Long-Term Submergence-Induced Elongation in *Rumex palustris* Requires Abscisic Acid-Dependent Biosynthesis of Gibberellin$_1$[1]

Joris J. Benschop[2,3], Jordi Bou[2,4], Anton J.M. Peeters, Niels Wagemaker[5], Kerstin Gühl, Dennis Ward, Peter Hedden, Thomas Moritz, and Laurentius A.C.J. Voesenek*

Plant Ecophysiology, Institute of Environmental Biology, Utrecht University, Utrecht, Sorbonnelaan 16, 3584 CA, The Netherlands (J.J.B., J.B., A.J.M.P., N.W., K.G., L.A.C.J.V.); Rothamsted Research, Harpenden, AL5 2JQ Herts, United Kingdom (D.W., P.H.); and Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S–90183 Umea, Sweden (T.M.)

Rumex palustris (*polygonceae*) responds to complete submergence with enhanced elongation of its youngest petioles. This process requires the presence of gibberellin (GA) and is associated with an increase in the concentration of GA_1 in elongating petioles. We have examined how GA biosynthesis was regulated in submerged plants. Therefore, cDNAs encoding GA-biosynthetic enzymes GA 20-oxidase and GA 3-oxidase, and the GA-deactivating enzyme GA 2-oxidase were cloned from *R. palustris* and the kinetics of transcription of the corresponding genes was determined during a 24 h submergence period. The submergence-induced elongation response could be separated into several phases: (1) during the first phase of 4 h, petiole elongation was insensitive to GA; (2) from 4 to 6 h onward growth was limited by GA; and (3) from 15 h onward underwater elongation was dependent, but not limited by GA. Submergence induced an increase of GA_1 concentration, as well as enhanced transcript levels of *RpGA3ox1*. Exogenous abscisic acid repressed the transcript levels of *RpGA20ox1* and *RpGA3ox1* and thus inhibited the submergence-induced increase in GA_1. Abscisic acid had no effect on the tissue responsiveness to GA.

In several semiaquatic species ethylene promotes shoot extension to rates many times faster than normal (Musgrave et al., 1972; Kende et al., 1998; Voesenek et al., 2003a). In these plants, ethylene accumulates to high levels inside the submerged tissue and acts as a signal for submergence (Voesenek et al., 1993). The enhanced elongation response in these species functions as part of an underwater escape mechanism that enables submerged shoots to emerge from the water and function as snorkels, thus restoring gas exchange between the atmosphere and the submerged plant tissues (Voesenek et al., 2004).

The dicot *Rumex palustris* also responds to submergence with this strong enhancement in cell elongation of petioles (Vreeburg et al., 2005). The entire response can be mimicked by supplying ethylene to air-grown plants (Voesenek et al., 1990), while inhibition of ethylene perception completely abolished the response in submerged plants (Benschop et al., 2005). Ethylene was found to act by stimulating cellular elongation through increasing the concentration of and sensitivity to gibberellins (GAs). The stimulation of GA biosynthesis by ethylene is considered a crucial factor in the submergence-induced elongation since application of GA to nonsubmerged plants mimics underwater elongation, while reducing GA content by inhibiting its biosynthesis strongly inhibits submergence-induced elongation (Rijnders et al., 1997). Another hormone interaction that occurs during submergence is the repression of endogenous levels of abscisic acid (ABA) by elevated ethylene concentrations. This is an important signal transduction step since ABA was found to operate as a negative regulator of underwater elongation (Benschop et al., 2005). Although several players in underwater elongation in *R. palustris* are now identified, surprisingly little is known about the regulation of GA biosynthesis, the dynamics of mRNAs coding for GA-biosynthetic and -catabolic genes, and the identity of the interaction between GA and ABA during submergence. The importance of the ABA-GA

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[2] These authors contributed equally to the paper.

[3] Present address: Department for Physiological Chemistry, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.

[4] Present address: Laboratori de Genètica Molecular Vegetal, Consorci Consejo Superior de Investigaciones Científicas-Institut de Recerca i Technologia Agroalimentàries, Jordi Girona, 18–26, 08034 Barcelona, Spain.

[5] Present address: Institute for Water and Wetland Research, Faculty of Science, Radboud University, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands.

* Corresponding author; e-mail l.a.c.j.voesenek@bio.uu.nl; fax 31–30–2518366.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Laurentius A.C.J. Voesenek (l.a.c.j.voesenek@bio.uu.nl).

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interaction is already indicated by work on deepwater rice (*Oryza sativa*), in which reduced ABA levels increase the responsiveness to GA (Hoffmann-Benning and Kende, 1992).

The biosynthetic pathway for GA_1, which is the major bioactive GA in Rumex (Rijnders et al., 1997), is now well characterized. cDNAs for many of the enzymes are cloned and orthologs have been identified in a number of different species (for review, see Hedden and Phillips, 2000). GAs are derived from geranylgeranyl diphosphate, which is converted to *ent*-kaurene and then by oxidation to yield GA_{12} and its 13-hydroxylated analog GA_{53}. In the final stages of GA biosynthesis GA_{12} and GA_{53} are converted to GA_4 and GA_1, respectively. GA_{53} is converted by GA 20-oxidase (GA20ox) via three consecutive steps to GA_{44}, GA_{19}, and GA_{20}, from which the bioactive GA_1 is formed by GA 3-oxidase (GA3ox). Finally, deactivation of GA_1 by conversion to GA_8 is catalyzed by GA 2-oxidase (GA2ox; Hedden and Phillips, 2000).

Regulation of GA levels takes place predominantly through the activities of GA20ox, GA3ox, and GA2ox. Overexpression of genes encoding earlier enzymes in the biosynthetic pathway generally does not affect plant stature: Feedforward and feedback regulation of *GA20ox* and *GA2ox* transcript levels enables the plant to maintain stable GA levels despite large changes in earlier metabolites (Fleet et al., 2003). In contrast, manipulation of the transcription of *GA20ox*, *GA3ox*, and *GA2ox* genes did result in large alterations in plant growth and stature (Coles et al., 1999; Hedden and Phillips, 2000; Hedden, 2003; Radi et al., 2006). Transcription of *GA20ox*, *GA3ox*, and *GA2ox* is regulated by many environmental and endogenous cues, including light quality, temperature, and auxin (Kamiya and Garcia-Martinez, 1999; Ross et al., 2000; Stavang et al., 2005). Recent work showed that phytochrome-regulated petiole elongation in Arabidopsis (*Arabidopsis thaliana*) depends on transcriptional up-regulation of *GA20ox* (Hisamatsu et al., 2005). In *R. palustris*, changes in light quality and ethylene seem to stimulate petiole growth in a comparable manner (Pierik et al., 2005), suggesting that ethylene may also act via *GA20ox*.

In this project we investigated whether submergence and ABA regulate GA biosynthesis in petioles of *R. palustris*. For this work, orthologs for *GA20ox*, *GA3ox*, and *GA2ox* were cloned from this species. Subsequently, the kinetics of transcription of these genes was measured in detail during 24 h of submergence and was compared to changes in concentration of bioactive and inactive GAs and to a detailed analysis of elongation growth. In addition, the effect of submergence and ABA on GA sensitivity was investigated. The results show that the underwater elongation response could be separated in distinct GA-insensitive and GA-dependent phases. Submergence induced an accumulation of GA_1 and enhanced transcript levels of *RpGA3ox1*. These increases in GA_1 content and *RpGA3ox1* transcript levels were inhibited when submerged plants were treated with ABA, whereas ABA did not affect GA sensitivity.

RESULTS

Submergence-Induced Elongation

Upon complete submergence of *R. palustris*, leaf elongation rates increased strongly, after a lag phase of 2 h (Fig. 1), to a rate almost 4 times that of control plants (after 4 h of submergence). After this point, the growth rate decreased slightly, but increased again after 8 h, after which the elongation rate remained 2- to 3-fold faster than air-grown plants for at least 22 h. Separate measurements showed that after 2 d of submergence, petiole length increased almost 3-fold (Fig. 1 insert), whereas leaf blades grew only by 30%.

Analysis of GA Concentrations

To examine whether the enhanced elongation in petioles correlated with enhanced GA biosynthesis, the levels of bioactive GA_1 as well as its precursors (GA_{53}, GA_{19}, and GA_{20}) and first catabolite (GA_8) were measured. The level of GA_{53} was increased during submergence after a lag phase of 2 h (Fig. 2A). Changes in GA_{19} and GA_{20} concentration followed a similar pattern: an increase in GA_{19} was observed after 4 to 6 h, followed by an increase in GA_{20} after 8 h (Fig. 2, B and C). The concentration of bioactive GA_1 increased 2-fold in submerged petiole tissue after only 4 h (Fig. 2D). After this point the concentration remained above that in air-grown plants during the remainder of the treatment period. GA_8, the first catabolite of GA_1, was present at a relatively high concentration (Fig. 2E). Apart from a transient increase in submerged plants between 4 and 8 h of treatment, GA_8 decreased gradually in both submerged and air-grown tissue during the treatment period.

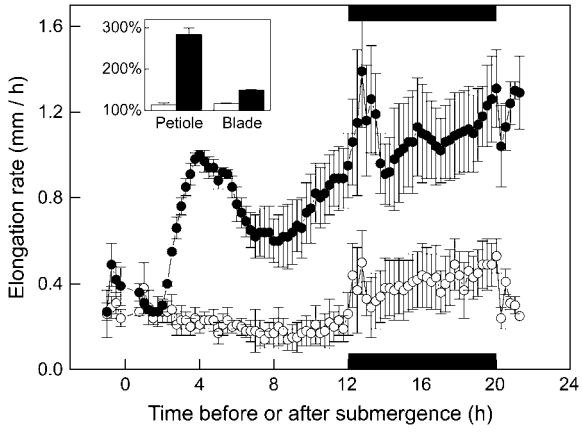

Figure 1. Leaf elongation rate of *R. palustris* growing in air (white circles) or submerged at $t = 0$ (black circles). Means of three replicates with SES. Black bars at the *x* axis represent dark periods. Insert: Relative growth of petioles and leaf blades after 48 h in air (white bars) or submergence (black bars). Means of 12 replicates with SES.

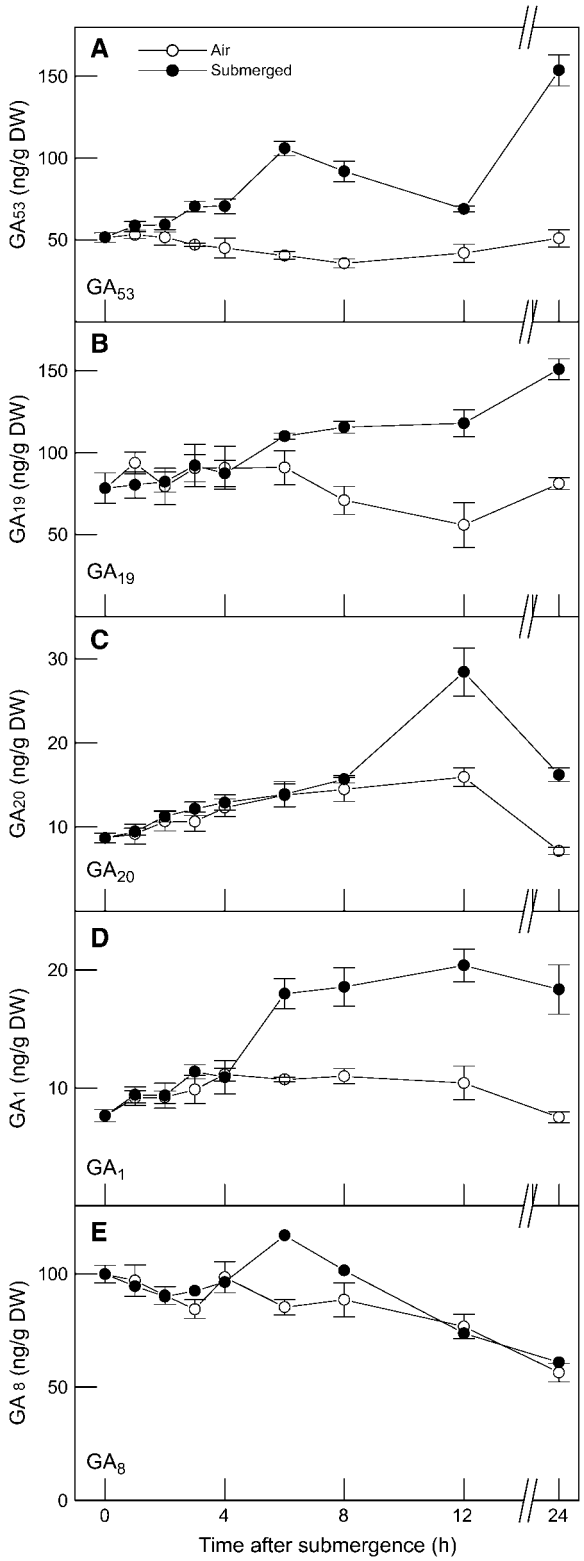

Figure 2. Concentrations of GA_{53}, GA_{19}, GA_{20}, GA_1, and GA_8 in petioles of *R. palustris* during submergence. White circles: controls in air; black circles: submerged at $t = 0$ h. Means of four replicates with SES.

Regulation of GA Biosynthesis during Submergence

To determine if the submergence-induced changes in GA concentration resulted from enhanced transcript levels of GA-biosynthetic genes, orthologs from GA20ox, GA3ox, and GA2ox were cloned from *R. palustris* and their mRNA levels were analyzed by means of real-time reverse transcription (RT)-PCR in submerged and air-grown plants. To isolate cDNA clones, PCR was performed on a cDNA library from *R. palustris* shoots submerged for 24 h, using degenerated oligonucleotides based on conserved domains of orthologs from Arabidopsis and potato (*Solanum tuberosum*). Full-length cDNAs were obtained by screening the cDNA library and extension by PCR. We named these genes *RpGA20ox1*, *RpGA3ox1*, and *RpGA2ox1*, respectively. Their predicted amino acid sequences contained the residues necessary for the binding of Fe^{2+} and the cofactor 2-oxoglutarate conserved in all 2-oxoacid-dependent dioxygenase proteins (Prescott and John, 1996; Valegard et al., 1998; Hedden and Phillips, 2000). The three clones were each closely related to corresponding dicot GA oxidases (data not shown). To ensure that these transcripts encode functional enzymes full-length clones were transferred to pET32C expression vectors and expressed in *Escherichia coli*. Bacterial lysates were incubated with the appropriate ^{14}C-labeled GA substrate and shown to contain the expected enzymatic activity (data not shown).

Real-time RT-PCR analysis of *RpGA20ox1*, *RpGA3ox1*, and *RpGA2ox1* showed that submergence induced distinct alterations of transcript levels of the three genes. Transcript levels of *RpGA20ox1*, whose product catalyzes the consecutive conversions from GA_{53} via GA_{44} and GA_{19} to GA_{20}, decreased upon submergence (Fig. 3A). After 4 h of submergence *RpGA20ox1* transcript levels increased again gradually to levels equal to or above those found in air-grown plants. Transcript levels of *RpGA3ox1*, whose product catalyzes the conversion of GA_{20} to GA_1, increased rapidly inside the submerged tissue. After a lag phase of about 1 h, *RpGA3ox1* transcript abundance peaked to a level 4-fold higher than that of air-grown plants (Fig. 3B). A partial fragment of a second *RpGA3ox* clone demonstrated no change in transcript levels upon submergence (data not shown). Transcript levels of *RpGA2ox1*, whose product inactivates GA_1 by conversion to GA_8, also increased in submerged plants (Fig. 3C). However, after 4 to 6 h of submergence, *RpGA2ox1* transcript levels decreased to levels below those in air-grown plants.

Effect of ABA on GA Biosynthesis and Catabolism

Externally applied ABA has been shown to inhibit the increase in GA_1 concentration in submerged plants (Benschop et al., 2005). To investigate the cause of this effect, the concentrations of various GA_1 precursors and of the catabolite GA_8 were measured in plants submerged in the presence or absence of ABA. This

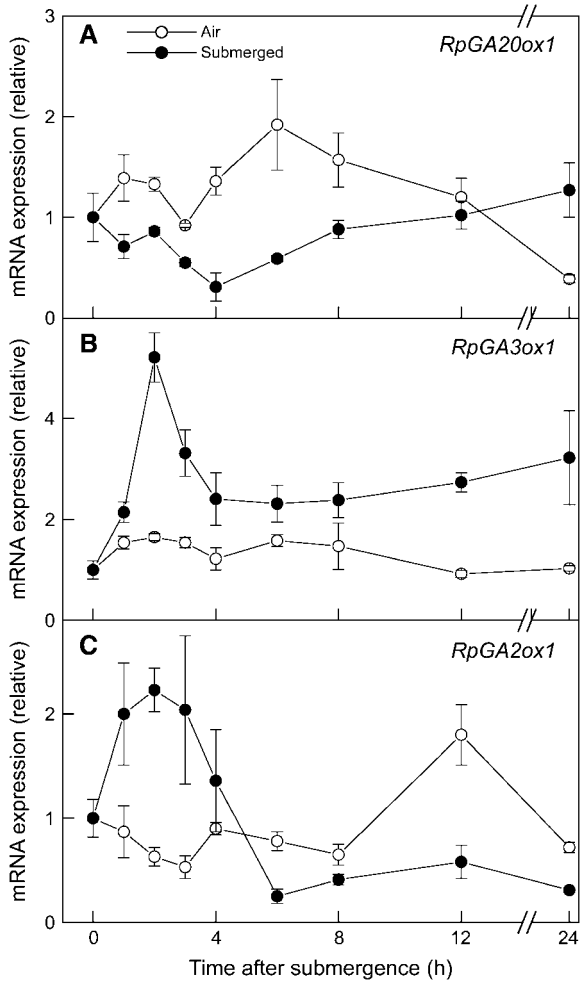

Figure 3. Relative transcription of *RpGA20ox1*, *RpGA3ox1*, and *RpGA2ox1* mRNA in petioles of *R. palustris* during submergence measured with real-time RT-PCR. White circles: controls in air; black circles: submerged at $t = 0$. Expression of mRNA was quantified relative to the value obtained at $t = 0$. Means of four replicates with SES.

showed that ABA did not affect the concentrations of GA_{53}, GA_{19}, GA_{20}, or GA_8 in petioles (Fig. 4). However, the increase in levels of bioactive GA_1, which was observed in plants submerged without ABA, was completely absent when ABA was present in the submergence solution. This could occur if ABA reduced GA3ox activity. Evidence for this was obtained by determining the effect of ABA on transcript levels of *RpGA20ox1*, *RpGA3ox1*, and *RpGA2ox1* (Fig. 5). As observed in an independent experiment (Fig. 3), submergence resulted in increased *RpGA3ox1* transcript levels in petioles. However, this increase in *RpGA3ox1* transcript levels did not occur when plants were submerged in the presence of ABA. In addition, ABA also inhibited transcript levels of *RpGA20ox1* in submerged petioles (which was already lower than that of air-grown plants), while transcript levels of *RpGA2ox1* were not affected after 4 h. After 8 h ABA addition resulted in a *RpGA2ox1* transcript level that was intermediate between air and submergence.

Effect of ABA on GA Sensitivity

To investigate whether ABA influenced GA sensitivity as well as GA biosynthesis, plants were submerged in solutions containing a matrix of concentrations of GA and ABA, an approach similar to that described for deepwater rice (Hoffmann-Benning and Kende, 1992).

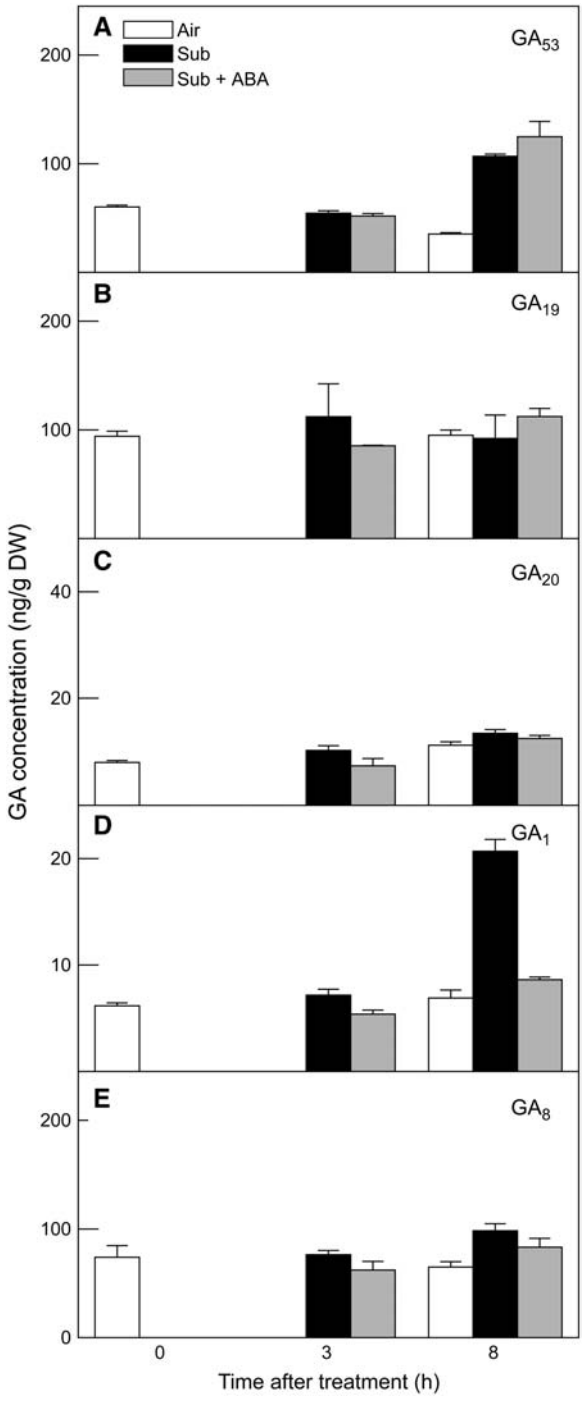

Figure 4. Concentrations of GA_{53}, GA_{19}, GA_{20}, GA_1, and GA_8 in petioles during submergence. White bar: controls in air; black bar: submerged (Sub) at $t = 0$ h; gray bar: submerged at $t = 0$ h in the presence of 10 μM ABA. Means of four replicates with SES.

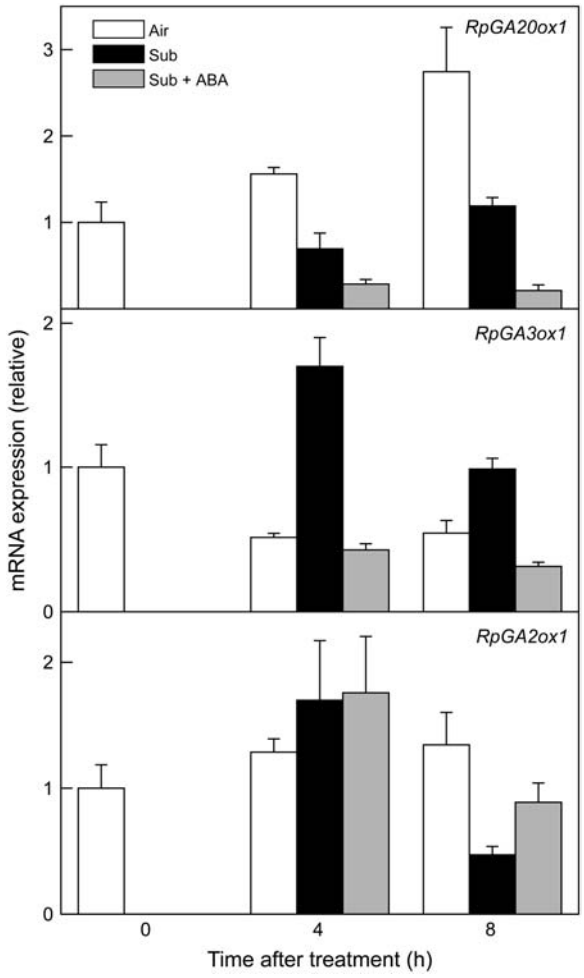

Figure 5. Relative transcription of *RpGA20ox1*, *RpGA3ox1*, and *RpGA2ox1* mRNA in petioles during submergence in the presence or absence of 10 μM ABA. White bar: controls in air; black bar: submerged at $t = 0$ h; gray bar: submerged at $t = 0$ h in the presence of 10 μM ABA. Transcription was quantified relative to the value obtained at $t = 0$. Means of four replicates with SES.

All plants were pretreated with paclobutrazol to avoid an effect of ABA on GA production. After 48 h, the combined effect of these hormones on petiole growth was determined (Fig. 6). This showed, as expected, that externally applied ABA negatively affected petiole elongation (see also Benschop et al., 2005) and that GA stimulated petiole elongation in submerged plants (see also Rijnders et al., 1997). In this experiment no significant interaction between GA and ABA was observed; GA stimulated petiole elongation to the same extent in the absence or presence of additional ABA. This result suggests that ABA does not affect GA responsiveness in submerged *R. palustris* petioles.

Manipulation of in Vivo GA and ABA Concentrations

Submergence induced an enhancement of petiole elongation after a lag time of 2 h (Fig. 1), but the increase in GA_1 concentration could not be observed until at least 4 h of submergence (Fig. 2). Therefore, the increase in growth rate during the initial hours of submergence could not be explained by increased GA_1. Nevertheless, the enhanced growth that took place immediately following submergence might still be mediated by GA because sensitivity to GA was shown previously to increase upon submergence (Rijnders et al., 1997). This was reinvestigated by measuring the kinetics of underwater elongation in plants treated with bioactive GA_3 or with paclobutrazol, an inhibitor of GA biosynthesis.

Underwater elongation was not affected by GA treatment during the initial growth phase (2–4 h; Fig. 7A). However, the temporary slowing of growth rate that was observed in untreated plants during the period of 4 to 10 h of submergence was partly prevented by externally applied GA (Fig. 7A). After 12 h of submergence the growth rate of GA-treated plants was only slightly higher than or even equal to the rate found in untreated plants.

Pretreating plants with paclobutrazol showed that the initial growth phase (2–6 h after submergence) was almost unaffected by this treatment (Fig. 7B). Only a very high dose (100 μM) of paclobutrazol was able to reduce growth somewhat during this period. However, this concentration was likely to have affected processes other than GA biosynthesis, as the growth rate of these leaves was also inhibited before plants were submerged (see Fig. 7B; $t < 0$), and separate experiments showed that the inhibitory effect on growth by this high concentration of paclobutrazol could not be fully rescued by external GA in these plants (data not shown). During the second period of fast elongation (6–18 h) growth was significantly inhibited even at low concentrations of paclobutrazol. Although some underwater elongation still remained, the growth rates were only marginally faster than

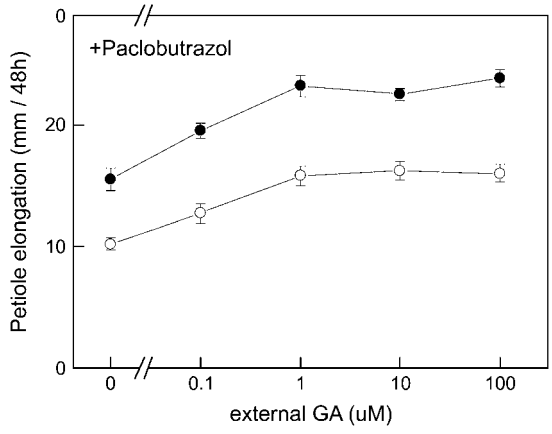

Figure 6. Effect of different concentrations of GA (0–100 μM) and ABA (0 μM [black circle] or 10 μM [white circle]) on petiole elongation in submerged *R. palustris* pretreated with 10 mL 100 μM paclobutrazol 96 h prior to submergence. The length of the second youngest petiole was measured before and after 48 h of treatment and the increase in length was calculated as the difference. Means of 12 replicates with SES.

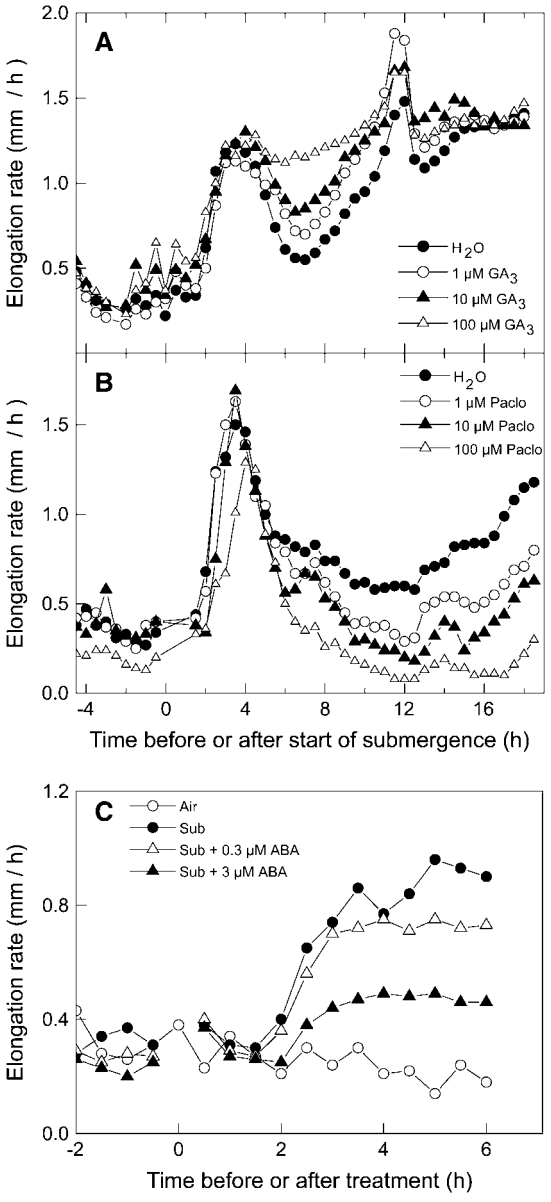

Figure 7. Effect of paclobutrazol (paclo), GA, and ABA on submergence-induced leaf elongation in *R. palustris* measured using linear displacement transducers. Paclobutrazol was administered 96 h prior to submergence. Plants were submerged at $t = 0$ h. A, Plants were submerged in water (black circles) or in water containing 1 μM GA (white circles), 10 μM GA (black triangles), or 100 μM GA (white triangles). Average SE was 0.06 mm h^{-1} (four replicates). B, Plants were submerged in water after pretreatment with 10 mL 0.1% ethanol (black circles), or 10 mL 1 μM (white circles), 10 μM (black triangles), or 100 μM (white triangles) paclobutrazol. Typical SES were 0.06 mm h^{-1} (four replicates). C, Plants remained in air (white circles) or were submerged (Sub) in water (black circles), or in water containing 0.3 μM ABA (white triangles) or 3 μM ABA (black triangles). Average SES were 0.05 mm h^{-1} (six replicates).

those measured just before the start of submergence (Fig. 7B). Taken together, these results suggest that the enhanced elongation response consist of an initial, GA-insensitive phase (0–4 h), a second phase of GA-limited growth (4–15 h), and a third phase that is also dependent, but not limited by GA signaling (from 15 h onward).

Although GA did not influence enhanced elongation during the first few hours after submergence, growth in this period was inhibited by ABA (Fig. 7C). Application of 3 μM ABA to submerged plants reduced the enhanced elongation response by more than 50%.

DISCUSSION

We present here a detailed analysis of regulation of GA_1 biosynthesis in petioles of *R. palustris* during the first 24 h of submergence. The submergence treatment induced changes in transcript levels of a number of different GA biosynthesis genes, and these were reflected in the changes of endogenous GA levels, ultimately leading to a 2-fold increase in bioactive GA_1. This increase in GA_1 was consistent with the onset of a GA-dependent growth phase after 4 h, and was dependent on ethylene-induced down-regulation of ABA levels in submerged petioles.

Submergence Affects Multiple Steps in GA Biosynthesis

We observed a sharp increase in *RpGA3ox1* transcript levels in petioles within 2 h of submergence, indicating an enhanced capacity for GA_1 biosynthesis (Fig. 3). Consistent with this observation, the concentration of GA_1 increased after 4 h of submergence (Fig. 2). The increase in *RpGA3ox1* transcript levels following submergence was accompanied by a decrease in transcript levels of *RpGA20ox1* and an increase in that of *RpGA2ox1* (Fig. 3). These patterns in transcription upon submergence were confirmed in an independent experiment (Fig. 5).

The reduced *RpGA20ox1* transcript levels may explain the accumulation of GA_{53} (Fig. 2), but there is no corresponding decrease in the concentrations of GA_{20} and GA_{19}, which suggested that there may be enhanced activity (concentration and/or flux) at upstream steps in the pathway earlier than GA_{53}. The changes in transcript levels of *RpGA20ox1* and *RpGA2ox1*, which are opposite to what would be expected to increase GA biosynthesis, may be the consequence of negative feedback and positive feedforward regulation, respectively, in response to increased GA signaling, which allows GA homeostasis (for review, see Hedden and Phillips, 2000). A comparable effect was noted for dark-grown pea (*Pisum sativum*) seedlings after exposure to light when a rapid reduction in GA_1 content due to decreased transcript levels of *PsGA3ox* and increased transcript levels of *PsGA2ox* was followed by increased transcription of *PsGA3ox* and *PsGA20ox* genes (Reid et al., 2002). In this case, however, the changes in Rp*GA20ox* and *RpGA2ox* transcript levels occurred before the increase in GA_1 concentration was detected, making a homeostatic regulation via GA concentration unlikely (Figs. 2 and 3). Since submerged Rumex

petioles increase in GA sensitivity upon submergence (Rijnders et al., 1997), we hypothesize that this sensitivity change might be the input signal for GA homeostasis.

Alternatively, it could be argued that more orthologs of each gene might be present in the Rumex genome, which might be regulated differently from the ones presented here. Although it is impossible to fully exclude this possibility, it should be noted that the genes presented here for *R. palustris* were the sole members that could be identified from a screen on cDNA from submerged petiole tissue and genomic DNA. The lack of additional members is in agreement with the notion that GA biosynthesis and breakdown genes are generally members of small gene families in species completely sequenced so far (Sponsel and Hedden, 2004).

ABA Affects GA Biosynthesis Rather Than GA Sensitivity

In paclobutrazol-pretreated plants no significant interaction between ABA and GA was observed (Fig. 6). This suggests that externally applied ABA is not affecting the responsiveness of *R. palustris* petioles to GA. This contrasts with work in the submergence-tolerant deepwater rice, in which it was suggested that ABA inhibits sensitivity to GA (Hoffmann-Benning and Kende, 1992).

Instead of reducing GA sensitivity, ABA inhibited transcription of *RpGA20ox* and *RpGA3ox* (Fig. 5). As a result of this, the increase in GA_1 that takes place in submerged petioles is absent in ABA-treated plants (Fig. 4). This increase in GA_1 is crucial for enhanced underwater elongation as long-term extension growth depends strongly on GA (Fig. 7B). In previous work we showed that ABA declines rapidly in petioles of *R. palustris* upon submergence or ethylene treatment (Benschop et al., 2005). The timing of this decline correlated well with the observed kinetics of up-regulation of *RpGA3ox1* transcription (Fig. 3B). We now show that externally applied ABA inhibits this up-regulation of *RpGA3ox1*. However, as ABA also influences enhanced growth during the first few hours after the onset of submergence (Fig. 7C) ABA seems to have more than one mode of action. The nature of this secondary regulatory mechanism is yet unidentified, but was shown recently not to be related to two other growth-stimulating mechanisms that occur in submerged *R. palustris*: the up-regulation of expansins or the increased apoplastic acidification in petioles (Vreeburg et al., 2005).

GA Is Important for Long-Term Elongation But Does Not Influence Initial Underwater Growth

The observation that submergence-induced elongation precedes an up-regulation of GA_1 (Fig. 1 versus Fig. 2) indicated that elongation growth during this phase was not dependent on this increase in GA. This was confirmed by the observation that paclobutrazol and external GA could not alter elongation growth during this initial period (Fig. 7, A and B). In contrast, elongation growth during this period was inhibited by ABA (Fig. 7C). The pharmacological treatments showed that submergence-induced elongation in *R. palustris* can be separated into several phases. The first phase starts 2 h after submergence and is characterized by a rapid increase in elongation rate. This initial burst in growth is insensitive to GA as it remains unaffected by manipulations of GA concentration. Although our paclobutrazol-pretreated plants showed a very severe GA-deficient phenotype we can, however, not completely exclude that trace amounts of GA_1 still present play a role in petiole elongation during the first hours of submergence. The second growth phase is between 5 and 15 h of submergence. Here elongation is limited by GA and can be directly manipulated by changing the GA content (Fig. 7, A and B). A third phase, from 15 h onward, is dependent on, but not limited by GA signaling. In this phase the GA concentration and GA sensitivity seem to be saturated, since adding extra GA does not further enhance elongation. Removing GA during this phase does, however, inhibit elongation severely, indicating a continued dependency on GA.

CONCLUSION

We examined the role of GA during submergence-induced elongation in *R. palustris* at a molecular, biochemical, and physiological level. Submergence induced an increase in GA_1 content in petioles of *R. palustris* probably through enhanced transcription of *RpGA3ox1*. This up-regulation is mediated by ABA, which acts as an inhibitor of *RpGA3ox1* transcription. High levels of ethylene, that accumulate upon submergence, induce a rapid decline in ABA (Benschop et al., 2005). This process relieves the inhibitory effect on *RpGA3ox1* and allows the concentration of GA_1 to increase, which is a requirement for long-term elongation in submerged Rumex plants.

MATERIALS AND METHODS

Plant Growth and Elongation Measurements

Rumex palustris plants were grown according to Benschop et al. (2005).

For measurements of leaf elongation rates, linear displacement transducers were used according to Voesenek et al. (2003b; type ST 2000; Schlumberger Industries). Growth rates were subsequently calculated by fitting lines through intervals of 10 to 30 min.

Measurements of GA Concentrations

Levels of GAs in petiole and leaf samples were analyzed using 10 mg dry weight of previously ground and freeze-dried tissue. [$^2H_2$]GAs were added to the samples as internal standards, in amounts of 375 pg for GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, and GA_{34} and 750 pg for GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_{29}, GA_1, and GA_8. GAs were extracted in 80% methanol with 0.02% diethyl dithiocarbamate

as antioxidant with vigorous shaking for 1 h at 4°C. The mixture was centrifuged at 2,800 rpm for 10 min at 4°C. The supernatant was transferred into kimble tubes and dried under reduced pressure. The residue was redissolved in 50 μL methanol; 300 μL hexane was added and the mixture was applied to a Silica (1 g) column previously conditioned with hexane and equilibrated with hexane/ethyl acetate (80:20). GAs were eluted with methanol containing 1% acetic acid. The eluate was dried under reduced pressure and methylated with ethereal diazomethane. Dried residues were redissolved in 200 μL 30% methanol containing 1% acetic acid. When necessary samples were filter centrifuged through an Ultrafree-MC centrifugal filter device (0.22 μm; Millipore) before separation on a C_{18}-reverse HPLC column, in a gradient of 30% methanol to 100% methanol. Fractions were grouped, dried under reduced pressure, and transferred to gas chromatography vials. Samples were trimethylsilylated and GA analysis was carried out by gas chromatography/mass spectrometry selected reaction monitoring using a JEOL/SX/102A four-sector mass spectrometer (JEOL) according to Moritz and Olsen (1995).

Isolation of Genes and Real-Time RT-PCR

The isolation of cDNAs encoding GA-biosynthetic enzymes was carried out by PCR amplification using degenerate primers based on the most conserved regions of known orthologs of Arabidopsis (*Arabidopsis thaliana*). For GA20ox primers 5′-GAGAAGCTTACNGGNCCNCAYWSNGAYCC-3′ and 5′-GCGAATTCNCCDATRTTNACNACYAA-3′ (from the conserved amino acid sequences TGPHCDP and LVVNIG) were used. For GA3ox primers 5′-GCSATGGGSCTSGCSGCSCAYACNG-3′ and 5′-CCGTTHGASAGRTGGAASAGRTCNCC-3′ (corresponding to MGLAAHTD and GDLFHIL) were used, and for GA2ox primers 5′-AAYGGNGAYNTNGGNTGG-3′ and 5′-CTRAANCCRTTNGTCATNAC-3′ (corresponding to NGDIGW and FGEHTDP) were used. PCR products amplified from genomic DNA or cDNA from *R. palustris* shoots were cloned in a pGEM-T easy vector (Promega) and sequenced. Positive clones were labeled as probes to screen the cDNA library and the isolated partial cDNAs were extended to full length by nested PCR using specific primers from the gene and from the vector.

For each sample, RNA of five 3rd petioles was extracted using a modified method of Kiefer et al. (2000). Fifty microliters of Nucleon Phyto Pure DNA extraction resin was used and samples were washed with 70% ethanol after each isopropanol precipitation. DNAse treatment was repeated four times with 7 units DNaseI (Amersham Pharmacia Biotech no. 27–0514–02). The effectiveness of the treatment was checked by PCR amplification of genomic specific sequences. One microgram of RNA was used for cDNA synthesis (Stratagene cDNA synthesis kit no. 200401–5). Real-time RT-PCR was performed on a ABI Prism (Applied Biosystems) using the Taqman kit (Perkin Elmer). A set of two primers and a gene-specific probe were used. For GA20ox we used the primers 5′-GACCCCACCTCCCTTACCAT-3′ and 5′-CACACGCCATCGACAAACAC-3′ and the probe CTCCACCAGGATCATGTTAACGGCCTT. For GA3ox we used the primers 5′-GCGCTTTCTTCGGTTAGGCTAT-3′ and 5′-CTAACTACCAACTTCAACTATGC-3′ and the probe TGGACCGCCATTGATCCGATCGGACC. For GA2ox we used the primers 5′-CCCCATGGATTCCATCAAGA-3′ and 5′-GGACCCGCCTTCTCCTTCT-3′ and the probe AGGCCGTCAAGTTCTTCTCCCTGCC. Relative mRNA values were calculated using the comparative Ct method described by Livak and Schmittgen (2001), expressing mRNA values relative to 18S RNA. All transcript levels were presented relative to the value obtained at $t = 0$ h.

Functional Analysis GA Genes

Preparation of Lysate

Cultures (50 mL) of *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL transformed with the *RpGA20ox1*, *RpGA3ox1*, and *RpGA2ox1* coding regions in pET32c were grown with shaking at 37°C in 2 × YT broth, containing 100 μg mL^{-1} ampicillin and 34 μg mL^{-1} chloramphenicol, to OD600 = approximately 0.3. Expression was then induced by addition of isopropylthio-β-galactoside (final concentration 1 mM) and the cultures grown for a further 3 h at 25°C. The cells were then pelleted by centrifugation at 5,000 rpm at 4°C for 5 min, the pellets frozen and thawed, and then incubated in 1 mL of freshly prepared lysis buffer containing 100 mM Tris at pH 7.5, 5 mM dithiothreitol, and 1 mg mL^{-1} lysozyme, for 15 min at room temperature. The lysate was then incubated for a further 15 min after the addition of 20 units DNase. The lysate was then centrifuged at 15,000*g* for 10 min and the supernatant transferred to a separate tube.

Enzyme Assays

The supernatants (90 μL) were incubated shaking at 30°C for 2 h with shaking with [17-^{14}C]GA_9 (synthesized by Dr R. Brown) for analysis of GA2ox and GA3ox activities and [17-^{14}C]GA_{12} for the assay of GA20ox activity (obtained from Prof. L. Mander, Australian National University, Canberra, Australia; 300 Bq/150 pmol). The treated supernatants were subsequently added in 5 μL methanol and 5 μL cofactor mixture as described in Williams et al. (1998). The products were analyzed by HPLC with online radiomonitoring as described by MacMillan et al. (1997).

Manipulation of in Vivo GA Concentrations

GA_3 (Duchefa), (±)-ABA (Sigma), and paclobutrazol (Duchefa) were dissolved in ethanol or acetone to a stock concentration of 25 mM (for ABA) or 100 mM (for GA and paclobutrazol) and diluted at least 1,000-fold with distilled water. Paclobutrazol pretreatment occurred once, 96 h before measurements, with 10 mL 100 μM paclobutrazol or (for controls) with 0.1% (v/v) ethanol.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: *RpGA20ox1* (DQ641498); *RpGA3ox1* (DQ641497); and *RpGA2ox1* (DQ641499).

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