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The stimulating effects of ethylene and auxin on petiole elongation and on hyponastic curvature are independent processes in submerged *Rumex palustris*

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ABSTRACT

The flooding-tolerant plant species *Rumex palustris* (Sm.) responds to complete submergence with stimulation of petiole elongation mediated by the gaseous hormone ethylene. We examined the involvement of auxin in petiole elongation. The manipulation of petiolar auxin levels by removing the leaf blade, or by addition of synthetic auxins or auxin transport inhibitors, led to the finding that auxin plays an important role in submergence-induced petiole elongation in *R. palustris*. A detailed kinetic analysis revealed a transient effect of removing the auxin source (leaf blade), explaining why earlier studies in which less frequent measurements were taken failed to identify any role for auxin in petiole elongation. We previously showed that the onset of stimulated petiole elongation depends on a more upright petiole angle being reached by means of hyponastic (upward) curvature, a differential growth process that is also regulated by ethylene and auxin. This raised the possibility that both ethylene and auxin stimulate elongation only indirectly by influencing hyponastic growth. We show here that the action of ethylene and auxin in promoting petiole elongation in submerged *R. palustris* is independent of the promoting effect that these hormones also exert on the hyponastic curvature of the same petiole.

Key-words: time-lapse photography.

INTRODUCTION

Plants growing in river flood plains are regularly exposed to complete submergence. One of the main stresses imposed by submergence is impeded gas exchange between the plant and the atmosphere because of the slower diffusion of gases in water compared to air (Jackson & Ram 2003). This slower gas exchange can lead to oxygen deficiency and ethylene accumulation (reviewed in Voesenek & Blom 1999).

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The submergence-tolerant species *Rumex palustris* (Sm.) is characterized by two important growth responses upon complete submergence that enable the leaves to restore contact with the atmosphere, thus normalizing gas exchange. These two responses are hyponastic (upward) reorientation of the petioles (Voesenek & Blom 1989; Banga *et al*. 1997; Cox *et al*. 2003, 2004) and stimulation of petiole elongation (Voesenek & Blom 1989; Peeters *et al*. 2002; Voesenek *et al*. 2003). Stimulated petiole elongation in *R. palustris* is distributed almost equally along the petiole and results entirely from cell elongation (Voesenek *et al*. 1990). This faster petiole elongation is initiated by the accumulation of the gaseous plant hormone ethylene in submerged leaves (Voesenek & Blom 1989). The plant hormones abscisic acid and gibberellin also play important roles in the regulation of petiole elongation in submerged *R. palustris* (Voesenek *et al*. 2003; Benschop 2004). In addition, previous studies have shown that the upregulation of the gene coding for the putative cell wall loosening protein expansin correlates closely with the onset of submergence-induced petiole elongation in this species (Vriezen *et al*. 2000; Vreeburg 2004).

An earlier work has shown that auxin, together with ethylene, also plays an important role in submergence-induced elongation responses of several semiaquatic and aquatic plant species, such as *Hydrocharis morsus-ranae* (Cookson & Osborne 1978), *Nymphoides peltata* (Malone & Ridge 1983), *Ranunculus sceleratus* (Horton & Samarakoon 1982) and *Regnellidium diphyllum* (Walters & Osborne 1979). However, previous research indicated that auxin is not involved in petiole elongation in submerged *R. palustris*. These earlier studies showed that pretreatment with the auxin transport inhibitor naphthylphtalamic acid (NPA), or removal of the leaf blade (a putative source of auxin) (Ljung, Bhalerao & Sandberg 2001), did not affect petiole length when this was measured after 48 h of submergence (Blom *et al*. 1994; Rijnders *et al*. 1996). However, because petiole length was measured over several days, it was possible that transient effects of these manipulations had been overlooked. Furthermore, it was not known at that time whether leaf blade removal affects the indole-3-acetic acid (IAA) concentration in petioles of *R. palustris*. Accordingly, we embarked on a more detailed study on the involvement of auxin in stimulating petiole elongation in submerged *R. palustris*.

The work gained further impetus from our recent finding that petiole elongation in submerged *R. palustris* depends on a threshold petiole angle being reached by hyponastic curvature (Cox *et al*. 2003), a process that also requires the presence of both ethylene and auxin (Cox *et al*. 2004). When petioles were kept at an angle below 40 degrees with the horizontal, no elongation occurred, whereas a fast elongation was observed at petiole angles higher than 50 degrees. This new insight raises the possibility that both hormones are only required to induce hyponastic growth and therefore only indirectly play a role in the initiation or maintenance of submergence-induced petiole elongation. We tested this hypothesis by studying the impact of ethylene and auxin on elongation in petioles that were manipulated artificially to an almost vertical orientation. The orientation of these manipulated petioles was above the threshold angle, meaning that the true effects of ethylene and auxin on petiole elongation could be studied.

In the present study, we identified a role for auxin throughout ethylene-mediated, submergence-induced petiole elongation in *R. palustris*. Furthermore, we showed that the action of ethylene and auxin in promoting petiole elongation was independent of the effect that these hormones exert on the hyponastic curvature of the petiole under submerged conditions.

MATERIALS AND METHODS

Plant material and growth conditions

R. palustris (Sm.) plants were grown as described by Cox *et al*. (2003). The third oldest petiole of 27-day-old plants was studied, and all the experiments were started between 0800 and 1000 h under the conditions described by Cox *et al*. (2003).

Petiole length measurements

The length of the adaxial petiole surface was measured with time-lapse photography as described by Cox *et al*. (2003). For all time points, the increase in petiole length compared to the start of the treatment ($t = 0$) was calculated. Unless stated otherwise, the experiments were performed in continuous light to make photography possible. To acclimatize, the plants were placed singly in open-glass cuvettes ($18.5 \times 24.5 \times 25.5$ cm) in the camera system on the day before the experiment.

Angle manipulation

The experiments in which the petiole angle was manipulated were performed in glass cuvettes ($15.0 \times 17.5 \times 29.0$ cm) fitted with a metal ring that holds each pot. This ring could be tilted to position the petiole at any chosen angle with respect to the horizontal. With this manipulation, the petiole could move freely.

Submergence treatment

To completely submerge the plants, tap water (20 °C) was gently pumped into the cuvettes until a water depth of 20 cm (from the soil surface) was reached. Control plants rested on a moist irrigation mat in the cuvettes and were not submerged.

Ethylene, ACC and 1-MCP treatment

A 5 μL L^{-1} concentration of ethylene or air (70% relative humidity) was flushed through the glass cuvettes ($15.0 \times 17.5 \times 29.0$ cm) at 75 L h^{-1}. This ethylene concentration is known to saturate ethylene-induced petiole elongation in *R. palustris* (Voesenek & Blom 1989). The ethylene concentration inside the cuvettes was regularly checked with a gas chromatograph (GC955, Synspec, Groningen, the Netherlands) and remained constant throughout the experiment. Treatment with ACC took place by submerging the plants in tap water containing 1×10^{-5} M 1-aminocyclopropane-1-carboxylic acid (ACC).

The gaseous ethylene perception inhibitor 1-methylcyclopropene (1-MCP) was released from Ethylbloc (Floralife, Walterboro, SC, USA) containing 0.14% 1-MCP by dissolving it in water in an airtight container at 40 °C for 12 min. The 1-MCP gas was then collected from the head space with a syringe and injected into an airtight cuvette (for 1 μL L^{-1}: 1.6 g Ethylbloc m^{-3}) for a 3 h pretreatment. A longer pretreatment did not affect the results.

Auxin (inhibitor) treatment and removal of the leaf blade

Intact plants were pretreated with 125 μL 1×10^{-3} M NPA (17 and 2 h before submergence), 125 μL 1×10^{-3} M 1-naphthalene acetic acid (NAA) (2 h before submergence) or 125 μL 1×10^{-4} M 2,4-dichlorophenoxy acetic acid (2,4-D) (2 h before submergence) per plant, by brushing a pretreatment solution on the third oldest petiole and the basal part of the third leaf blade. The ethanol concentration in the pretreatment solution did not exceed 3%, and the pH was set to 7.5–8.0. Tween 20 (0.1%) was added to ensure an even distribution of the solution over the plant tissue. A pretreatment solution without the synthetic auxins/inhibitor but containing 3% ethanol and 0.1% Tween was used as a control. The pretreated plants were submerged in a solution containing 2.5×10^{-5} M NPA (0.2% ethanol) with or without 1×10^{-5} M NAA or 5×10^{-6} M 2,4-D. The ethanol concentration in the submergence solutions did not exceed 0.03%, and the pH was set to 7.5–8.0. All chemicals were obtained from DUCHEFA (Haarlem, the Netherlands).

To reduce the endogenous auxin concentration in the petiole, the leaf blade of the third oldest petiole was removed with scissors, and silicone vacuum grease was applied to the leaf wound surface. No acclimatization period was inserted after the removal of the leaf blade, because it did not influence the response (data not shown).

De-bladed plants were submerged in tap water (20 °C) with or without NAA or 2,4-D in the concentrations described earlier.

Paclobutrazol and gibberellin A_3 treatment

Intact plants were pretreated for 3 d (twice daily) with 5 mL 1×10^{-4} M paclobutrazol (DUCHEFA) to the soil and subsequently submerged in a solution containing 1×10^{-4} M paclobutrazol. The treatment with paclobutrazol resulted in plants with a compact stature and dark green leaves. For submergence in gibberellin A_3 (GA_3) (DUCHEFA), a concentration of 1×10^{-6} M was used.

Statistical analysis

The lag phase for the start of petiole elongation was calculated as described by Cox *et al*. (2003). The data were compared by using a one-way analysis of variance (ANOVA) and a Bonferroni's *post hoc* test (Figs 1–3) or an independent sample *t*-test (Fig. 4) using the program SPSS version 10 (SPSS, Chicago, IL, USA).

RESULTS

Depletion of auxin levels in the petiole delays submergence-induced elongation

We previously showed that removal of the leaf blade causes endogenous IAA concentrations in the petiole to decrease strongly. A drop in endogenous IAA concentration of 60% is apparent in plants in air and under water within 2 h of blade excision and is sustained for up to 24 h (Cox *et al*. 2004). In the present study, we investigated the effect of this auxin depletion on petiole elongation, by removing the leaf blade of the third leaf at the start of submergence. Figure 1a shows that the removal of the leaf blade lengthened the lag phase that precedes stimulated petiole elongation by 7 h (1.5–8.5 h, $P < 0.01$).

The start of petiole elongation in plants with intact leaf blades is dependent upon a threshold petiole angle being reached by means of hyponastic growth (Cox *et al*. 2003). The removal of the leaf blade, however, also strongly delays the onset of this differential growth process in submerged plants (Cox *et al*. 2004), thus raising the possibility that the longer lag phase of elongation shown in Fig. 1a is the outcome of slower hyponasty. To test this possibility, a tilting treatment was given in which an angle of 80 degrees from the horizontal was imposed at the onset of submergence (Fig. 1a). This is the maximum angle that is reached by hyponastic growth, and manipulation to this angle ensured that the threshold for petiole elongation was reached at the start of treatment. These tilted petioles without a leaf blade still showed a delay in submergence-induced elongation that was statistically identical to that of non-tilted petioles without a leaf blade (Fig. 1a). Thus, the longer lag phase for elongation caused by leaf blade excision was not the result

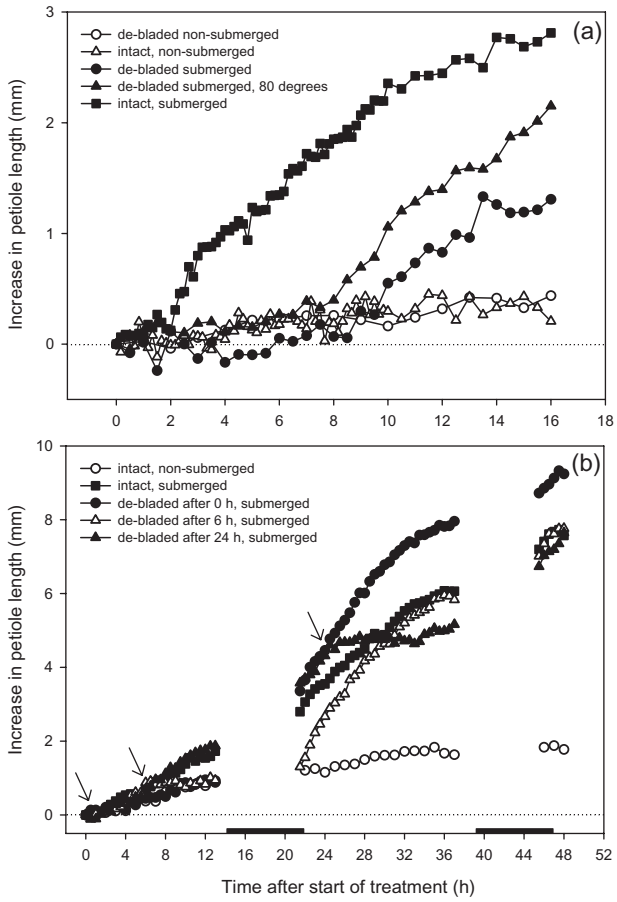

Figure 1. The effect of leaf blade removal on submergence-induced elongation of the third oldest petiole of 27-day-old *Rumex palustris* plants. The third leaf blade was removed: (a) at the start of the 16 h treatment, (b) after 0, 6 and 24 h of the 48 h submergence treatment (leaf blade removal indicated by arrows). The petiole manipulation in (a) consisted of adjusting the orientation of the petiole to an angle of 80 degrees with the horizontal at the start of submergence. The data are the means of 3–4 replicate plants. The mean standard error (SE) did not exceed 0.3 mm for (a) and 0.5 mm for (b). The lag phase for stimulated petiole elongation (a): 1.5 h (SE 0.2 h) for intact, submerged; 8.5 h (SE 1.2 h) for de-bladed submerged and 6.7 h (SE 0.9 h) for de-bladed submerged and manipulated to an angle of 80 degrees. The black boxes in (b) represent the night period.

of its impact on hyponasty but was directly related to the reduced auxin concentration in petioles.

To test if leaf blade removal had an inhibiting effect even after petiole elongation had already started, the leaf blades of the submerged petioles were removed at the start of submergence and after 6 and 24 h. Delaying the leaf blade excision until after 6 h of submergence resulted in an immediate arrest of the previously initiated elongation (Fig. 1b). However, after 24 h, elongation of these petioles had returned to the level of intact submerged petioles. It is notable that once elongation resumed, its rate was at least as fast as that recorded for submerged plants with intact leaf blades. Removing the leaf blades after 24 h of submergence had a similar effect (Fig. 1b). This experiment also revealed that

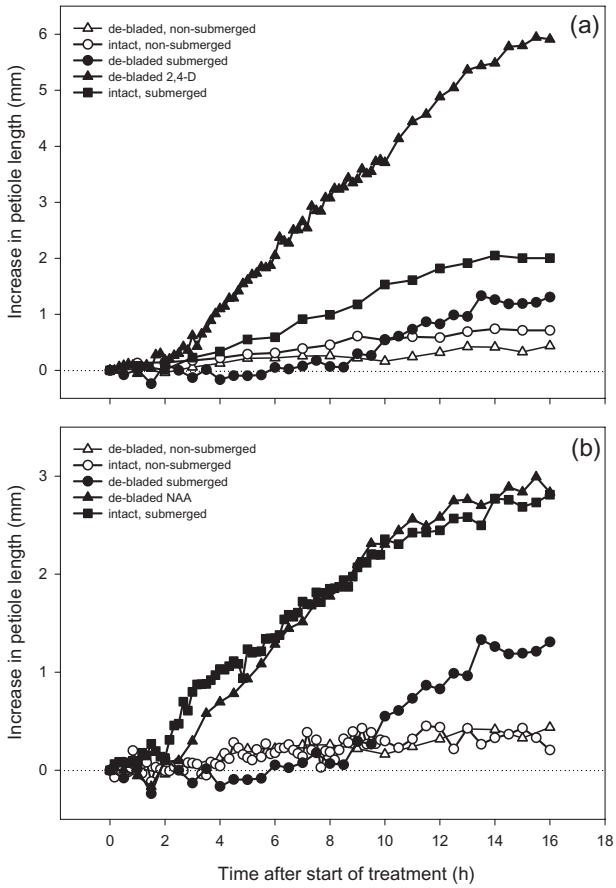

Figure 2. The elongation of the third oldest petiole of 27-day-old *Rumex palustris* plants whose leaf blades were removed at the start of treatment, submerged in a solution containing (a) 5×10^{-6} M 2,4-dichlorophenoxy acetic acid (2,4-D), or (b) 10^{-5} M 1-naphthalene acetic acid (NAA). The responses of non-submerged and submerged petioles of plants with intact leaf blades and those whose leaf blades have been removed (not treated with 2,4-D or NAA) are also shown. The data are the means of four replicate plants. The mean standard error (SE) did not exceed 0.4 mm for (a) and 0.3 mm for (b). The lag phase for stimulated petiole elongation: (a) 1.7 h (SE 0.4 h) for intact, submerged; 8.5 h (SE 1.2 h) for de-bladed submerged and 2.2 h (SE 0.5 h) for de-bladed treated with 2,4-D; and (b) 1.5 h (SE 0.2 h) for intact, submerged, 8.5 h (SE 1.2 h) for de-bladed submerged and 1.8 h (SE 0.5 h) for de-bladed treated with NAA.

the longer elongation lag phase that resulted from the removal of the leaf blade at the start of submergence had only a short-lived effect on petiole elongation. By the following day, total extension acheived underwater was at least as high as that of intact leaves (Fig. 1b).

Synthetic auxins can restore elongation in submerged petioles without leaf blades

The delay in the elongation of submerged petioles caused by the removal of the leaf blades at the start of the experiment could be restored by submerging the plants in a solution of 2,4-D or NAA. Figure 2a shows that the submergence in 2,4-D restored the elongation lag phase of petioles without leaf blades to that of petioles with leaf blades attached (approximately after 2 h). Moreover, the submergence in 2,4-D had a dramatic, promoting effect on the elongation of the petioles of plants whose leaf blades have been removed, which was approximately three times faster compared to submerged petioles of plants with intact leaf blades ($P < 0.01$) (Fig. 2a). The synthetic auxin NAA also restored the elongation lag phase of petioles of plants whose leaf blades have been removed to the level of intact submerged petioles (Fig. 2b). NAA, in contrast to 2,4-D, did not increase the level of elongation above that of submerged plants with intact leaf blades (Fig. 2b).

GA_3, ACC or ethylene cannot restore elongation in submerged petioles of plants without leaf blades

In contrast to the restoration by synthetic auxins of the elongation in submerged petioles of plants whose leaf blades have been removed, the submergence of petioles

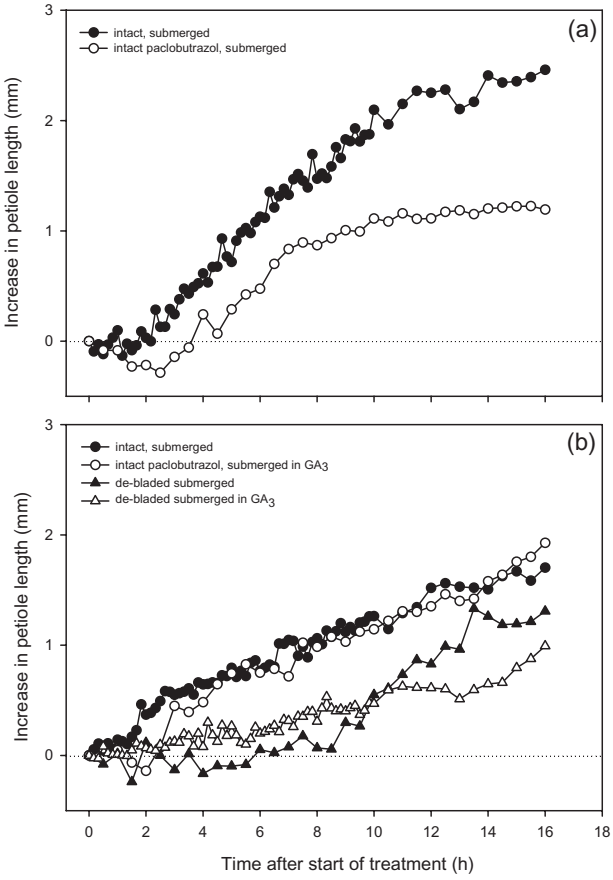

Figure 3. (a) The effect of paclobutrazol treatment (1×10^{-4} M) on the submergence-induced petiole elongation of intact 27-day-old *Rumex palustris* plants. (b) Submergence of plants whose leaf blades were removed at the start of treatment, or paclobutrazol-treated intact plants in a solution containing 1×10^{-6} M gibberellin A_3 (GA_3). The data are the means of four replicate plants. The mean standard error did not exceed 0.2 mm for (a) and 0.3 mm for (b).

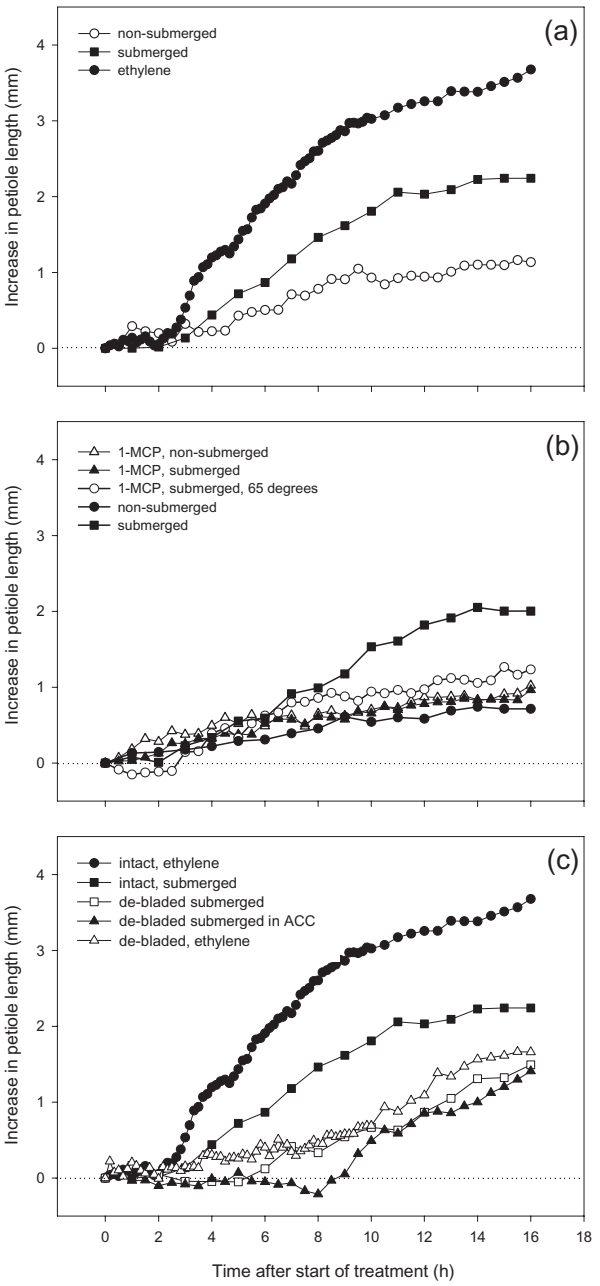

Figure 4. The elongation of the third oldest petiole of 27-day-old *Rumex palustris* plants exposed to: (a) submergence or 5 μL L^{-1} ethylene in a flow-through system, intact plants; (b) a 3 h pretreatment with 1 μL L^{-1} 1-methylcyclopropene (1-MCP) in closed cuvettes and subsequently air or submergence treatment, intact plants; and (c) submergence with or without 1-aminocyclopropane-1-carboxylic acid (ACC) (1×10^{-5} M) or ethylene treatment of intact plants and those whose leaf blades have been removed. The petiole manipulation in (b) consisted of adjusting the orientation of the petiole to an angle of 65 degrees with the horizontal at the start of submergence. The data are the means of 3–8 replicate plants. The mean standard error (SE) did not exceed 0.3 mm for (a), 0.2 mm for (b) and 0.3 mm for (c). The lag phase for stimulated petiole elongation: (a) 2.0 h (SE 0.6 h) for submerged, 2.1 h (SE 0.1 h) for treatment with ethylene; (b) 1.7 h (SE 0.4 h) for submerged.

without leaf blades in a solution of 1×10^{-6} M GA_3 did not restore elongation (Fig. 3b). Similar results were obtained when plants without leaf blades were submerged in a solution of 1×10^{-5} GA_3 (data not shown). However, the GA_3 solution used was capable of influencing petiole elongation in intact plants. Figure 3 shows that treatment of intact plants with paclobutrazol, an inhibitor of GA_3 biosynthesis, decreased the submergence-induced petiole elongation (Fig. 3a) and that this decrease could be restored by GA_3 (Fig. 3b).

The submergence of plants whose leaf blades have been removed in a solution containing 1×10^{-5} M ACC, or treatment with 5 μL L^{-1} ethylene in air, also did not shorten the excision-induced increase in lag phase and did not restore petiole elongation to the level of submerged plants with intact leaf blades (Fig. 4c). Similar results were obtained when 1×10^{-6} M ACC was used (data not shown).

Inhibition of polar auxin transport delays submergence-induced petiole elongation

The treatment of intact plants with the auxin efflux inhibitor NPA almost doubled the lag phase for submergence-induced petiole elongation compared to that of submerged plants without NPA ($P < 0.05$) (Fig. 5). Although there is a trend towards a larger increase in petiole length for NPA-treated plants after 12 h of treatment compared with submerged plants, this difference is not statistically significant at all time points between 12 h and the end of the experiment at 16 h. The delay in the onset of stimulated elongation caused by NPA could be overcome by NAA or 2,4-D

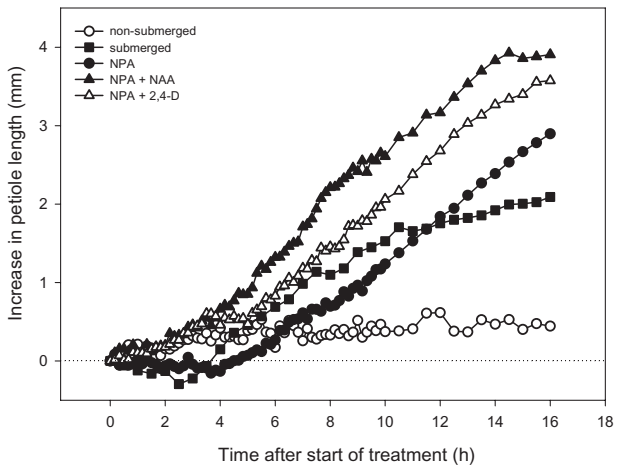

Figure 5. The effect of naphthylphtalamic acid (NPA) (2.5×10^{-5} M) and 1-naphthalene acetic acid (NAA) (10^{-5} M) or 2,4-dichlorophenoxy acetic acid (2,4-D) (5×10^{-6} M) on submergence-induced elongation of the third oldest petiole of intact 27-day-old *Rumex palustris* plants. The data are the means of 3–8 replicate plants. The mean standard error (SE) did not exceed 0.2 mm. The lag phase for stimulated petiole elongation: 2.6 h (SE 0.2 h) for submerged, 4.8 h (SE 0.5 h) for treatment with NPA, 1.6 h (SE 0.3 h) for treatment with NPA and NAA and 1.7 h (SE 0.1 h) for treatment with NPA and 2,4-D.

(Fig. 5). 2,3,5-Triiodobenzoic acid (TIBA), another inhibitor of the auxin efflux carrier, inhibited the lag phase in a similar fashion as NPA, although the effect of TIBA was generally weaker (data not shown). 1-Naphthoxyacetic acid (1-NOA), an inhibitor of the auxin influx carrier, was tested at a number of concentrations (3×10^{-5}, 1×10^{-4} M), but did not affect the kinetics of submergence-induced petiole elongation (data not shown).

Ethylene is essential for submergence-induced petiole elongation

A continuous supply of 5 μL L^{-1} ethylene to non-submerged *R. palustris* plants stimulated elongation of the third petiole (Fig. 4a). Moreover, ethylene-induced petiole elongation was more pronounced than that induced by submergence. After 16 h, the ethylene-treated petioles were almost twice as long as the submerged petioles ($P < 0.05$). However, elongation in both ethylene-treated and submerged plants showed a lag phase of 2 h (Fig. 4a).

The pretreatment of plants with the ethylene perception inhibitor 1-MCP (1 μL L^{-1}) for 3 h completely inhibited the submergence-induced petiole elongation during the 16 h of treatment (Fig. 4b). The submergence of non-treated plants resulted in an increase in petiole length of approximately 2 mm after 16 h, whereas the elongation of 1-MCP pretreated submerged petioles did not differ from that of non-submerged petioles (Fig. 4b). Petiole elongation was also completely inhibited in 1-MCP pretreated plants that were exposed to ethylene, indicating that 1-MCP can completely block ethylene effects (data not shown). Because hyponastic growth also requires ethylene, the lack of elongation in 1-MCP-treated plants could have been caused by the absence of hyponastic growth (Cox *et al*. 2004), and thus by the absence of the appropriate petiole angle required for petiole elongation. To test this, we tilted 1-MCP pretreated plants to 65 degrees at the start of their submergence. This angle is sufficiently upright for petiole elongation to take place (Cox *et al*. 2003). However, this manipulation did not result in a significant stimulation of petiole elongation in 1-MCP pretreated plants (Fig. 4b), demonstrating that ethylene directly affects elongation, rather than working via the angle of the petiole.

DISCUSSION

The digital camera system used in this study allowed us to examine the role of the plant hormones auxin and ethylene in submergence-induced petiole elongation of *R. palustris* in much more detail than was possible in previous studies. Using this method, we previously showed that the onset of stimulated petiole elongation upon submergence depends on a certain petiole angle being reached via the process of hyponastic growth (Cox *et al*. 2003). Because both ethylene and auxin have also been implicated in this hyponastic growth (Cox *et al*. 2004), an important goal of the present work was to establish if these hormones together exerted a promoting influence on petiole elongation indirectly through angle adjustment or whether they also possessed a distinctly separate and direct effect on petiole elongation *per se*.

Auxin is required throughout submergence-induced petiole elongation

We have shown in this study that the presence of auxin is required for the onset of petiole elongation. The removal of the leaf blade, which results within 2 h in a 60% decline in IAA concentration of the petiole (Cox *et al*. 2004), had a dramatic effect on the kinetics of submergence-induced petiole elongation (Fig. 1a). The elongation lag phase of petioles of plants without leaf blades (removed at the start of the experiment) was delayed by approximately 7 h compared with that of submerged plants with intact leaf blades ($P < 0.01$).

Our finding on the involvement of auxin in submergence-induced petiole elongation is supported by our recent study (Cox *et al*. 2004) that showed an increase in IAA in the outer cell layers of the submerged petiole, which correlated with the onset of both petiole elongation and hyponastic growth. Morelli & Ruberti (2000, 2002) proposed a model for the elongation of shaded hypocotyls that involves the lateral distribution of auxin from the vasculature to the epidermal and cortical cells. It is possible that a similar mechanism takes place in submerged *R. palustris* petioles, resulting in a larger amount of IAA in the outer cell layers upon submergence. Figure 1a shows that the elongation of submerged petioles of plants without leaf blades eventually started after approximately 8 h, although we have previously shown that the endogenous IAA concentration of the petioles of plants whose leaf blades have been removed is still low at this time (Cox *et al*. 2004). Although the petioles of plants whose leaf blades have been removed had low IAA levels, it is possible that the submergence-induced redistribution of auxin to the outer cell layers still took place in these petioles, but at a much slower rate because of the lack of auxin source (leaf blade). This would explain why petiole elongation eventually started after an initial delay.

The dependence of submergence-induced petiole elongation on auxin was confirmed by studies carried out over a longer time period during which the leaf blade was removed at different time points after the onset of submergence (Fig. 1b). The removal of the leaf blade when petiole elongation had already started (6 or 24 h after the start of submergence) resulted in a transient arrest of petiole elongation that was restored within 24 h to the level of petioles of intact plants (Fig. 1b). From these experiments, we conclude that the dependence of submergence-induced petiole elongation on auxin remains during the entire elongation response and is not restricted to the onset of the process. Figure 1 also explains why in previous studies on submergence-induced petiole elongation (Rijnders *et al*. 1996), no role for auxin in petiole elongation was found. In these previous studies, the leaf blade was removed at the start of submergence and petiole elongation was only measured

after 48 h of submergence. Because the effect of leaf blade removal is transient (Fig. 1b), it is not surprising that no effect was observed after 48 h.

Removal of the leaf blade is a crude treatment that can also influence the transport of growth-inducing substances other than auxin. The excision experiments alone do not necessarily prove that the decline in IAA is the cause of the delay in growth. However, we also showed that the delayed onset of stimulated petiole elongation in submerged petioles of plants whose leaf blades have been removed could be restored by the addition of the synthetic auxins NAA or 2,4-D (Fig. 2). It is known that 2,4-D is a much more effective synthetic auxin than NAA (Nutman & Thornton 1945; Slade, Templeman & Sexton 1945). This explains the much stronger promotion of petiole elongation by 2,4-D in our experiments, although NAA was also able to restore elongation to at least the level of intact plants as well.

GA_3 (Fig. 3), ACC (Fig. 4c) and ethylene (Fig. 4c) could not restore the delay in petiole elongation, indicating that the effect of the leaf blade removal on petiole elongation is related to the presence of auxin and not to the presence of these other hormones. Taken together, these results strongly implicate auxin in the effect of leaf blade removal and indicate the importance of auxin in submergence-induced petiole elongation. In addition, the fact that neither ACC nor ethylene could restore elongation in submerged petioles of plants whose leaf blades have been removed (Fig. 4c), whereas ethylene strongly stimulates elongation in petioles of intact plants (Fig. 4a), indicates that the effect of ethylene on this growth process is auxin-dependent. A similar auxin dependency was found for the role of gibberellin in the elongation of submerged petioles. GA_3 was unable to restore elongation in auxin-deficient petioles of plants whose leaf blades were removed (Fig. 3b), whereas the GA_3 concentration used in the leaf blade-removal experiment was sufficient to restore petiole elongation in intact plants treated with the gibberellin synthesis inhibitor paclobutrazol (Fig. 3). Rijnders *et al*. (1997) also demonstrated the role of gibberellin (similar GA_3 concentrations as used in our experiment) in petiole elongation of intact *R. palustris* plants. This finding that the effect of gibberellin on petiole elongation seems to be auxin-dependent is consistent with the results of Fu & Harberd (2003), who showed that auxin is necessary for gibberellin-mediated control of root growth in *Arabidopsis thaliana*. However, an auxin requirement in gibberellin-induced growth is not shown consistently in all the plant species studied. For example, Ross, O'Neill & Rathbone (2003) showed that the decapitated internodes of *Pisum sativum*, which are auxin-deficient, responded strongly to gibberellin application.

Auxin transport to the petiole

We studied the role of polar auxin transport (PAT) in the submergence-induced elongation response of *R. palustris* petioles, using synthetic inhibitors of the influx and the efflux carriers. It has been previously shown that the efflux carrier inhibitor NPA inhibits PAT in *R. palustris* petioles (Visser *et al*. 1995). Figure 5 shows that NPA treatment almost doubled the lag phase of submergence-induced petiole elongation, indicating that the right timing of petiole elongation depends on a functional PAT pathway.

The delay in the lag phase of petiole elongation caused by NPA could be restored by either of the synthetic auxins we tested (Fig. 5). The restoration with 2,4-D of NPA-treated petioles was expected because this synthetic auxin is taken up by the influx carrier but is not a substrate for the efflux carrier (Delbarre *et al*. 1996). In contrast, NAA enters the cell by passive diffusion (Delbarre *et al*. 1996) and is secreted by the efflux carrier, which is blocked in NPA-treated plants. Thus, the ability of NAA to overcome the NPA-induced delay in submergence-induced elongation was not anticipated. The explanation for this unexpected result may be that NPA-treated petioles were surrounded by a solution containing NAA, and the elongating cells were able to take up NAA from the medium by diffusion, thus bypassing active auxin transport. This could be an explanation for the stimulation of overall petiole elongation by both synthetic auxins (Fig. 5). TIBA, which also inhibits the auxin efflux carrier, influenced petiole elongation in a similar manner to NPA, although the effect was less pronounced (data not shown). An inhibitor of the influx carrier, 1-NOA, had no influence on submergence-induced petiole elongation (data not shown).

Auxin does not stimulate petiole elongation via its influence on hyponastic growth

In addition to delaying submergence-induced petiole elongation, removing the leaf blade at the start of submergence also delays the onset of hyponastic growth (Cox *et al*. 2004). Given the recently discovered interaction between hyponastic growth and petiole elongation in submerged *R. palustris* petioles (Cox *et al*. 2003), we explored whether auxin stimulates petiole elongation via its influence on hyponastic growth. A petiole angle higher than 40–50 degrees from the horizontal is required for submergence-induced petiole elongation to start (Cox *et al*. 2003). However, imposing an angle of 80 degrees in a tilting experiment still failed to illicit a prompt elongation response in auxin-depleted petioles (brought about by the removal of the leaf blade) (Fig. 1). This indicates that the influence of auxin on the elongation of submerged petioles is independent of its effect on hyponastic growth and petiole angle.

Ethylene

Treatment of *R. palustris* with ethylene promoted petiole elongation (Fig. 4a), a result consistent with earlier findings (Voesenek & Blom 1989; Banga *et al*. 1997). However, in the present study, ethylene had a stronger effect on petiole elongation than did submergence (Fig. 4). This is in contrast with previous studies on the same species where ethylene could induce only approximately 70% of the submergence

response (Voesenek & Blom 1989; Voesenek *et al*. 1997). This difference may have arisen because petioles of different developmental stages were studied. In the present work, the third oldest petioles of 27-d-old plants were used; in previous studies, the fifth oldest petioles from plants of the same age were used. Horton (1992) also observed a developmental difference in response to ethylene based on leaf age in *Ranunculus pygmaeus*.

Ethylene requirement for petiole elongation during submergence is independent of its role in hyponastic growth

The role of ethylene in submergence-induced petiole elongation in *R. palustris* has been extensively documented (Voesenek & Blom 1989; Banga *et al*. 1997). However, previous studies on the involvement of ethylene in this species have not taken into account that petiole elongation can only start when a certain angle of the petiole has been reached (Cox *et al*. 2003), and that ethylene plays a role in achieving this angle through the stimulation of hyponastic growth (Cox *et al*. 2004). In the present work, we uncoupled ethylene action in each of these events as follows. Ethylene is an important factor responsible for stimulated elongation of submerged petioles, because submerged plants pretreated with the ethylene perception inhibitor 1-MCP do not show petiole elongation (Fig. 4b). However, 1-MCP also strongly delays hyponastic growth (Cox *et al*. 2004). Therefore, ethylene could either stimulate petiole elongation itself or establish the appropriate angle necessary for the onset of elongation by stimulating hyponasty. We tested this using plants pretreated with 1-MCP that were manipulated to an angle higher than the threshold petiole angle for elongation. Figure 4b shows that 1-MCP still inhibited underwater elongation to the same extent in these plants, indicating that ethylene can act to promote petiole elongation independently of its effect on petiole angle.

CONCLUSIONS

In this study, we have shown for the first time an important role for auxin in submergence-induced petiole elongation in *R. palustris*. In addition, we present evidence that the combined actions of ethylene and auxin on hyponastic curvature and on straight petiole extension are independent processes. That is, the two hormones play an active role in both growth responses, and the effect of both auxin and ethylene on stimulated petiole elongation is not confined to merely the establishment of the appropriate angle necessary for the onset of elongation by the stimulation of hyponasty. We conclude that submergence-induced elongation of *R. palustris* petioles starts when two input conditions have been met. The first is a submergence signal, thought to be an increase in internal ethylene brought about by water entrapment. The second is the cooperative action of ethylene with endogenous auxin to induce an upright orientation (40–50 degrees from the horizontal) by means of hyponastic growth (Cox *et al*. 2004). Only when this angle has been achieved are ethylene and auxin able to stimulate petiole elongation.

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