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Contrasting interactions between ethylene and abscisic acid in *Rumex* species differing in submergence tolerance

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Summary

Complete submergence of flooding-tolerant *Rumex palustris* plants strongly stimulates petiole elongation. This escape response is initiated by the accumulation of ethylene inside the submerged tissue. In contrast, petioles of flooding-intolerant *Rumex acetosa* do not increase their elongation rate under water even though ethylene also accumulates when they are submerged. Abscisic acid (ABA) was found to be a negative regulator of enhanced petiole growth in both species. In *R. palustris*, accumulated ethylene stimulated elongation by inhibiting biosynthesis of ABA via a reduction of *RpNCED* expression and enhancing degradation of ABA to phaseic acid. Externally applied ABA inhibited petiole elongation and prevented the upregulation of gibberellin A_1 normally found in submerged *R. palustris*. In *R. acetosa* submergence did not stimulate petiole elongation nor did it depress levels of ABA. However, if ABA concentrations in *R. acetosa* were first artificially reduced, submergence (but not ethylene) was then able to enhance petiole elongation strongly. This result suggests that in *Rumex* a decrease in ABA is a prerequisite for ethylene and other stimuli to promote elongation.

Keywords: abscisic acid, elongation, ethylene, environmental stress.

Introduction

Temporary flooding of riverside habitats is a worldwide phenomenon that has a severe impact on terrestrial plant life as well as on human activities. One of the acclimations of higher plants to complete submergence of the shoot and the associated shortages of carbon dioxide, oxygen and light (Jackson and Ram, 2003; Voesenek *et al.*, 1997) is stimulated elongation of shoot parts such as internodes and petioles. This adaptive trait has been documented in a variety of monocot and dicot species (Kende *et al.*, 1998; Ridge, 1987; Voesenek *et al.*, 1992). It is under hormonal control and has been the subject of physiological and molecular analysis for over 30 years (Jackson, 1990). It enables shoot parts to emerge from the water and function as 'snorkels', so that gas exchange between the atmosphere and the submerged plant tissues can be restored before the plant asphyxiates.

The genus *Rumex* includes submergence-tolerant (e.g. *Rumex palustris*) as well as submergence-intolerant species (e.g. *Rumex acetosa*; Blom and Voesenek, 1996). When submerged, the elongation rate of the petioles of the submergence-tolerant *R. palustris* increases strongly within a few hours. In *R. acetosa*, the elongation-based escape mechanism is absent and accordingly this species is intolerant to prolonged complete submergence of its shoot system (Voesenek *et al.*, 1997). In common with many other aquatic and semiaquatic species, enhanced elongation of submerged petioles by *R. palustris* requires an upsurge of endogenous ethylene. In submerged plants, ethylene accumulates because the surrounding water imposes a barrier to outward diffusion of gas that slows the escape of ethylene, which continues to be synthesized (Voesenek *et al.*, 1993). Enhanced underwater elongation can be mimicked by applying ethylene to non-submerged plants, while inhibitors of ethylene action such as silver ions strongly inhibit fast underwater elongation (Voesenek and Blom, 1989). In

R. palustris, ethylene-promoted growth is associated with increased biosynthesis of gibberellic acid A_1 (GA_1) and greater sensitivity to gibberellins. By contrast, in submergence-intolerant *R. acetosa*, submergence or ethylene de-sensitize petioles to gibberellic acid A_3 (GA_3) and fail to increase GA biosynthesis. Still, ethylene accumulates in submerged shoots of this species and externally applied GA_3 stimulates elongation in air-grown plants (Rijnders *et al.*, 1997). Therefore, the different responses to submergence by *R. acetosa* and *R. palustris* appeared to result primarily from different effects of ethylene on the biosynthesis and action of GA. Overall, these results indicate that ethylene rather than GA is the primary regulator of underwater elongation.

In extension to these observations, there have been indications that ABA is also involved in regulating underwater elongation of *R. palustris*. Abscisic acid is a known negative regulator of extensional vegetative growth, and a decline in internal levels of ABA is often a prerequisite for germination (Bacon *et al.*, 1998; Bentsink and Koornneef, 2003; Trewavas and Jones, 1991). Furthermore, levels of ABA can be regulated by ethylene during vegetative growth. For example, Zeevaart (1983) found that applied ethylene depresses endogenous ABA concentrations in rehydrated *Xanthium* leaves. Subsequent work in submergence-tolerant monocots (deep-water rice, *Scirpus micronatus* and lowland rice seedlings) showed that endogenous ABA levels decreased strongly upon submergence (Hoffmann-Benning and Kende, 1992; Lee *et al.*, 1996; Ram *et al.*, 2002). Because this effect of submergence could be mimicked with ethylene treatment and because external ABA was able to inhibit submergence-induced elongation, Hoffmann-Benning and Kende (1992) proposed that in deep-water rice tissue sensitivity to GA was inhibited by ABA. An ethylene-induced decrease in ABA caused by submergence would then enhance sensitivity to GA, thus giving rise to faster elongation. However, these observations are in contrast with the suggestion of Hansen and Grossmann (2000) and Grossmann and Hansen (2001) that increased levels of ethylene stimulate biosynthesis of ABA and that this stimulation is a general component of interaction between these two hormones. These contrasting views raise the question as to how close the correlation between ABA, ethylene and submergence-induced elongation really is. The link between them may be revealed more certainly by asking whether ethylene and ABA are regulated differently by submergence in flooding-tolerant and flooding-intolerant *Rumex* species and whether ethylene regulates ABA in a similar manner to submergence in each of these species. Therefore, we determined: (i) the relationships between the presence or absence of submergence-induced elongation in *R. palustris* and *R. acetosa* and changes in internal ABA; (ii) if ethylene is a necessary and sufficient factor affecting ABA levels in submerged plants; (iii) if submergence- or ethylene-mediated changes in endogenous ABA are an outcome of altered biosynthesis and/or catabolism of ABA; and (iv) which interactions exist between ABA and GA.

We show here that ABA is an inhibitory factor for underwater elongation in both *R. palustris* and *R. acetosa*; the difference between the two species being that only in *R. palustris* does submergence relieve this inhibition by means of a ethylene-mediated decrease in ABA. This decrease is shown to involve both a severe slowing of biosynthesis of ABA and a promotion its breakdown. The reduction in ABA is thought to enable ethylene-induced enhancement of GA_1, a prerequisite for enhanced growth.

Results

Submergence or ethylene treatment each rapidly depress internal concentrations of ABA

Upon submergence, leaf elongation in *R. palustris* increased rapidly after a lag phase of approximately 2 h (Figure 1a). After 6 h, the rate of leaf elongation was four times that of controls. Concentrations of ABA in petioles of submerged plants declined with a lag phase of only 15 min (Figure 1b). Concentrations of ABA then decreased further and were sixfold lower than in controls after 80 min of submergence. Plants submerged for 24 or 48 h in this same experiment retained these low levels of internal ABA (data not shown). Applying 5 $\mu l\ l^{-1}$ of ethylene (growth-saturating level) to air-grown plants affected levels of ABA in a similar way to submergence (Figure 1c). A rapid decline in the endogenous ABA concentration was observed within 60 min of treatment. Overall, the kinetics of decline of ABA in ethylene-treated plants was similar to that observed in submerged plants. Separate measurements of different plant parts showed that decline of ABA was not restricted to the fastest growing petiole (the second oldest leaf) – it was also observed in all other petioles, leaf blades and roots after 4 h of submergence (Figure 2).

ABA inhibits elongation growth

When *R. palustris* was submerged for 48 h, the presence of ABA in the solution severely inhibited underwater elongation (Figure 3a). A significant inhibition of elongation was observed at ABA concentrations above 0.1 μM. However, even at extremely high concentrations of ABA (100 μM), the rate of elongation of submerged plants was still somewhat higher than that of air-grown plants.

To test the interaction between ethylene and ABA on petiole elongation, plants were grown in hydroculture. In this set-up, ABA (0–10 μM) was administered through the roots and 5 $\mu l\ l^{-1}$ ethylene was applied to the shoot in a flow-through set-up. A strong inhibitory effect of ABA on ethylene-promoted petiole elongation was observed (Figure 3b).

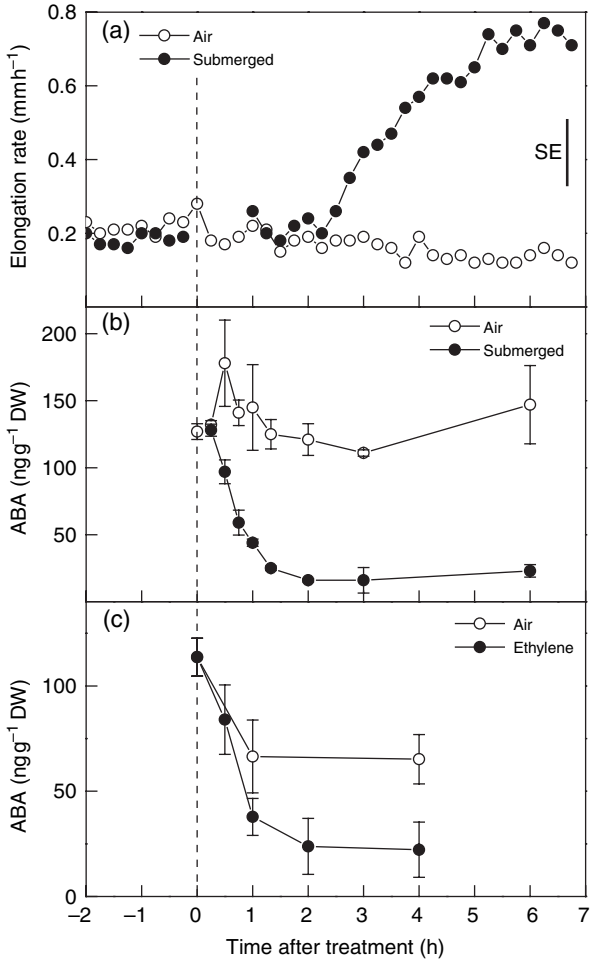

Figure 1. Leaf elongation rate (a) and ABA concentration (b, c) in petioles of *Rumex palustris* during submergence (a, b) or during treatment with 5 μl l^{-1} ethylene (c). Open circles, air controls; filled circles, submerged or ethylene-treated starting at $t = 0$ h (dashed line). Means of six replicates (a) with a typical standard error (SE) shown; three replicates (b) or four replicates (c) ±SEs.

The scale of the effect was similar to that seen in submerged plants.

Ethylene is the principal factor depressing levels of ABA in submerged plants

Although ethylene alone is sufficient to mimic the effect of submergence by stimulating elongation (Figure 3) and decreasing internal ABA (Figure 1), it remains possible that, in submerged plants, the faster extension rate and large decrease of ABA could be the result of other influences. These might include a decrease in transport of root-derived ABA or a decrease in biosynthesis due to a lack of oxygen. To investigate these possibilities, submerged plants were pre-treated with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception. If factors other than ethylene were

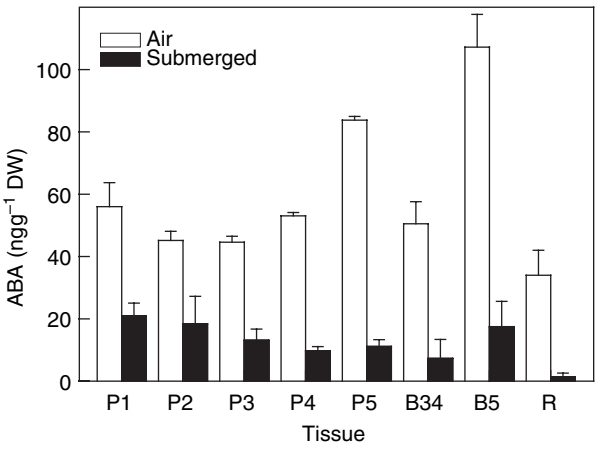

Figure 2. ABA concentrations in different parts of *Rumex palustris* plants placed in air (open) or submerged for 4 h (filled). P1–P5, petiole 1 (oldest) to petiole 5 (youngest); B34, blade of leaf 3 and 4; B5, blade of the fifth (youngest) leaf; R, roots. Means of four replicates with standard errors.

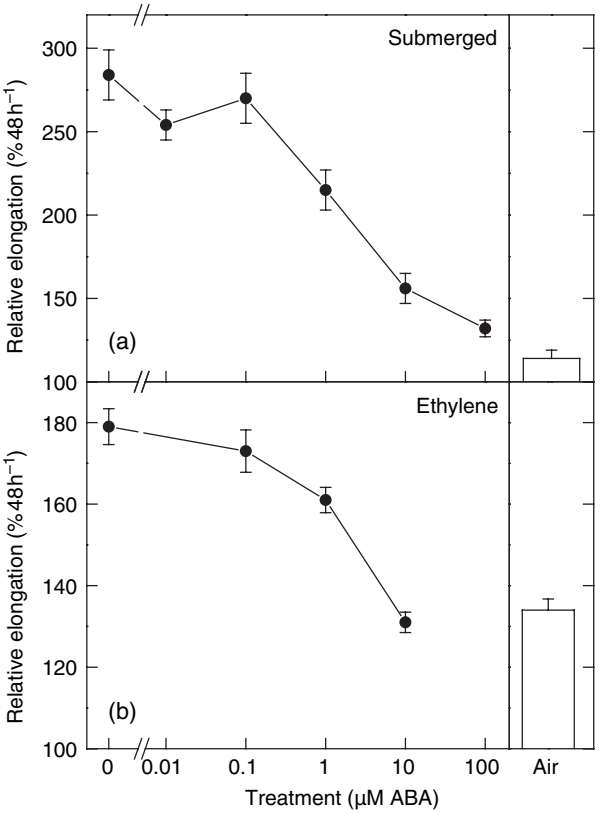

Figure 3. Effect of treating *Rumex palustris* plants with increasing concentrations of ABA on the petiole elongation response to (a) submergence or (b) 5 μl l^{-1} ethylene. Treatments lasted 48 h. The length of the second youngest petiole was measured before and after treatment and increase in length calculated as the difference. For (a) plants were grown in pots and submerged in solutions containing 0–100 μM ABA. For (b) plants were grown in hydroculture where the root environment was treated with 0–100 μM ABA and the shoot placed in a cuvette through which air or 5 μl l^{-1} ethylene flowed at 30 l min^{-1}. Bars indicate the relative elongation of air-grown plants during the same experimental period. Means of 12 plants with standard errors.

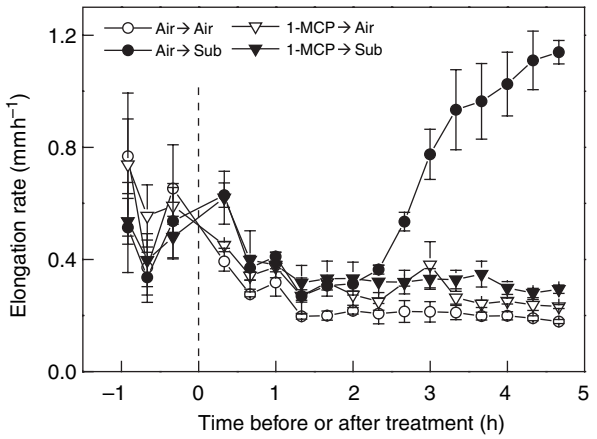

Figure 4. Effect of submergence (Sub) and 1-MCP on leaf elongation rate in *Rumex palustris*.
Plants were placed in desiccators for 1 h in the absence (circles) or presence (triangles) of 1 μl l^{-1} 1-MCP. Leaf extension was then measured in the second youngest leaf using linear displacement transducers after plants were submerged at $t = 0$ h (filled symbols) or placed in the open air (open symbols). Means of three replicates with standard errors.

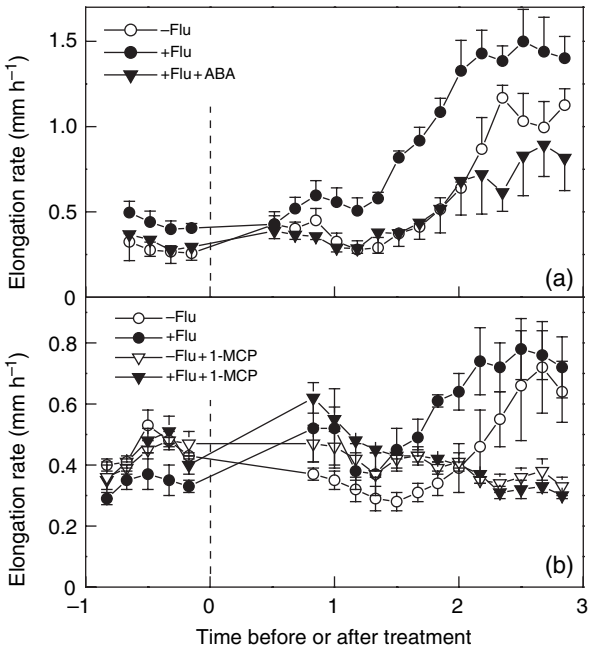

Figure 5. Effect of fluridone, ABA and 1-MCP on submergence-induced leaf elongation in *Rumex palustris* measured with linear displacement transducers. Plants were treated with fluridone (Flu, filled symbols) or water (open symbols) 72 h prior to submergence. Plants were submerged at $t = 0$ h (dashed line).
(a) Plants submerged in water (circles) or in water containing 5 μM ABA (triangles).
(b) To administer volatile 1-MCP, all plants were enclosed in desiccators for 1 h in the presence (triangles) or absence (circles) of 1 μl l^{-1} 1-MCP before measurements started. Means of four replicates with standard errors.

Table 1 Effect of submergence and 1-MCP on the concentration of ABA in petioles of *Rumex palustris*. Plants were placed in desiccators for 1 h in the presence or absence of 1-MCP. After this, plants were placed back in the growth room in air or submerged in water. After 4 h, petioles were harvested for ABA extraction

Treatment	ABA (ng g^{-1} DW)
Air	80.3 ± 12.9
Submerged	36.3 ± 3.8
Submerged + 1-MCP	93.6 ± 11.5

Means of three replicates with standard errors.

responsible for stimulating elongation and depressing ABA during submergence, 1-MCP should not prevent their occurrence. However, pre-treatment with 1-MCP resulted in the complete absence of enhanced elongation by submerged plants for at least 5 h (Figure 4) and endogenous ABA concentrations remained high and comparable with those of non-submerged plants (Table 1).

Effects of manipulation of ABA levels on underwater extension

In order to start a submergence experiment with a reduced endogenous ABA concentration, *R. palustris* plants were pretreated for 72 h with 10 ml of 100 μM fluridone, an inhibitor of ABA biosynthesis. This treatment reduced ABA concentrations in the petiole by 20% (data not shown) but had no significant effect on petiole elongation in air-grown plants (Figure 5a, $t < 0$). However, upon submergence of these same plants the 2-h lag time for enhanced elongation that existed in plants not treated with fluridone was shortened to only 60 min (Figure 5a). When internal ABA concentrations in submerged fluridone-treated plants were restored by adding 5 μM ABA, the normal lag phase for elongation was rescued and extension slowed to rates similar to those of submerged plants not given fluridone (Figure 5a).

The experiment was repeated in plants also treated with 1-MCP prior to submergence (Figure 5b). In these plants, fluridone treatment no longer stimulated underwater elongation. This shows that fluridone specifically interferes downstream of ethylene (as opposed to other potential growth-stimulating factors such as changes in O_2 or CO_2 supply (Voesenek *et al.*, 1997), and indicates that a reduction in ABA is required for enhanced growth, but not sufficient to promote elongation in the absence of ethylene.

In separate experiments, ABA was applied to plants that had already been submerged for 24 h. This approach was intended to reveal whether ABA inhibits only the time of onset of underwater elongation or whether it is capable of inhibiting elongation in petioles once the enhanced elongation mechanism is fully engaged. The treatment should also indicate the length of time taken for external ABA to enter the plant tissue and affect elongation growth. Figure 6 shows that when ABA was given to submerged *R. palustris* plants 24 h after the start of submergence, an inhibitory

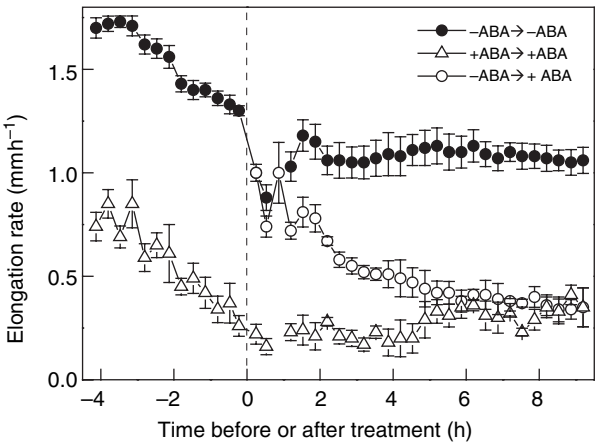

Figure 6. Effect of ABA on submergence-induced leaf elongation in *Rumex palustris* measured with linear displacement transducers.
Plants were submerged at $t = -24$ h in water (filled circles) or in water containing 5 μM ABA (open triangles). At $t = 0$ h (dashed line), plants were drained rapidly and resubmerged in water (filled circles) or water containing 5 μM ABA (open circles, open triangles). Means of three replicates with standard errors.

effect was evident after only 45 min. Within 5 h of treatment, the elongation rate in these plants was indistinguishable from that of plants treated with ABA at the start of submergence.

ABA affects underwater elongation in Rumex acetosa*, a submergence-intolerant species*

The genus *Rumex* contains both submergence-tolerant and submergence-intolerant species. In contrast to tolerant *R. palustris*, the rate of petiole elongation in intolerant *R. acetosa* is not promoted by submergence or ethylene treatment (Voesenek and Blom, 1989). We therefore studied the ability of submergence to decrease internal ABA levels in *R. acetosa* and examined the effects of fluridone and thus of endogenous ABA on elongation growth in this species. These experiments showed that, in contrast to *R. palustris*, ABA levels in *R. acetosa* were not decreased by submergence. Petiolar concentrations remained similar to those in non-submerged plants for up to 48 h (Figure 7a). The elongation rate of *R. acetosa* petioles during the first 6 h of submergence was similar to the growth rate observed before submergence. However, when plants were pre-treated with fluridone, the growth response to submergence was converted to that seen in submergence-tolerant *R. palustris*, i.e. a strong increase in elongation rate took place that was about three times that of non-submerged controls and followed a distinct 1.5 h lag phase (Figure 7b). To establish whether increased ethylene levels were involved in this enhanced underwater elongation in *R. acetosa* the experiment was repeated after plants were pre-treated with 1-MCP. In contrast to *R. palustris* (Figure 4) we found that

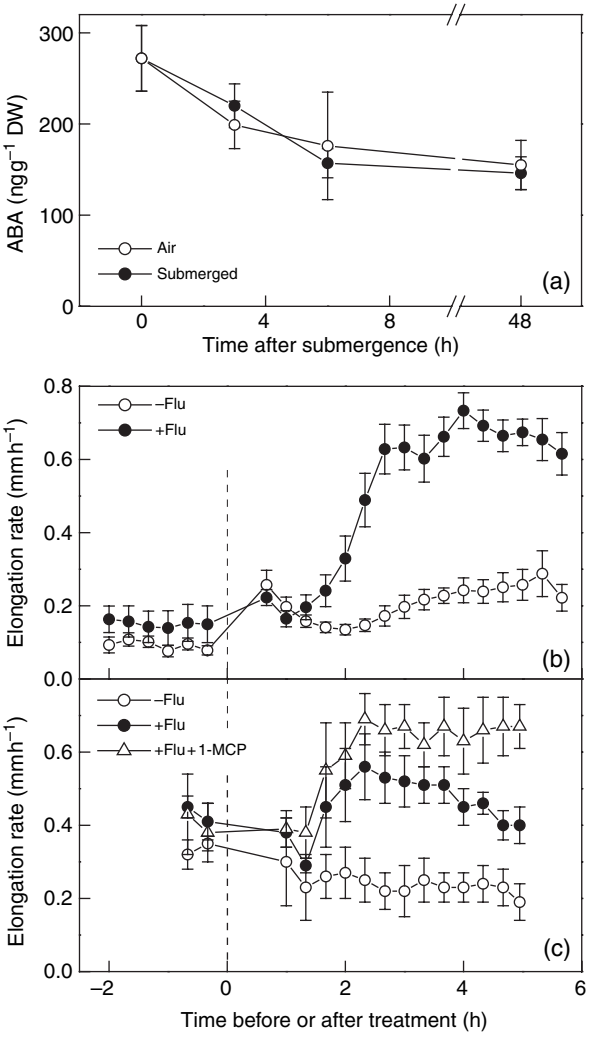

Figure 7. (a) Effect of submergence on ABA concentrations in petioles of *Rumex acetosa*. Open symbols, air controls; filled symbols, submerged at $t = 0$ h (means of four replicates ± standard errors).
(b, c) Effect of fluridone and 1-MCP on submergence-induced leaf elongation in *R. acetosa* measured with linear displacement transducers. Fluridone (Flu) was administered 72 h prior to submergence. Plants were submerged at $t = 0$ h (dashed line). To administer volatile 1-MCP (c) all plants were enclosed in desiccators for 1 h in the presence (triangles) or absence (circles) of 1 μl l^{-1} 1-MCP before measurements started. Means of six (b) or four (c) replicates with standard errors.

the underwater elongation rate in *R. acetosa* treated with fluridone was not inhibited by 1-MCP but was, in fact, even higher than that found in plants that were still able to sense ethylene (Figure 7c).

Relations between ABA and GA

Rijnders *et al.* (1997) showed that submergence and ethylene treatment enhanced levels of bioactive GA_1 in *R. palustris* but not in *R. acetosa*. As external ABA is very potent in inhibiting underwater elongation, we hypothesize

that ABA may act via the inhibition of the GA synthesis required for submergence-induced growth. Therefore, levels of GA_1 were determined in *R. palustris* plants grown in air or submerged in the presence or absence of 10 μM ABA for 8 h. In this experiment, submergence (without ABA) induced a threefold increase in GA_1 in the petioles [20.7 ± 1.1 ng g^{-1} dry weight (DW) versus 6.9 ± 0.7 ng g^{-1} DW in air-grown plants]. However, in submerged plants also treated with ABA, levels of GA_1 were not increased but remained similar to those observed in air-grown plants (8.6 ± 0.2 ng g^{-1} DW).

Ethylene decreases internal ABA levels by decreasing biosynthesis and increasing breakdown

Although at least five different enzymatic steps are involved in the biosynthetic pathway of ABA, it is believed that, in vegetative tissue, ABA biosynthesis is regulated principally through 9-*cis*-epoxycarotenoid dioxygenase (NCED), which catalyses the first committed step of ABA biosynthesis (Schwartz *et al.*, 1997). Using degenerate primers, 11 distinct 550 bp long sequences were isolated which showed 70 to 80% amino acid sequence homology to known NCED orthologues from other plant species. These genes were named *RpNCED1–11* and were clustered in five groups of one to three homologues. Within each cluster nucleotide sequence homology was over 95% (not shown).

The mRNA expression of each cluster was determined using quantitative reverse transcriptase (RT)-PCR. For this, specific primer pairs were created that matched all members of one cluster but no members of other clusters. Using PCR, each of these primer pairs yielded one product of the correct length when tested on cDNA or plasmids containing the intended homologues. No product was observed when plasmids containing other homologues were used as a template. Quantitative RT PCR was performed for each cluster on cDNA of *R. palustris* petioles that were submerged for up to 8 h (Figure 8). To ensure that any observed changes in expression did not result from naturally occurring diurnal variations, control samples of plants grown in air were taken at each time point. Of the five gene clusters, four showed measurable expression in the petioles of submerged and air-grown plants. The expression level in each of these was strongly affected by submergence. Within 2 h of treatment, mRNA levels decreased between 86–95% and remained at these low values during the experimental period. Separate measurements of *RpNCED1* expression showed that mRNA levels remained low for at least 48 h (data not shown). A reduction of expression was also observed in air-grown plants for the second and third primer pair (*RpNCED2*, -3, -6, -7 and -8) but these decreases were significantly less pronounced then those observed in submerged plants. The failure to detect expression of the fifth gene cluster (*RpNCED5* and *RpNCED11*) was not due to experimental limitations, as lowering of the annealing temperature did not increase product levels (although it did yield non-specific amplification), while a product of the correct size was observed at the (higher) experimental temperature when plasmids containing the intended homologues were used as a template.

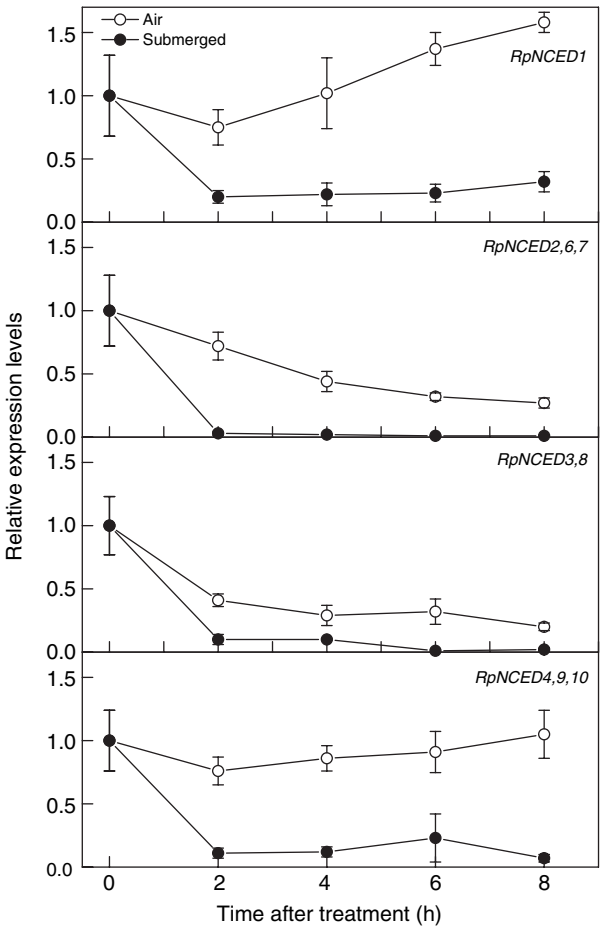

Figure 8. Relative expression of *RpNCED* mRNA in submerged and air-grown plants: controls in air (open symbols); submerged at $t = 0$ h (filled symbols). Expression of mRNA was quantified relative to the value obtained at $t = 0$ h. Means with standard errors of four biological replicates with two to four technical replicates each.

Phaseic acid (PA), the primary breakdown product of ABA, and ABA–glucose ester, a common conjugate, were analysed in plants that were submerged or given ethylene for 1 h (Table 2). Again, this treatment reduced internal ABA levels to almost 70% lower than in air-grown plants. In contrast, PA levels increased by 10–25% in the same time period. No ABA–glucose ester could be detected in either control or submerged plants (data not shown).

To further investigate the relevance of catabolism to the rapid decrease of ABA in submerged *R. palustris*, submerged plants were treated with an analogue of ABA, 8′-acetylene ABA. This synthesized compound acts as a specific inhibitor of ABA 8′-hydroxylase, an enzyme that

Table 2 Effect of ethylene and submergence on concentrations of ABA and PA in petioles of *Rumex palustris*. Rows 1 and 2 show levels of ABA and PA from plants in air or treated with 5 μl l^{-1} ethylene for 1 h. Rows 3 and 4 show levels of ABA and PA from plants in air or submerged for 1 h

Treatment	ABA (ng g^{-1} DW)	PA (ng g^{-1} DW)
Air	113.7 ± 9.0	150.2 ± 10.7
Ethylene	37.8 ± 8.8	188.2 ± 6.3
Air	81 ± 6.4	171.8 ± 4.0
Submerged	28.6 ± 3.0	189.7 ± 2.6

Means of four replicates with standard errors.

catalyses the breakdown of ABA to PA (Cutler *et al.*, 2000; Rose *et al.*, 1997) by binding irreversibly to the enzyme thus rendering it inactive. Submerged *R. palustris* plants were treated for 48 h with ABA and the analogue 8′-acetylene ABA after which the effect on petiole extension was determined. This showed that 8′-acetylene ABA is about 10 times more active in inhibiting underwater growth than natural ABA (Table 3), indicating that even during this longer period of submergence the inhibition of degradation of ABA is effective in inhibiting underwater extension growth.

The significance of catabolism was further illustrated in plants that were submerged and later desubmerged (Figure 9). In petioles of these desubmerged plants a strong enhancement of NCED expression was observed within 1 h. However, this increase in biosynthesis capacity failed to increase levels of ABA for at least 6 h after desubmergence. Desubmergence did effectively halt enhanced elongation, again showing that a reduction in ABA alone cannot bring about enhanced elongation in air-grown plants.

Table 3 Effect of ABA and 8′-acetylene-ABA on submergence-induced petiole elongation in *Rumex palustris*. Plants were treated for 48 h in air or submerged with or without ABA or 8′-acetylene-ABA (acABA). The length of the second youngest petiole was measured before and after treatment, and the increase in length was calculated. Inhibition values represent the percentage inhibition of elongation, normalized for plants that were air grown (100%) and submerged without hormones (0%). Average starting length was 13.1 mm

Treatment	Increase (mm)	Inhibition %
Air	4.6 ± 0.42	(100)
Submerged		
No ABA or acABA	20.3 ± 1.18	(0)
0.1 μM ABA	16.8 ± 0.62	22
0.1 μM acABA	12.9 ± 0.67	47
1 μM ABA	13.5 ± 0.46	43
1 μM acABA	10.4 ± 0.46	63
10 μM ABA	9.3 ± 0.53	70

Means of eight replicates with standard errors.

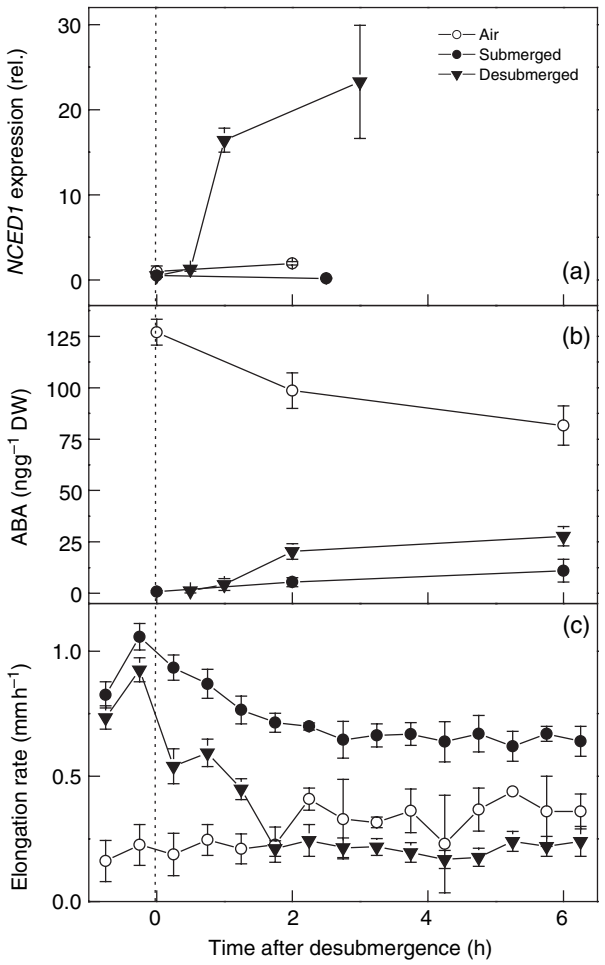

Figure 9. Effect of desubmergence on the expression of *RpNCED1* (a), levels of endogenous ABA (b) and petiole elongation rate (c) from plants grown in air (open circles), submerged (filled circles) or submerged and desubmerged at $t = 0$ h. Submergence treatment lasted at least 10 h. Expression of mRNA was quantified relative to the value obtained at $t = 0$ h. Means with standard errors of three (a, b) and four (c) replicates.

Discussion

ABA is a factor inhibiting enhanced elongation

Our results support the hypothesis that the rate of petiole elongation in submerged *R. palustris* plants is determined not only by growth-promoting stimuli from ethylene and GA but also by the growth-inhibiting factor ABA. The first hours of submergence of *R. palustris* are characterized by a sharp decline in ABA concentration in the petiole. This happens well in advance of any measurable increase in elongation rate (Figure 1a,b). Furthermore, when internal ABA is kept artificially high with exogenous ABA, elongation can be effectively inhibited (Figure 3a). This inhibitory effect is present both at the start of submergence-induced growth and throughout at least 24 h of submergence (Figure 6), indicating that reduced levels of ABA are a requirement for

both the onset and the maintenance of enhanced elongation.

Fluridone, an inhibitor of carotenoid biosynthesis, suppresses production of ABA in plants and seeds (Gamble and Mullet, 1986; Yoshioka *et al.*, 1998). In the present work application of fluridone resulted in a modest decline (20%) in the concentration of ABA in petioles, but no wilting was observed even though stomatal regulation may well have been affected. This reduction in endogenous ABA was associated with a significantly shorter lag phase of submergence-induced elongation (Figure 5), an effect that was reversible by supplying ABA. This suggests that the lag phase of elongation that followed submergence can – at least partially – be explained by a blockage of elongation growth by ABA that is terminated when tissue concentrations decline below some critical threshold.

Apart from a build-up of internal ethylene, submergence results in a large number of other environmental and internal changes, including changes in light climate and the concentration of O_2 and CO_2 (Rijnders *et al.*, 2000). The data presented here show that during submergence increased ethylene within the tissues is both required and sufficient to induce the observed decrease in internal ABA levels (Figure 1c, Table 1). However, these experiments also showed that the enhanced elongation response is not merely the result of less ABA acting alone. Air-grown controls treated with fluridone did not show enhanced elongation (Figure 5b, $t < 0$) and rate of elongation did not increase in upon submergence after treatment with 1–MCP (Figure 5b, $t > 0$). These results indicate that, in addition to a loss of ABA, the presence and action of growth-stimulating amounts of ethylene are also needed.

Submergence and ethylene induce an increase in the level of GA_1 in *R. palustris* (Rijnders *et al.*, 1997). Furthermore, Vreeburg *et al.* (2005) showed, in *R. palustris*, that ethylene stimulates transcription of cell wall loosening expansins as well as the activity of proton efflux carriers, resulting in a notable apoplastic acidification. The inhibitory action of ABA on biosynthesis of GA_1 may very well be a key function of this hormone in the process of underwater elongation, as ABA was found not to inhibit the enhancement of expression of the expansin gene or to hamper apoplastic acidification, a process required for expansin activity and known to be negatively regulated by ABA in other model species (Balsevich *et al.*, 1994; Roelfsema *et al.*, 1998; Vreeburg *et al.*, 2005).

Submergence does not affect internal levels of ABA in R. acetosa

The submergence-induced downregulation of the ABA concentration in petioles of flooding-tolerant *R. palustris* is absent in flooding-intolerant *R. acetosa*; levels of ABA in the petioles of submerged *R. acetosa* plants remain similar to those of non-submerged plants for at least 48 h (Figure 7a). Thus, our observations in *R. acetosa* do not support our hypothesis that ABA biosynthesis is induced by increased levels of ethylene in submergence-intolerant species. Submergence of *R. acetosa* was shown to result in saturating levels of internal ethylene (Voesenek *et al.*, 1993), but levels of ABA failed to increase (Figure 7a).

When tissue ABA in *R. acetosa* is artificially depressed using fluridone this species responds to submergence with accelerated petiole extension. In sharp contrast to *R. palustris*, in which submergence-induced growth is completely dependent on ethylene (Figure 4), underwater elongation in *R. acetosa* does not rely on increased ethylene levels (Figure 7c). In fact, inhibition of ethylene perception using 1-MCP was found to enhance underwater growth rates in comparison with untreated ethylene-sensing plants.

The petiole elongation rate in *R. acetosa* is not affected by fluridone in non-submerged control plants (Figure 7b, $t < 0$) suggesting that a reduction in ABA *per se* is insufficient to trigger enhanced elongation. Inhibition of ethylene perception with 1-MCP also does not enhance petiole growth in non-submerged plants (Figure 7c, $t < 0$). As ethylene perception is not required for enhanced underwater elongation in this species (Figure 7c, $t > 0$) another elongation-inducing signal must be present, as is also the case for the submerged aquatic *Potamogeton pectinatus*, an aquatic species that is constitutively unable to synthesize ethylene (Jackson *et al.*, 1996). This signal is unlikely to be GA, as Rijnders *et al.* (1997) showed that submergence did not influence levels of GA in *R. acetosa* and reduced the sensitivity to GA in this species. Possible alternatives include low levels of O_2 or high levels of CO_2 (Azuma *et al.*, 2001; Voesenek *et al.*, 1997). However, as our experiments were performed in the light and light quality or intensity were not significantly altered by the shallow water layer, a depletion of O_2 or build up of CO_2 is very unlikely in these experiments. A reduction in the level of CO_2 level (which is likely to take place under our experimental conditions) may possibly act as a signal, but such a mechanism has not been previously described in plants. It is interesting to note that this unknown signalling mechanism, so clearly active in *R. acetosa*, is unable to induce elongation in submerged *R. palustris* when ethylene signalling is absent (Figure 4).

Regulation of the concentration of ABA

It is well established that ABA in higher plants is derived from C_{40}-carotenoids, with the key regulatory step being the oxidative cleavage of C_{40}-epoxycarotenoids (Qin and Zeevaart, 1999; Schwartz *et al.*, 1997). The enzyme responsible for this step, NCED, catalyses the first unique step in ABA biosynthesis to produce xanthoxal (Schwartz *et al.*, 1997). Upregulation of NCED genes in response to drought

has been found in many species (e.g. Burbidge *et al.*, 1999; Schwartz *et al.*, 1997).

In *R. palustris*, expression of several *RpNCED* genes decreased quickly upon submergence and remained at a low level for at least 8 h. Still, the rapid decrease of ABA levels observed upon submergence or ethylene treatment (Figure 1) may be difficult to explain in terms of decreased biosynthesis alone. Although upregulation of *NCED* expression might well induce a rapid increase of the levels of NCED, and thus ABA, the impact of downregulation of gene expression on enzyme activity would be tempered by the half-life of NCED. Therefore, a decrease in biosynthesis and an increase in catabolism may both be required to achieve the rapid decrease in ABA that is observed in ethylene-treated *R. palustris* plants. The strong regulatory capacity of the catabolic pathway is also indicated by the observation that, in desubmerged plants, a rapid enhancement in the capacity for ABA biosynthesis fails to induce an equally rapid increase in the concentration of this hormone (Figure 9).

The predominant pathway for ABA catabolism is through oxidative breakdown to 8′-hydroxy-ABA, with subsequent conversion to PA and dihydrophaseic acid (DPA) (Cutler and Krochko, 1999; Zeevaart and Creelman, 1988). The enzyme involved, ABA 8′-hydroxylase, is a cytochrome P450 monooxygenase (Krochko *et al.*, 1998) and is encoded by *CYP707As* in Arabidopsis (Kushiro *et al.*, 2004; Saito *et al.*, 2004). In addition to this process, conjugation to ABA–glucose ester can also deplete the endogenous titre of ABA (Vernieri *et al.*, 1994). Activity of ABA 8′-hydroxylase is rapidly induced by ABA in corn suspension cells (Krochko *et al.*, 1998). Zeevaart (1983) found increased metabolism of ABA to PA upon recovery of drought stress in *Xanthium* leaves. This process was found to be enhanced by external ethylene, although ethylene was unable to decrease levels of ABA to below those of turgid leaves. In *R. palustris*, we find a small but significant increase in the concentration of PA after 1 h of submergence or treatment with ethylene. At this time internal ABA decreased by almost 70%. These changes indicate a substantial shift in the concentration ratio between the two substances, indicating increased activity of the catabolic pathway. The observation that the total increase in PA is smaller then the decrease in ABA is not surprising. Apart from glucosidation and 8′-hydroxylation via PA, ABA is also known to be metabolized via 7′-hydroxylation (Lehmann and Schwenen, 1988) and 9′-hydroxylation (Zhou *et al.*, 2004). Furthermore, PA itself is metabolized to DPA, which in turn is probably metabolized further (either by conjugation or cleavage, A. Cutler, personal communication). The discrepancy between the increase in PA and the decrease in ABA suggests that one or more additional catabolic processes are also enhanced in submerged *R. palustris* plants.

The use of ABA analogues to inhibit the activity of ABA 8′-hydroxylase with a high degree of specificity has been used in a variety of studies (Abrams *et al.*, 1997; Cutler *et al.*, 2000; Lamb *et al.*, 1996; Schmitz *et al.*, 2002) and has illustrated the importance of ABA metabolism during dormancy and germination. Here we used 8′-acetylene ABA (Rose *et al.*, 1997) as an irreversible inhibitor of the breakdown of ABA to show that enhanced elongation during submergence can be inhibited by slowing the breakdown of ABA. This effect is present after 48 h of submergence, while *NCED* expression and ABA levels are decreased within the first 5% of that period (2 h). The capacity for the inhibition of ABA catabolism to restrict prolonged underwater elongation indicates that, despite a strong decrease in biosynthesis, a considerable flux of ABA remains present in submerged plants. Thus, a decrease in *NCED* expression alone seems insufficient to keep the amount ABA in submerged plants at these very low levels (Table 3), similar to it being unable to fully restore levels of ABA in desubmerged plants (Figure 9). While *NCED* regulation is undoubtedly crucial in the regulation of levels of ABA in submerged and air-grown *R. palustris* plants, it seems that the regulation of catabolism is equally important in determining the resulting levels of ABA during submergence.

Abscisic acid and ethylene have been shown to act antagonistically in a number of different processes. Ethylene was found to reduce ABA sensitivity in roots and imbibed seeds (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000), while application of ABA enhanced elongation in water-stressed roots by inhibiting ethylene production (Sharp and LeNoble, 2002). Ethylene has also been shown to induce ABA catabolism during rehydration (Zeevaart, 1983), and it is quite feasible that such a regulatory mechanism is present in many plant species. The presence of an ethylene-induced reduction of ABA in submergence-tolerant monocots (Hoffmann-Benning and Kende, 1992; Lee *et al.*, 1996; Ram *et al.*, 2002) would imply a similar adaptation, although it is not known how ABA is regulated by ethylene in these species.

Conclusions

We show that ABA is a key regulator of submergence-induced elongation in *R. palustris* and that internal ABA concentrations are depressed by entrapped ethylene. The decrease in ABA takes place well before enhanced elongation starts, suggesting a cause and effect relationship. This notion is supported by our finding that externally applied ABA can fully inhibit ethylene-induced elongation. A clear-cut lag phase of approximately 2 h is a feature of submergence-induced petiole elongation in *R. palustris* and appears to arise, in part, from the time taken for internal concentrations of ABA to decline. This conclusion is supported by the observation that the lag phase can be much shortened experimentally by decreasing internal ABA with fluridone and can be restored with an exogenous ABA

supplement. As a decline in ABA by itself does not induce elongation, it is unlikely that ethylene-induced elongation in *R. palustris* is simply a result of an ethylene-induced reduction in levels of ABA. Enhanced growth is absent in plants treated with both 1-MCP and fluridone prior to submergence. Rates of elongation decrease in desubmerged plants without a notable increase in levels of ABA. Thus, ABA-independent pathways, such as an increase in levels of expansin or a reduction in apoplastic pH (Vreeburg *et al.*, 2005) are considered to be equally important for this physiological response. Enhanced elongation is ultimately dependent on the growth-promoting action of GA (Rijnders *et al.*, 1997). Upon submergence, entrapped ethylene is thought to induce enhanced elongation at least partly via an increase in the concentration of GA_1. This increase is dependent on a (ethylene-induced) reduction in ABA, as levels of GA_1 do not increase in submerged plants also treated with ABA.

The critical role for the decreased ABA in enabling enhanced elongation is further highlighted by the response of *R. acetosa* to submergence. Normally, this species does *not* respond to submergence with faster petiole extension rates nor does it show any decrease in ABA during this time. The observed enhancement of underwater elongation in *R. acetosa* treated with fluridone is not stimulated by ethylene. Nevertheless the process is inhibited by ABA, as fluridone treatment permits this species to respond to submergence with faster petiole elongation (Figure 7b). Therefore, ABA may act as an inhibitory factor that is not just restricted to ethylene-induced elongation but also affects other sorts of growth responses, and would imply that any stimulus that promotes enhanced elongation in *Rumex* requires a decrease in internal levels of ABA before it can be realized.

Experimental procedures

Growth of plants

Seeds of *R. palustris* (Sm.) and *R. acetosa* (L.) were germinated on black polyethylene beads (Elf Atochem, Vlissengen, The Netherlands), floating on tap water in a transparent container for 10 days [12 h light, 25°C, 70 μmol m^{-2} sec^{-1} photosynthetic photon flux density, and 12 h dark, 10°C]. Germinated seedlings were transplanted singly into plastic pots (70 ml) containing a mixture of potting soil and sand (2:1, v:v), enriched with 0.14 mg MgO-CaO per pot. Prior to seedling transfer, each pot was saturated with 20 ml nutrient solution containing 7.5 mM $(NH_4)SO_4$, 15.0 mM KH_2PO_4, 15.0 mM KNO_3, 86 μM Fe-EDTA, 4.3 μM $MnSO_4$, 1.8 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 42 μM H_3BO_3 and 0.53 μM Na_2MoO_4. All chemicals were PA grade (Merck, Amsterdam, The Netherlands). Plants were grown for 19 days on irrigation mats (Maasmond-Westland, De Lier, The Netherlands) in a growth chamber [20°C, 70% relative humidity (RH), 16 h light 200 μmol m^{-2} sec^{-1} PPFD]. The mats were automatically watered with tap water to saturation twice a day, and the excess water was drained away. For some experiments, plants were transplanted to hydroponics and grown according to Poorter and Remkes (1990). All plants were selected for homogeneity with respect to the developmental stage of the youngest leaf (the fifth) prior to the start of the experiments.

Application of chemicals

Abscisic acid (Sigma Zwijndrecht, The Netherlands) was dissolved in a few drops of 5 M KOH, diluted to 25 mM with distilled water and titrated back to pH 7 with HCl. Fluridone (Sigma) was dissolved in acetone to a concentration of 100 mM and diluted 1000-fold with distilled water. Plants were treated once, 72 h before measurements, with 10 ml 100 μM fluridone or (for controls) with 0.1% (v/v) acetone applied to the soil. For short-term treatments with ethylene and 1-MCP (Ethylblock, Floralife, Walterboro, SC, USA), plants were placed in closed 24 l glass containers, and 1-MCP and ethylene were administered with an end concentration of 1 μl l^{-1} for 1 h (1-MCP) or 5 μl l^{-1} for 1–4 h (ethylene). For longer-term treatments with ethylene, up to 24 plants were placed in 500 l flow-through containers in which air containing 5 μl l^{-1} ethylene was flushed at a rate of 30 l min^{-1}. This concentration of ethylene was observed in submerged *R. palustris* plants in previous experiments (Voesenek *et al.*, 1993).

Plant growth measurements

Petiole elongation was measured according to Rijnders *et al.* (1997). For measurements of leaf elongation rates, linear displacement transducers (Schlumberger Industries, Bognor Regis, UK; type ST 2000) were used according to Voesenek *et al.* (2003). Growth rates were subsequently calculated by fitting lines through intervals of 10 to 30 min.

Measurements of ABA, PA, GA_1 and methyl esters

For ABA and PA, samples consisting of 20–60 mg of freeze-dried petiole tissue were homogenized in liquid nitrogen and extracted in 10 ml 80% (v/v) methanol containing 20 mg l^{-1} butylated hydroxytoluene. Subsequently 6 ng deuterated ABA and/or 20 ng deuterated PA were added. The methanol fraction was removed under reduced pressure and the sample passed through a nylon filter and loaded onto a C18 column (Sep-Pak WAT051910; Waters, Etten-Leur, The Netherlands). After rinsing with water, ABA was eluted with 60% (v/v) methanol. Methanol was removed under reduced pressure and the remaining water fraction was acidified to pH 4 and extracted three times with 1 ml ethyl acetate. The combined ethyl acetate fraction was dried, dissolved in 50 μl of methanol and methylated with diazomethane, redissolved in ethyl acetate and passed through an aminopropyl SPE column (Bond-Elut product no 12102014; Varian, Middelburg, The Netherlands). The resulting flow-through was then subjected to gas chromatography–mass spectroscopic (GC-MS) analysis (Agilent 5890 MSD, Amstelveen, The Netherlands). Ions at *m/z* 190 and 162 (ABA), 193 and 165 ($^2H_3$-ABA), 194 and 176 (PA) and 197 and 179 ($^2H_3$-PA) were monitored under conditions described by Whitford and Croker (1991).

Glucose esters of ABA and PA were assayed according to published methods (Daeter and Hartung, 1990; Neill *et al.*, 1983; Vernieri *et al.*, 1994). In some cases samples were split into two halves, with half placed at pH 12 at room temperature and half at pH 12 at 60°C, and glucose esters were measured as the difference in

ABA or PA concentration between the treated and non-treated samples. In other cases, hormones were extracted from the water fraction with diethyl ether prior to the C18-column step. The water phase containing glucose esters was then hydrolysed at pH 12 and 60°C for 2 h. $^2H_3$-ABA was then re-added as an internal standard or left out, thereby allowing it to be used as an indicator of ABA carry-over from the diethyl ether fraction.

Levels of GA_1 were determined according to Coles *et al.* (1999). For each sample 10 mg freeze-dried material was used.

Isolation and expression measurements of RpNCED *homologues*

Degenerate primers were designed based on those constructed by Burbidge *et al.* (1999), but adjusted by using the consensus sequence from conserved regions of a wider range of known NCED orthologues taken from a number of monocots and dicots. The forward primer was 5′-TTCGACGGNGACGGNATGGT-3′ and the reverse primer 5′-ATNGCGAARTCRTGVATCAT-3′. With these primers, a 550 bp fragment was amplified by PCR using *R. palustris* cDNA and genomic DNA as template. This PCR product were subsequently cloned into pGEM-T (Promega, Leiden, The Netherlands) and transformed into *Escherichia coli*. Analysis of 60 clones (using the T7 and SP6 fragments present in the pGEM-T vector) repeatedly revealed 11 distinct genes. Each of these had 70–80% amino acid homology to known NCED orthologues; they were subsequently named *RpNCED1* to *RpNCED11*. Genes were clustered according to sequence homology and primer pairs were developed that amplified mRNA from all members of one distinct cluster.

For each sample, RNA of five third petioles was extracted using a modified method of Kiefer *et al.* (2000). For each time point four biological replicates were extracted. Fifty microlitres of Nucleon Phytopure DNA extraction resin (Amersham RPN8511; Amersham, Little Chalfont, UK) was used to remove the high amount of polysaccharide. Treatment with DNase was repeated three times using 2U DNaseI each time (DNA-free kit no 1606; Ambion, Austin, TX, USA). The effectiveness of the treatment was checked by gel electrophoresis. One microgram of RNA was used for cDNA synthesis using Random Hexamers (50 μM) (Amersham, no 27-2166-01), RNA guard RNase inhibitor, Porcine (Amersham, no 27-0816-01) and Superscript III reverse transcriptase (100 U) (Invitrogen, Breda, The Netherlands; no 18080-085).

Real-time RT-PCR was performed on a MyiQ terminal cycler (Bio-Rad, Veenendaat, The Netherlands). Each sample was performed in duplicate using the iQ SYBR Green Supermix (Bio-Rad, no 170-8882). The annealing temperature was optimized for each primer pair resulting in specific amplification of the transcript of interest. All primer combinations showed no dimerization or non-specific amplification. The primer sequences were 5′-TTCTCCGGCCAGCTCAACT-3′ and 5′-CGAACATTTCTTTGGTGACGG-3′ (for *RpNCED1*, at 66°C), 5′-GTCGGAAGACGACGTTCCTTAT-3′ and 5′-CCTTTGAAATCGTATCTGCCGA-3′ (for *RpNCED2*, -6 and -7, at 68°C), 5′-CCATGATCGCCCACCCGAAA-3′ and 5′-CTTCTTCACAACGTCGTAGCTA-3′ (for *RpNCED3* and -8, at 68°C), 5′-TGGTTCACGCTGTCAAGTTCC-3′ and 5′-GGAATACCGGTCGGCCGAGT-3′ (for *RpNCED4*, -9 and -10, at 68°C) and 5′-CCGAAGGCTATAGGTGAGCTCCAT-3′ and 5′-CGCACATGCCACGGGAGAGAAAC-3′ (for *RpNCED5* and -11, at 72°C). Relative mRNA values were calculated using the comparative Ct method described by Livak and Schmittgen (2001), expressing mRNA values relative to 18S RNA. All expression levels were presented relative to the value obtained at $t = 0$ h.

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