Diagnostics Nederland, Almere, the Netherlands) and M-MLV reverse transcriptase (superscript III), according to the manufacturer's instructions (Invitrogen, Breda, the Netherlands). The reaction was performed in an Eppendorf master cycler gradient programmed to the following conditions; 5 min at 25°C and then 60 min at 50°C, with the reaction terminated by heating to 70°C for 15 min. Thereafter the cDNAs were diluted to 100 μl with water and 5 μl was used per real-time RT-PCR analysis.

### *Real-time RT-PCR analysis*

Real-time RT-PCR analysis was performed with probes and primers designed with either Applied Biosystems software or the PRIMERS3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using the sequences deposited in GenBank. To date, 19 expansin A genes from *R. palustris* have been cloned (Colmer *et al.*, 2004; Vriezen *et al.*, 2000). The nomenclature of these genes is according to Kende *et al.* (2004).

Real-time RT-PCR (Heid *et al.*, 1996) for *RpEXPA1*, *8*, *10*, *15* and *18* used an ABI Prism 7700 (Applied Biosystems, Foster City, CA, USA) or a Bio-Rad MyiQ single colour real-time PCR detection system (Bio-Rad, Veenendaal, the Netherlands) with FAM and TAMRA-labelled fluorescent TaqMan probes. The chemicals used were as recommended by Applied Biosystems, and the PCR reaction conditions were 2 min at 50°C, 10 min at 95°C and thereafter 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each PCR assay was performed twice. A cDNA for 18S ribosomal RNA isolated from a *R. palustris* cDNA library was used as an internal sample control. For each gene the optimal primer/probe concentration was determined according to the manufacturer's instructions (TaqMan universal PCR master mix protocol; Applied Biosystems). The primers and probes used are listed in Colmer *et al.* (2004) and were obtained from Isogen (Maarssen, the Netherlands). The primer and probe combinations showed no, or minimal, cross-amplification with other expansins, tested on plasmid sequences (data not shown).

Additionally, real-time RT-PCR measurements were performed for *RpEXPA1*, *2*, *3*, *5*, *6*, *7*, *9*, *10*, *15*, *17*, *18* and *19* with a Bio-Rad MyiQ single colour real-time PCR detection system with SYBR-green as fluorescent intercalating dye (Bio-Rad iQ SYBR-green super mix). Primer sequences are shown in Table 3 and were obtained from Isogen. Much effort was put into the design of primers and PCR circumstances used. We deliberately have chosen to make primers on the coding sequences and not on the 3 or 5′ UTR, as for these we had sequence information for all of the 19 genes. Efficiency, melting curves and agarose gels were obtained for all the primer combinations used. Due to high homology within the expansin gene family, it was, in some cases, impossible to identify gene-specific primer pairs. In addition, annealing temperatures had to be raised in some cases to enable specific amplification (see Table 3, annealing temperature). As a result of this, cross-amplification within the primer and cDNA pool was shown to be absent or at a very low level at the annealing temperatures used (data not shown). Apart from the annealing temperature (Table 3) the standard PCR protocol was 30 sec at 95°C, 30 sec at the appropriate annealing temperature and 1 min at 72°C.

*RpEXPA1*, *10*, *15* and *18* were analysed with both real-time RT-PCR methods. No significant differences in transcription patterns were detected (data not shown).

Relative mRNA values were calculated using the comparative $2_T^{-\Delta\Delta C}$ method described by Livak and Schmittgen (2001), expressing mRNA values relative to 18S RNA. To compare the relative abundances of the different *R. palustris* expansins, PCR efficiency was determined using known amounts of plasmid expansin DNA. The relationship between the amount of DNA and the Ct value was used to obtain the relative amounts of the selected expansin genes in one sample. The data set was corrected for these ratios, defining *RpEXPA1* at $t = -2$ as 1.

### *Protein extraction, Western blot analysis and protein activity*

Crude cell wall protein extracts were made according to Rochange *et al.* (2001), modified to extract five petioles in 1.5 ml reaction tubes. Cell wall material was separated by centrifugation at 21 000 ***g***. After extraction, the cell wall residue was washed with water, 70% ethanol and acetone, all solutions being kept on ice. Starch was removed from the samples by treating them overnight

**Table 3** Sequences of primer combinations for the expansin A genes used in the SYBR-green assay

| Gene | Forward primer 5′-…-3′ | Reverse primer 5′-…-3′ | Annealing temperature (°C) |
|---|---|---|---|
| *RpEXPA1* | agacgttcactcggtgtcgat | cagttctggccccaattcc | 60 |
| *RpEXPA2* | tttgggtaggaggagcatgt | ccttgacatggttgcccatc | 68 |
| *RpEXPA3* | ctctgttcaacaacggcttg | ccctccctttcttctgcaat | 65 |
| *RpEXPA5* | gtcattactttctcaaatat | cctcgacatcggctgccacc | 68 |
| *RpEXPA6* | taatcacgcaatcactcact | ccggctcatgctcatccagt | 65 |
| *RpEXPA7* | cacgaggctttctcttacaa | tccggcgccacccacattgg | 65 |
| *RpEXPA9* | atcaccgtgacagccaccaa | gagggtaggccttaggcttt | 70 |
| *RpEXPA10* | cgaggaggaatcaggttcac | caactgcgggctacaagatt | 68 |
| *RpEXPA15* | caaacaccgccttaccaaat | ttcctcctgatttcctgcac | 65 |
| *RpEXPA17* | gcacgatgggaggagcttgt | cgcggtactcagcaatcttc | 70 |
| *RpEXPA18* | ctacggcggcggagatgctt | aagagtgtccgttgattgta | 65 |
| *RpEXPA19* | cgccctcccaagcgataacg | aggttgaagtaacggaaacc | 65 |
| *Rp18S* | ccgttgctctgatgattcatga | gttgatagggcagaaatttgaatgat | 60 |

with 75 U bacterial α-amylase (Sigma A6380) at 20°C in 125 μl 20 mM of potassium phosphate buffer at pH 7, 10 mM of NaCl and 0.05% chlorbutol. After de-starching, the cell wall material was washed again with cooled 70% ethanol and acetone, dried in a speed-vac and weighed. The mean cell wall dry weight (DW) per set of four petioles of control and submerged petioles did not differ (data not shown). Proteins were loaded on gel relative to the cell wall DW in order to represent the amount of proteins in a physiologically relevant way (Cho and Kende, 1997c).

A 15% SDS-PAGE was used to separate the proteins. The proteins were blotted on reinforced nitrocellulose paper. The blots were labelled with 1:2000 LeEXPA2 antibody and 1:20 000 antirabbit IgG, peroxide conjugate (A0545; Sigma). For detection we used Pierce Super Signal Ultra, chemiluminescent substrate (no. 34075; Pierce Biotech Inc., Rockford, IL, USA) and Kodak Biomax Light films (no. 8194540; Eastman Kodak Company, New Haven, CT, USA). Band intensities were determined using custom-made software on an image analysis set-up (KS 400, version 3.0; Carl Zeiss Vision, Jena, Germany). To overcome film saturation artefacts, samples were loaded in three dilutions and multiple exposure times were used. Each blot contained an air-grown sample and a submerged sample both taken at the same time. In order to compare different blots, submerged values were expressed as a ratio to the air-grown values. Because the values used are ratios, the values were log-transformed before standard errors were calculated, after which the values were back-transformed.

The predicted protein sizes from the cDNA sequences were obtained using LASERGENE DNA software (DNA Star Inc., Madison, WI, USA). The signal peptide sequences were predicted *in silico* using Signal P (http:/www.cbs.dtu.dk/services/SignalP-2.0/; Nielsen *et al.*, 1997). Protein sizes from gels were calculated using kaleidoscope pre-stained standards (no. 161-0324; Bio-Rad) as reference.

*Acetobacter xylinus*-derived cellulose/xyloglucan composites were grown as described by Whitney *et al.* (2000), with an incubation time of 5 days. Crude cell wall protein extracts (method given above) were re-suspended in 50 mM of sodium acetate buffer at pH 4.5, relative to the cell wall DW (see Western blot analysis). The protein extracts were added to 2 × 12 mm strips of cellulose/xyloglucan composites in an extensometer with a pulling force relative to 20 g.

### *Hormone treatments*

Ethylene (analytical grade; Hoekloos, Schiedam, the Netherlands) was applied in a flow-through system with 70% RH, 20°C and 200 μmol $m^{-2}$ $sec^{-1}$ photosynthetic photon flux density. Plants were treated with 5 μl $l^{-1}$ ethylene or with air without added ethylene. 1-MCP (Ethylbloc; Floralife Inc.) 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) 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 water.

Paclobutrazol (Duchefa, Haarlem, the Netherlands) was prepared as 0.1 M stock in 96% ethanol and diluted to 50 μM in water. Soil-grown plants were treated with 10 ml of 50 μM of paclobutrazol 4 days before the experiment. Non-paclobutrazol-treated plants were pre-treated with water with the same concentration of ethanol. $GA_3$ (Duchefa) 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 of $GA_3$, 0.1% Tween 20 solution and were fed 10 ml of 50 μM of $GA_3$ to the soil. Submerged GA-treated plants were flooded in 10 μM of $GA_3$. Non-GA-treated plants were treated with similar solutions with $GA_3$ omitted.

1-Naphthalene acetic acid (Duchefa) 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 of 1-NAA, 0.1% Tween 20 solution and 10 ml of 50 μM 1-NAA was added to the soil. Submerged 1-NAA-treated plants were flooded in 10 μM of 1-NAA. Non-1-NAA-treated plants were treated with similar solutions with 1-NAA omitted.

### *Susceptibility of petiole cell walls to crude cell wall protein extracts of celery*

Frozen/thawed *R. palustris* petioles were boiled for 15 sec to inactivate native expansin activity, put in extensometers and the extension of 6 mm segments was recorded in 50 mM of sodium acetate buffer at pH 4.5 for 30 min. The incubation solution was then changed to a de-salted celery crude cell wall protein extract re-suspended in the same buffer, relative to cell wall DW.

### *Statistical analyses*

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, IL, USA). Statements about differences in the ABA, GA and 1-NAA experiments (Figures 5 and 6) are statistically valid for $P = 0.05$.

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