Expansins in submergence-induced petiole elongation of *Rumex palustris*: kinetics and regulation

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General introduction

Introduction

Plant cell elongation is fundamental for determining plant size and form. Plant cells are encapsulated in a complex polymeric network forming the cell wall. The tensile strength of the cell wall withstands the turgor pressure of the symplast and both act in concert to support external loads in herbaceous plants. Cell wall properties and turgor pressure also work cooperatively in the process of cell elongation. The interplay between the two has been clearly formulated by Lockhart (1965), describing cell elongation with cell wall properties and turgor pressure as parameters. Although both turgor pressure and cell wall properties are involved in achieving cell elongation, the cell wall is thought to be the main regulatory site for cell elongation (Bacon, 1999; Cosgrove, 2000a; Kutschera, 2001). The following sections will describe briefly the composition of the cell wall and cell wall modifying mechanisms.

Model of the plant cell wall

The primary cell wall of dicots is an assembly of cellulose microfibrils, xyloglucans and pectin polymers (Carpita and Gibeaut, 1993). Cellulose microfibrils are tightly packed bundles of several â-1,4-linked D-glucose polymers, with high tensile strength. The surface of the cellulose microfibrils are coated with xyloglucan polymers, which are attached to each other with hydrogen bonds (McCann and Roberts, 1994; Pauly *et al.*, 1999; Davies and Harris, 2003). The most widely accepted model of the cell wall proposes that the xyloglucans span the intervening space between the cellulose microfibrils and link the cellulose microfibrils together (Carpita and Gibeaut, 1993; McCann and Roberts, 1994). The cross-linking xyloglucans are considered to be the load bearing molecules in the longitudinal axis of an elongating cell (Carpita and Gibeaut, 1993).

A second network, next to the cellulose/xyloglucan matrix, is the pectin network. Pectin is composed mainly of galacturonans, and several domains can be classified in pectin polymers, such as homogalacturonan, rhamnogalacturonan I and II, and xylogalacturonan (Vincken *et al.*, 2003). The carboxyl groups of the galacturonosyl residues of homogalacturonan can be ester linked to a methyl group, or unesterified, by pectin methyl esterase (Vinken *et al.*, 2003). With the right distribution of unesterified carboxyl groups, the homogalacturonan domains can cross link with calcium to form Ca^{2+}-pectate gels (Vinken *et al.*, 2003). The pectic network fills the spaces between the cellulose/xyloglucan network and determines the pore size of the cell wall, varying from 4 to 10 nm (Carpita and Gibeaut, 1993). Although the cellulose/xyloglucan network dominates the response of plant cell walls to mechanical stress, pectins also contribute to the tensile strength of the cell wall (Ryden *et al.*, 2003).

A third network co-existing in the cell wall is a network of structural proteins. A major group of structural proteins in cell walls are extensins, a family of hydroxyproline rich glycoproteins. Expression of extensins is correlated with growth cessation and pathogen attack (Wilson and Fry, 1986). The rod-like proteins lock the primary wall into shape by forming a cross-linked network (Wilson and Fry, 1986; Carpita and Gibeaut, 1993). Inter- and intra-protein cross-links are formed between extensins by peroxidases, but covalent cross-links have also been reported between extensin and pectin (José-Estanyol and Puigdomenech, 2000, and references therein).

Proline-rich proteins, glycine-rich proteins and arbinogalactan proteins are also considered structural cell wall proteins (José-Estanyol and Puigdomenech, 2000; Ringli *et al.*, 2001; Showalter, 2001). The function of these proteins in the cell wall, however, is not as well understood as of extensins.

Measuring cell wall loosening: the constant load extensometer

A devise often used for determination of changes in cell extension in response to enzymes or other cell wall modifying mechanisms, is the extensometer, or

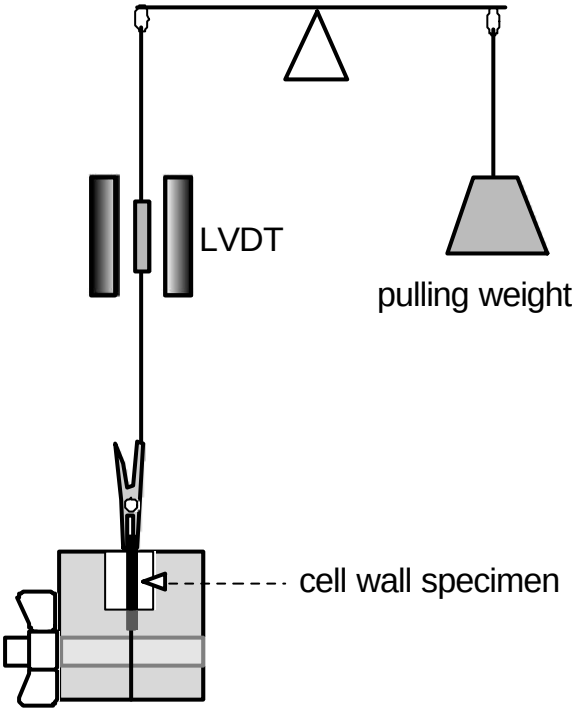


Figure 1. Schematic diagram of an extensometer used for experiments that are described in this thesis.

extensiometer (Figure 1; Cosgrove, 1989; Schopfer, 2001). The extensometer measures the extension of a cell wall specimen that is secured between two clamps. The specimen is located in a small chamber, so that it can be bathed in a buffer. The buffer can be changed, for example, with a buffer of different pH, or with buffers containing cell wall protein. One of the clamps is connected to a pulling weight, pulling on the specimen with a force relative to 5 to 30 g weight. The extension of the specimen is recorded with a Linear Variable Differential Transformer (LVDT). Positional data obtained from the LVDT can be analysed by a computer.

When a piece of plant tissue is used as cell wall specimen, the tissue is first frozen and thawed, so that the tissue is killed, but most cell wall protein activity remains intact. The cuticle of the tissue is abraded to access the apoplast, and the tissue is pressed under a weight to squeeze out excess fluid. The plant specimen can eventually be boiled to inactivate native proteins and test tissue susceptibility to cell wall loosening enzymes.

Several cell wall modifying mechanisms are now known to induce extension on a frozen/thawed and boiled tissue when assayed in an extensometer (see below). The constant load extensometer was, and still is, a major tool for the study of cell wall properties in relation to elongation growth.

Cell wall modifying mechanisms

Irreversible cell elongation is achieved by plastic deformation of the cell wall. For plastic expansion of the cell wall to occur, the carbohydrate network has to be loosened. Several mechanisms have been found to modify the cellulose/xyloglucan network. Some of these enzymatic and non-enzymatic mechanisms related to elongation growth will be discussed below.

Endo-b-1,4-glucanases

Endo-â-1,4-glucanases, also named cellulases, break 1,4-â-bonds between glucose residues. Such bonds are present in cellulose, but also in xyloglucans. The expression of these enzymes correlates with fruit softening and tissue abscission (Bonghi *et al.*, 2000; Woolley *et al.*, 2001). Initial experiments showed that when the action of these enzymes was tested in a constant load extensometer, the tissue broke with hardly any preceding extension (Cosgrove and Durachko, 1994). Therefore, this enzyme was thought to be only involved in cell wall degradation. However, purified endo-â-1,4-glucanase from blue mussel (*Mytilus edulis*) and from a fungus (*Trichoderma reesei*) did show extension activity in a constant load extensometer (Xu *et al.*, 2000; Yuan *et al.*, 2001). The cellulase activity tested *in vitro* showed a broad pH activity with a maximum at pH 5 (Xu *et al.*, 2000). However, the extension activity of this endoglucanase, tested on cellulose/xyloglucan matrix (Whitney *et al.*, 2000) hardly showed a pH dependency (Vreeburg and Janson, unpublished results). Other endo-â-1,4-glucanases have been purified with slightly different pH optima (Smriti and Sanwal, 1999; Woolley *et al.*, 2001), which are not tested for extension activity yet.

Xyloglucan endotransglucosylase/hydroxylases (XTHs)

A second family of proteins hydrolysing the 1,4-â-bond between glucose molecules are the xyloglucan endotranglucosylase/hydrolases (XTHs). This is a family of proteins containing the enzymes formerly known as xyloglucan endotransglycosylases (XETs) and endoxyloglucan transferases (EXGTs). These proteins break xyloglucan polymers and paste one of the strings to the end of another xyloglucan string (Rose *et al.*, 2002). In this way, the tension of load bearing xyloglucan polymers can be released. The endotransglucosylation activity of XTHs depends on pH and has a pH optimum at pH 5.8 (Fry *et al.*, 1992; Nishitani and Tominaga, 1992). McQueen-Mason *et al.* (1993) found no extension activity of XTHs when tested in a constant load extensometer. A more recent report described a XTH with xyloglucan endotranshydrolase activity that did induce increased extensibility in a similar *in vitro* extension assay (Kaku *et al.*, 2002).

Yieldins

Yieldins are a family of proteins with an unknown mode of action on the biochemical level. They lower the yield threshold of the cell wall, that is the minimum of tensile force needed to extend the cell wall (Okamoto-Nakazato *et al.*, 2000a). Homology searches revealed that yieldins show high homology to acidic

endochitinases (Okamoto-Nakazato *et al.*, 2000b). This high homology of yieldins with endochitinases makes it difficult to clone yieldins form other organisms. Only a functional assay would reveal whether the target gene is a yieldin or an endochitinase. Yieldins are activated at low pH (Okamoto-Nakazato *et al.*, 2000a), and are expressed in hypocotyl tissue prior to elongation (Okamoto-Nakazato *et al.*, 2001).

Hydroxyl radicals

A cell wall loosening mechanism not depending on enzymatic breaking of cell wall bonds is the extension activity induced by hydroxyl radicals. These hydroxyl radicals can be produced from hydrogen peroxide, catalysed by reduced copper or iron (Fry, 1998). Induction of hydroxyl radical production in frozen thawed tissue in a constant load extensometer, resulted in similar extension kinetics as applying expansins to the tissue (compare McQueen-Mason *et al.*, 1992 and Schopfer, 2001). Formation of hydroxyl radicals from hydrogen peroxide also showed a pH dependency, with a pH optimum at pH 4.5 (Fry, 1998). The extension activity of hydroxyl radicals is thought to be by scission of cell wall polymers, such as xyloglucans (Fry, 1998; Fry *et al.*, 2002). Hydroxyl radicals are highly reactive with many substrates, and when produced in the cell wall, will react with the polymer close to its site of origin (Fry, 1998). The high reactivity enables specific localisation of hydroxyl radical action in the cell wall.

Expansins

Expansins were found as proteins inducing the acid-induced extension in etiolated cucumber hypocotyls (McQueen-Mason *et al.*, 1992), and are now found in a large amount of other species, from various genera (Li *et al.*, 2002). Two main groups can be distinguished, á-expansins and â-expansins (McQueen-Mason and Rochange, 1999), both consist of large gene families. A third group of expansins, ã-expansins, has been proposed by Li *et al.* (2002). Since no member of this group has shown expansin activity yet, the assignment of the name expansin has been considered to be premature, and the name expansin-like (EXPL) has been proposed (Cosgrove *et al.*, 2002).

Expansins show a pH dependent extension activity in a constant load extensometer, with a pH optimum between pH 3.5 and 4.5 (McQueen-Mason *et al.*, 1992), and the expression of the gene is correlated with fruit softening (Catala *et al.*, 2000), meristem development (Pien *et al.*, 2001) and elongation growth (Cosgrove *et al.*, 2002, and references therein). The role of expansins in elongation has been further elucidated with transgenic plants. Initial studies contained contra-intuitive results, constitutive expression of a cucumber expansin protein in tomato plants, for example, lead to reduced growth (Rochange *et al.*, 2001). Expansin knock out studies in the bryophyte *Physcomitrella patens* did not reveal a phenotype (Schipper *et al.*, 2002). These results could be explained by a negative feedback of plant growth by altered cell wall properties (Rochange *et al.*, 2001), or by the redundancy of expansins present, compensating for knock outs. To overcome compensating feedback mechanisms triggered by constitutive over or under expression of expansin genes, inducible over or under expresser lines could

be used. Indeed, inducing the expression of the á-expansin *OsEXP4* in rice seedlings resulted in enhanced growth, while induction of an *OsEXP4* anti-sense resulted in reduced elongation (Choi *et al.*, 2003).

Expansins are hypothesised to break the hydrogen bonds between hemicellulose and cellulose (McQueen-Mason and Cosgrove, 1994). This hypothesis is based on the observation that expansins lack hydrolytic activity on cellulose or xyloglucans (McQueen-Mason and Cosgrove, 1994) and induced extension on tissues composed of only cellulose and xyloglucan (Whitney *et al.*, 2000). The hydrogen bonds as specific target for expansin action is further indicated by a decreased extension activity of expansins on tissues in which water is replaced by deuterated water, the latter forming stronger hydrogen bonds (McQueen-Mason and Cosgrove, 1994).

Interactions of cell wall modifying mechanisms

The localisation of cell wall loosening mechanisms in the same compartment, the cell wall, suggests that these mechanisms show interactions with each other. Indeed, pre-treatment of cell walls with pectinases or cellulases resulted in stronger expansin mediated extension of these walls (Cosgrove and Durachko, 1994). However, information about such interactions is scarce.

The *Rumex* system for studying cell elongation

The terrestrial dicoteledous plant *Rumex palustris* is a submergence tolerant species that occurs in environments that are periodically flooded. As *R. palustris* is submerged completely, the petioles show a remarkable morphological response: the orientation of the rosette leaves change from rather horizontal to almost vertical (hyponastic growth), followed by enhanced elongation of the petiole (Voesenek and Blom, 1989; Banga *et al.*, 1997; Cox *et al.*, 2003). The submergence-induced elongation is caused solely by cell elongation and is distributed equally over the entire petiole (Voesenek *et al.*, 1990; Rijnders *et al.*, 1996). When submerged plants are de-submerged by lowering the water level, the petiole elongation rate declines to those of air-grown plants (Voesenek *et al.*, 2003b). The equal distribution of elongation over the petiole, exclusion of cell division, and the reversibility upon de-submergence, makes the submergence-induced petiole elongation of *R. palustris* a good model system to study the regulation of elongation growth.

Hormonal regulation

As plants are submerged, the exchange of gases is severely limited, since gases diffuse 10 000 times slower in water than in air (Jackson, 1985). This causes an entrapment of gases produced by the plant and therefore leads, among other changes, to a rise in the endogenous concentration of the gaseous hormone ethylene (Voesenek and Blom, 1989). Due to the fast accumulation of ethylene upon submergence (Banga *et al.*, 1996a), and the ethylene inducibility of petiole

elongation (Voesenek and Blom, 1989), ethylene is seen as a very early signal in submergence-induced petiole elongation. Both submergence and ethylene exposure resulted in a rapid decrease in the endogenous concentration of abscisic acid (ABA; Voesenek *et al.*, 2003a; Benschop *et al.*, unpublished results). Moreover, ABA added to submerged *R. palustris* plants inhibited elongation of the petiole, leading to the hypothesis that ABA is a negative regulator of the submergence-induced petiole elongation (Voesenek *et al.*, 2003a; Benschop *et al.*, unpublished results).

Submergence-induced petiole elongation in *R. palustris* also depends on the concentration of gibberellins (GAs). Inhibition of GA biosynthesis by the GA biosynthesis-inhibitor paclobutrazol reduced the elongation response upon submergence (Rijnders *et al.* 1997). This reduced elongation could be rescued by addition of GA_3 to the plants (Rijnders *et al.*, 1997). Submergence and ethylene exposure also induced an increase in the concentration of the bioactive GA_1 (Rijders *et al.*, 1997).

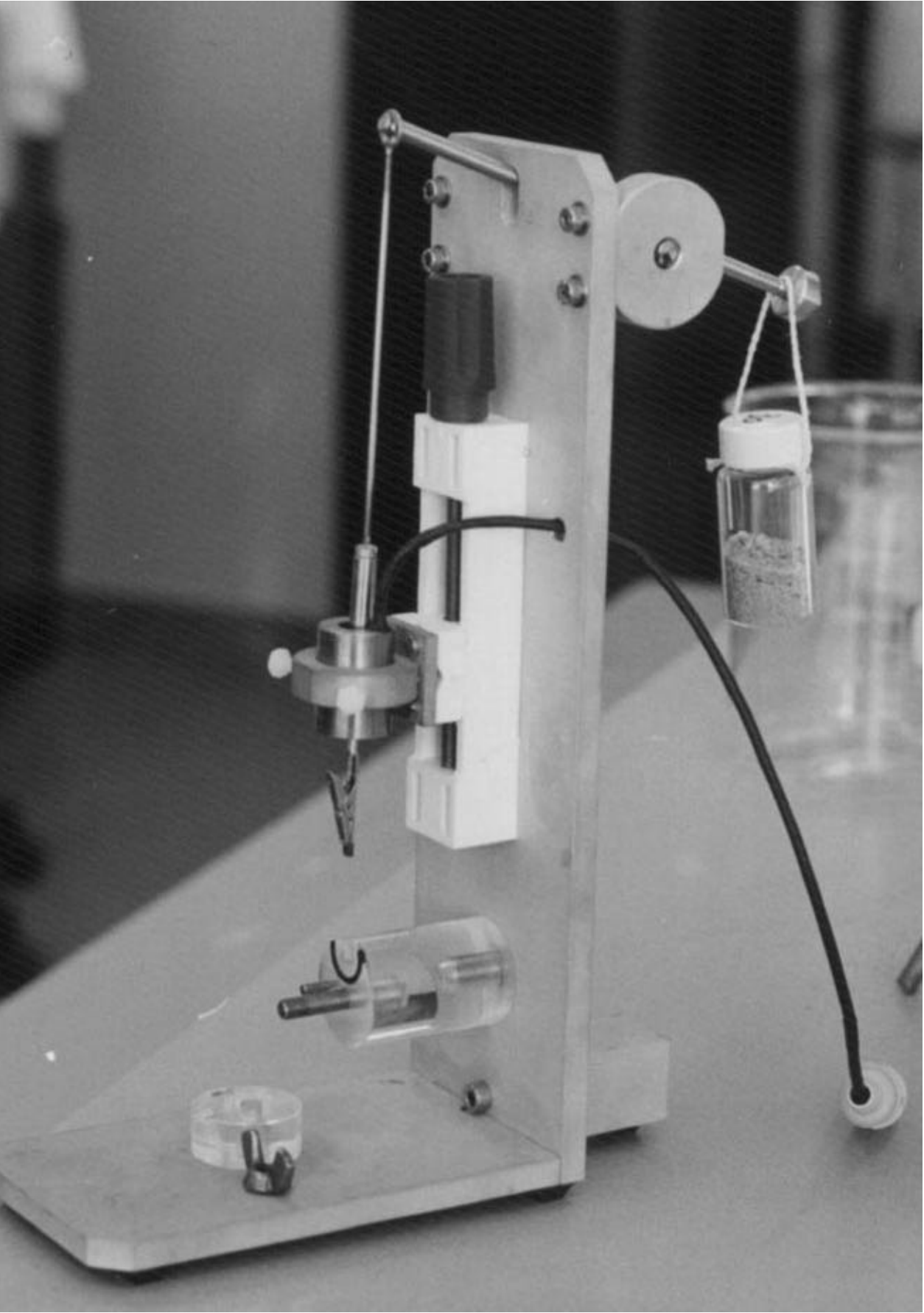
In addition to ABA and GA, auxins are also likely to be involved in the submergence-induced petiole elongation response of *R. palustris*. Removal of the leafblade resulted in a more than 50% decline of endogenous indole-3-acetic acid (IAA) in petioles, within 2 h (Cox *et al.*, unpublished results). This treatment was accompanied by a reduced submergence-induced elongation for 8 h, after which the submerged, decapitated petioles started to elongate (Cox *et al.*, unpublished results). Application of the synthetic auxin 1-naphtalene acetic acid (1-NAA) restored the elongation rate to that of submerged petioles with leafblade (Cox *et al.*, unpublished results). Submergence also induced a rise in endogenous IAA in *R. palustris* petioles, mainly localised in the peripheral tissues (Cox *et al.*, unpublished results). Auxin is therefore likely to be needed for submergence-induced petiole elongation of *R. palustris*.

Since cellular elongation is thought to be regulated by cell wall properties (Cosgrove, 2000a; Kutschera, 2001), it is expected that submergence-induced petiole elongation of *R. palustris* will also be caused by cell wall loosening. Vriezen *et al.* (2000) showed with northern blot analyses that both submergence and ethylene exposure resulted in enhanced *RpEXP1* transcript levels. At least 20 different expansin genes are present in *R. palustris*, showing high homology (Vriezen *et al.*, 2000; Peeters *et al.*, unpublished results). The high homology of the known *R. palustris* expansin genes (Peeters *et al.*, unpublished results), makes is unlikely that the northern blot analyses of Vriezen *et al.* (2000) specifically detected *RpEXP1* transcript levels. In order to overcome the non-specific detection of *Rumex* expansins, a gene specific method was developed using *real time* RT-PCR (Heid *et al*., 1996; Peeters *et al.*, unpublished results).

Research questions and chapter introduction

Vriezen *et al.* (2000) showed that expansin transcript levels are up-regulated upon submergence and ethylene exposure. These observations made expansins a prime candidate involved in submergence-induced cell wall loosening in *R. palustris*. This thesis describes results of an in-depth study on the role of expansins in the submergence-induced petiole elongation of *R. palustris*, studying expansin mRNA and protein expression, combined with activity measurements.

We extended the research started by Vriezen *et al.* (2000) by measuring e.g. the transcript abundance of specific expansins with *real time* RT-PCR, expansin protein levels and expansin activity. **Chapter 2** shows the results of a study into the kinetics of submergence-induced petiole elongation, expansin transcript and protein abundance, and expansin activity. **Chapter 3** evaluates how the observed submergence and ethylene-induced increase in expansin transcript abundance is hormonally regulated downstream of ethylene. **Chapter 4** uses the unique property of the *R. palustris* system to alter the rate of petiole elongation by manipulating petiole angle (Cox *et al.*, 2003) and describes the altered growth kinetics by asymmetric distribution of acid-induced extension. Since the activity of many cell wall loosening mechanisms depend on the apoplastic pH, **chapter 5** presents a study into the regulation of apoplastic pH of *R. palustris* petioles in response to submergence.



Chapter 2

Elucidating the role of expansins in submergence-induced petiole elongation: kinetics of growth, expansin mRNA and protein levels

with Cornelis A.M. Wagemaker[§], Catherine P. Darley[1], Simon J. McQueen-Mason[1], Laurentius A.C.J. Voesenek, and Anton J.M. Peeters

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Abstract

Rumex palustris petioles show a submergence-induced enhancement of their elongation rate. This faster elongation growth enables the plant to regain air-contact and restore gas exchange with the atmosphere. In this study we investigated the involvement of expansins, a family of cell wall loosening proteins, in the elongation response. The submergence-induced petiole elongation coincides closely with a rise in acid-induced extension, with an up-regulation of one expansin transcript, *RpEXP1*, and with more expansin proteins of the size group containing RpEXP1. All of these submergence-induced responses could be reversed by de-submergence. The detailed growth measurements showed that the period of enhanced elongation of submerged petioles was followed by a period in which both submerged and air-grown petioles showed similar elongation rates (in the time-frame from 12 to 18 h of submergence). During this period, the acid-induced extension, *RpEXP1* transcript abundance and amount of the RpEXP1 containing protein group were still at elevated levels, indicating that more expansins do not necessarily lead to faster elongation. Alternative mechanisms that could over-rule expansin action and thus explain the temporary uncoupling of elongation and expansin levels are discussed.

Introduction

Plant cell elongation is not only part of the plant developmental programme, but is also a mechanism by which plants can escape stressful conditions. In the dicot *Rumex palustris*, complete submergence triggers an upward bending of the petiole (hyponastic growth) and an enhancement of the petiole elongation rate (Cox *et al.*, 2003). The combination of these responses enables the plant to regain contact with the atmosphere, thereby restoring effective gas exchange with the air environment (Voesenek and Blom, 1989; Banga *et al.*, 1997). In contrast to the flooding-tolerant monocot deepwater rice (Kende *et al.*, 1998), and in common with other similarly responding dicots such as *Callitriche playcarpa* (Musgrave *et al.*, 1972), submergence-induced petiole elongation in *R. palustris* is achieved by cell elongation alone (Voesenek *et al.*, 1990). The enhanced cellular elongation in submerged *R. palustris* is equally distributed over the entire length of the petiole (Rijnders *et al.*, 1996).

The gaseous hormone ethylene is a prime signal for the submergence-induced petiole elongation in *R. palustris* (Voesenek and Blom, 1989). When submerged, shoots of *R. palustris* produce ethylene at a rate that is similar to that of non-submerged controls (Voesenek *et al.*, 1993; Banga *et al.*, 1996b). A slower diffusion rate of the ethylene in water compared to air (Jackson, 1985), traps ethylene in the plant, leading to a rise in internal concentration within 1 h to approximately 1 $\mu l\ l^{-1}$ (Banga *et al.*, 1996b), a concentration that is saturating for the elongation response (Banga *et al.*, 1996a). When previously submerged *R. palustris* plants are de-submerged so that the whole shoot is exposed to air again, accumulated ethylene rapidly diffuses out of the plant and the submergence signal is released (Voesenek *et al.*, 1993). The accumulated ethylene in submerged *R. palustris* enhances the levels of, and sensitivity towards, the growth promoting hormone gibberellic acid (GA, Rijnders *et al.*, 1997). Overall, the ethylene-mediated submergence-induced signal transduction pathway finally leads to faster cellular elongation.

Cell walls are considered to be the main regulatory sites for plant cell elongation (Cosgrove, 2000a; Kutschera, 2001) and their properties can be modified by several enzymes, such as endo-1,4-β-D-glucanases, XTH's (xyloglucan endotransglucosylase/hydolase, Rose *et al.*, 2002), and expansins (for reviews see Cosgrove, 2000b and Kutschera, 2001). Of these enzymes, expansins are thought to be of major importance in regulating cell elongation by means of cell wall loosening (Cosgrove, 2000a). This hypothesis is based on the observed acid-induced extension activity of expansins *in vitro* (McQueen-Mason *et al.*, 1992) and the correlation between the spatial distribution of cell wall elongation and expansin expression in deepwater rice (Cho and Kende, 1998), graviresponding maize roots (Zhang and Hasenstein, 2000), and cucumber hypocotyls (McQueen-Mason,1995). In addition to the spatial correlation with growth, expansin expression also shows a temporal correlation with elongation growth (Cho and Kende, 1997a; Vriezen *et al.*, 2000).

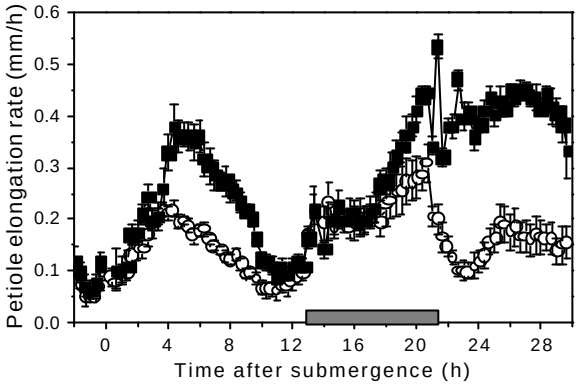

Figure 1. Elongation rates of the 3rd petioles of 25 d old *R. palustris* grown in air (open circles), or submerged at t=0 (filled squares, ± se, n=6). Growth rates are calculated every 20 min from length data obtained with a transducer setup. Gray horizontal bar indicates 8 h dark period.

This study presents a detailed examination of growth kinetics, expansin mRNA abundance, protein levels and activity in petioles *R. palustris* in response to submergence. Such a multi-level approach enabled us to examine if the kinetics of submergence-induced expansin expression match the kinetics of submergence-induced petiole elongation. Our results indicate that the initiation of the submergence-induced petiole elongation of *R. palustris* corresponds closely in time with the onset of expansin gene expression and protein levels suggesting that expansins underlie the onset of the growth response to submergence. However, we also show that, in the same system, up-regulation of elongation related expansins and enhanced expansin protein levels do not necessarily lead to enhanced petiole elongation.

Results

Submergence induces petiole elongation

Rumex palustris petioles showed an increase in elongation 3 h (± 0.3, n=6) after the start of submergence compared to the growth rate of air-grown petioles (Figure 1). This stimulation of growth lasted for approximately 12 h after the onset of submergence. A second period of faster petiole elongation started after 18 h of submergence and lasted until the end of the experiment (30 h after submergence, Figure 1). During this latter period, extension rates of air-grown petioles decreased while the elongation rate of submerged petioles increased slightly. Overall, the daily repeating phases of faster petiole elongation in submerged plants resulted in significantly longer petioles (data not shown).

RpEXP1 expression is up-regulated during submergence

Since expansins are thought to play a key role in the regulation of cell wall loosening and thus cell expansion (Cosgrove, 2000a), we investigated the closeness of the link between expression of expansins in petioles and submergence-induced growth. Twenty α-expansins cDNAs were cloned from *R. palustris* (Vriezen *et al.*, 2000; Peeters *et al.*, unpublished results), which aligned into three groups when clustered based on deduced amino-acid sequences (data

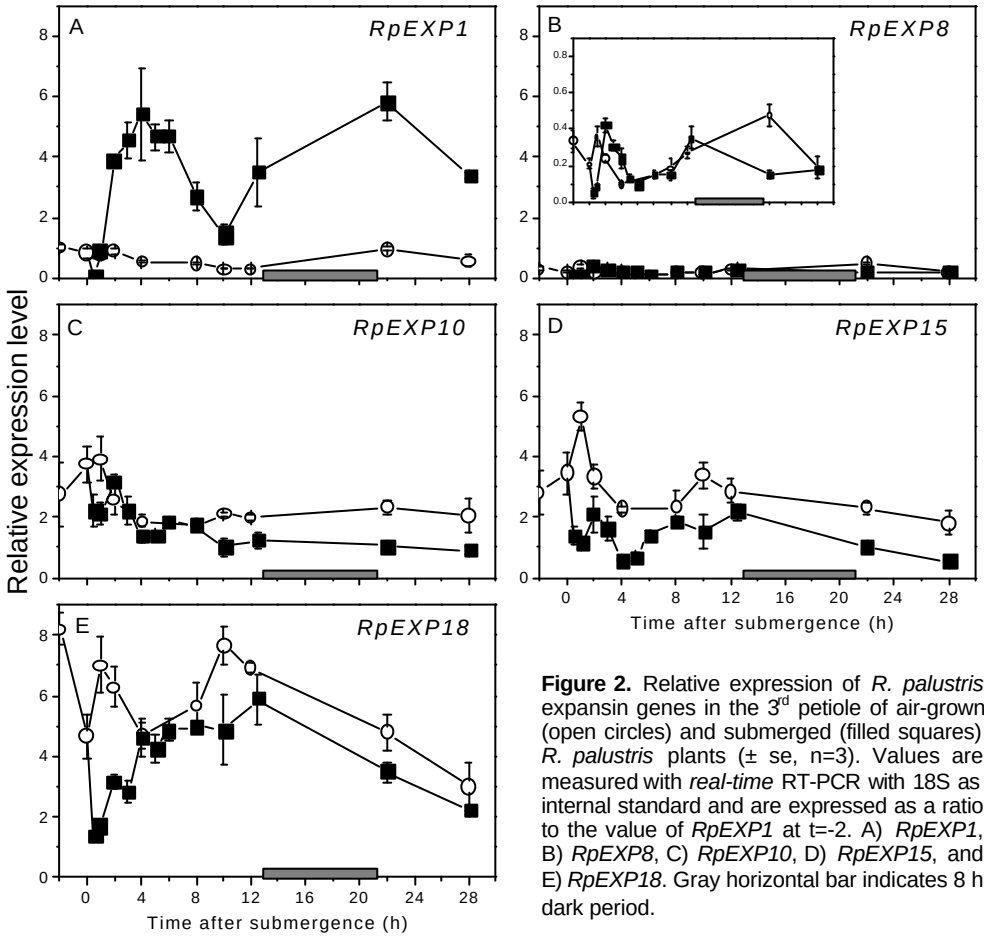

Figure 2. Relative expression of *R. palustris* expansin genes in the 3rd petiole of air-grown (open circles) and submerged (filled squares) *R. palustris* plants (± se, n=3). Values are measured with *real-time* RT-PCR with 18S as internal standard and are expressed as a ratio to the value of *RpEXP1* at t=-2. A) *RpEXP1*, B) *RpEXP8*, C) *RpEXP10*, D) *RpEXP15*, and E) *RpEXP18*. Gray horizontal bar indicates 8 h dark period.

not shown). To obtain an overall mRNA expression profile of expansins in submerged and air-grown *R. palustris* petioles, one or two genes per cluster were selected (*RpEXP1*, *8*, *10*, *15* and *18*) for a detailed study using *real-time* RT-PCR.

RpEXP1 transcript levels in petioles of air-grown control plants might indicate a diurnal rhythm exhibiting most expression at the start of the light period (Figure 2A). Submergence induced an up-regulation of *RpEXP1* transcripts within 2 h. As in air-grown plants, *RpEXP1* mRNA levels in submerged petioles also suggest a diurnal pattern, with most accumulation observed early in the morning.

Figure 2B shows transcript levels of *RpEXP8*, which were present at an order of magnitude less than those of *RpEXP1*. Submergence had no effect on the expression of *RpEXP8*, except after 22 h, when expression dipped below that of air-grown controls. mRNA of both *RpEXP10* and *RpEXP15* did not show a pronounced response to submergence (Figure 2C and 2D), although there was a trend towards a decrease in transcript levels during submergence. A reduction in

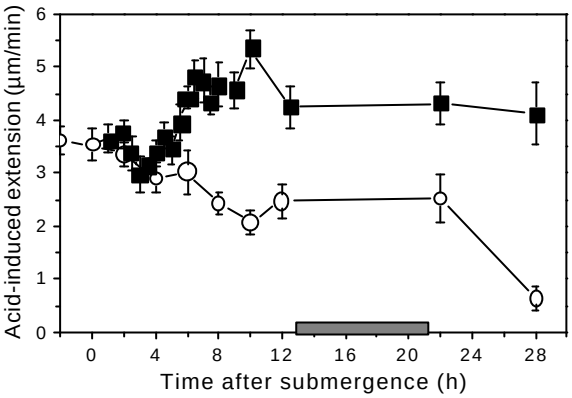

Figure 3. Acid-induced extension (AIE) of *R. palustris* 3rd petioles of air-grown (open circles) and submerged (filled squares) plants (± se, n=10). The AIE was measured in an extensometer with a constant load of 30 g, and is calculated as the extension rate in a 10 min interval after a pH-change from pH 6.8 to 4.5, minus the extension rate in a 10 min interval before the pH-change. Gray horizontal bar indicates 8 h dark period.

mRNA concentrations upon submergence was also observed for *RpEXP18*. This effect was especially prominent during the first 3 h of submergence (Figure 2E).

Acid-induced extension is increased and maintained at a high level during submergence

Expansins induce tissue elongation in a pH-dependent manner *in vitro* (McQueen-Mason *et al.*, 1992). Acid-induced extension (AIE) of a tissue can therefore be seen as a reflection of the expansin activity present *in planta* (McQueen-Mason *et al.*, 1995). To investigate whether submergence not only leads to an increase in *RpEXP1* accumulation, but also to higher AIE, the AIE of segments of *R. palustris* petioles was measured with a constant load extensometer. AIE in air-grown petioles decreased during the 28 h of the experiment (Figure 3) with all of this decrease restricted to the light period. In contrast, the AIE of submerged petioles increased after a lag phase of 4 h, and this elevated level was maintained for the duration of the experiment.

Submergence induces an increase in expansin protein levels

The observed rise in AIE of *R. palustris* petioles could be the resultant of a rise in expansin protein levels, a rise in activity per unit of expansin protein or an increased susceptibility of the cell walls to expansin activity, making the cell wall extend more with similar amounts of expansins.

To test the first of these three possibilities, the amount of expansin proteins were determined by Western blot analysis. Several bands were detected when a Western blot of a crude cell wall protein extract (CCWE) of *R. palustris* petioles was incubated with LeExp2 antibodies (Figure 4). To determine which bands were expansin proteins, the predicted protein sizes of the full length *R. palustris* expansin cDNAs were deduced. Nine of the 20 *R. palustris* expansin cDNA clones were full length and three groups (24.1-24.6 kD, 25.7 kD, and 27.2 kD) could be distinguished based upon the predictable protein sizes excluding the signal peptide (Table 1). A group of three major bands with similar size distribution was found on the LeExp2-antibody-incubated Western blot (24, 26 and 28 kD, Figure 4) and were designated to correspond to the deduced expansin size groups. Binding of a

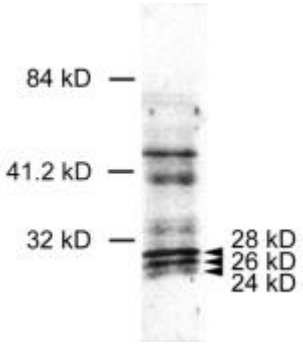

Figure 4. *R. palustris* expansin proteins detected in a crude cell wall protein extract of petioles, with LeEXP2 antibodies on a Western blot. The marker bands are indicated at the left and the expansin sizes next to the arrowheads are calculated using the marker.

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

Size Range		
24.1-24.6 kD	**25.7 kD**	**27.2 kD**
RpEXP1	**RpEXP10**	**RpEXP18**
RpEXP7	RpEXP11	
RpEXP8	RpEXP12	
RpEXP13		
RpEXP14		

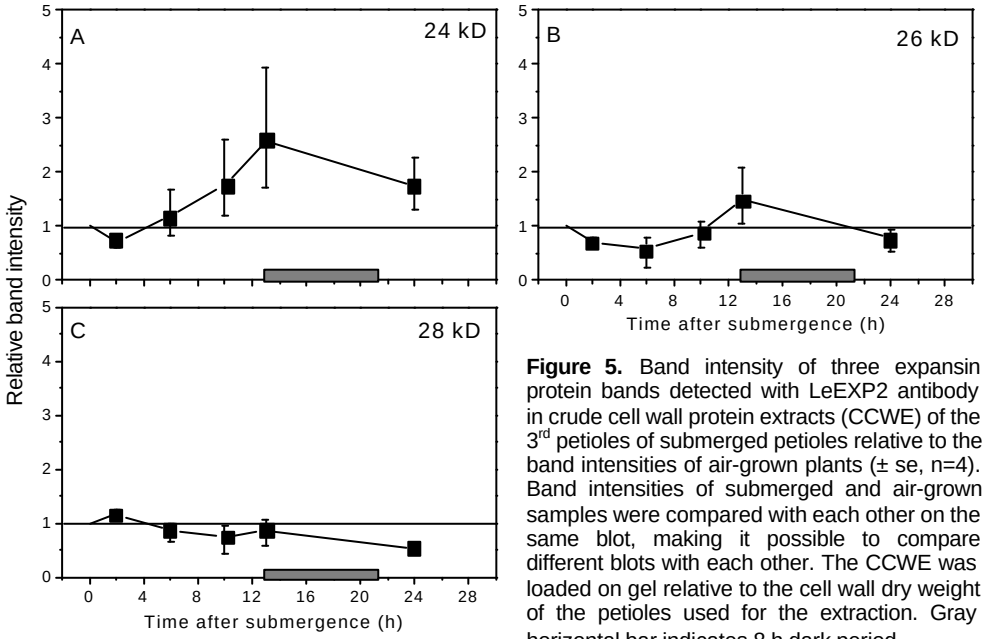

Figure 5. Band intensity of three expansin protein bands detected with LeEXP2 antibody in crude cell wall protein extracts (CCWE) of the 3rd petioles of submerged petioles relative to the band intensities of air-grown plants (± se, n=4). Band intensities of submerged and air-grown samples were compared with each other on the same blot, making it possible to compare different blots with each other. The CCWE was loaded on gel relative to the cell wall dry weight of the petioles used for the extraction. Gray horizontal bar indicates 8 h dark period.

polyclonal expansin antibody to proteins in the expected size class, as well as larger proteins, has also been observed for other dicot CCWE, such as for tomato (Rose *et al.*, 2000; McQueen-Mason, unpublished results).

Figure 5 shows the intensity of the three different expansin bands in submerged samples plotted relative to the intensity in air-grown controls. The relative intensity of the 24 kD band (predicted to include the RpEXP1 and 8 proteins) increased significantly after 10 h of submergence (Figure 5A). In contrast, the intensities of

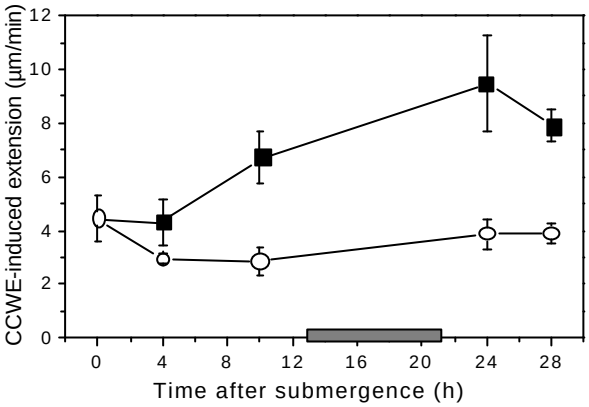

Figure 5. Band intensity of three expansin protein bands detected with LeEXP2 antibody in crude cell wall protein extracts (CCWE) of the 3rd petioles of submerged petioles relative to the band intensities of air-grown plants (± se, n=4). Band intensities of submerged and air-grown samples were compared with each other on the same blot, making it possible to compare different blots with each other. The CCWE was loaded on gel relative to the cell wall dry weight of the petioles used for the extraction. Gray horizontal bar indicates 8 h dark period.

Table 2. Susceptibility of *R. palustris* 3rd petiole segments to a crude cell wall protein extract (CCWE, ± se, n=5). The extension of 15 seconds boiled petioles, lacking native expansin activity, after addition of celery CCWE was measured in an extensometer with 30 g load.

Time after Submergence (h)	CCWE-induced Extension (µm/min)	
	Air-grown	Submerged
6	2.40 ± 0.30	1.96 ± 0.21
10	1.45 ± 0.22	1.59 ± 0.37
24	1.50 ± 0.16	1.61 ± 0.20

both the 26 kD (containing e.g. *RpEXP10*) and the 28 kD bands (containing e.g. *Rp-EXP18*) did not show significant changes relative to the control.

Crude cell wall protein extracts (CCWE) were also assayed for expansin activity using *Acetobacter* derived cellulose/xyloglucan composites. These composites have molecular and ultra-structural features in common with dicot cell walls (Whitney *et al.*, 1995), but are more sensitive to expansins than plant tissues, such as cucumber hypocotyls, commonly used for these assays (Whitney *et al.*, 2000). The extractable expansin activity of *R. palustris* petioles, tested on these cellulose/xyloglucan composites, was increased relative to air-grown controls after only 4 h submergence and remained elevated for the duration of the experiment (Figure 6).

This increase in expansin activity following submergence does not rule out the possibility that the observed increase in AIE of submerged petioles and the decrease of AIE of air-grown petioles could in part be caused by changes in the cell wall susceptibility to expansins. To examine this possibility, heat inactivated petiole segments from submerged and non-submerged plants were assayed for extension in response to the addition of equal amounts of extracted expansin activity. These experiments revealed no clear differences in cell wall susceptibility between submerged and air-grown petioles (Table 2).

Table 3. Effects of de-submergence on the submergence-induced changes in growth and expansin expression (± se). Plants were submerged for 10 h, followed by at least 14 h of lowered water levels in which the shoots were exposed to air.

		Air-grown	Submerged	De-submerged
Growth rate	mm/h	0.10 ± 0.02	0.20 ± 0.01	0.11 ± 0.02
RpEXP1 expression	rel.[1]	0.57 ± 0.17	3.38 ± 0.12	1.06 ± 0.11
RpEXP8 expression	rel.[1]	0.19 ± 0.03	0.18 ± 0.01	0.30 ± 0.06
RpEXP10 expression	rel.[1]	2.06 ± 0.59	0.88 ± 0.14	2.67 ± 0.32
RpEXP15 expression	rel.[1]	1.80 ± 0.41	0.50 ± 0.07	3.13 ± 0.65
RpEXP18 expression	rel.[1]	3.00 ± 0.79	2.20 ± 0.13	3.40 ± 0.36
A.I.E.	µm/min	0.64 ± 0.21	4.13 ± 0.57	1.07 ± 0.20
24 kD protein levels	rel.[1]	1	3.4 ± 0.5	1.3 ± 0.3
extractable expansin activity	µm/min	3.92 ± 0.37	7.91 ± 0.62	5.50 ± 0.60

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

Submergence-induced responses can be reversed by de-submergence

To gain further insight into the regulation of expansin expression and protein levels in relation to submergence-induced petiole elongation, plants were de-submerged to soil level (waterlogging) to withdraw the submergence signal. To this end, plants were first submerged for 10 h, followed by de-submergence. When the submergence signal was withdrawn, petiole growth rates dropped to the level of air-grown control plants (Table 3). *RpEXP1* transcript levels, which rose during submergence (Figure 2A), also decreased after de-submergence to the values of air-grown controls (Table 3). In contrast, for *RpEXP8*, *RpEXP10*, *RpEXP15* and *RpEXP18*, de-submergence increased transcript accumulation to amounts found for the air-grown controls (*RpEXP10* and *Rp-EXP18*) or above (*RpEXP8* and *RpEXP15*, Table 3). Both AIE and the amounts of 24 kD class of proteins showed enhanced levels after submergence, and this was reversed to air-grown control values following de-submergence (Table 3).

Discussion

The submergence-induced petiole elongation of *R. palustris* has proved to be a useful system to study inducible elongation growth without interference from cell division. Petiole elongation rates can be measured in detail, allowing lag-phases and elongation kinetics to be monitored with great accuracy. A study in which the kinetics of growth are compared with those of expansin transcript and protein levels enhances a better understanding of the role expansins play in regulating elongation growth.

Growth measurements shown in Figure 1 demonstrate that submergence-induced growth in *R. palustris* over a period of 30 h consists of two phases in which submerged petioles show a higher elongation rate than air-grown petioles. The

onset of the first phase of submergence-induced elongation that started after 3 h of submergence, was preceded by the up-regulation of *RpEXP1* transcript levels, which occurred within 2 h of submergence (Figure 2A). The other four studied expansin genes showed either no change in transcript levels (*RpEXP8*) or a trend to decline (*RpEXP10, 15, 18*) upon submergence (Figure 2B-E). Such a differential response of expansin genes to one stimulus has also been found for α- and β-expansins in deep water rice (Lee and Kende, 2001; 2002) and for α-expansins in parasitic angiosperms (O'Malley and Lynn, 2000), indicating a specific role for individual expansins in different cell wall related processes. *RpEXP1* was cloned by Vriezen *et al.* (2000) from a cDNA library of 24 h submerged *R. palustris* shoots. Northern blot analysis showed that *RpEXP1* up-regulation upon submergence was located mainly in the petioles and less in the leaf blades (Vriezen *et al.*, 2000), indicating that *RpEXP1* is expressed in the main elongating shoot tissue (Voesenek and Blom, 1989). The importance of the rapid rise in *RpEXP1* transcript levels for the submergence-induced petiole elongation of *R. palustris* is strengthened by the reversibility of the gene expression upon de-submergence (Table 3). Similar correlations between (partial) submergence-induced growth and expansin transcript levels have been found in the monocot deepwater rice (Cho and Kende, 1997a; Huang *et al.*, 2000).

Submerged *R. palustris* petioles showed an increase in AIE relative to air-grown petioles (Figure 3), as would be expected from the rise in *RpEXP1*. The enhancement of the AIE of submerged petioles was observed after 4 h of submergence, lagging behind the up-regulation of *RpEXP1* transcript levels, but coinciding closely with the onset of enhanced petiole elongation. Since the AIE of a tissue is the summation of the amount of expansin proteins, activity, and the susceptibility of the tissue for the enzymes, we studied these parameters during submergence. The susceptibility of submerged *R. palustris* petioles to a cell wall protein extract did not differ from the susceptibility of air-grown petioles (Table 2), indicating that the rise in AIE of submerged petioles could be accounted for solely by increased amounts or activities of expansin proteins. Western blot analysis revealed that only the expansin size group containing RpEXP1 (24 kD) increased during submergence (Figure 5A), while in the other size groups no increase was observed, in agreement with the changes in transcript abundance.

After the initial rise in the petiole elongation rate of submerged *R. palustris*, starting 3 h after the onset of submergence, elongation rates gradually declined to values similar to those of air-grown petioles after 12 h of submergence (Figure 1). Extension growth of submerged and air-grown petioles remained similar from 12 h until 18 h after the onset of submergence (end of light period to middle of dark period). During this period of similar elongation, the AIE, *RpEXP1* transcript and expansin protein levels were much larger in submerged petioles compared to those in air-grown controls. Since growth did not differ, even though expansin protein levels differ substantially in this period, another yet unidentified mechanism must be modulating elongation. As faster elongation resumes after 18 h of submergence, it is unlikely that an active cell wall stiffening process, such as lignification or extensin incorporation, is involved. It seems more likely that another, reversible, elongation regulating mechanism is limiting elongation in this phase in

submerged and air-grown petioles (discussed below). The period of similar elongation rate was followed by a period in which the elongation rates of submerged petioles were again substantially faster than those of air-grown petioles (Figure 1). The relation between elongation growth and expansin activity was thus restored during this third period.

Although elongation growth and expansin expression show close correlations in our data, we cannot exclude other cell wall loosening mechanisms also playing a role in submergence-induced petiole elongation. These mechanisms, if present, might modulate petiole elongation of submerged plants in the period of 12 to 18 h after submergence. Apoplastic pH may be involved in the submergence-induced petiole elongation of *R. palustris*, since the activity of expansins and cell wall extensibility are pH sensitive (McQueen-Mason *et al.*, 1992). Apoplastic pH is known to be regulated by ethylene in the aquatic plant *Nymphoides peltata*, a plant in which, like in *R. palustris*, the submerged status of the plant is sensed by a rise in internal ethylene concentrations (Malone and Ridge, 1983). Preliminary results show that an acidification of the apoplast might also follow submergence in *R. palustris* petioles (data not shown). The reduction in elongation rate of air-grown and submerged petioles, which is initiated already 5 h after applying the submergence treatment, could reflect alkalisation of the apoplast. Next to apoplastic pH, proteins, other than expansins, could influence cell wall properties and thus growth in *R. palustris* petioles. For example, Xu *et al.* (2000) and Yuan *et al.* (2001) showed that endo-β-1,4-glucanases purified from blue mussel (*Mytillus edulis*) and a fungus (*Trichoderma reesei*) also showed *in vitro* cell wall extension activity when assayed in an extensometer. A second class of plant cell wall loosening enzymes are yieldins. Yieldins lower the yield threshold of cell walls in a pH dependent manner (Okamoto-Nagazato *et al.*, 2000a; 2000b) and in this way can make the cell wall more extensible. A third expansin independent cell wall loosening mechanism has been proposed by Fry (1998) and Schopfer *et al.* (2002), which involves the scission of polysaccharides by hydroxyl radicals. Induction of extension by hydroxyl radicals in frozen/thawed maize coleoptiles showed similar rates of extension as those seen by the addition of expansins in cucumber hypocotyl sections (compare data in McQueen-Mason *et al.*, 1992, and Schopfer *et al.*, 2002). Although these cell wall modifying mechanisms might not be responsible for the observed submergence-induced petiole elongation of *R. palustris*, they might, with other mechanisms, modulate the extension activity of expansins, resulting in equal petiole elongation rates of submerged and air-grown plants in the period from 12 to 18 h of submergence.

The kinetics of the onset of submergence-induced petiole elongation and the up-regulation of expansins coincide closely. A lag-phase of 3 h was determined before the submergence-induced petiole elongation was initiated. The up-regulation of *RpEXP1* transcript abundance was observed after 2 h of submergence (Figure 2), at least 1 h before the initiation of enhanced elongation, most likely reflecting the time needed for mRNA translation and transport of the proteins to the cell wall. Expansin protein levels of the 24 kD size class containing RpEXP1 do rise upon submergence, but no significant increase was observed until 10 h of submergence (Figure 5). However, the increase in RpEXP1 might be masked, since the 24 kD

size class contains at least 5 other expansin proteins (Table 1) of which several might decline in protein abundance upon submergence. A strong indication for increased active expansins comes from the higher extension activity of *R. palustris* CCWE after 4 h of submergence (Figure 6). Furthermore, the AIE rise after 4 h of submergence, coincided with the submergence-induced petiole elongation of *R. palustris*.

From the timing of the submergence-induced petiole elongation and expansin expression upon submergence, we conclude that both are closely linked, a connection that is retained when de-submergence slows petiole extension (Table 3). These links apply to *RpEXP1* transcript, expansin protein and activity levels. In the same system that showed a close relation between petiole elongation and expansin expression, an uncoupling of expansin levels and elongation was observed, since no difference in elongation rate between submerged and air-grown petioles was observed after 12 h of submergence, while submerged petioles contained elevated expansin mRNA (*RpEXP1*), protein and activity levels. We therefore propose that an up-regulation of *RpEXP1* is required for the submergence-induced petiole elongation in *R. palustris*, but that other elongation modulating mechanisms override the submergence-induced expansin activity during periods when petiole elongation was equal in submerged and air-grown plants.

Material and Methods

Plant material and treatments

Rumex palustris seeds were sown on black polyethylene beads (Elf Atochem, Marseille, France, baked at 95 ^{o}C for at least one week) floating on tapwater in a transparent container. Seeds were let to germinate for 9 d with 12 h light, 20 ^{o}C, 70 µmol m^{-2} s^{-1} photosynthetic photon flux density, and 12 h dark, 10 ^{o}C. Seedlings were potted in plastic pots of 80 ml in a mixture of 2:1 (v/v) potting soil and sand, with 2.8 g MgOCaO (Magkal, 17 % MgO, Vitasol BV, Cruquius, The Netherlands) per litre potting mixture. Before potting of the seedlings, pots were placed in a tray with nutrient solution and each pot was allowed to take up on average 24 ml of nutrient solution containing: 7.5 M $(NH_4)_2SO_4$, 15 mM KH_2PO_4, 15 mM KNO_3, 4.3 µM $MnSO_4$, 1.8 µM $ZnSO_4$, 0.32 µM $CuSO_4$, 43 µM H_3BO_3, 0.53 µM Na_2MoO_4, and 86 µM Fe-EDTA. Plants were grown 15 d in a growth chamber (16/8 h light/dark, 20 ^{o}C, 70% relative humidity, and 200 µmol m^{-2} s^{-1} photosynthetic photon flux density). Pots with seedlings were kept in trays covered with glass for 2 d after potting, after which the pots were placed on two layers of irrigation mat. The mats were automatically watered with tap water to saturation twice a day, after which water was drained. The 24 d old plants had an expanding 3rd leaf and a newly emerging 4th leaf. Plants were submerged in 50 l tanks with water that had been acclimated to the growth room conditions overnight. Air-grown control plants were watered at the time of submergence and before the dark period. In all experiments, time of submergence was 10.00 a.m. For de-submergence, the water was siphoned out of the tanks to soil level. Each replicate consisted of a different set of plants with a similar distribution in plant size.

Plant growth measurements

Petiole elongation was measured with linear variable differential transformers as described by Voesenek *et al.* (2003b) adjusted with clamps designed to measure only elongation of the petiole and with a net pulling weight of 5 gr. Growth rates were calculated by fitting lines through 20 min intervals. Lag phases of the submergence-induced elongation were determined using the data-fitting procedure as described by Cox *et al.* (2003) on individual length traces.

RNA extraction

For each sample, RNA of five 3rd petioles was extracted using a modified method of Kiefer *et al.* (2000). 50 µl 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 U DNaseI (Amersham Pharmacia Biotech inc. no. 27-0514-02). 1 µg RNA was dissolved in 30 µl de-ionised water, checked for DNA with PCR using primers designed to amplify expansins non-specifically: 5'-ACCGCCACCAACTTCTGC-3' and 5'-CATTGGTGATCAGGACTAGG-3' and 1 µg RNA was used for cDNA synthesis (stratagene cDNA synthesis kit, no. 200401-5).

Real-time PCR measurements of expansin mRNA

Twenty expansins were cloned by Vriezen *et al.* (2000) and Peeters *et al.* (unpublished data). The sequences are submitted to the GenBank database and were designated with the accession numbers: *RpEXP1*; AF167360, *RpEXP2*; AF167361, *RpEXP3*; AF167362, *RpEXP4*; AF167363, *RpEXP5*; AF167364, *RpEXP6*; AF167356, *RpEXP7*; AF428174, *RpEXP8*; AF428175, *RpEXP9*; AF428176, *RpEXP10*; AF428177, *RpEXP11*; AF428178, *RpEXP12*; AF428179, *RpEXP13*; AF428180, *RpEXP14*; AF428181, *RpEXP15*; AF428182,

RpEXP16; AF428183, *RpEXP17*; AF428184, *RpEXP18*; AF428185, *RpEXP19*; AF428186, and *RpEXP20* will be submitted in forthcoming weeks.

Real-time PCR (Heid *et al*., 1996) was performed on a ABI Prism 7700 (Applied Biosystems), with FAM and TAMRA labelled fluorescent probes, using standard cycle temperatures. The primers and probes used are:
RpEXP1; primers 5'-GGCGCGTGCTTCGAGATA-3' and 5'-GAGGGCAGAAGTTAGTGGCTGTAA-3', probe 5'-ATTGCACGCGCGACCCACG-3'.
RpExp8; primers 5'-CAACTCTTACCTTAACGGCCAAAC-3' and 5'-CCCCAACGTAAGTCTGACCAAA-3', probe 5'-CTGTCATTCTCCGTTACCTCTAGCGACGGA-3'.
RpExp10; primers 5'-TTGTGGATTCGGCAACTTGTACT-3' and 5'-TGGGTCGTCAGCGCACTT-3', probe 5'-CGCCGCAGCTCATCCCGTTG-3'.
RpExp15; primers 5'-CCCTCCCCTGCACCACTT-3' and 5'-AGGGATGATGCCGGCTTT-3', probe 5'-TCGCCCACCCTGTCTTCCAGCA-3'.
RpExp18; primers 5'-TGTGTGGCTGACCGGAAAT-3' and 5'-TGTTCGGCGGGCAGAA-3', probe 5'-CCGTGACCACAATGCTGCCGG-3'.
18S; primers 5'-CCGTTGCTCTGATGATTCATGA-3' and 5'-GTTGATAGGGCAGAAATTTGAATGAT-3', probe 5'-ACCTCGACGGATCGCACGGCC-3'.
The primer and probe combinations showed no or minor amplification of other expansins, tested on plasmid sequences (data not shown).

Relative mRNA values were calculated using the comparative Ct method described by Livak and Schmittgen (2001), expressing mRNA values relative to 18S RNA. To compare the relative abundance of the different *R. palustris* expansins, the PCR efficiency was determined using known amounts of plasmid expansin DNA. The relation between the amount of DNA and the Ct value was used to obtain the relative amounts of the five selected expansin genes in one sample. The data set was corrected for these ratios, defining *RpEXP1* at t = -2 as 1.

Acid-induced extension

Acid-induced extension of 6 mm segments of the 3rd petioles was measured in a custom built constant load extensometer, modified from the design presented by Cosgrove (1989), with a pulling force relative to 30 g. Petioles were first incubated in 160 µ 50 mM HEPES pH 6.8 for 30 min, after which the buffer was changed to 50 mM sodium acetate, pH 4.5. The acid-induced extension was calculated by fitting a line through a 10 min interval starting 1 min after the observed bending point, minus the slope of a line fitted through a 10 min interval starting 11 min before the bending point.

Protein extraction and Western blot analysis

Crude cell wall protein extracts were made according to Rochange *et al.* (2001), modified to extract from five petioles in 1.5 ml reaction tubes. Cell wall material was separated by centrifugation. After extraction, the cell wall residue was washed with cold water, cold 70% ethanol and cold acetone. Starch was removed from the samples by treating them overnight with 75 U bacterial α-amylase (Sigma A6380) at 20°C in 125 µl 20 mM phosphate buffer pH 7, 10 mM NaCl, and 0.05% chlorbutol. After de-starching, the cell wall material was washed again, dried, and weighed. The mean cell wall dry weight of control and submerged petioles did not differ (data not shown). Proteins were loaded on gel relative to the cell wall dry weight in order to represent the amount of proteins in a physiologically relevant way (Cho and Kende, 1997c).

A 15% SDS-PAGE was used to separate the proteins. The proteins were blotted on reinforced nitrocellulose paper. The blots were labelled with 1:2000 LeEXP2 antibody (kindly

provided by Dr. A.B. Bennett and Dr. A.L.T.Powell, University of California, Davis) and 1:20000 anti-rabbit IgG, peroxide conjugate (Sigma, A0545). For detection we used Pierce Super Signal Ultra, chemiluminiscent substrate (Pierce, no. 34075) and Kodak Biomax Light films (Kodak, no 8194540). Band intensities were determined using custom-made software on an image analysis set-up (KS 400, version 3.0, Carl Zeiss Vision, Germany). To overcome film saturation artefacts, samples were loaded in three dilutions and multiple exposure times were used. Each blot contained an air-grown sample and a submerged sample of the same sample time. In order to compare different blots, submerged values were taken as a ratio to the air-grown values. Because the values used are ratios, the values were log transformed before standard errors were calculated, after which the values were back-transformed.

The predicted protein sizes from the cDNA sequences were obtained from DNA Star. The signal peptide sequences were located using Signal P (Nielsen *et al*., 1997). Protein sizes from the gel were calculated using the kaleidoscope prestained standard (Bio-Rad, no. 161-0324) as reference.

Extractable protein activity

Acetobacter xylinus derived cellulose/xyloglucan composites were grown as described by Whitney *et al.* (2000), with an incubation time of 5 d. CCWE (see Western blot analysis) were re-suspended in 50 mM sodium acetate buffer pH 4.5 relative to the cell wall dry weight of the samples (see Western blot analysis). The protein extracts were added to 2x12 mm strips of cellulose/xyloglucan composites in an extensometer with a pulling force relative to 20 g.

Susceptibility experiment

Frozen/thawed *R. palustris* petioles were boiled for 15 s to inactivate native expansin activity, put in the extensometer and incubated in 50 mM sodium-acetate buffer of pH 4.5 for 30 min. The incubation solution was then changed to a desalted CCWE re-suspended in the same buffer. The desalted CCWE was derived from commercially obtained celery by Dr. S.J. McQueen-Mason (University of York).

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Chapter 3

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

with Cornelis A.M. Wagemaker[§], Anton J.M. Peeters, and Laurentius A.C.J. Voesenek

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Abstract

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

Introduction

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

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

The river-plain species *Rumex palustris* shows a striking morphological response upon submergence, first the petioles bend upwards (hyponastic growth), followed by an enhanced elongation of the petioles (Banga *et al.*, 1997; Cox *et al.*, 2003). These traits restore air contact and gas exchange with the atmosphere and improve plant survival in flooding-prone environment. Submergence-induced elongation of *R. palustris* petioles is a good model to study tissue and cellular extension, since i) the elongation is achieved by cell elongation, without involvement of cell division (Voesenek *et al.*, 1990), ii) the enhanced cellular extension is distributed equally over the entire petiole (Rijnders *et al.*, 1996) and iii) the elongation response can be switched on and off in a reliable way by submergence and ethylene treatments, allowing comparative studies of elongation kinetics and cell extension mechanisms, like expansins (Chapter 1).

Submergence-induced petiole elongation of *R. palustris* is under control of various plant hormones. When submerged, the basal production of ethylene of *R. palustris* shoot tissue is maintained (Voesenek *et al.*, 1993; Banga *et al.*, 1996b), leading, together with the lower diffusion rate of ethylene in water than in air (Jackson, 1985), to ethylene concentrations of 1 µl l^{-1} within one hour of submergence. This concentration is already saturating the petiole elongation response (Banga *et al.*, 1996a). Both submergence and ethylene exposure result in a rapid decline in abscisic acid (ABA) concentrations, preceding the start of petiole elongation. Moreover, ABA added to submerged plants severely inhibits the elongation response (Voesenek *et al.*, 2003a; Benschop *et al.*, unpublished results). Next to ABA, gibberellic acid (GA) also acts downstream of ethylene in the elongation response. Submergence of *R. palustris* results in increased GA concentrations, while submerged plants pre-treated with GA biosynthesis inhibitors result in retarded petiole elongation, which could be rescued by addition of GA_3 (Rijnders *et al.*, 1997). In addition to ABA and GA, auxins are also involved in the ethylene-driven submergence response of *R. palustris*. A rise in the native auxin indole-3-acetic acid (IAA) was observed upon 4 h of submergence (Cox *et al.*, unpublished results). Removal of the leafblade, in this way reducing internal IAA concentrations, inhibited petiole elongation and could be rescued by addition of the synthetic auxin 1-naphtolene acetic acid (1-NAA; Cox *et al.*, unpublished results).

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

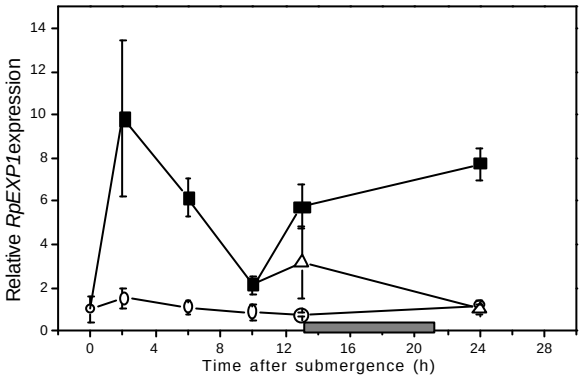


Figure 1. *RpEXP1* expression in 3rd petioles of air-grown (open circles), submerged (filled squares), or de-submerged (open triangles) *R. palustris* plants, relative to air at t=0. During the de-submergence, after 10 h of submergence, water was lowered to soil level. mRNA concentrations were measured using *real-time* RT-PCR with 18S RNA as internal standard. Mean ± se, n=3. Grey horizontal bar indicates 8 h dark period.

Results

RpEXP1 transcription is up-regulated by submergence

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

RpEXP1 transcription is regulated by ethylene

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

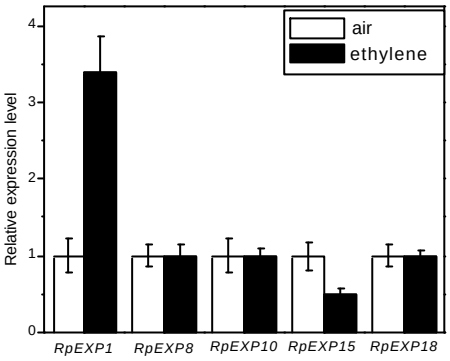


Figure 2. Relative expression of selected *R. palustris* expansin genes in the 3rd petiole after 6 h of 5 µl l^{-1} ethylene treatment, or at the same time in air without ethylene, measured using *real-time* RT-PCR. Values are relative to air-control for each expansin gene. Mean ± se, n=4.

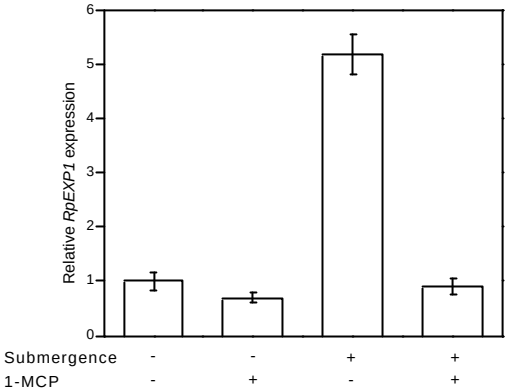


Figure 3. Effect of 1 µl l^{-1} 1-MCP pre-treatment on the relative *RpEXP1* expression in 3rd petioles of 6 h submerged or air-grown *R. palustris* measured using *real-time* RT-PCR. Values are relative to air-control. Mean ± se, n=3.

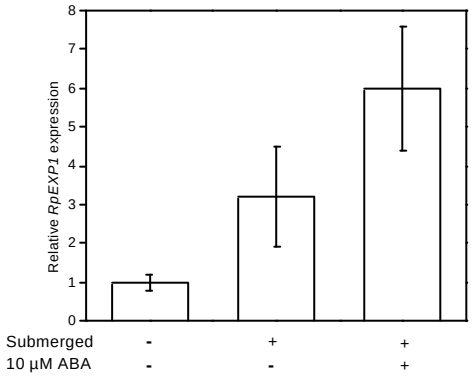


Figure 4. *RpEXP1* expression in 3rd petioles of air-grown, submerged, or ABA-submerged *R. palustris*, measured using *real-time* RT-PCR. Values are relative to air-control. Mean ± se, n=2.

ABA is not involved in submergence-induced *RpEXP1* transcription

Since *RpEXP1* is the only expansin gene that so far showed enhanced transcript concentrations in petioles of *R. palustris* upon submergence (Chapter 1) and ethylene treatment (Figure 2), the expression profile of this gene was further analysed for its response to other hormones acting downstream of ethylene in the submergence-induced elongation response of *R. palustris*. Next to a rapid rise in ethylene (Banga *et al.*, 1996a), ABA concentrations also showed a fast decrease upon submergence and ethylene exposure (Voesenek *et al.*, 2003a; Benschop *et al.*, unpublished results). Plants submerged in water containing 10 µM ABA to keep internal ABA concentrations in petioles high, showed a reduced elongation response to submergence (Benschop *et al.*, unpublished results). Based on these results we hypothesised that ABA acts as a negative regulator of *RpEXP1* transcript abundance. However, when plants were submerged in water containing 10 µM ABA, no decline in *RpEXP1* abundance was observed (Figure 4).

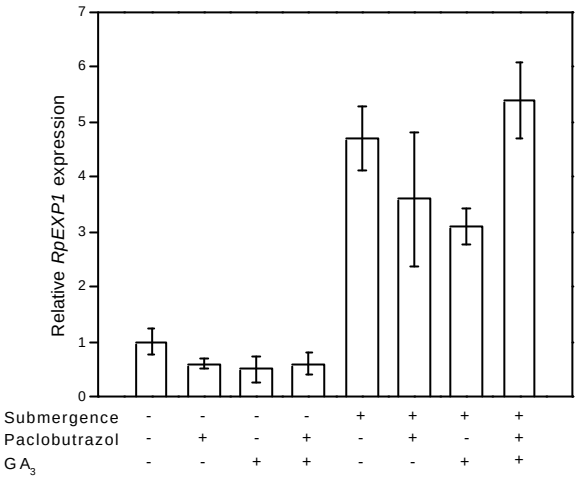


Figure 5. Effect of paclobutrazol pre-treatment and GA addition on the *RpEXP1* expression in 3rd petioles of 6 h submerged or air-grown *R. palustris*, measured using *real-time* RT-PCR. Values are relative to non-paclobutrazol pre-treated air control. Mean ± se, n=3.

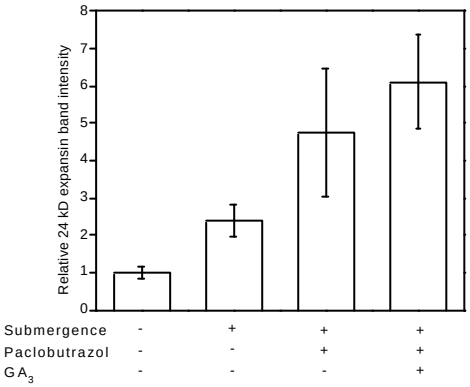


Figure 6. Intensity of the 24 kD expansin protein band upon 13 h submergence with or without paclobutrazol pre-treatment or GA_3 addition in *R. palustris* 3rd petioles. Values are relative to air control. Mean ± se, n=4.

GA manipulations do not show an effect on *RpEXP1* transcription

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

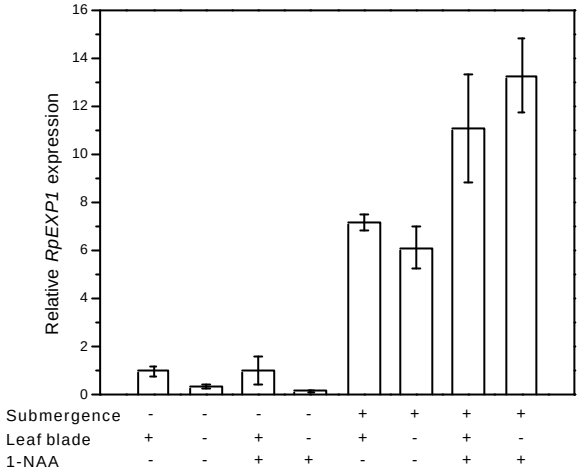


Figure 7. Effect of excision to leaf blades and 1-NAA addition on the relative *RpEXP1* expression in 3rd petioles of 6 h submerged or air-grown *R. palustris*, measured using *real-time* RT-PCR. Values are relative to air control. Mean ± se, n=3.

Paclobutrazol induces a GA_3 independent increase in expansin protein

GA and paclobutrazol did not affect the mRNA concentration of *RpEXP1*. We can not, however, exclude that GA affects the translation of *RpEXP1* mRNA. Therefore, we also studied the regulation of expansins protein expression in response to paclobutrazol and GA treatments. When Western blots of *R. palustris* crude cell wall extracts were incubated with LeEXP2 antibody, three major bands were observed of 24, 26 and 28 kD (Chapter 1). Only the 24 kD band, containing e.g. RpEXP1, increased upon submergence with the largest increase 13 h after submergence (Chapter 1). Figure 6 shows the relative intensity of the 24 kD expansin band in *R. palustris* crude cell wall protein extracts. A submergence period of 13 h increased the amount of 24 kD expansin protein, which unexpectedly increased even further when the plants were pre-treated with paclobutrazol. When paclobutrazol pre-treated plants were flooded in a 10 µM GA_3 solution, no reversion of the paclobutrazol-induced increase was observed. Paclobutrazol nor GA_3 showed an effect on the amount of 26 kD or 28 kD expansin proteins (data not shown), indicating that the paclobutrazol-induced increase is specific for the 24 kD expansin and not an artefact of loading. The *RpEXP1* mRNA profiles of the 13 h treated plants was similar to the results obtained after 6 h (Figure 6, data not shown).

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

Auxin is required for submergence-induced petiole elongation (Cox *et al.*, unpublished results). Removal of the leaf blade resulted in 36 to 62 % lower auxin concentrations in the petiole and a lack of submergence-induced petiole elongation for 8 h (Cox *et al.*, unpublished results). The growth reduction could be restored by application of the synthetic auxin 1-NAA to the submergence-water (Cox *et al.*, unpublished results). Detachment of the leaf blade from the petiole led to an overall significant reduction of *RpEXP1* transcript abundance (P=0.005) and was most pronounced in air-grown plants (Figure 7). Addition of auxin to intact or leaf-

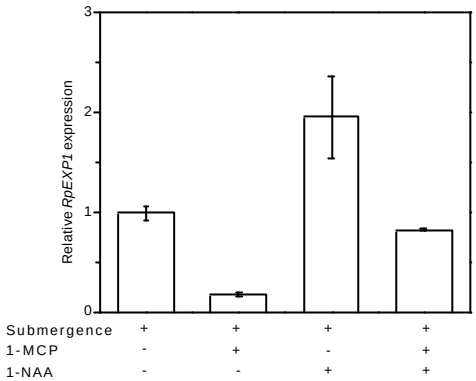


Figure 8. Effect of 1-MCP on the 1-NAA-induced increase in *RpEXP1* expression in submerged *R. palustris* 3rd petioles, measured using *real-time* RT-PCR. Values are relative to non-1-MCP pre-treated submerged plants. Mean ± se, n=3.

detached plants did not cause a change in *RpEXP1* transcript abundance (Figure 7), while air-grown plants treated with 1-NAA did show typical 1-NAA-induced bending of the leaves (data not shown). These results indicate that, in petioles of air-grown plants, the inhibiting effect of leaf blade removal on *RpEXP1* transcript abundance is not a specific auxin effect. Petioles of submerged plants showed a marked increase in *RpEXP1* transcript levels (Figure 7). Addition of 1-NAA to the submergence water increased the transcript levels of *RpEXP1* even further (Figure 7). Since auxin is known to stimulate ethylene production (Yang and Hoffman, 1984), we tested whether the auxin stimulation of *RpEXP1* transcription was due to a stimulation of ethylene production by blocking the ethylene signalling with 1-MCP. Figure 8 shows that 1-MCP did lower the amount of *RpEXP1* transcription, but that blocking the ethylene pathway with a 1-MCP pre-treatment could not block the whole stimulatory effect of the submergence-auxin interaction.

Discussion

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

Ethylene, as the prime signal in the submergence response, enhanced, next to the petiole elongation rate, also the *RpEXP1* transcript concentration in *R. palustris* petioles (Figure 2). The regulation of *RpEXP1* mRNA concentration by ethylene is further strengthened by inhibition of the submergence-induced increase in *RpEXP1* transcript levels by the ethylene perception-inhibitor 1-MCP (Figure 3). The rachis of the fern *Regnellidium diphyllum* also has the capacity to elongate strongly when

submerged or exposed to ethylene (Cookson and Osborne, 1978). Kim *et al.* (2000) showed that both treatments induced increased concentrations of the á-expansin gene *RdEXP1* in this species. Transcription levels of a second á-expansin gene, *RdEXP2*, did not change upon submergence nor ethylene exposure, indicating that, like in *R. palustris*, submergence and ethylene induce the expression of a specific expansin gene. In *R. acetosa*, a species of the same genus as *R. palustris*, but not showing submergence-induced petiole elongation, ethylene exposure lead to decreased concentrations of *RaEXP1* (Vriezen *et al.*, 2000). These results indicate that ethylene exposure does not necessarily induce expansin transcript expression and that ethylene-induced transcript expression coincides positively with the growth response of the plant species to ethylene. Since ethylene is a prime submergence signal for many plant species (Voesenek and Blom, 1999; Jackson and Ram, 2003) and results in a cascade of changing hormone concentrations and sensitivities (Kende *et al.*, 1998, Voesenek *et al.*, 2003a), we studied whether the observed induction of *RpEXP1* mRNA was directly regulated by ethylene, or via downstream hormones (Figure 9).

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

Since GA promotes petiole elongation in *R. palustris* (Rijnders *et al.*, 1997) and GA is the key hormone in the submergence-induced internode elongation of deep water rice (Kende *et al.*, 1998), inducing more transcripts of á- and â-expansins in rice (Lee and Kende, 2001; 2002), we expected an inhibiting effect of the GA biosynthesis-inhibitor paclobutrazol and a stimulatory effect of GA on *RpEXP1* transcript levels. Pre-treatment of *R. palustris* plants with paclobutrazol, nor addition of GA_3, had any effect on *RpEXP1* transcript levels (Figure 5). Four other studied expansin genes (*RpEXP8*, *10*, *15* and *18*) also showed no increase in transcript concentrations upon treatment with GA. Next to the expansin transcript levels, the amount of expansin proteins also did not decrease upon paclobutrazol pre-treatment (Figure 6). These results indicate that in *R. palustris*, the stimulatory effect of GA on petiole elongation growth is not via a stimulation of the amount of expansin transcript or proteins (Figure 9).

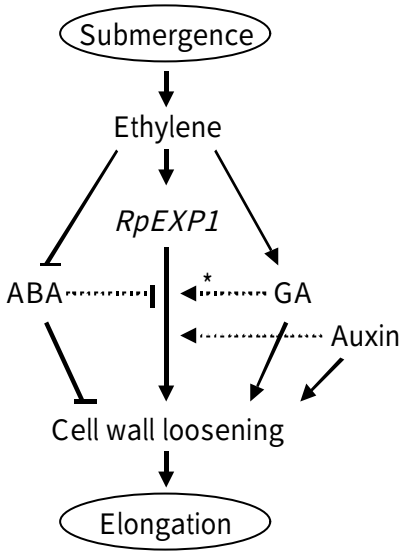


Figure 9. Partial signal transduction and response network covering the hormones involved in submergence-induced petiole elongation of R. palustris in relation to RpEXP1 expression. Arrows represent positive regulation and bars represent negative regulation. Dashed lines are hypothetical interactions. * Not via regulation of translation.

Pre-treatment with the GA biosynthesis inhibitor paclobutrazol led to higher amounts of the 24 kD protein in submerged *R. palustris* petioles (Figure 6), while such an increase was not observed for two other expansin protein bands (26 kD and 28 kD, data not shown). The accumulation of a 24 kD expansin protein by paclobutrazol could be caused by higher translation rates, slower turn-over of the proteins, or increased transcription of expansins other than RpEXP1 and RpEXP8 (expansins studied with real-time RT-PCR and belonging to the 24 kD size group). No distinction between these mechanisms could be made with the present data, nor whether the observed paclobutrazol-induced increase of the 24 kD expansin protein is caused by GA biosynthesis inhibition, or by an unknown action of paclobutrazol. As an alternative, paclobutrazol might also act indirectly via the reduced growth of plants that are treated with paclobutrazol 4 d in advance (Rijnders *et al.*, 1997), triggering a feed-back loop to maintain tissue elongation via more expansin proteins while the GA-regulated elongation mechanism is inhibited and which is not yet reversed after 13 h GA treatment. GA has shown to alter various cell wall properties, such as inducing reorientation of cellulose microfibril arrangement more traverse to the cell axis in Zea mays and in deep water rice (Mita and Katsumi, 1986; Sauter *et al.*, 1993), and increasing xyloglucan endotransglycosylation (XET) activity in cucumber hypocotyls (Potter and Fry, 1994). In peas, GA showed an effect on the yield threshold of the cell walls (Cosgrove and Sovonick-Dunford, 1989). Whether these mechanisms cause the GA-induced petiole elongation in *R. palustris* remains to be elucidated.

Submergence induced a rise in IAA concentration in the peripheral tissues of *R. palustris* (Cox *et al.*, unpublished results). A lower IAA concentration induced by cutting off the leaf blade resulted in a temporal reduction of submergence-induced petiole elongation rate for at least 8 h, and could be rescued by addition of 1-NAA (Cox *et al.*, unpublished results). The auxin dependency of the elongation response is not the result of a dependency of expansin expression on auxin, since no 1-

NAA-induced *RpEXP1* transcription was observed after 6 h of treatment (Figure 7, Figure 9). However, in combination with submergence, auxin did show a stimulatory effect on *RpEXP1* mRNA abundance. Auxin is known to enhance ethylene production (Yang and Hoffman, 1984). We therefore studied whether the observed interaction of submergence and 1-NAA, leading to enhanced *RpEXP1* transcript abundance, involved ethylene. Pre-treatment of plants with the ethylene antagonist 1-MCP reduced the *RpEXP1* transcript levels in petioles of plants submerged with 1-NAA (Figure 8), but not to levels of 1-MCP pre-treated submerged plants without 1-NAA (Figure 8). These results indicate an ethylene independent interaction between submergence and 1-NAA that has an additive effect on *RpEXP1* transcript abundance. The nature of this interaction remains to be elucidated.

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

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

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

Materials and methods

Plant material and growth conditions

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

Hormone treatments

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

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

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

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

RNA extraction and analysis

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

Western blot analysis

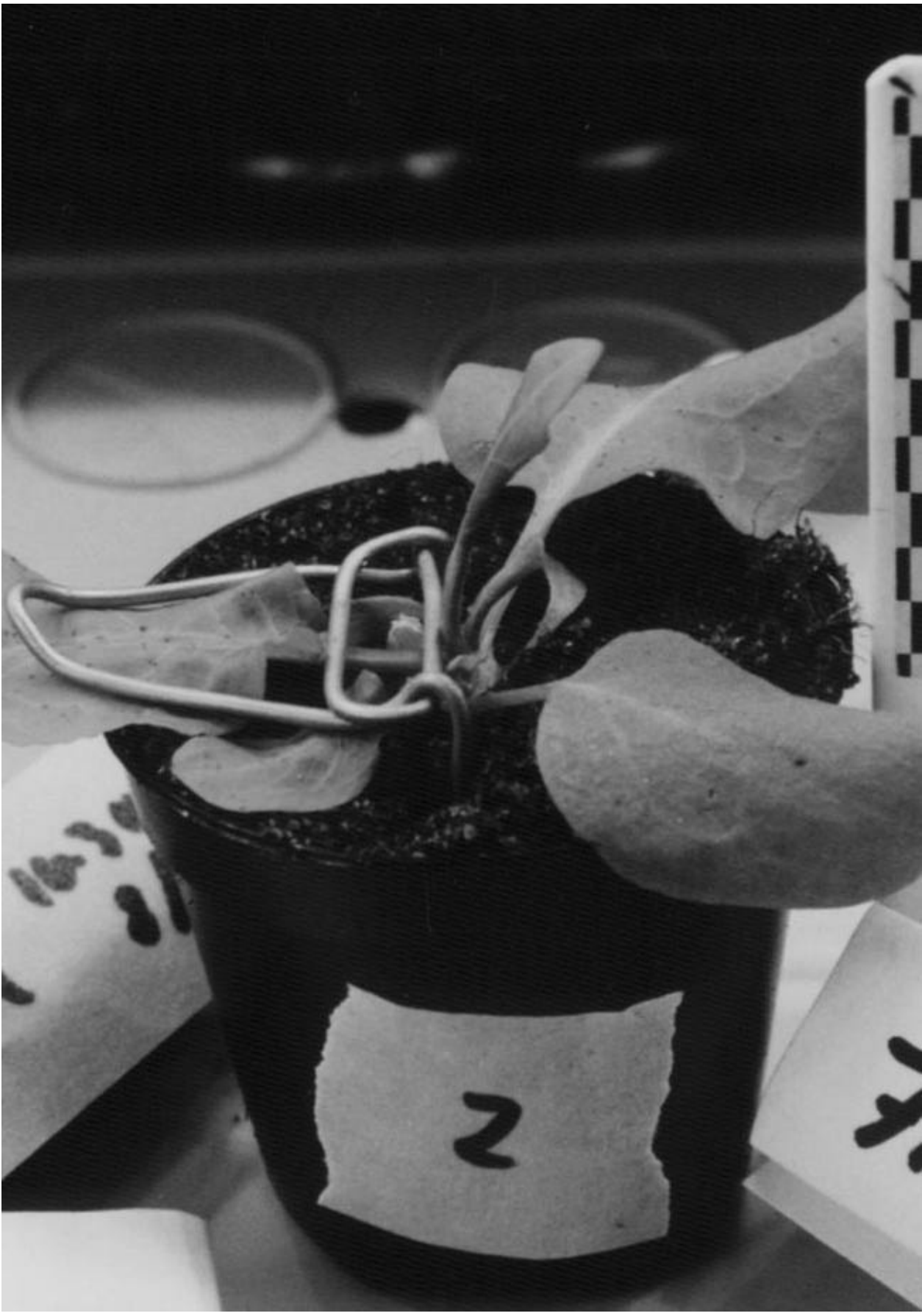
Crude cell wall protein extracts were made according to Rochange *et al.* (2001) with modifications described in chapter 1. Samples were loaded on a 15 % SDS-PAGE relative to the cell wall dry weight to gain a physiological representation of the amount of expansin protein (Cosgrove and Li, 1993; Cho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

Statistical analysis

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

Acknowledgements

We thank Joris Benschop for advice on hormone treatments and, together with Marjolein Cox, for giving us insight in their data, and Dr. T.D. Colmer for valuable advise on the manuscript. This research was supported by the Dutch Science Foundation, PIONIER grant no. 800.84.470.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Chapter 4

Angle manipulation of *Rumex palustris* petioles induces asymmetric distribution of cell wall extensibility

with Marc M. Matthies, Henri W. Groeneveld, Simon J. McQueen-Mason[1], Anton J.M. Peeters, and Laurentius A.C.J. Voesenek

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Abstract

When submerged, *Rumex palustris* plants show an upward bending of the petioles (hyponastic growth), followed by enhanced elongation of the petioles. Cox *et al.* (2003) showed that submergence-induced petiole elongation is influenced by the angle of the petiole. In the present study, we fixed the petioles in a horizontal position, below the original angle, and studied submergence-induced petiole elongation, expansin expression and cell wall extensibility. The extensibility was also determined for angular collenchyma strands isolated from the abaxial and adaxial side of the petiole. We found that fixation of petioles in a horizontal position, led to an altered distribution of cell wall extensibility in the petiole, proposed to be caused by asymmetric expression of expansins. We hypothesised a model that explained the development of this asymmetric distribution, assuming a gravitropic set-point angle (GSA) that will be adopted by the petiole. The asymmetric distribution of cell wall extensibility induced by regaining the GSA is likely to also affect total petiole elongation.

Introduction

Rumex palustris has a striking submergence response to survive periods of flooding during high river water levels. Submerged *R. palustris* plants first show an upward bending of petioles (hyponastic growth), followed by an enhanced petiole elongation (Banga *et al.*, 1997; Cox *et al.*, 2003). The combination of these two growth responses can bring leaf blades above the water surface, and thus restore gas exchange with the atmosphere. Hyponastic growth is related to the expansion of a limited number of cells at the base of the abaxial side of the petiole (Cox *et al.*, unpublished results). Submergence-induced petiole elongation of *R. palustris* is caused by faster cell elongation equally distributed over the entire petiole (Voesenek *et al.*, 1990; Rijnders *et al.*, 1996).

Expansins are a family of cell wall proteins proposed to play a key role in the regulation of tissue elongation (Cosgrove, 2000a). Vreeburg *et al.* (Chapter 1) showed that expansin action is involved in the submergence-induced elongation response of *R. palustris* petioles. The amount of *RpEXP1* transcript (see also Vriezen *et al.*, 2000), as well as the amount of expansin proteins of the size class containing RpEXP1, were up-regulated upon submergence (Chapter 1). The acid-induced extension (AIE) of petioles, a property determined by *in planta* amounts of expansins (McQueen-Mason, 1995), also showed a close coincidence with submergence-induced petiole elongation (Chapter 1). The strong association between submergence-induced petiole elongation and expansin up-regulation in *R. palustris* petioles was further strengthened by the decline of expansin RNA, protein, activity levels, and slowed petiole elongation upon releasing the submergence signal by lowering the water to soil levels (Chapter 1).

The gaseous phytohormone ethylene is a primary signal stimulating under water petiole elongation in *R. palustris* (Voesenek and Blom, 1989; Voesenek *et al.*, 1992), and also induces enhanced transcript levels of the submergence-induced *RpEXP1* gene (Vriezen *et al.*, 2000; Chapter 2). Cox *et al.* (2003) proposed that the submergence-induced petiole elongation of *R. palustris* is not only regulated by the endogenous concentration of ethylene, but that a second signal is also required to induce fast petiole elongation. They observed severely reduced petiole elongation rates when the petioles of submerged plants were restricted in their hyponastic response by keeping the petioles at their original angle. Releasing the restriction on the hyponastic response resulted in a rapid upward bending of the petiole and restored fast petiole elongation (Cox *et al.*, 2003). Therefore, they proposed a model in which, next to submergence, the petiole also had to achieve a certain angle before enhanced elongation would occur. The ‘critical angle’ that had to be reached was determined to be 40 to 50 degrees relative to the horizontal plane (Cox *et al.*, 2003). Since the submergence-induced petiole elongation depends on petiole angle as well as ethylene, the rate of petiole elongation in submerged *R. palustris* can be manipulated independent of ethylene. We used this unique tool of angle manipulation to reduce the submergence-induced elongation of *R. palustris* petioles and studied whether altered elongation kinetics could be explained by altered cell wall extensibility and expansin expression. This study also

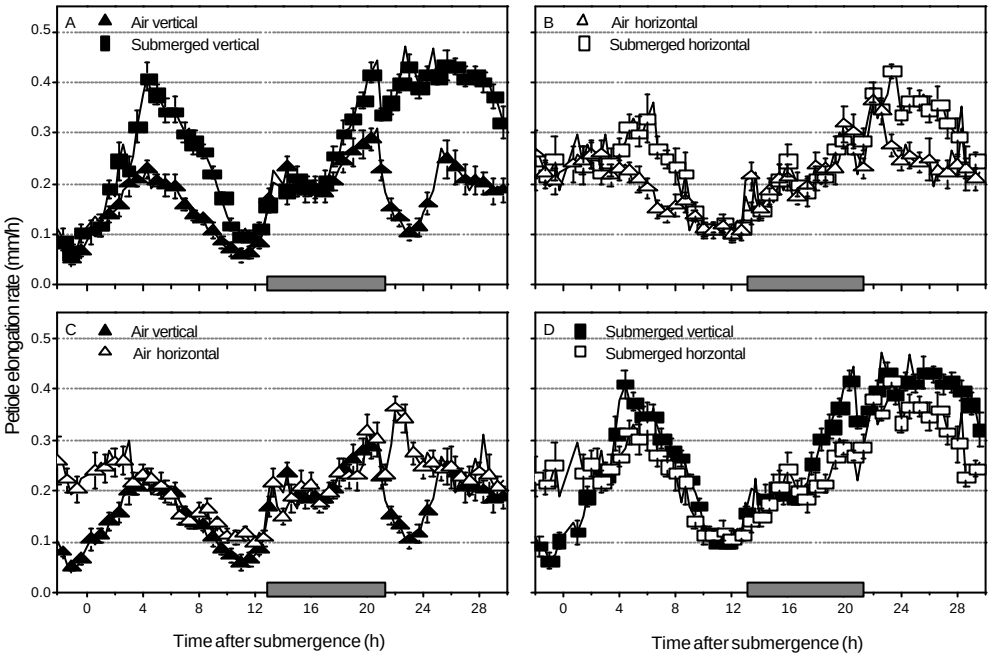

Figure 1. Petiole elongation rates of 25 d old *R. palustris* 3rd petioles which are grown in air (triangles), or submerged at t=0 (squares), and are kept in a vertical position (filled symbols), or in a horizontal position (open symbols). A) Elongation rates of vertical, air-grown and submerged petioles. B) Elongation rates of horizontal, air-grown and submerged petioles. C) Elongation rates of air-grown, vertical and horizontal petioles. D) Elongation rates of submerged, vertical and horizontal petioles. Growth rates are calculated every 20 min from length data obtained with a transducer set-up, symbols are plotted every two data points. Grey horizontal bar indicates 8 h dark period (mean ± se, n=10).

involved measuring the extensibility of angular collenchyma strands isolated from the ab- and adaxial sides of petioles of *R. palustris*.

Results

Horizontally-fixed petioles show reduced submergence-induced elongation

In order to study the effect of a horizontal angle on petiole elongation, we fixed the petiole at a low, near horizontal angle (0 – 30 degrees), or at a high, near vertical angle (80 – 90 degrees). Petioles in a vertical orientation showed a marked induction of submergence-induced petiole elongation after 3 h of submergence (Figure 1A). Submergence-induced petiole elongation reached a maximum of 0.4 mm h^{-1} after 4 h of submergence (Figure 1A). The elongation rate of submerged petioles fell back to air-grown rates after this maximum, and elongation rates of air-grown and submerged petioles were similar after 13 h of submergence (Figure 1A). Petiole elongation rates diverged again during the night period (Figure 1A). Petioles in a horizontal orientation also showed a submergence-induced growth enhancement, starting after 4 h of submergence (Figure 1B). This increase in

petiole elongation rate was, however, not as large as of vertical, submerged petioles, reaching a maximum elongation rate of 0.35 mm h^{-1} (Figure 1D). Although the petiole elongation rate of horizontal, submerged petioles did rise after 23 h submergence (Figure 1B, D), this rise was later and smaller than that of vertical submerged petioles (Figure 1D).

For plant kept in air, petioles that were fixed in a horizontal position started with higher elongation rates than petioles that were kept vertical (Figure 1C). Petioles were clamped to a horizontal position 16 h before the onset of the submergence treatment (t=-16). The difference in elongation rate at t=-2 between vertical petioles and petioles that were put horizontally resulted from a decrease in petiole elongation rate of air-grown, vertical petioles at the end of the dark period (light switched on at t=-3). A similar decrease was also observed at t=21 (Figure 1A, C). Such a decrease in petiole elongation rate at the end of the dark period (at t=-3 and t=21) was absent for horizontal petioles of air-grown plants (data not shown for t=-3; Figure 1B, C).

The vertical or horizontal orientations of *R. palustris* petioles did not significantly alter the increase in petiole length measured over 24 h in air-grown plants (4.3 ± 0.3 mm and 5.0 ± 0.3 mm, n=10, P=0.14, for vertical and horizontal petioles, respectively). However, when *R. palustris* plants were submerged, the horizontal position of the petiole resulted in a significant decrease of the length obtained in 24 h, when compared with submerged vertical petioles (6.9 ± 0.3 mm and 5.9 ± 0.3 mm, n=10, P<0.005 for vertical and horizontal petioles, respectively).

Horizontal fixation affects the basal transcript level, but not the submergence-induced increase, of *RpEXP1* abundance

Of the five expansin genes studied (*RpEXP1*, *8*, *10*, *15* and *18*), only *RpEXP1* showed an increase in amount of transcript in vertical petioles upon submergence (Figure 2), a finding consistent with previous observations (Chapter 1). *RpEXP1* mRNA abundance increased about 4-fold within 6 h of submergence and remained higher for the duration of the experiment. In vertical petioles, the transcript levels of both *RpEXP8* and *RpEXP18* did not respond upon submergence, while for *RpEXP10* and *RpEXP15*, submergence induced a decrease in amounts of transcripts. When petioles were kept in a horizontal position, the basal levels of *RpEXP1*, *8* and *10* transcripts increased, while no change or a decrease in basal levels were observed for *RpEXP15* and *18*. For *RpEXP1*, the only gene showing an up-regulation upon submergence in vertical petioles, submergence also induced a further, 2-fold, increase in mRNA levels when petioles were kept horizontally. A transient submergence-induced up-regulation (8-fold) in horizontal petioles was also observed for *RpEXP8*, which did not show such a submergence-induced rise in vertical petioles. No submergence-induced increase was observed in horizontally fixed petioles for the amounts of *RpEXP10*, *15* or *18* transcripts.

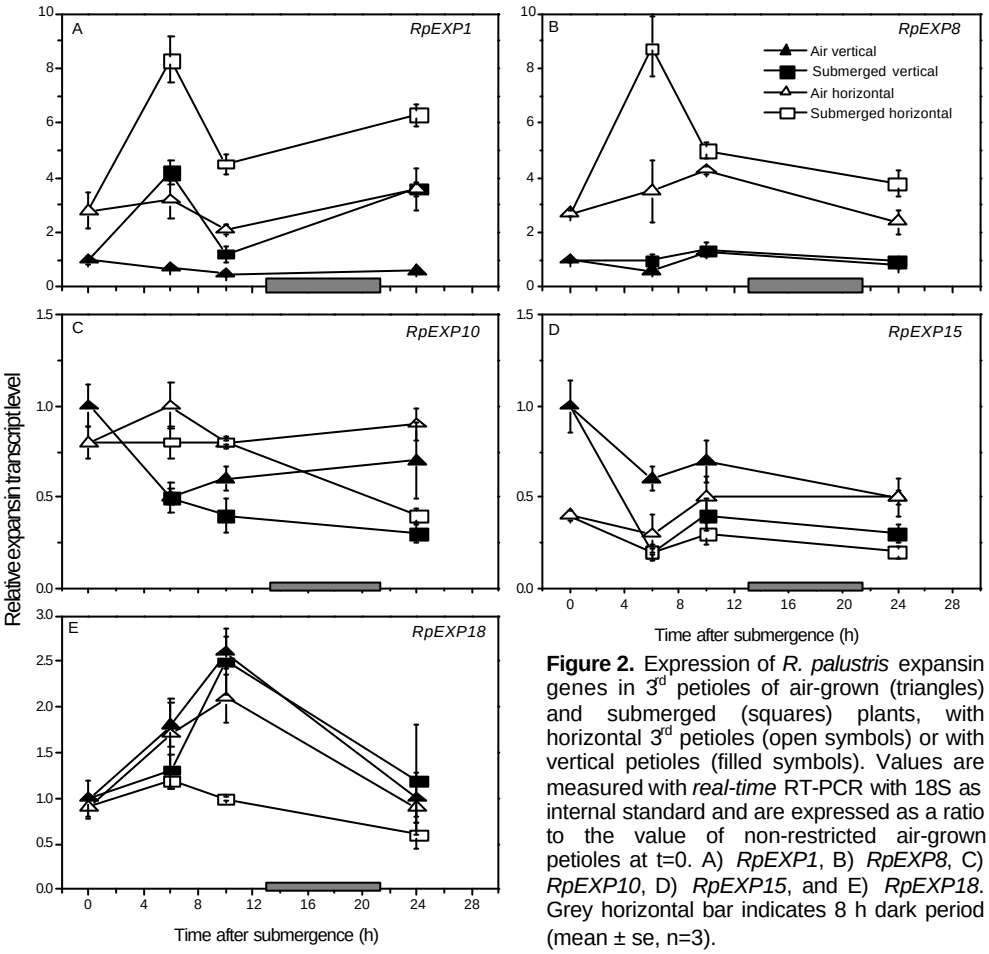

Figure 2. Expression of *R. palustris* expansin genes in 3rd petioles of air-grown (triangles) and submerged (squares) plants, with horizontal 3rd petioles (open symbols) or with vertical petioles (filled symbols). Values are measured with *real-time* RT-PCR with 18S as internal standard and are expressed as a ratio to the value of non-restricted air-grown petioles at t=0. A) *RpEXP1*, B) *RpEXP8*, C) *RpEXP10*, D) *RpEXP15*, and E) *RpEXP18*. Grey horizontal bar indicates 8 h dark period (mean ± se, n=3).

Fixation to a horizontal position does not affect the extractable expansin activity of submerged petioles

The effects of vertical and horizontal positions and submergence were tested, next to expansin RNA abundance, also for expansin activity. An indicator for expansin activity present in the cell wall, is the extension activity of cell wall protein extracts on cellulose/xyloglucan composites. Cellulose/xyloglucan composites produced by *Acetobacter xylinus spp.* share molecular and ultra-structural features with dicot cell walls (Whitney *et al.*, 1995), but are more responsive to expansins than plant tissues (Whitney *et al.*, 2000). Figure 3 shows the extension of cellulose/xyloglucan composites when crude cell wall protein extracts (CCWE) of *R. palustris* petioles were added. The CCWE of submerged, vertical petioles induced more extension than the CCWE of air-grown, vertical petioles after 4 h of submergence and remained higher during the experiment (Figure 3). CCWE of air-grown petioles that were kept horizontally showed a slight enhanced extension activity relative to extracts from vertical, air-grown petioles (Figure 3). However, no difference in

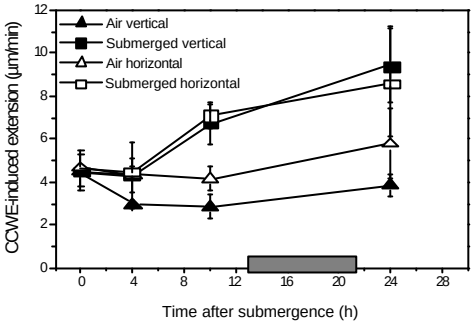

Figure 3. Extractable expansin activity of crude cell wall protein extracts (CCWE) from *R. palustris* 3rd petioles of air-grown (triangles) and submerged (squares) plants, with horizontal 3rd petioles (open symbols) or vertical petioles (filled symbols). The CCWE was tested on *Acetobacter*-derived cellulose/xyloglucan composites clamped in an extensometer with 20 g constant load (± se, n=5). CCWE of *R. palustris* 3rd petioles was re-suspended in 50 mM Na-acetate pH 4.5 buffer relative to the cell wall dry weight of the petioles and added to cellulose/xyloglucan composites. Grey horizontal bar indicates 8 h dark period.

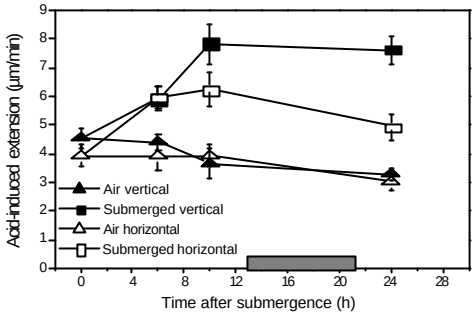

Figure 4. Acid-induced extension (AIE) of *R. palustris* 3rd 6 mm petiole segments of air-grown (triangles) and submerged (squares) plants, with horizontal 3rd petioles (open symbols) or with a vertical petiole (filled symbols). The AIE was measured in an extensometer with a constant load of 30 g, and was calculated as the extension rate in a 10 min interval after a pH-change from pH 6.8 to 4.5, minus the extension rate in a 10 min interval before the pH-change. Grey horizontal bar indicates 8 h dark period (mean ± se, n=6-10).

CCWE-induced extension could be observed between horizontal and vertical petioles when submerged (Figure 3).

Horizontal fixation affects acid-induced extension of submerged petioles

Expansins are activated by an acidic pH (McQueen-Mason *et al.*, 1992). Therefore, the acid-induced extension (AIE) of a tissue is also an indication for the expansin activity present in the cell walls of that tissue (McQueen-Mason, 1995). Air-grown petioles showed a constant AIE with no difference between horizontally held and vertical petioles (Figure 4). When *R. palustris* plants were submerged, the AIE was increased 6 h upon submergence, both for vertical and horizontal petioles (Figure 4). However, when submerged for a longer period (10 and 24 h), the acid-induced extension of vertical petioles increase further while it decreased for petioles that were kept horizontal (Figure 4).

Fixation to a horizontal position has a differential effect on AIE of abaxial and adaxial sides of petioles

Horizontally-fixed, submerged petioles show a reduced AIE relative to vertical, submerged petioles after 10 and 24 h of submergence (Figure 4). However, no difference in extractable expansin activity was observed for this treatment (Figure 3). Both assays are an indication for potential expansin activity, with AIE measured on intact petioles, while the CCWE is extracted from homogenized petioles. The difference observed between the two ways of determining expansin activity might

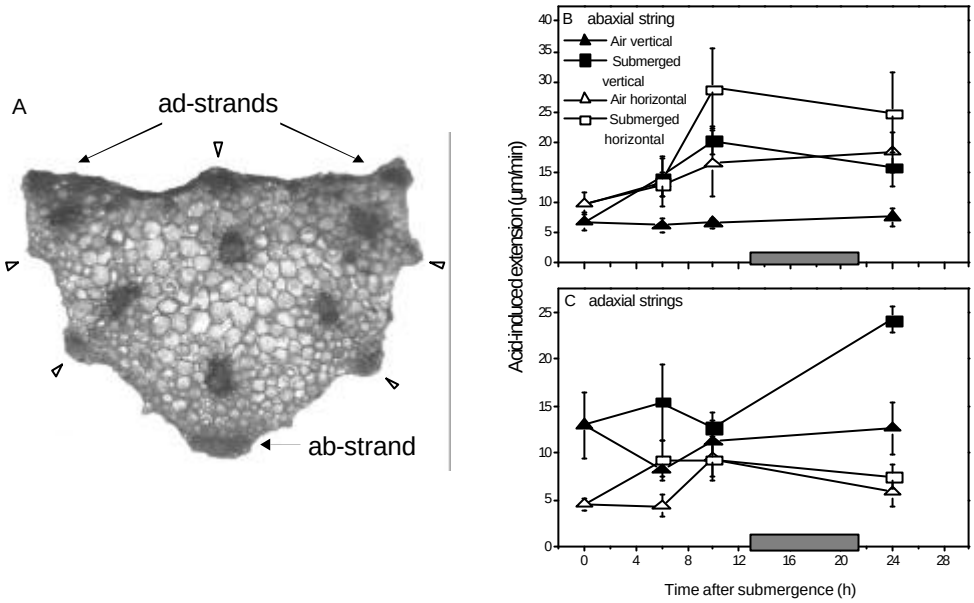

Figure 5. A) Cross-section of 3rd petiole of air-grown *R. palustris*. Angular collenchyma strands are indicated with open arrow heads. The upper, adaxial strands (ad-strands) and lower, abaxial strand (ab-strand) used in the experiments are indicated. B,C) Acid-induced extension (AIE) of 6 mm segments of angular collenchyma strands from *R. palustris* 3rd petioles of air-grown (triangles) and submerged (squares) plants, with horizontal 3rd petioles (open symbols) or vertical petioles (filled symbols). The AIE was measured in an extensometer with a constant load of 5 g. B) AIE of ab-strands. C) AIE of ad-strands. Grey horizontal bar indicates 8 h dark period (mean ± se, n=5-17).

be caused by the spatial distribution of expansins in the petiole, which is maintained in the AIE measurements, but is cancelled out when making a CCWE.

R. palustris petioles contain 8 angular collenchyma strands positioned at the outside of the petiole (Figure 5A). The collenchyma strand at the lower, abaxial side (ab-strand) and both strands at the upper, adaxial side (ad-strands, see Figure 5A) can easily be removed from the petiole and tested for their AIE in an extensometer. The AIE of the ab-strands of both horizontal and vertical petioles increased 2 to 3-fold upon submergence (Figure 5B). When petioles were kept horizontally, the AIE of ab-strands of air-grown petioles was higher than of the ab-strands of vertical petioles (Figure 5B). The AIE of ab-strands from horizontal petioles increased between 6 to 10 h after submergence, while the AIE of ab-strands from vertical increased within 6 h of submergence. Similar to the AIE of ab-strands, the AIE of ad-strands from vertical petioles also increased upon submergence (Figure 5C). The AIE of ad-strands from horizontally held petioles was lower than that of vertical petioles, and only showed an increase upon 6 h of submergence (Figure 5C).

Discussion

In the present study, horizontal petioles were clamped to angles at least 20 degrees below their original angles. This treatment resulted in an asymmetric distribution of cell wall extensibility (Figure 5) and a higher expression of three expansin genes (Figure 2). In the following paragraphs we explain these observations by assuming petioles of *R. palustris* have a gravitropic set-point angle (GSA) as proposed for shoots of other species by Digby and Firn (1995). The GSA is defined as "... the angle with respect to gravity at which an organ shows no gravity-induced differential growth in order to correct its orientation" (Digby and Firn, 1995).

Submerged petioles that are not restricted in their hyponastic growth response bend at the base of the petiole (Figure 6A; Cox *et al.*, unpublished results). When petioles are fixed to a horizontal orientation below their starting angle, however, the area which will bend to an upwards position becomes distributed over the entire petiole and is most pronounced in horizontally kept, submerged petioles (Figure 6B). The upward bending of air-grown petioles that are restricted to a lower than original angle, could be explained by assuming that a mechanism is induced to maintain a certain angle with the horizontal plane (i.e. a GSA). Such a 'set-point' was proposed by Digby and Firn (1995) for the internodes of the hanging plants *Tradescantia fluminensis* and *Oplismenus hirtellus*, and named the gravitropic set-point angle (GSA). The internodes of these hanging plants maintain a specific, developmentally and environmentally controlled angle with the gravitropic vector (Digby and Firn, 1995; 2002).

Upward bending of the petiole, induced by its horizontal fixation, is also reflected in the distribution of AIE in the petiole. The AIE of the abaxial collenchyma strands (ab-strands, Figure 5A) of horizontal air-grown petioles was generally higher than those from vertical petioles (Figure 5C), whereas the AIE of adaxial collenchyma strands (ad-strands, Figure 5A) was lower for horizontal petioles than it was for vertical petioles (Figure 5B). Submergence might increase the GSA of petioles. In case of the petiole being fixed in a horizontal position when submerged, the GSA will increase, while the fixed petiole remains at a lower angle. The difference in GSA and petiole angle will increase and a steeper distribution of cell wall loosening activity between the adaxial and abaxial side is expected. Indeed, submergence induced a rise in AIE of the ab-strands of both vertical and horizontal petioles (Figure 5B), while for the ad-strands submergence induced a rise in AIE of those from vertical petioles, and not from horizontal petioles (Figure 5C). Submergence, thus, results in a larger difference in cell wall loosening properties between the ad- and abaxial sides in horizontally fixed, than in vertical petioles.

Submergence-induced AIE enhancement of horizontal petioles was less than in vertical petioles at t=10 and t=24 (Figure 4). The total pool of extractable expansin activity on a whole petiole basis, however, remained similar (Figure 3). The reduced submergence-induced rise in AIE of horizontal petioles might be caused by the lack of enhanced extension of the collenchyma strands in the adaxial side of horizontal petioles (Figure 5C). A change in distribution of AIE between the two

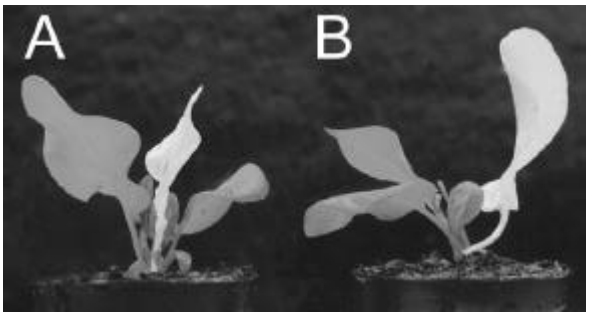

Figure 6. Side-view picture of *R. palustris* plants that were submerged for 10 h without a clamp (A), or with the 3rd petiole fixed to a horizontal position (B). The clamp was removed just before the picture was taken. The 3rd leaf of both plants were digitally highlighted.

sides of petioles, thus influences the AIE of the whole petiole, and is likely to affect petiole elongation *in vivo*. This led us to hypothesize that a change in spatial distribution of AIE is underlying the altered growth kinetics of horizontally fixed petioles (Figure 1).

The abundance of expansin transcripts, including *RpEXP1*, *8*, and *10*, were enhanced by the horizontal fixation of the petioles (Figure 2A, B, C). Transcript abundance of two other studied expansins genes were either not affected (*RpEXP15*), or decreased (*RpEXP18*) when the petioles were fixed in a horizontal position (Figure 2D, E). The enhanced expression of *RpEXP1*, *8*, and *10* in the whole petiole, might reflect their up-regulation in the abaxial side of horizontal petioles. Asymmetric distribution of expansins in relation to gravitropic bending of tissues have been observed in maize roots. Expansin mRNA was more abundant at the faster elongating side of the root (Zhang and Hasenstein, 2000). Fixation of the petiole in a horizontal position did not, however, result in a large change in submergence-induced expansin activity tested *in vitro* on cellulose/xyloglucan composites (Figure 3). These results indicate that the up- and down-regulation of different expansin genes by horizontal fixation (Figure 2) did not result in a large difference of total expansin action, and that different expansins act redundantly with respect to extension activity.

Clamping the petiole to a lower than original angle induced changes that would not be expected for petioles fixed at their original (i.e. pre-submergence) angle. The submergence-induced rise in *RpEXP1* observed in horizontally clamped petioles might be located at the abaxial side of the petiole and result in enhanced AIE of ab-strands (Figure 5B). We can not exclude that such a rise will be absent when petioles are fixed at their original angle. Severe inhibition of submergence-induced petiole elongation by fixing the petiole angle in its original position, but below 40 to 50 degrees (Cox *et al.*, 2003) might thus be caused by inhibition of *RpEXP1* up-regulation.

We propose that submergence increases the GSA of *R. palustris* petioles, and that the GSA is adopted by the petiole by bending at its base. Submergence-induced petiole elongation commences when petioles reach an angle of 40 to 50 degrees relative to the horizontal plane (Cox *et al.*, 2003). The mechanism by which a low angle (< 40 to 50 degrees) is inhibiting elongation is unresolved, but might be by inhibition of submergence-induced *RpEXP1* expression. When petioles are

restricted in that their angle is kept below their GSA, an asymmetric distribution of AIE over the entire length of the petiole was initiated, probably caused by asymmetric expression of expansin proteins, to let the whole petiole bend instead of only the basal part.

Materials and methods

Plant material

Rumex palustris plants were grown as described in chapter 1. The 24 d old plants had an expanding 3rd leaf and a newly emerging 4th leaf. For the RNA, AIE and CCWE experiments, galvanized iron clamps to keep the 3rd petiole in a horizontal position were put on the petiole the afternoon in advance of the experiment. Plants indicated as vertical were not clamped. Plants were submerged in 50 l tanks with water that had been acclimated to the growth room conditions (20 ^{o}C) overnight. Air-grown control plants were kept on irrigation mats and watered at the time of submergence and before the dark period. In all experiments, time of submergence was 10.00 a.m. Each replicate consisted of a different set of plants with a similar distribution in plant size and petiole length.

Plant growth

Petiole elongation was measured with linear variable differential transformers as described by Voesenek *et al.* (2003b) in a vertical or horizontal orientation. The transducers were adjusted with clamps designed to measure elongation of only the petiole. The pulling force of both horizontal and vertical grown plants was relative to 5 gr. Growth rates were calculated over 20 min time intervals. Growth measurements with a digital camera set-up (Cox *et al.*, 2003), showed that petiole growth of vertically kept petioles in the transducer set-up showed similar responses to submergence as plants that were not clamped (data not shown). We therefore indicated non-restricted petioles as vertical.

RNA extraction and analysis

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

Acid-induced extension assay

Acid-induced extension of 6 mm segments of the 3rd petioles was measured in a custom built constant load extensometer, modified from the design presented by Cosgrove (1989), with a pulling force relative to 30 g. Petioles were first incubated in 160 µl 50 mM HEPES pH 6.8 for 30 min, after which the buffer was changed to 50 mM sodium acetate, pH 4.5. The acid-induced extension was calculated by fitting a line through a 10 min interval starting 1 min after the observed bending point, minus the slope of a line fitted through a 10 min interval starting 11 min before the bending point. Angular collenchyma strands were pulled of the petiole with tweezers and were put in the extensometer with a pulling force relative to 5 g.

Active expansin extraction and activity measurement

Crude cell wall protein extracts (CCWE) were made according to Rochange *et al.* (2001), modified to extract from five petioles in 1.5 ml reaction tubes as described in chapter 1. Protein extracts were tested for activity (see below) relative to the cell wall dry weight in order to represent the amount of proteins in a physiologically relevant way (Cho and Kende, 1997).

Acetobacter xylinus derived cellulose/xyloglucan composites were grown as described by Whitney *et al.* (2000), with an incubation time of 5 d. CCWE were re-suspended in 50 mM sodium acetate buffer pH 4.5 relative to the cell wall dry weight of the samples. The protein extracts were added to 2x12 mm strips of cellulose/xyloglucan composites in an extensometer with a pulling force relative to 20 g.

Acknowledgements

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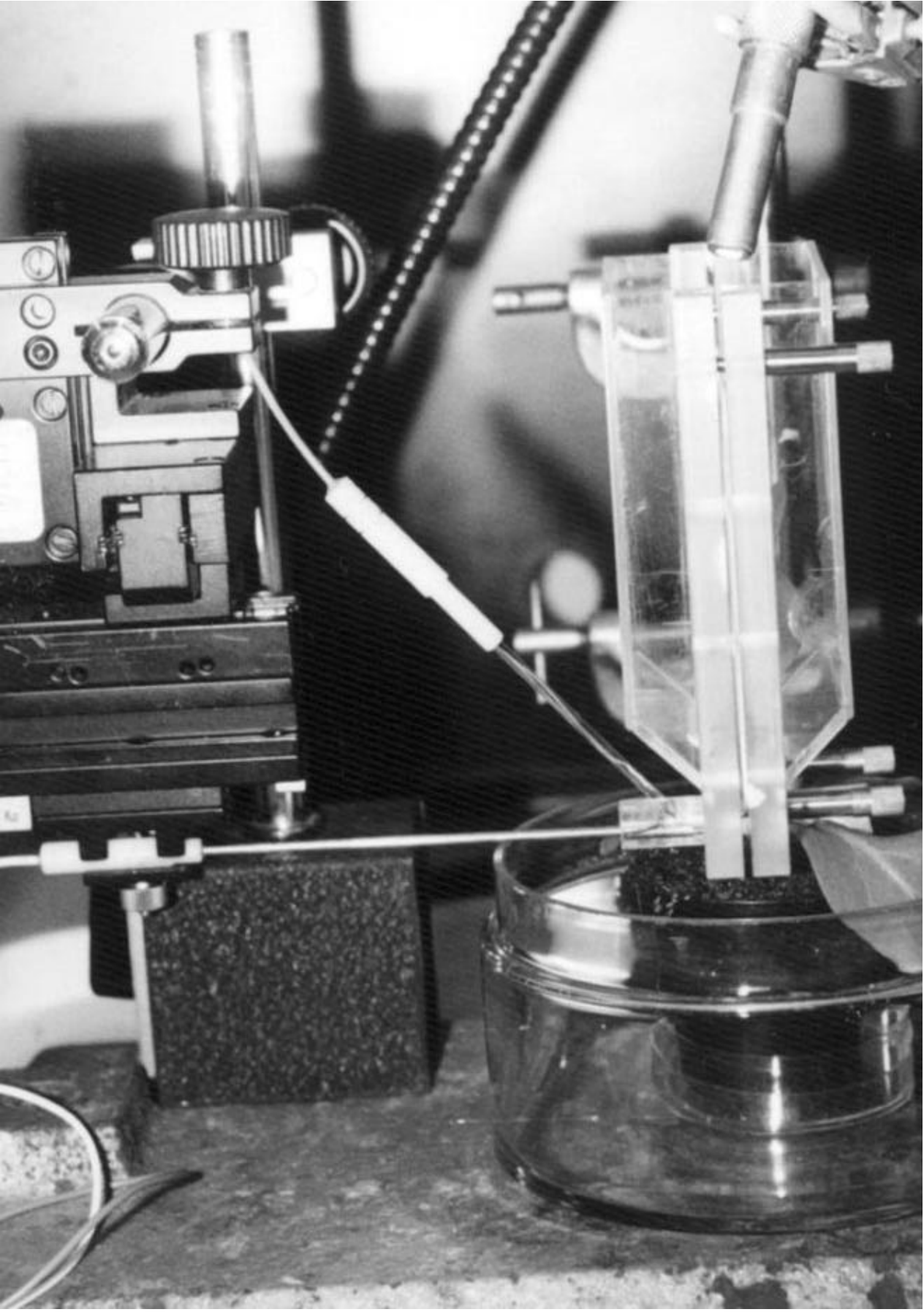

Chapter 5

Submergence-induced apoplastic acidification: hormonal regulation and its relation to elongation growth

with Joris J. Benschop[*], Marten Staal[1], J. Theo M. Elzenga[1], and Laurentius A.C.J. Voesenek.

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Abstract

Plant cell extension is thought to be mainly regulated by cell wall properties. The activity of many known cell wall loosening mechanisms depends on pH. In the *Rumex palustris* system, enhanced elongation growth is initiated in the petioles when the plant is submerged. A prime stimulatory signal for the submergence-induced petiole elongation is the gaseous hormone ethylene, while abscisic acid (ABA) acts as a negative regulator of this elongation response. Earlier work has indicated a close coincidence of the initiation of the enhanced petiole elongation with the expression of an expansin gene (*RpEXP1*). In the present study, we found that manipulations of apoplastic pH of *R. palustris* petioles influence petiole elongation, suggesting a role of apoplastic pH in regulating elongation growth *in vivo*. Furthermore, an ethylene-dependent acidification of the apoplast was observed upon submergence. From the kinetics of acidification, we hypothesize that submergence-induced apoplastic acidification facilitates the action of a cell wall loosening enzyme, proposed to be *RpEXP1*.

Introduction

Plant cell elongation is a resultant of both cell turgor pressure, as driving force, and cell wall loosening, which is thought to regulate cell extension (Cosgrove, 2000b; Kutschera, 2001). Much attention has been given to study the role of cell wall modifying mechanisms in relation to elongation growth, such as expansins (Vriezen *et al.*, 2000; Cosgrove *et al.*, 2002), xyloglucan endotransglycosylases/hydrolases (Potter and Fry, 1994), hydroxyl radicals (Schopfer, 2001), and yieldins (Okamoto-Nakazato *et al.*, 2001). The general approach to relate the activity of cell wall loosening enzymes with tissue elongation has often been to determine the transcript or protein abundance of the enzyme, or to test the activity of cell wall extracts *in vitro*. The activity of most of the cell wall modifying enzymes, however, is regulated by pH (such as expansins (McQueen-Mason *et al.*, 1992), XET activity (Fry *et al.*, 1992; Nishitani and Tominaga, 1992), and yieldins (Okamoto-Nakazato *et al.*, 2000a)). Since *in vivo* activity of cell wall modifying mechanisms not only depends on enzyme abundance, but also on the pH of the cell wall, apoplastic pH is a major determinant for cell wall loosening and thus elongation growth of plant tissues.

Submergence-induced petiole elongation of *Rumex palustris* provides a good physiological system to study plant cell elongation. *R. palustris* petioles show enhanced elongation when submerged (Voesenek and Blom, 1989; Chapter 1), which could be reversed by lowering of the water levels and thus releasing the submergence signal (Voesenek *et al.*, 2003b). Furthermore, the petiole elongation is distributed equally over the entire petiole (Rijnders *et al.*, 1996), and is achieved by cell elongation only (Voesenek *et al.*, 1990), excluding the interference of cell division. Submergence-induced petiole elongation is part of a strategy of *R. palustris* plants to survive periods of high water, which they encounter in their natural habitat in river flood-plains (Blom *et al.*, 1994).

Submergence of *R. palustris* petioles is related with enhanced concentrations of endogenous ethylene (Voesenek and Blom, 1989), caused by the reduced diffusion of ethylene out of the plant when surrounded by water while ethylene production is continued (Voesenek *et al.*, 1993; Banga *et al.*, 1996b). Both submergence and ethylene exposure induce the up-regulation of an expansin gene (*RpEXP1*) in *R. palustris* petioles (Vriezen *et al.*, 2000; Chapters 1 and 2). This indicates a role of RpEXP1 in the submergence-induced petiole elongation of *R. palustris*. In contrast to the stimulatory effect of ethylene on *R. palustris* petiole elongation (Voesenek and Blom, 1989), abscisic acid (ABA) inhibits the submergence-induced petiole elongation (Benschop *et al.*, unpublished results). This growth inhibition was not due to decreased *RpEXP1* transcript levels, since submergence of *R. palustris* in an ABA solution did not result in lowered *RpEXP1* transcript abundance (Chapter 2). ABA has been shown to inhibit apoplastic acidification or induce alkalisation of apoplastic pH in *Vicia faba* and *Zea mays* shoot tissues (Balsevich *et al.*, 1994; Goh *et al.*, 1996; Felle and Hanstein, 2002). Therefore, ABA might inhibit enhanced underwater elongation in *R. palustris* by restricting apoplastic acidification in the petiole tissue.

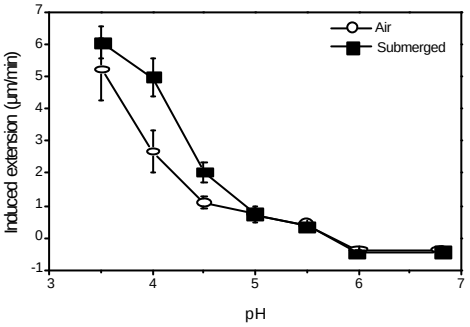

Figure 1. Extension of 6 mm *R. palustris* petiole segments induced by 50 mM citric acid/1 M K_2HPO_4 buffers measured with a constant load extensometer. Before the buffer of the pH indicated on the x-axis was added, petiole segments were incubated at pH 6.8. Petioles used were submerged for 6 h (open circles) or kept in air and harvested at the same time (filled squares; mean ± se, n=5).

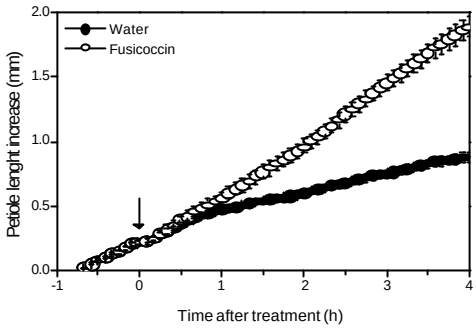

Figure 2. Increase in petiole length air-grown *R. palustris* petioles treated with 10 µM fusicoccin (filled symbols) or non-treated controls (open symbols). Arrow indicate time of fusicoccin addition (mean ± se, n=3).

This paper addresses the question whether submergence induces a physiological significant decrease in apoplastic pH in *R. palustris* petioles, and how such a submergence-induced apoplastic acidification is regulated. We show here, that *R. palustris* petiole elongation depends on apoplastic pH. Furthermore, submergence induces a decrease in apoplastic pH in *R. palustris* petioles, an effect that could be reversed by inhibiting ethylene reception.

Results

In vitro R. palustris petiole extension is pH sensitive

Figure 1 shows the extension of segments of *R. palustris* petioles induced by buffers of various pH after initial incubation in pH 6.8, measured with a constant load extensometer. Petioles of both air-grown and submerged plants showed increased extension when incubated in buffers with low pH. Air-grown and submerged petioles showed increased extension already at pH 5.5 (Figure 1). Below pH 5, the increased extension of submerged petioles was most pronounced and was higher than the extension of petioles from air-grown plants. The extension of petioles from air-grown plants started to increase rapidly at pH values below pH 4.5 (Figure 1).

In vivo elongation can be stimulated by acidifying the apoplast

Elongation of air-grown *R. palustris* petioles was stimulated by application of 10 µM fusicoccin (Figure 2), a fungal toxin stimulating the plasma membrane H^+-ATPase and acidifying the apoplast. Furthermore, injection of 50 mM succinate buffer of pH 4 in air-grown *R. palustris* petioles, induced a direct stimulation of the petiole elongation (Figure 3A). Injection of a 50 mM succinate buffer of pH 6 did not show

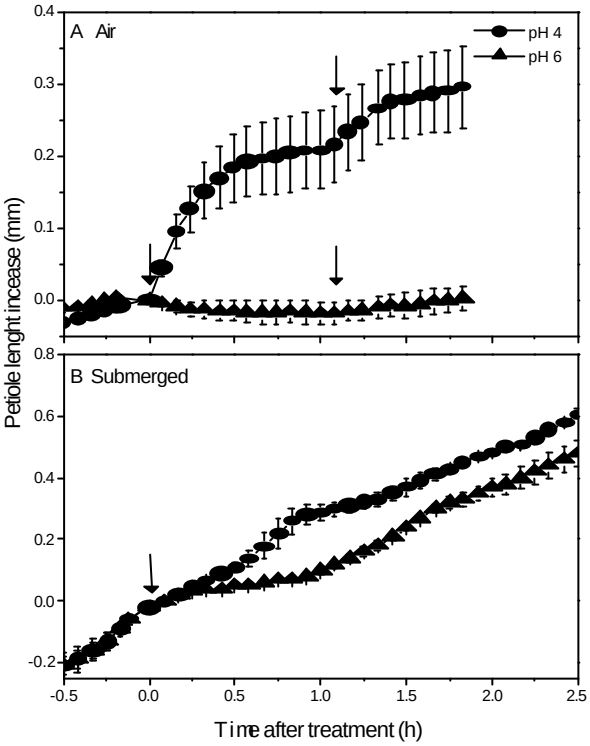

Figure 3. Increase in petiole length of *R. palustris* petioles injected with 50 mM succinate buffer of pH 4 (circles) or pH 6 (triangles). A) Air-grown petioles, n=4. B) 4 h submerged petioles, n=2. Arrows indicate time of injection (mean ± se).

a stimulatory effect on petiole elongation (Figure 3A), indicating that the effect is most likely due to a change in apoplastic pH and not by a change in osmolarity of the apoplast. The pH 4-induced elongation could be repeated in the same petiole (second arrow), although the stimulating effect was reduced (Figure 3A).

Submergence-induced petiole elongation is inhibited by alkalisation of the apoplast

Submergence-induced petiole elongation of *R. palustris* shows a maximum after 4 h of submergence (Benschop *et al.*, unpublished results). Injection of a 50 mM succinate buffer of pH 6 inhibited enhanced petiole elongation when applied after 4 h of submergence (Figure 3B). In contrast, injection of a 50 mM succinate buffer of pH 4 did not show an effect on submergence-induced petiole elongation (Figure 3B). The inhibitory effect of pH 6 injection was temporal and elongation rates were restored after 1 h (Figure 3B). This indicates an active regulation of apoplastic pH.

Submergence induces acidification of the apoplast, which could be inhibited by 1-MCP and not by ABA

The apoplastic pH in petioles of air-grown plants decreased during the experiment with 0.44 pH units in 4 h (Figure 4). In petioles of submerged plants, the apoplastic pH showed a much faster acidification, leading to a decrease of almost 0.96 pH unit in 4 h (Figure 4), decreasing from an initial pH of 5.9 (± 0.2, n=6), to a pH of 5.0 (± 0.2, n=6).

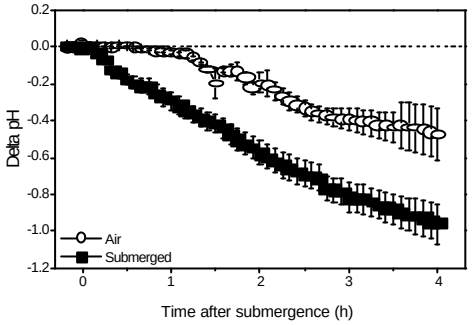

Figure 4. Apoplastic pH increase of *R. palustris* petioles. The petioles were grown in air (open circles, n=4), or submerged at t=0 (filled squares, n=8; mean ± se).

Table 1. Change in apoplastic pH of *R. palustris* petioles. Plants were grown in air, submerged, submerged with a pre-treatment with 1-MCP, or submerged in ABA. Values are mean changes after 4 h of treatment, ± se (n).

Treatment	Delta pH	
Air	-0.44 ± 0.14	(4)
Submerged	-0.96 ± 0.11	(8)
Submerged + 1-MCP	-0.43 ± 0.21	(2)
Submerged + ABA	-0.84 ± 0.14	(4)

Pre-treatment of *R. palustris* plants with the 1 Methylcyclopropene (1-MCP), a known inhibitor of ethylene perception, blocked submergence-induced decrease in apoplastic pH (Table 1). Submergence of *R. palustris* in 20 µM ABA, a concentration inhibiting submergence-induced petiole elongation, did not result in an inhibition of submergence-induced apoplastic pH acidification after 4 h of submergence (Table 1).

Discussion

Acidification of the apoplast of air grown *R. palustris* petioles increased petiole elongation (Figures 2 and 3A), while injection of a pH 6 buffer in submerged petioles inhibited the enhanced elongation response (Figure 3B). These results indicate a regulatory role for pH in submergence-induced petiole elongation of *R. palustris*. Indeed, apoplastic pH showed a decrease upon submergence of almost a full pH unit in 4 h (Figure 4; Table 1). Although the pH measured with a surface pH electrode might differ slightly from the actual pH in the cell wall (see below), the observed decrease in apoplastic pH from 5.9 to pH 5.0 in 4 h can be considered to be of physiological importance for modulating cell wall loosening. At an initial pH of 6 or less, an acidification of about 1 pH unit will lead to more extension of frozen-thawed *R. palustris* petioles in a constant load extensometer (Figure 1). Although the petioles were kept in a water environment to enable pH measurements, the decrease in apoplastic pH of air-grown petioles is not a result of a partial induction of the submergence-induced apoplast acidification. Blocking the perception of ethylene, the key hormone in the submergence signalling pathway (Voesenek and Blom, 1999), resulted in a similar drop in apoplastic pH as that found in air-grown plants (Table 1). This also shows that the submergence-induced decrease of apoplastic pH is actively regulated by ethylene (Table 1; Figure 5).

Submergence of *R. palustris* plants in 20 µM ABA inhibited the submergence-induced petiole elongation (Benschop *et al.*, unpublished results), but did not result in an inhibition of submergence-induced apoplastic acidification (Table 1). ABA

also showed no inhibitory effect on the submergence-induced increase of *RpEXP1* transcript abundance (Chapter 2). Submergence lead to a fast decrease in endogenous ABA (Benschop *et al.*, unpublished results), whereas inhibition of ABA biosynthesis with fluridone decreased the lag phase for enhanced underwater elongation (Benschop *et al.*, unpublished results). These results suggest that a decrease in endogenous ABA levels is a requirement for enhanced elongation to commence. The nature of this growth-inhibiting effect of ABA, however, remains to be elucidated (Figure 5)

Submergence-induced apoplastic acidification started almost immediately upon submergence (Figure 4). If the apoplastic pH is the key factor determining submergence-induced petiole elongation, this gradual decrease in apoplastic pH upon submergence combined with the gradual increase in tissue extensibility with decreasing pH (Figure 1), would suggest a gradual increase of petiole elongation rate immediately after the onset of submergence. However, the petiole elongation rate of *R. palustris* plants does not show a gradual, but a steep increase upon submergence, after a lag phase of 3 h (Chapters 1 and 3). The contrasting kinetics of submergence-induced petiole elongation and apoplastic acidification could be explained in two ways; i) apoplastic acidification is involved in an elongation stimulating process for which a certain threshold has to be reached before it is activated, and ii) the kinetics of enhanced elongation are determined by enhanced biosynthesis of factors constituting a pH dependent cell wall loosening mechanism. Apoplastic acidification will enhance the activity of this mechanism, causing its up-regulation to result in more effective cell wall loosening action. Both alternatives are discussed below.

Although plant cell growth is thought to be regulated by cell wall properties (Cosgrove, 2000b; Kutschera, 2001), the relevant force making plant cells elongate is turgor. Submergence-induced apoplastic acidification is likely to be achieved by enhanced excretion of protons by the H^+-ATPase. Excretion of positively charged protons will result in a hyperpolarisation of the plasma membrane. The enhanced inwardly directed driving force for positively charged ions created by membrane hyperpolarisation, is suggested to result in the uptake of cations and increased turgor pressure (Van Volkenburgh, 1999; Figure 5). This proposed system includes the action of cation channels, of which the gating allows a threshold hyperpolarisation to be reached before they open and facilitate uptake of cations, a property shown to exist for the KAT1 and KAT2 potassium channels in *Arabidopsis* (Pilot *et al.*, 2001). High turgor pressure might not only result in enhanced cell elongation by the increased extensive force, but a high turgor pressure will also make cells elongate more efficiently in response to cell wall loosening. In this way, high turgor pressure facilitates the action of other cell extension mechanisms (Figure 5). *R. palustris* petioles become more turgid upon submergence (Vreeburg and Benschop, unpublished results). However, the magnitude, timing and regulation of this submergence-induce increase in tissue turgor pressure is still under investigation. The discrepancy in kinetics of acidification and submergence-induced petiole elongation is based on the observation that a gradual decrease of apoplastic pH (Figure 4) will lead to a gradual increased extensibility (Figure 1). The presence of a constant external force in the experiments presented in figure 1,

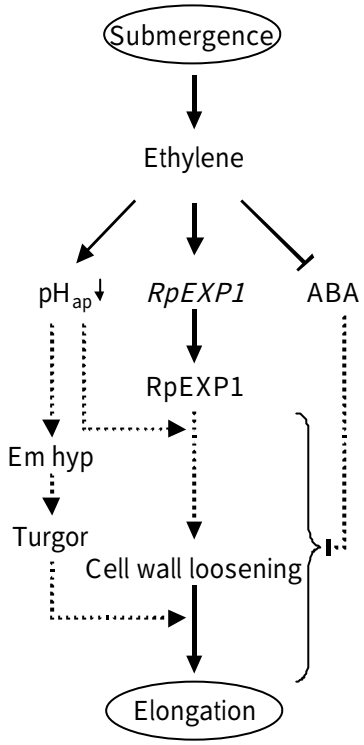

Figure 5. Partial signal transduction and response network positioning the putative roles of RpEXP1 and apoplastic pH (pH_{ap}) in the submergence-induced petiole elongation of *R. palustris*. Arrows represent positive regulation and bars represent negative regulation. Dashed lines are hypothetical interactions. Em hyp stands for plasma membrane hyperpolarization.

mimicking turgor, instead of an increasing turgor expected *in planta*, could thus also partially be responsible for the discrepancy.

Next to initiating a cell extension mechanism with a certain threshold, apoplastic acidification could also facilitate the action of cell wall loosening mechanisms. In this way, up-regulation of these cell wall loosening mechanisms will control the onset of submergence-induced petiole elongation in *R. palustris*. A good candidate for this cell wall loosening mechanism is the á-expansin RpEXP1. Up-regulation of *RpEXP1* transcript coincides closely with the onset of submergence-induced petiole elongation of *R. palustris* (Chapter 1). Since expansins are more active at low pH (McQueen-Mason *et al.*, 1992), submergence-induced acidification of the apoplast could create an environment in which the enhanced levels of RpEXP1, induced by submergence, can work more efficiently (Figure 5).

Acidification of the apoplast can also induce the activity of other cell wall modifying processes, which in turn could facilitate expansin action (Cosgrove and Durachko, 1994). The activity of many cell wall modifying enzymes, such as yieldins, endoglucanases, and xyloglucan endotrasglycosylase/hydrolases, alter cell wall properties in a pH dependent manner (Fry *et al.*, 1992; Nishitani and Tominaga, 1992; Okamoto-Nakazato *et al.*, 2000a; Xu *et al.*, 2000; Woolley *et al.*, 2001). Although the production of cell wall loosening hydroxyl radicals also depend on pH (Fry, 1998), preliminary results with hydroxyl radical scavengers indicate that the action of hydroxyl radicals is probably not involved in the submergence-induced elongation of *R. palustris* petioles (Vreeburg, unpublished results). Acidification of

the apoplast will also alter the ionic environment in the cell wall (Grignon and Sentenac, 1991), which will influence the properties of the pectic network in the cell wall (Ishii *et al.*, 1999), likely to effect expansin-induced extension activity (Cosgrove and Durachko, 1994).

Measurements of apoplastic pH with surface electrodes hold the risk that the pH measured is not the actual pH of the cell wall. By abrasion of the cuticle, a free diffusional pathway is created from the epidermal plasma membrane to the outer medium, allowing access to the apoplast and the possibility to manipulate the ionic composition of the cell wall compartment. Proton pumping by the proton-pumping ATPase will create a proton gradient, and since the position of the surface electrode in that gradient is not known, the pH readings of the electrode might not represent the pH in the cell wall. The observed decrease in pH upon submergence, however, still reflects an acidification of the apoplast, but the measurements might underestimate the degree of acidification in the cell wall.

This study showed that apoplast acidifies upon submergence, and the apoplastic pH is likely under control of ethylene (Table 1; Figure 5). The observed decrease in apoplastic pH was large enough to induce enhanced extension of freeze-thawed *R. palustris* petioles (Figure 1). Apoplastic acidification is a crucial element of underwater elongation (Figures 2, and 3). However, as the initiation of the acidification does not correlate well with the onset of enhanced elongation of submerged petioles, we suggest the rate of elongation under water is not a direct function of apoplastic pH. We propose that apoplastic acidification modifies cell wall properties such that the action of a cell wall loosening enzyme is facilitated (Figure 5). The prime candidate for this enzyme is RpEXP1, since up-regulation of *RpEXP1* transcript levels coincide very closely with the onset of submergence-induced petiole elongation (Chapter 1). The cell wall extension action of RpEXP1 will be directly stimulated by a lower pH, or indirectly by altered cell wall characteristics or enhanced turgor pressure (Figure 5).

Materials and methods

Plant growth

Rumex palustris plants were grown as described in chapter 1. All measurements were done on the 3rd petioles. Because the different techniques used in this study required different sizes of the 3rd petiole, we had to use plants of different ages. Plants were potted 9 or 10 days after sowing. For the extensometer assay we used plants 15 d after potting, and for the petiole elongation and the pH measurements we used plants 18 d after potting.

Extensometer assay

The extension of a 6 mm petiole segment was measured in a constant load extensometer with a pulling force relative to 30 gr. Petioles were first incubated in 50 mM succinate/1 M K_2HPO_4 buffer of pH 6.8 for 30 min, after which the buffer was changed to similar buffer with a lower pH. To ensure the initial buffer would not alter the pH of the second buffer, the well was rinsed once with the new buffer. The buffer-induced extension was calculated by determining the extension rate in a 10 min time frame, 1 min after the buffer change, subtracted with the extension rate of the petiole in a 10 min time frame, 1 min before the buffer change.

Petiole elongation measurements

Petiole elongation was measured with linear variable differential transformers as described by Voesenek *et al.* (2003b), adjusted with clamps to measure petiole elongation only. Petiole length was averaged every 5 min. To some plants, fusicoccin (Sigma-Aldrich, Zwijdrecht, The Netherlands) was applied as 10 µM in a 0.1% Tween 20 solution, to abraded petioles. Control petioles were also abraded and treated with a 0.1% Tween 20 solution.

In the petioles of some plants, 7 µl of 50 mM succinate buffer pH 4 or 6 was injected using a 10 µl syringe with a conical needle tip. The buffer was injected in air-grown petioles, or to petioles that were submerged for 4 h.

pH measurements

Apoplastic pH was measured with a surface pH electrode (MI-406, Microelectrodes Inc., Bedford, USA) in a custom made cuvet in which the 3rd petiole could be bathed in a 1 mM KCL solution while the root, leafblade and other leaves could be in non-submerged conditions. The pH was measured at the abaxial side of the petiole that was abraded with carborundum powder just before the start of the experiment. The petiole was bathed in a 1 mM KCl solution that bridged the circuit to the reference electrode (MI-401, Microelectrodes Inc., Bedford, USA). When submerged, the root and leaf compartment were filled with 1 mM KCl. Some plants were pre-treated with 3 µl l^{-1} 1-Methylcyclopropene (1-MCP, Ethylbloc, Floralife inc., Walterboro, USA) for at least 40 min. This treatment inhibited the submergence-induced hyponastic response (Cox *et al*., 2003) of plants treated in the same batch, indicating that ethylene perception was blocked (data not shown). Some plants were submerged in 20 µM ABA (Acros, 's-Hertogenbosch, The Netherlands, dissolved in 1 M KCl), by filling the root and leaf compartment and change the solution of the measuring compartment with the ABA solution. For submerged plants, the change in pH is plotted as the change relative to the pH at the time of submergence. Air-grown plants showed an initial increase in apoplastic pH when the measurements were started, followed by a decrease. The change in pH of air-grown petioles was plotted relative to the highest pH reached during the transient pH increase. This procedure will result in a maximum pH decrease in air-grown

petioles. Therefore, a larger submergence-induced acidification relative to the observed acidification in air-grown petioles will be more likely to indicate a significant difference.

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Chapter 6

General discussion

General discussion

Plants react in various ways on environmental stress conditions. The riverplain species *Rumex palustris* responds to submergence with an upward bending of petioles (hyponastic growth) and enhanced petiole elongation (Voesenek and Blom, 1989). Expansin expression is closely related to elongation in various systems (Cosgrove *et al.*, 2002). This close relationship of expansin expression and elongation, combined with the ability of expansins to induce cell wall loosening *in vitro* (McQueen-Mason *et al.*, 1992), make expansins a prime candidate for the enzyme controlling cell extension (Cosgrove, 2000a). Submergence-induced petiole elongation of *R. palustris* has been linked to expansin gene expression by Vriezen *et al.* (2000), with the aid of Northern blot analyses. At least 20 different expansin genes have now been cloned from *R. palustris*, all with high homology to each other (Vriezen *et al.*, 2000; Peeters *et al.*, unpublished results). For expansins to be involved in the cell wall loosening process leading to enhanced petiole elongation of submerged *R. palustris*, the kinetics of submergence-induced growth has to correspond to the kinetics of expansin up-regulation.

With gene-specific primer and probe combinations (Peeters *et al.*, unpublished results) we found that the up-regulation of *RpEXP1* coincided closely with the start of submergence-induced growth enhancement (Chapter 2). Acid-induced extension (AIE) of petiole segments measured with a constant load extensometer, also increased with the same kinetics upon submergence as the petiole elongation rate (Chapter 2). Since the acid-induced extension could be considered as a measure of expansin activity, the rise in AIE is likely caused by higher expansin abundance, most likely RpEXP1. Western blot analysis with an expansin antibody raised against a tomato expansin, revealed three protein bands that are, according to their size, likely to be expansins (Chapter 2). The protein sizes predicted from the full length cDNAs of *R. palustris* expansins can be arranged in three size groups corresponding with the sizes of the three bands observed on the blot. Only the 24 kD expansin band, containing e.g. RpEXP1, increased upon submergence, while the intensities of the 26 and 28 kD bands did not change relative to air-grown plants (Chapter 2). All the submergence-induced increases in expansin expression were, as the petiole elongation rate, reversed to air levels when submerged plants were de-submerged (Chapter 2). *RpEXP1* transcript expression was under direct control of ethylene, without a direct effect of the known down-stream hormones abscisic acid (ABA), gibberellic acid (GA), and auxins (Chapter 3). Since the kinetics of expansin (*RpEXP1*) transcript and activity up-regulation upon submergence coincides closely with submergence-induced elongation rates, we conclude that expansins, more specifically RpEXP1, is involved in the initiation of submergence-induced petiole elongation of *R. palustris*, and that the up-regulation is under direct control of ethylene (Figure 1).

The detailed growth analysis of submerged and air-grown petioles showed that after 12 to 13 h of submergence, the petiole elongation rates of submerged and air-grown plants were similar (Chapters 2 and 4). During this phase with similar elongation rates, expansin mRNA (*RpEXP1*), protein and activity levels were higher in submerged petioles than in air-grown petioles (Chapter 2). This led us to

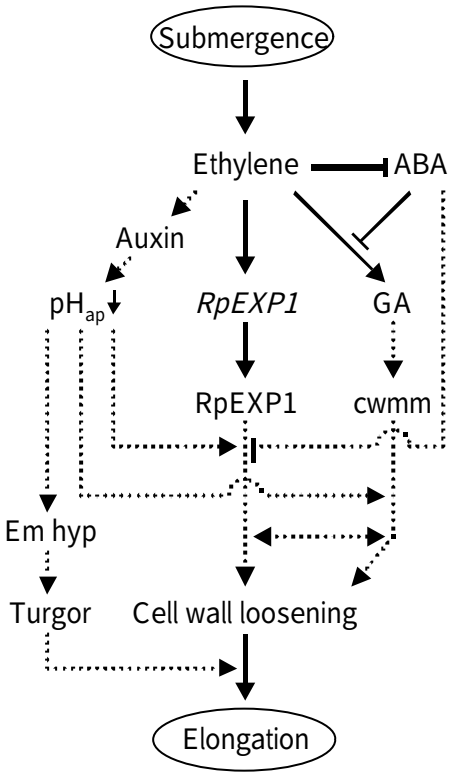

Figure 1. Partial signal transduction pathway and response network of the submergence-induced petiole elongation of *R. palustris*. pH_{ap} stands for apoplastic pH, Em hyp stands for plasma membrane hyperpolarisation, cwmm stands for cell wall modifying mechanism. Arrows represent positive regulation and bars represent negative regulation. Dashed lines are hypothetical interactions.

the conclusion that high expansins levels do not necessarily lead to enhanced elongation growth, and that other mechanisms might modulate expansin action. The involvement of cell elongation mechanisms other than expansins in the submergence-induced petiole elongation was also pointed out by the results in chapter 3. Hormonal treatments that influence the submergence-induced petiole elongation (e.g. GA, ABA and auxin), did not alter *RpEXP1* transcript abundance. Faster elongation rates are thus not necessarily related to high *RpEXP1* levels. These results do not exclude expansins as cell elongation regulatory factor, but they do indicate that expansins are not the sole cell expansion regulating mechanism. Expansins are likely to act in a concerted way with other cell wall modifying mechanisms. Some of these mechanisms have also shown, like expansins, to act as cell loosening factor on its own (Xu *et al.*, 2000; Okamoto-Nakazato *et al.*, 2000a; Kaku *et al.*, 2002; Schopfer, 2001). GA stimulates the submergence-induced petiole elongation, and a rise in endogenous GA concentration is observed after 4 to 6 h of submergence (Benschop and Bou, unpublished results). Inhibition of GA biosynthesis with paclobutrazol, nor GA addition showed an effect on *RpEXP1* transcript levels (Chapter 3). The role of GA in regulating the elongation response suggest that GA regulates a *RpEXP1* independent cell wall modifying mechanism (Figure 1), which is involved in maintaining fast elongation after the initial action of RpEXP1.

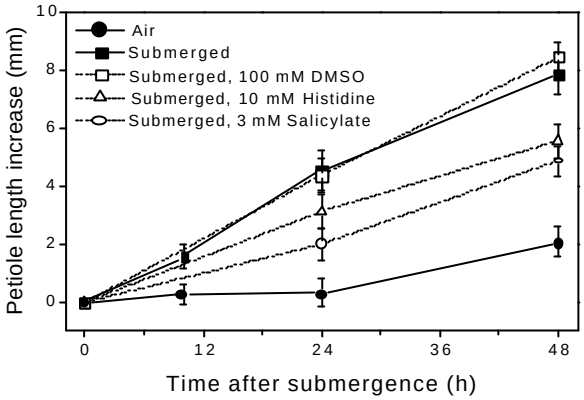

Figure 2. Petiole length increase of *R. palustris* 3rd petioles that are submerged (filled squares), kept in air (filled circles), submerged in 3 mM salicylate (open circles), submerged in 10 mM histidine (open triangles), or submerged in 100 mM dimethyl sulfoxide (DMSO; open squares). Mean ± se, n=8.

Other cell wall modifying mechanisms that might be involved in modulating submergence-induced petiole elongation and are under control of GA (Figure 1), are xyloglucan endotransglucosylase/hydroxylase (XTH), or cellulase activity. Cellulose microfibril re-orientation has also been suggested as growth regulating, GA stimulated mechanism (Sauter *et al.*, 1993). Growing tissue is often correlated with cellulose microfibrils in a transverse orientation, while cellulose microfibrils are often oriented longitudinally in tissue that stopped elongating. However, there is still a debate whether the microfibril orientation is a cause, or an effect of cell elongation (Fischer and Schopfer, 1998; Himmelspach and Nick, 2001). Production of cell wall loosening hydroxyl radicals has also been found to be related with elongation growth (Schopfer, 2001). When this mechanism is involved in the submergence-induce petiole elongation of *R. palustris*, scavenging hydroxyl radicals with biocompatible scavengers would reduce submergence-induced petiole elongation. Histidine, salicylate and dimethyl sulfoxide (DMSO) are examples of such biocompatible scavengers (Schopfer, 2001). Submergence-induced petiole elongation was inhibited by histidine and salicylate, but not by DMSO (Figure 2). DMSO is a potent hydroxyl radical scavenger when tested *in vitro* (Fry, 1998). Although the results are not fully conclusive, the lack of inhibition by DMSO led us to hypothesize that hydroxyl radical production is probably not involved in the submergence-induced petiole elongation. The inhibitory effect of histidine and salicylate might be caused by the pH buffering capacity of these solutions, or interference with plant metabolism. The action of the above stated cell wall modifying mechanisms could interact and modulate each others cell wall extension activity (Cosgrove and Durachko, 1994; Figure 1)

The activity of expansins and of many other cell wall loosening activities, are regulated by the pH of their environment. Apoplastic pH is a good candidate for modulating expansin action, and for activating other cell wall loosening mechanisms. Submergence induced an ethylene dependent decrease in pH (Chapter 5). The timing of this submergence-induced pH decrease, however, did not coincide with growth kinetics of submerged petioles. We therefore concluded that the submergence-induced apoplastic pH decrease is not the key factor determining submergence-induce petiole elongation, but might be involved in facilitating the action of other processes, such as the activity of expansins (Figure 1).

Enhanced proton excretion from the symplast into the apoplast will result in a hyperpolarisation of the plasma membrane potential. The hyperpolarisation might result in uptake of cations, such as potassium, and increase turgor pressure (Figure 1). High turgor pressure will lead to an increased extensive force acting on the cell wall and will facilitate cell extension when cell walls are loosened (Figure 1). *R. palustris* petioles become more turgid upon submergence (data not shown). Not only does a higher turgor pressure facilitates cell expansion, it also gives the petiole more stiffness. Submerged *R. palustris* show, next to enhanced petiole elongation, also an upward bending of the petiole (hyponastic growth; Banga *et al.*, 1997; Cox *et al.*, 2003). Petiole stiffness is required for the effectiveness of the hyponastic growth. When the petiole is not stiff enough, hyponastic growth at the base of the petiole, will not result in a vertical orientation of the leaf blade.

Submergence of *R. palustris* plants with ABA added to the submergence water, inhibited petiole elongation (Benschop *et al.*, unpublished results). Endogenous ABA levels decreased rapidly upon submergence (Benschop *et al.*, unpublished results), indicating that the inhibitory effect of ABA is released upon submergence. Since apoplastic pH can reversibly modulate enzyme activity, we hypothesised that ABA might inhibit submergence-induced apoplastic acidification. However, no inhibition of submergence-induced apoplastic acidification was observed (Chapter 5). ABA inhibits the synthesis of GA (Benschop *et al.*, unpublished results) and will inhibit petiole elongation by preventing up-regulation of GA stimulated extension mechanisms (Figure 1). However, ABA also inhibited growth before a rise in GA concentrations was detected (Benschop *et al.*, unpublished results), ABA therefore also interferes with an extension mechanism early in the submergence-induced petiole elongation. We propose that *RpEXP1* is involved in the initiation of submergence-induced petiole elongation (see above). Since ABA showed no effect on *RpEXP1* transcript abundance, we hypothesize that ABA interferes with the action of the RpEXP1 protein (Figure 1).

Submerged *R. palustris* petioles show, next to enhanced petiole elongation, also an upward bending of the petiole (hyponastic growth; Banga *et al.*, 1997; Cox *et al.*, 2003; Chapter 4). Hyponastic growth in submerged *R. palustris* petioles is located at the base of the petiole (Cox *et al.*, unpublished results; Chapter 4). Cox *et al.* (2003) observed that submergence-induced petiole elongation was severely inhibited when petioles were kept at an angle below 40 to 50 degrees relative to the horizontal plane. They proposed a model that submergence-induced petiole elongation would only start when the petiole angle reaches the ‘critical angle’ of 40 to 50 degrees. Petiole angle of submerged plants is thus a determinant for petiole elongation. Where the angle signal might be integrated in the signal transduction pathway leading to enhanced petiole elongation (Figure 1) is still unresolved, but it might be at the level of *RpEXP1* transcription. Restriction of the hyponastic growth response result in an upward bending distributed over the whole petiole (Chapter 4). The asymmetric elongation underlying the upward bending is related to higher AIE at the abaxial side of the petiole and reduced AIE at the adaxial side (Chapter 4). The enhanced AIE of the abaxial angular collenchyma strands is related to enhanced expression of *RpEXP1* and *RpEXP8* transcripts of the whole petiole (Chapter 4).

Synthesis

We propose a model (Figure 1) in which submergence leads, via ethylene, to an enhanced abundance of RpEXP1. The cell wall loosening action of RpEXP1 is likely to be regulating the initiation of the submergence-induced petiole elongation of *R. palustris*. Submergence of *R. palustris* petioles also result in an ethylene dependent acidification of the apoplast, which might involve the action of auxin. Acidification of the apoplast is likely to facilitate the action of expansins in the cell wall. Proton excretion to the apoplast will also result in a hyperpolarisation of the cell membrane potential and might result in higher cell turgor pressure. The increased cell extensive force is likely to facilitate cell extension when cell walls are loosened. The GA dependency of submergence-induced cell elongation is not regulated via RpEXP1 expression. We propose that GA induces an alternative cell wall loosening mechanism, such as cellulase, XTHs or pectin modifying enzymes. We can not rule out that expansin action and the GA-induced cell wall modifying mechanism interact and influence each others activity. ABA inhibits growth via inhibiting GA biosynthesis, but also via a GA-independent route. This alternative inhibitory effect might be by inhibition of RpEXP1 action.

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Nederlandse samenvatting

Moeraszuring (*Rumex palustris*) is een plantensoort die groeit in de uiterwaarden van de Nederlandse rivieren. Als de plant bij hoog water onder water komt te staan, vertoont deze een opmerkelijke respons. Allereerst klappen de petiolen en bladeren omhoog (hyponastische groei), gevolgd door versnelde strekkingsgroei van de petiolen. Het opklappen en versneld groeien van de petiolen kunnen ervoor zorgen dat de bladeren deels boven het wateroppervlak komen en gaswisseling met de lucht kunnen herstellen.

Om sneller te kunnen groeien, moeten de cellen in de petiool hun celwand verweken, zonder dat ze hun stevigheid verliezen. Een eiwit wat daar een grote rol in zou kunnen spelen is expansine. Expansines kunnen plantenweefsels uit laten rekken in een artificieel systeem en worden vaak aangetoond in strekkend weefsel. Vriezen *et al.* (2000) had al met Northern blots een verhoging in expansine RNA aangetoond. Dit proefschrift bouwt voort op dit werk en beschrijft een gedetaileerde studie naar de rol van expansines in de versnelde groei van petiolen van overstroomde moeraszuring.

Er zijn tenminste 20 expansine genen in moeraszuring. We hebben van vijf expansines de mRNA expressie bestudeerd. Slechts van één van deze expansines nam de hoeveelheid mRNA in petiolen toe als moeraszuring onder water werd gezet, en wel van *RpEXP1* (*Rumex palustris* expansine 1). Naast meer *RpEXP1* mRNA, werd bij overstroming ook meer expansine eiwit aangemaakt, van dezelfde grootte als verwacht voor RpEXP1. De expansine activiteit gemeten in de petiolen nam ook toe als de planten onder water werden gezet. Omdat de toename van zowel *RpEXP1* mRNA als expansine activiteit correleerden met de initiatie van de versnelde groei, nemen we aan dat RpEXP1 betrokken is bij de initiatie van de overstromings geïnduceerde groei van de petiolen van moeraszuring. Echter, er waren ook periodes dat de petiolen van overstroomde planten niet sneller strekten, terwijl er wel meer expansines aanwezig waren. Tezamen met de observatie dat versnelde strekking geïnduceerd kon worden met hormonen, zonder een toename van RpEXP1, leidde tot de conclusie dat meer factoren de strekkingsgroei van petiolen bepalen.

Een primair signaal in de signaaltransductieketen van overstromings geïnduceerde strekkingsgroei is het gasvormige hormoon ethyleen. Ethyleen induceert een signaleringsketen die de actie van andere hormonen, zoals gibberellinezuur en abscisinezuur, omvat. Echter, geen van de plantenhormonen waarvan bekend is dat ze een effect hebben op de strekkingsgroei van moeraszuring anders dan ethyleen, heeft een duidelijk effect op *RpEXP1* mRNA niveau's. De expressie van *RpEXP1* lijkt dus onder directe regulatie van ethyleen te staan.

De strekkingsactiviteit van expansines wordt gereguleerd door de zuurgraad van zijn omgeving. De pH van de celwand van petiolen daalt na overstroming, kan worden geremd door de ethyleen antagonist 1-MCP. De verzuring van de celwand start eerder dan de versnelde strekking van overstroomde petiolen. We nemen daarom aan dat de ethyleen afhankelijke verzuring van de celwand na overstroming geen direct regulerende factor is, maar dat de verzuring wel van

wezenlijk belang is voor de celstrekking na overstroming. Dit omdat de activiteit van expansines en andere celwand eiwitten hoger is bij een lage pH.

Als petiolen van moeraszuring worden gefixeerd zodat ze niet omhoog kunnen klappen bij overstroming, gaan de petiolen minder hard groeien. Of dit komt doordat geen RpEXP1 wordt aangemaakt is nog onbekend. Proeven hebben wel uitgewezen dat de interactie tussen de hoek van de petiool met de horizontaal en de versnelde groei erg complex is. Als de hoek van de petiolen lager dan de oorspronkelijke hoek wordt gehouden, vertonen de petiolen wel een versnelde groei bij overstroming. Deze groei wordt waarschijnlijk veroorzaakt door het aanmaken van meer expansines aan de onderzijde van de petiool.

De resultaten van deze studie naar de rol van expansines in de overstromings-geïnduceerde strekking van petiolen in moeraszuring, wijzen op een rol voor RpEXP1 in de initiatie van de strekkingsgroei. De expressie van *RpEXP1* staat onder controle van ethyleen, een hormoon dat in het begin van de signaaltransductieketen staat. Naast een opregulatie van *RpEXP1* is ethyleen ook betrokken bij de verzuring van de celwand welke optreedt na overstroming. Deze verzuring van de celwand verhoogt de activiteit van expansines en andere celwand eitwitten welke waarschijnlijk betrokken zijn bij de overstromings-geïnduceerde strekkingsgroei van moeraszuring petiolen.

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Robert

Curriculum vitae

Robert Vreeburg was born on the 7th of August 1976. He started his study biology in 1994 at Utrecht University. He specialised himself in botany, with research projects studying the amino acid uptake in roothairs in the former group of Transport Physiology at Utrecht University, the uptake of herbicides by willows at the former Long Ashton Research Station, and a molecular specialization in the group of Molecular Plant Physiology at Utrecht University. He received his degree in Biology in 1999, after which he directly started his Ph.D. in the Ecophysiology of Plants research group, under supervision of Prof. Dr. L.A.C.J. Voesenek. The aim of the project was to study the role of the cell wall loosening enzyme expansin in the submergence-induced petiole elongation of *Rumex palustris*. During his Ph.D., he collaborated with the group of Prof. Dr. S.J. McQueen-Mason from the University of York and the group of Prof. Dr. J.T.M. Elzenga from the Universtiy of Groningen. The results of his project are presented in this thesis and were also presented at various congresses as poster or oral presentation.

In December 2003, after handing in his thesis to the thesis committee, he started as a post doc in the group of Prof. Dr. S.C. Fry at the University of Edinburgh. In this project he will study the role of hydroxyl radicals in cell wall disassembly during fruit ripening.

Song

Though veiled in spires of myrtle wreath,
Love is a sword that cuts its sheath.
And through the clefts, itself has made,
We spy the flashes of the blade!

But through the clefts itself has made,
We likewise see love's flashing blade.
By rust consumed or snapt in twain:
And only hilt and stump remain.

"Work without hope" S.T. Coleridge