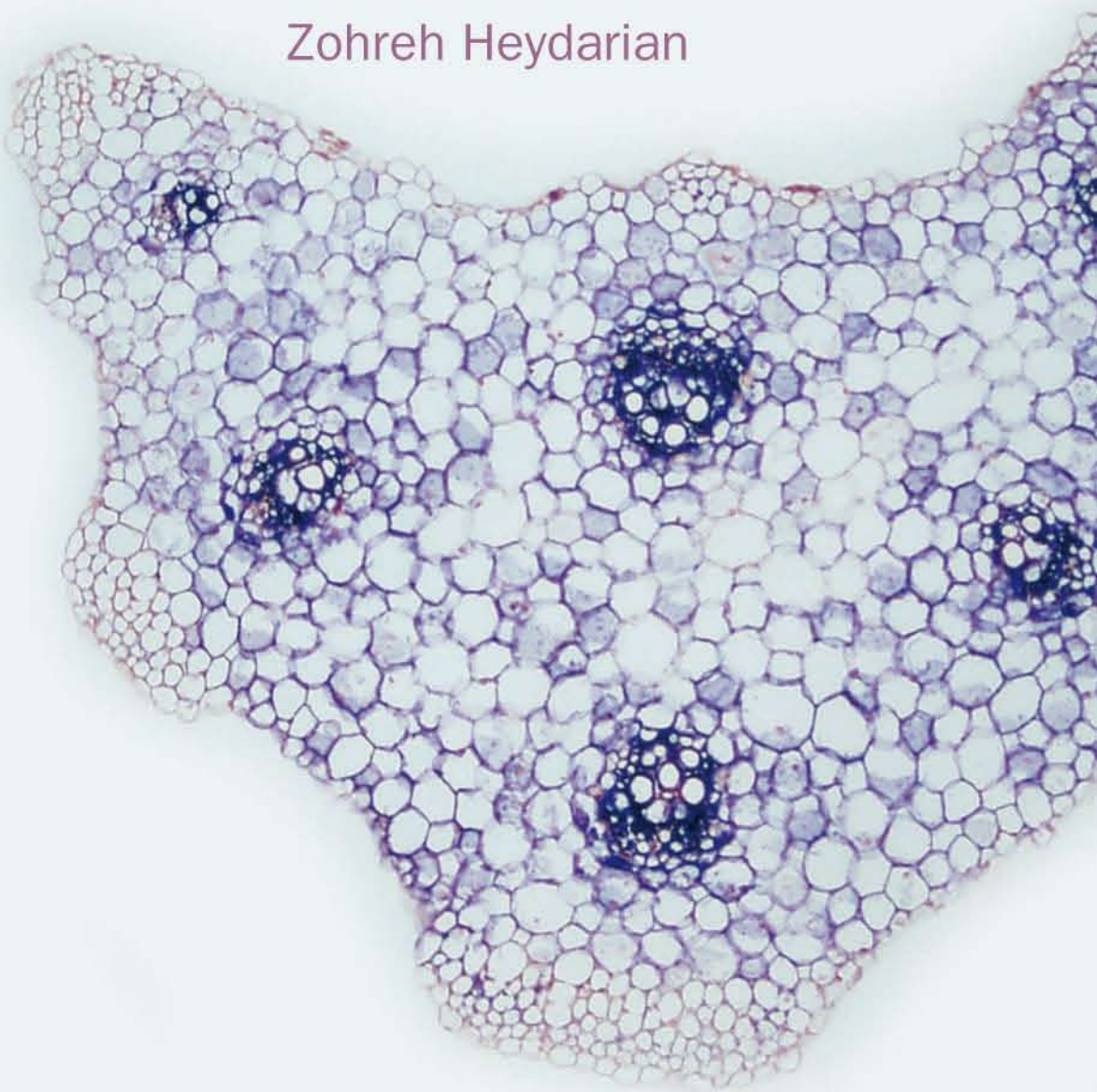
Transcriptome and kinome analysis during ethylene-induced growth in *Rumex palustris*

Zohreh Heydarian



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Transcriptome and kinome analysis during ethylene-induced growth in *Rumex palustris*

Genexpressie en fosforylering analyse gedurende door ethyleen geïnduceerde groei in *Rumex palustris*

(met een samenvatting in het Nederlands)

Proefschrift

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Thesis content

Chapter 1 **General introduction**

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Strategy of plants to survive flooding

Due to their sessile lifestyle plants are continuously threatened by biotic attackers and abiotic disturbances. Since they cannot escape their environment they have to adjust to changes appropriately to improve their chances for survival. Adaptations enable all living organisms, including plants, to cope with environmental stress. A biological adaptation can be an anatomical structure, a physiological process or a behavioral trait that has evolved over a period of time. In this research we study phenotypic plasticity, the ability of organisms to change their phenotype in response to changes in the environment.

One of the abiotic stresses that plants may encounter is flooding. Flooding can be defined as any situation with excess of water. Flooding belongs to the most important and damaging environmental stresses world wide. It has a dramatic effect on growth and yield of many plants, but especially on crop plants since most of these economically important species are very intolerant to flooding. World wide, approximately 10% of all farmland has been estimated to suffer from excess of water, decreasing crop yield by 20% (Voesenek *et al.*, 2006 and references therein). In the United States for instance, flooding damage accounts for 50 billion dollars crop loss over the past 25 years (Mittler, 2006). Upon flooding, plant growth is arrested immediately, senescence processes are switched on and most species die within a few days. However, some species have the capacity to tolerate unfavourable flooding conditions. In particular, species originating from semi-aquatic environments have the capacity to cope with flooding stress. They can survive complete submergence for weeks and some even have the capacity to grow vigorously and produce flowers and seeds in permanently water-saturated soils. A broad range of metabolic and morphological adaptations characterise these species. Flood-tolerant plants have developed the capacity to generate ATP without oxygen (continuation of glycolysis) and/or developed specific morphological traits (e.g. air channels, enhanced shoot elongation) that improve or restore the entrance of oxygen (Armstrong *et al.*, 1994; Colmer, 2003; Crawford, 1992; Drew *et al.*, 2000; Gibbs and Greenway, 2003; Jackson, 1985; Perata and Alpi, 1993; Sauter, 2000; Voesenek *et al.*, 2006). Some plant species have adapted to flooding and are able to survive flooding events by a 'quiescence' strategy (Voesenek *et al.*, 2006 and references therein), characterised by a reduction of redundant metabolic processes to limit the use of resources until the water retreats. Alternatively, some species exhibit an 'escape' strategy, shown as enhanced growth to restore gas exchange (Perata and Voesenek, 2007; Sauter, 2000; Voesenek and Blom, 1999).

Species from semi-aquatic environments are ideal to study phenotypic plasticity with respect to flooding tolerance and to find the morphological, physiological and underlying molecular mechanisms they have evolved to cope with flooding stress. It is evident that a better understanding of mechanisms that result in flooding tolerance will eventually lead to improved crops.

Hyponastic growth and shoot elongation

When plants are completely submerged, the rate of gas diffusion is decreased approximately 10,000 times compared to the situation in air (Armstrong, 1979). Together with the consumption of remaining oxygen in the plant this results in a net decrease of oxygen in many plant organs. A decrease from 21 to 3–10 kPa has been observed *in situ* in submerged petioles (Rijnders *et al.*, 2000). The concentration of the gaseous plant hormone ethylene increases due to physical entrapment. Furthermore, assuming that the experiment is performed in a normal day–night rhythm, the CO_2 concentration will vary accordingly (Voesenek and Blom, 1999). Sensing these changes in gas levels and subsequent induction of signal transduction cascades ultimately leads to adaptive responses.

The semi-aquatic plant *Rumex palustris* is able to survive complete submergence through a highly coordinated enhancement in upward growth of shoot organs, i.e. petioles. This growth response restores contact of the shoot with the atmosphere allowing the plant to resume aerobic metabolic activity needed for long term survival. In this way, the shoot functions as a 'snorkel' that facilitates the inward diffusion of oxygen. Enhanced petiole elongation in *R. palustris* is accomplished by two visibly distinguishable phases; the first being enhanced differential growth of the petiole to establish a lift of the leaf, resulting in a more upright position (hyponastic growth). The second phase is the enhanced growth of the entire petiole (and to a much lesser extend of the leaf blade) to bring the leaf tip above the water level, and thus restoring gas exchange (Voesenek *et al.*, 2006).

Hyponastic growth upon submergence was shown by Cox *et al.* (2004) to be the result of localised differential growth. In the basal region of the petiole, cells at the abaxial (lower) surface of plants submerged for 6 h were more elongated compared to cells of air-grown plants. Furthermore, there was no difference in cell length between these two treatments at the basal adaxial (upper) side. This observed differential growth was restricted to the basal region of the petiole; no significant differences in cell length were observed in the more apical regions (Cox *et al.*, 2004). The increase of cell length at the abaxial side of the petiole was shown to account for the observed change in angle (Cox *et al.*, 2004).

Cox *et al.* (2003) showed that the start of enhanced petiole growth, that is the non-differential vigorous growth of the petiole to reach the water surface, was dependent on the angle of the petiole. A minimal angle of 40-50 degrees was a prerequisite for induction of enhanced petiole growth (Cox *et al.*, 2003).

Signal transduction; role of ethylene and other plant hormones

As mentioned, oxygen, carbon dioxide and ethylene are gases that in submerged plants can change dramatically in concentration and are therefore potential sensors of submergence. Since the internal concentrations of O_2 and CO_2 are strongly influenced by both photosynthesis and respiration these are considered to

be not the most reliable signals, whereas ethylene on the other hand is considered as a far more reliable indicator (Voesenek and Blom, 1999).

Biosynthesis of ethylene in plants is well-characterised (Wang *et al.*, 2002) and depends on the presence of (some) oxygen (Kende, 1993). The production rate of ethylene during submergence is maintained at the same level in *R. palustris* (Voesenek *et al.*, 1993; Vriezen *et al.*, 1999), or, as was shown in the internode tissue of deepwater rice (Kende *et al.*, 1998), increased. Within the first hour upon submergence the level of ethylene increases 20 fold, up to approximately 1 $\mu L\ L^{-1}$ as a result of physical entrapment (Banga *et al.*, 1996; Voesenek *et al.*, 1993).

In flooding-tolerant Rumex species, a precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid concentration (ACC), strongly increased upon submergence, whereas the conversion to ethylene was inhibited. Also the expressions of ACC synthase genes, and the resulting enzyme activity, were significantly increased (Rieu *et al.*, 2005; Voesenek *et al.*, 2003a; 2003b; Vriezen *et al.*, 1999). The next step in ethylene biosynthesis involves ACC oxidases. These genes are strongly upregulated in *R. palustris* during submergence and this correlates well with an enhanced enzyme activity (*in vitro* assay) (Voesenek *et al.*, 2003a; 2003b; Vriezen *et al.*, 1999). Since the ACC oxidase step is oxygen dependent, the increased concentration of the ACC oxidase enzyme during submergence possibly counterbalances the reduced enzyme activity at lower O_2 concentrations, thus supporting ethylene production during submergence (Vriezen *et al.*, 1999).

In *Arabidopsis thaliana*, ethylene is perceived by a family of five receptors that possess sequence similarity with bacterial two-component histidine kinases. Using genetic techniques, their mode of action and the subsequent signal-transduction cascade have been characterised (Alonso and Stepanova, 2004; Chang, 2003; Guo and Ecker, 2004; Stepanova and Alonso, 2005). Putative orthologues of the receptors have been found in many plant species, including rice, carnation, peach, tomato (for a review see Stepanova and Alonso, 2005) and *R. palustris*. The *R. palustris* orthologue of the ethylene response sensor (*RpERS*) ethylene receptor gene was shown to be strongly upregulated upon submergence. Since ethylene receptors suppress downstream ethylene responses when no ethylene is bound, this results in a desensitisation of the tissue for ethylene (Vriezen *et al.*, 1997). This has probably no effect on the ethylene-induced responses, as ethylene concentrations were found to be saturated in the shoots of submerged plants (Banga *et al.*, 1996; Voesenek *et al.*, 1993).

Beside ethylene it was shown that abscisic acid (ABA), gibberellic acid (GA) and auxin play a role during the growth responses upon submergence. The increase in ethylene concentration causes a fast decrease in the ratio between two other plant hormones: ABA and GA (Benschop *et al.*, 2005; Cox *et al.*, 2004). In both deepwater rice and *R. palustris* a strong and rapid reduction of the endogenous ABA concentration was observed (Benschop *et al.*, 2005; Hoffmann-Benning and Kende, 1992). In *R. palustris* this decline is brought about by a downregulation of

several 9-cis-epoxycarotenoid dioxygenases (NCED), enzymes involved in a crucial step in ABA biosynthesis, and an enhancement of ABA catabolism (Benschop *et al.*, 2005).

The importance of this step was also shown by experiments using fluridone, an ABA biosynthesis inhibitor (Benschop *et al.*, 2005). Pretreatment with this chemical reduced the lag phase of submergence-induced petiole elongation in *R. palustris* by 50%, resulting in a very rapid start of the elongation response. Underwater elongation growth, however, was completely abolished when fluridone was applied in combination with a 1-MCP pretreatment (inhibitor of ethylene action). This indicates that a reduction of ABA by itself is insufficient to induce fast petiole elongation; the presence of elevated concentrations of ethylene is required (Benschop *et al.*, 2005). It is clear that in both *R. palustris* and deepwater rice, ABA acts as a negative regulator of underwater elongation. This raises the question as to how this is regulated at the physiological level. The decline of ABA is accompanied by an increase of GA (Cox *et al.*, 2004; Raskin and Kende, 1984; Rijnders *et al.*, 1997). Indications that ABA directly interferes with GA biosynthesis came from data obtained by Benschop and co-workers (2005). When submerged *R. palustris* plants were additionally treated with 10 μM ABA, no increase was observed in the endogenous GA1 concentration whereas this was evident for the submerged controls. Therefore, ABA probably inhibits the upregulation of the growth-promoting hormone GA (Benschop *et al.*, 2005). Recent evidence for this was found by Benschop *et al.* (2006) who showed that the GA biosynthesis genes *RpGA20ox1* and *RpGA3ox1* are actively downregulated by the presence of ABA while the catabolic gene *RpGA2ox1* is initially not affected (after 4 h) but is upregulated after 8 h of submergence compared to the submerged situation without ABA application.

Cell elongation

Not all petioles within one *R. palustris* plant are equally responsive, as was demonstrated in a study by Groeneveld and Voesenek (2003). In this study, both the very young and the oldest petioles possess only a very minimal competence to elongate, whereas intermediately aged petioles can potentially elongate up to 300% of their initial length, measured over 10 d of submergence. Differentiation in responsiveness was also described for internode elongation in deepwater rice where only the youngest internodes respond to submergence (Sauter, 2000). Shoot elongation under water requires energy and carbohydrates for the synthesis of new cell wall material for cell divisions and cell elongation. Evidence for these costs of elongation growth came from work on rice in which elongation under water without reaching the water surface occurs at the expense of survival (Setter and Laureles, 1996).

In *R. palustris*, petiole elongation is primarily accomplished by cell enlargement. The plant cell wall is a fibrous structure whose properties, besides other factors,

determine the form and the size of plant cells. Cell elongation in general is a complex process that involves changes in pH caused by auxin, changes in turgor of cells, GA, and enzymes that are able to loosen the cell wall, such as expansins (Cosgrove, 1999; McQueen-Mason *et al.*, 1992; Vreeburg *et al.*, 2005), xyloglucan endotransglycosylase/hydrolase (XTH) (Fry *et al.*, 1992) and endo-ß-1,4-D-glucanase (Xu *et al.*, 2000). The acid growth theory (Kutschera, 2001; Rayle and Cleland, 1992) states that within 10 min after auxin addition to cells, the walls are acidified and cell wall loosening and the onset of elongation occur. Acid-induced cell wall extension involving expansins has been observed in submerged *R. palustris* and deep water rice, but whether this involves auxin is not yet clear (Cho and Kende, 1997; Vreeburg *et al.*, 2005). As mentioned before expansins play a role during the stimulated elongation process in submerged *R. palustris*. Expansins A from *R. palustris* were isolated by Vriezen *et al.* (2000) and Colmer *et al.* (2004). Studies examining the transcription dynamics of *RpEXPA1* have shown that the expression of this gene is upregulated in the petioles of *R. palustris* upon submergence or treatment with 5 $\mu L\ L^{-1}$ ethylene (Cox *et al.*, 2004; Vreeburg *et al.*, 2005; Vriezen *et al.*, 2000). The observed increase of this messenger, and more significant, of the resulting protein *RpEXPA1*, coincides with the increased elongation rate of the petioles during submergence (Cox *et al.*, 2004; Vreeburg *et al.*, 2005; Vriezen *et al.*, 2000).

Outline of this Thesis

Flooding-induced shoot elongation that has been described for *R. palustris*, was also shown for other plant species, e.g. *Ranunculus repens, Caltha palustris, Leontodon taraxacoides* and *Paspalum dilatatum* and (Grimoldi *et al.*, 1999; Insausti *et al.*, 2001; Ridge, 1987; Voesenek and Blom, 1989). Moreover, hyponastic growth responses are also described in response to other environmental cues, such as shading and high temperature (Ballaré, 1999; Millenaar *et al.*, 2005; Pierik *et al.*, 2004; Pierik *et al.*, 2005). Since ethylene is considered the driving force behind the submergence response the question arises whether externally applied ethylene by itself, without the "specific submergence" factors potentially influencing the response, is capable of evoking an identical response. The work described in this thesis deals with the comparison of the hyponastic and petiole elongation response in *Rumex palustris* upon ethylene and submergence. Moreover, several methods will be applied to isolate, identify and (functionally) characterise differentially expressed genes possibly involved in either ethylene-induced hyponastic growth, enhanced petiole elongation or both.

In Chapter 2 the physiological description of the experimental system, *R. palustris* plants treated with ethylene is described. A comparison is made between plants treated with ethylene and those submerged. This chapter serves as the physiological basis of the rest of the thesis. Based on this descriptive study, time points for isolation of differentially expressed genes and expression kinetic studies were selected.

Chapter 3 has its focus on the isolation and identification of genes differentially expressed in petioles during different treatments and at different time points. The results of subtraction, cDNA-AFLP and micro-array analyses will be discussed.

In Chapter 4 we show the detailed expression kinetics of several *R. palustris* genes during ethylene- and submergence-induced hyponastic and enhanced petiole growth. Besides the temporal expression also the spatial localisation of some of these genes is examined. Furthermore, we show that some orthologous Arabidopsis genes originally isolated as differentially expressed in ethylene treated *R. palustris* petioles, have a function in Arabidopsis associated with ethylene-induced hyponasty.

In Chapter 5 we present and discuss changes in the kinome during ethylene-induced hyponasty and elongation growth and link these to the differentially expressed genes of Chapter 3. Relevant pathways involved in the responses will be discussed.

Chapter 6 integrates the main results and conclusions from the Chapters 2-5 and formulates general conclusions.

Chapter 2

The kinetics of hyponastic growth and petiole elongation upon ethylene treatment in *Rumex palustris.*

Zohreh Heydarian
Marjolein C.H. Cox
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Abstract

Complete submergence is an important stress factor for many terrestrial plant species. A limited number of species have evolved mechanisms to deal with these conditions. *Rumex palustris* is among these species and manages to outgrow the water and thus restore contact with the atmosphere, through upward leaf growth (hyponasty) followed by strongly enhanced petiole elongation. These responses are initiated by the gaseous plant hormone ethylene which accumulates inside plants due to physical entrapment. Here we show that hyponastic growth and petiole elongation responses to submergence can be mimicked by exposing plants to ethylene. However, ethylene induces a putative increase of elongation of the 20-80 most basal abaxial epidermal cells of a petiole, whereas during submergence this differential growth occurs in the 40 most basal epidermal cells. We further show here that the petiole elongation response to ethylene depends on the initial angle of the petiole. When petiole angles were artificially kept at 0 degrees, rather than the natural angle of 35 degrees, ethylene could not induce enhanced petiole elongation. This is very similar to submergence studies and confirms the idea that there are endogenous, angle-dependent signals that influence the petiole elongation response to ethylene. Our data suggest that hyponastic growth and enhanced petiole elongation responses of *Rumex palustris* in response to submergence and ethylene treatment are largely similar. However, there are some differences that may relate to the complexity of the submergence treatment as compared to an ethylene treatment.

Introduction

Plants encounter various environmental stresses, both biotic and abiotic. Acclimation to these stresses enable plants to survive during unfavourable conditions. Submergence is a severe abiotic stress (Peeters *et al.*, 2002) that has negative effects on growth and survival of most terrestrial plants, especially when it occurs during the growing season (Blom and Voesenek, 1996; Colmer *et al.*, 1998). A consequence of plant submergence is limitation of gas diffusion, which is about 10,000 times slower in water compared to air. This causes dramatic changes in O_2, CO_2 and ethylene concentrations in plant tissues (Armstrong, 1979; Rijnders *et al.*, 2000; Voesenek and Blom, 1989). There is much variation between plant species in tolerance to submerged conditions; some species, such as *Rumex acetosa* and *Lycopersicon esculentum* (Jackson and Campbell, 1975) are sensitive, whereas others such as *Rumex palustris* and *Oryza sativa* are tolerant (Voesenek and Blom, 1999; Voesenek *et al.*, 2004; Vriezen *et al.*, 2003). Flood-tolerant species show acclimations in their morphology, anatomy and metabolism in response to flooding (Jackson and Armstrong, 1999; Peeters *et al.*, 2002) and these have been subject of extensive research for more than 25 years (Blom and Voesenek, 1996). *R. palustris* belongs to the Polygonaceae family, and grows in flood-prone areas. One of the acclimations of *R. palustris* to submergence is a change in orientation of petioles from approximately 35 degrees to almost vertical (Voesenek and Blom, 1989). This adaptive growth response is called hyponastic (upward) growth. Subsequently, the elongation rate of entire petioles is enhanced. The combination of these responses together bring leaf blades above the water surface which allows the plant to restore gas exchange with the atmosphere (Cox *et al.*, 2003; Groeneveld and Voesenek, 2003; Peeters *et al.*, 2002; Voesenek and Blom, 1989).

Elongation growth at the abaxial petiole side or inhibition of growth at the adaxial side forces the leaf into a more upright position and both are called hyponastic growth (Kang, 1979). For *R. palustris* it was shown that hyponastic growth of the petioles is a prerequisite for subsequent submergence-induced elongation of the entire petiole (Cox *et al.*, 2003). The minimal petiole angle that has to be reached before petiole elongation starts is approximately 50 degrees (Cox *et al.*, 2003).

Recently, the effects of submergence on *R. palustris* were studied by Benschop *et al.* (2005; 2006), Cox *et al.* (2003; 2004; 2006) and Vreeburg *et al.* (2005). These authors showed that various plant hormones play a role at several stages of hyponastic growth and petiole elongation and their studies confirmed that ethylene is a key plant hormone in these processes.

Previous studies with *R. palustris* have shown that ethylene accumulates inside plant tissues to drastically elevated levels during submergence (Banga *et al.,* 1996). Furthermore, the growth responses induced by submergence can largely be mimicked by exogenous ethylene. However, pre-treatment with a specific inhibitor of ethylene action, 1-Methylcyclopropene (1-MCP), that completely inhibits

ethylene-induced petiole elongation in *R. palustris*, could not completely abolish the hyponastic response in submerged plants (Cox *et al,*. 2004). This result suggests that next to ethylene other factors contribute to submergence-induced hyponastic growth.

In this study, we investigate to what extent ethylene-induced hyponastic growth and petiole elongation in *R. palustris* are identical to the responses demonstrated during submergence.

Results

Submergence-induced hyponastic growth and petiole elongation are largely mimicked by ethylene

Exogenous application of 5 µL L^{-1} ethylene to 27 days old *R. palustris* plants with an initial angle of 35-40 degrees of the third petiole phenotypically mimicked the response to submergence and induced hyponastic growth and petiole elongation (Figure 1). Ethylene application resulted in a lag phase for angle change of 2.3 h (Figure 2, Table 1). Submergence treatment resulted in a slightly shorter lag phase of 1.8 h. The final angle for both ethylene application and submergence was identical and reached a maximum of 75-80 degrees. This maximum angle was reached within almost 5-6 h in both treatments. Enhanced petiole elongation as induced

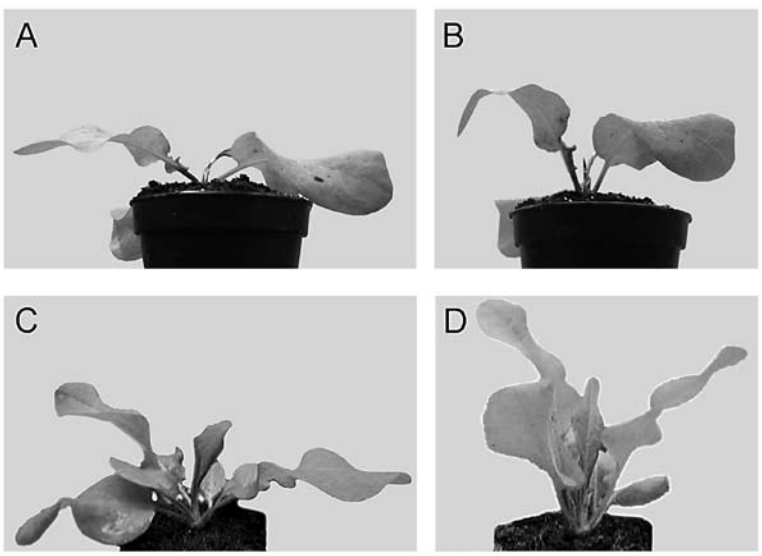

Figure 1. Hyponastic growth in *Rumex palustris*. Plants were exposed to air control conditions (A,C), 6 h of ethylene (B) and 6 h of submergence (D). Data in C and D are from Cox *et al.* (2003).

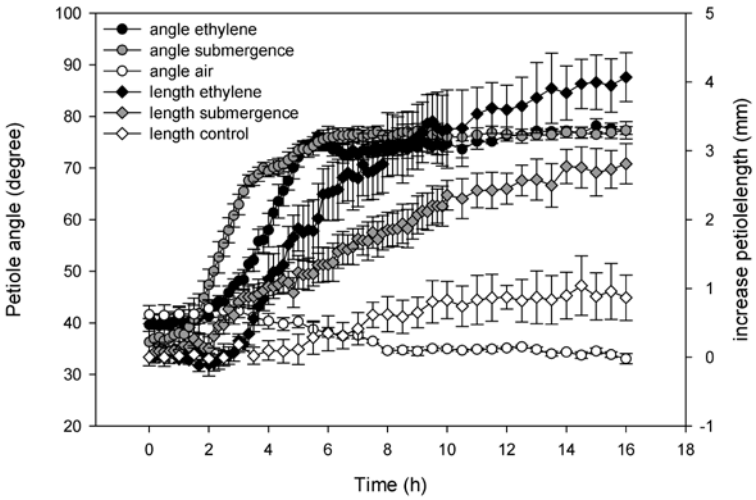

Figure 2. Ethylene- and submergence-induced hyponastic growth and petiole elongation in *Rumex palustris* plants with an initial petiole angle of 30–40 degrees. The means presented are calculated from 8 biological replicates (± se) for treatments and controls during 16 hours of growth in continuous light.

by ethylene showed a lag phase of 2.8 h and commenced when the angles of the petioles were close to 50 degrees (Figure 2, Table 1). Consistent with the earlier start of hyponastic growth during submergence also petiole elongation had a shorter lag-phase than during ethylene application. Our results suggest that ethylene- and submergence-induced hyponastic growth and enhanced elongation were almost similar. However, a slight difference in the kinetics of hyponastic growth and petiole elongation between submergence and ethylene responses was observed (Figure 2, Table 1).

Ethylene induces cell elongation at the abaxial basal half of petioles

To localise differential growth in petioles of ethylene treated plants, the lengths of epidermal cells from both abaxial and adaxial sides of petioles were measured. No differences were observed in cell lengths between controls and ethylene treated plants at the adaxial side (Figure 3A). However, abaxial epidermis cells from cell number 20 to 80 per cell file were slightly longer than cells in control plants (Figure 3B, Table 2). Since the entire petiole contains files of 140-160 epidermal cells hyponastic growth appears to be located at the abaxial, basal half of the petiole. The initial angle of the petiole was about 40 ± 2 degrees at the start of the treatment and after 4 h of ethylene application it reached an angle of 57.9 ± 3.2 degrees.

Table 1. Growth parameters measured during ethylene-induced hyponastic growth (A) and enhanced elongation growth (B) of the petioles of *Rumex palustris*. The values in bold square brackets [value (±se)], are from a submergence experiment (Cox *et al*.,2004). Values between normal brackets represent the standard errors of means of parameters which describe the kinetics of petiole elongation and hyponastic growth calculated by data fitting. Xo is the point of inflection and P represents the steepness of the curve. For manipulated treatments and controls 8 replicates were used. For non-manipulated treatments and controls 16 replicates were included.

Petiole angle (degrees)	-10 manipulated	40 natural angle		>65 manipulated	
A Hyponastic growth					
Lag-phase for hyponastic growth (h)	2.6 (0.4)	2.3 (0.1)	**[1.8 (0.1)]**	-	**[2.4 (0.2)]**
End hyponastic growth (h)	8.5 (0.3)	4.8 (0.8)	**[5.9 (0.2)]**	-	**[2.0 (0.2)]**
Xo angle	4.3 (0.1)	3.4 (0.1)	-		-
P angle	2.6 (0.4)	7.8 (0.4)	-		-
Final angle (degree)	67.4 (1.4)	77.5 (1.5)	**[78.6 (0.6)]**	72 (2.0)	**[80 (0.5)]**
B Elongation growth					
Lag-phase for petiole elongation (h)	1.8 (0.04)	2.8 (0.1)	**[2.1 (0.1)]**	1.9 (0.3)	**[1.2 (0.1)]**
Xo length	2.7 (0.04)	6.2 (0.7)	-	6.3 (1.3)	-
P length	2.7 (0.7)	6.6 (1.3)	-	3.2 (0.7)	-
Maximum length increase (mm)	3.1 (0.1)	4.1 (0.5)	**[3.1 (0.1)]**	4.1 (0.5)	**[3.0 (0.4)]**

We calculated if the difference in accumulated cell length at the abaxial side compared to the adaxial side could explain the observed angle change of 17.9 degrees after 4 h. To this end we applied a formula described in Cox *et al.* (2004) (Table 2). According to this calculation we expect an angle change to 56.8 degrees after 4 h of ethylene. This corresponded well with the observed angle (57.9) after 4 h of ethylene treatment. This result suggests that a very small ethylene-induced increase in the length of approximately 60 epidermal abaxial cells per file (cell number 20-80) of the petiole is responsible for the observed angle change.

Hyponastic growth and petiole elongation depend on the initial petiole angle

To study the dependence of ethylene-induced hyponastic growth and petiole elongation on the initial petiole angle, angle manipulation experiments were performed. Petioles manipulated to an initial angle of approximately -10 degrees showed, under both control and ethylene-enriched conditions, an immediate hyponastic growth response without a clear lag-phase. Up to approximately two hours no difference was observed between control and the ethylene-treated plants. However, after two hours the ethylene-treated plants showed an accelerated hyponastic

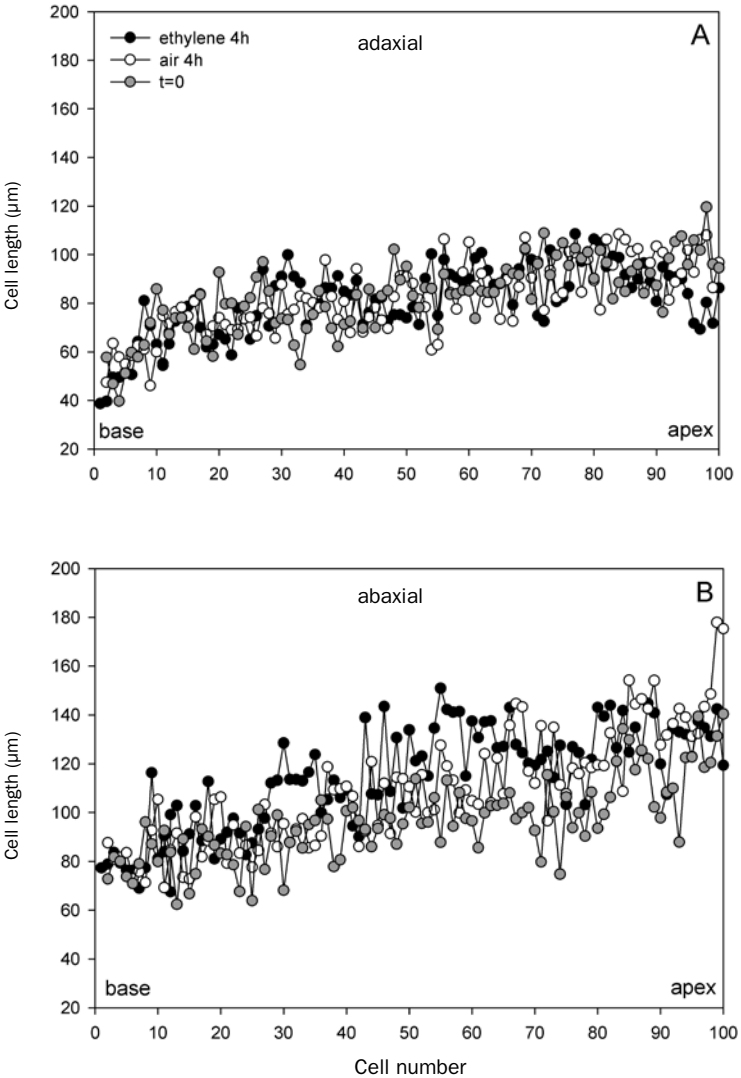

Figure 3. Epidermal cell lengths along the adaxial (A) and abaxial (B) side of the third petiole of *Rumex palustris* treated with ethylene for 4 h compared to control. The values presented are averages of 4 biological replicates. Differences between epidermal cell lengths during ethylene treatment were not significantly different from control in abaxial and adaxial side. Base is near the petiole base, apex close to the petiole apex. The total length of the petiole is around 140-160 cells.

Table 2. Cell lengths of the first 80 cells at the basal side of the petiole on the abaxial (lower) and adaxial (upper) side after 4 h of ethylene treatment or air control. Cell lengths were measured by studying imprints from 4 replicates of both sides of ethylene treated and control epidermal tissue of the same petiole. Cells were measured from base to apex. Means of total length of 4 replicates of 80 cells of the abaxial side were subtracted from the length at the adaxial side in both control and treatment (standard errors between brackets). Fold change was calculated by dividing the value of subtraction of the ethylene and control treatment.

Cell number	**80 cells**	
Petiole side	**abaxial**	**adaxial**
Ethylene treatment total length (µm) after 4 h	8,750 (249)	6,178 (206)
Control total length (µm) after 4 h	8,001 (180)	6,195 (171)
Abaxial - adaxial ethylene treatment total length (µm) after 4 h	2,572	
Abaxial - adaxial control total length (µm) after 4 h	1,806	
Ratio ethylene subtraction/control subtraction fold change	1.42	
Start angle X fold change= the end angle of the petiole after 4 h treatment	40x1.42 = 56.8	
Observed angle after 4h of ethylene treatment	57.9 (3.2)	

growth compared to controls (Figure 4A). The hyponastic response of the ethylene-treated plants continued until a final angle of almost 70 degrees was reached (Figure 4A, Table 1). Petioles for which the initial petiole angle was artificially set to -10 degrees and that were treated with ethylene, showed enhanced elongation growth with a biphasic pattern (Figure 4A).

The hyponastic response upon ethylene treatment was completely abolished when petioles were manipulated to an initial angle of approximately 70 degrees (Figure 4B). In contrast to these data a slight hyponastic growth with a lag-phase and a final angle was observed in submergence treatments (Table 1). Furthermore, the lag-phase of elongation and final end length of petiole were higher in ethylene-treated plants compared to submerged plants (Table 1).

Our results show that, similar to the response observed in submerged plants, the initial petiole angle has a strong impact on ethylene-induced hyponastic growth and petiole elongation.

Enhanced petiole elongation growth in response to ethylene

To quantify the rate of petiole elongation during ethylene application, linear displacement transducers were used (Voesenek *et al.*, 2003b). In this experiment, control and ethylene-exposed leaves were subjected to two additional treatments. The leaves were either positioned horizontal or vertical (Figure 5). The vertical petioles treated with ethylene, particularly during daytime, showed an enhanced elongation

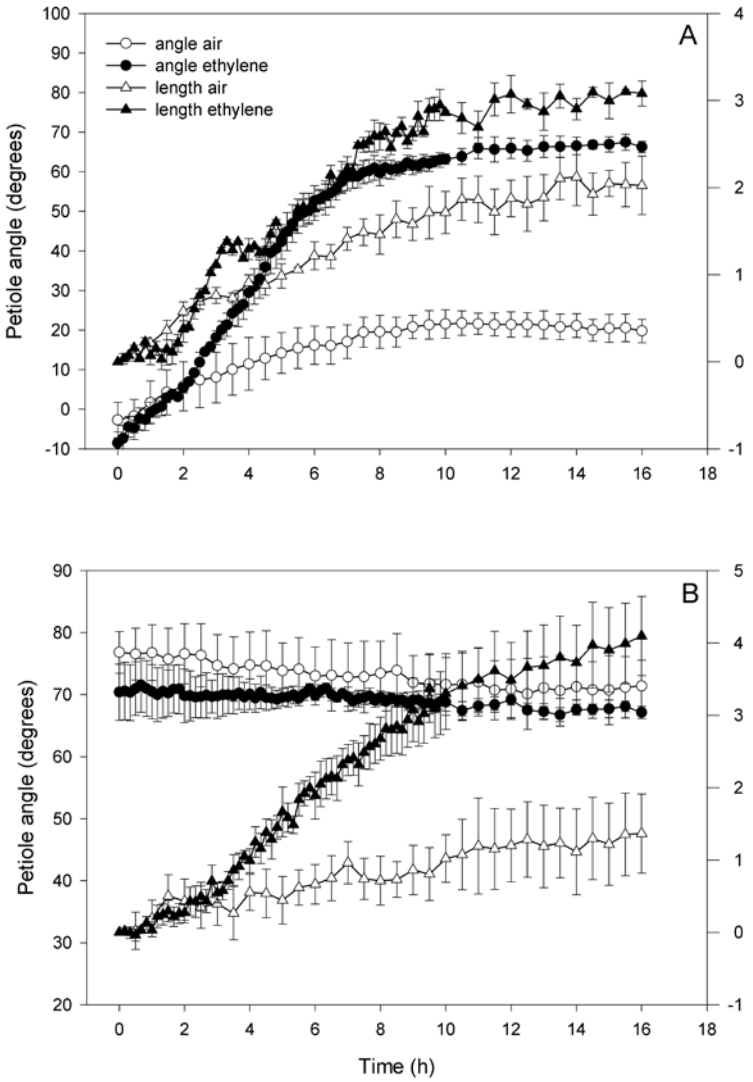

Figure 4. Ethylene-induced hyponastic growth and petiole elongation in *Rumex palustris* when the initial angles of the petioles were artificially manipulated to -10 degrees (A) or to 70 degrees (B) during 16 hours of treatment with ethylene. Manipulations were applied by changing the position of the pots with a ring holding the pots. The averages presented are calculated from means of 4 biological replicates (± se) in treatments and controls.

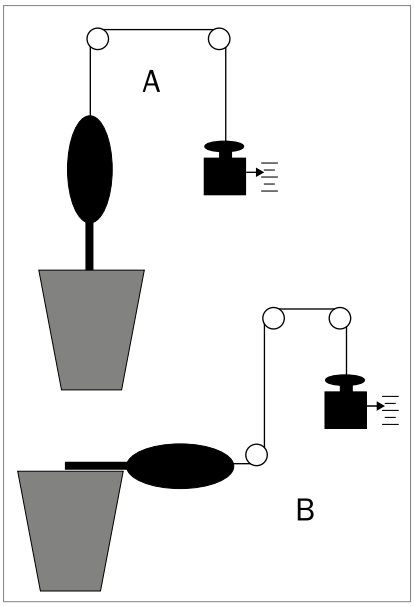

Figure 5. Schematic diagram of a linear variable displacement transducer set up with the target petiole in vertical (A) and horizontal (B) position.

rate when compared to the air control (Figure 6A). The maximum growth rate was calculated to be 0.56 (first day) to 0.69 (second day) mm h^{-1} for ethylene-treated petioles as compared to 0.35 (first day) to 0.39 (second day) for petioles in air (Figure 6A). The elongation of both control leaves as well as ethylene-treated leaves showed a diurnal pattern with the highest elongation rates during the day phase. The difference between control and ethylene-induced elongation rates were maximal during day time and almost zero during the night (Figure 6A). Periods of faster elongation, interrupted by phases in which elongation rates did not differ from control, re-occurred diurnally in experiments conducted over several days (data not shown). Interestingly, when petioles were kept horizontally in the transducer set-up, ethylene did not induce a significant enhancement of the elongation rate (Figure 6B).

These results again suggest that, like in submerged plants, ethylene-induced petiole elongation is dependent on the initial angle of the petiole. In addition, upon an appropriate angle manipulation, ethylene-induced hyponastic growth and petiole elongation of the leaves could be uncoupled.

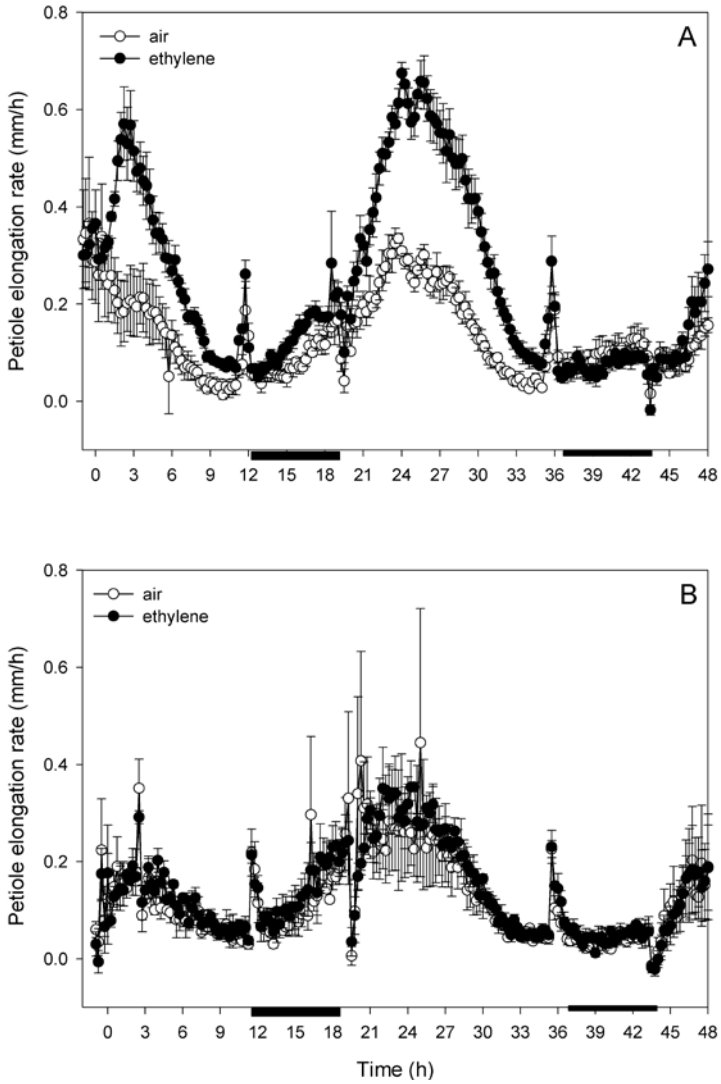

Figure 6. Elongation rates of 27-day old *Rumex palustris* petioles, air grown and ethylene treated plants. On the time axis the black boxes represent the night periods. Petioles were manipulated vertically (A) or horizontally (B). The values presented are the means of 8 biological replicates (± se).

Discussion

From previous studies, it was evident that the plant hormone ethylene plays a crucial role in hyponastic growth and petiole elongation responses of *R. palustris* upon flooding stress (Voesenek *et al.*, 2006). It was observed that plants treated with ethylene mimicked the phenotype of submerged plants, but the detailed kinetics of the response to ethylene were not yet described nor compared with submergence. Here we studied hyponastic growth and enhanced petiole elongation of *R. palustris* in response to ethylene and we compared the data with the results previously obtained with submerged plants.

Hyponastic growth

A time-lapse camera set-up was used to study the changes in petiole angle during ethylene treatment and submergence. This showed that ethylene-treated plants, without a manipulated angle, have a lag phase of 2.3 h (Figure 2, Table 1), which is slightly longer than submerged plants (1.8 h). However, both treatments resulted after 5-6 h in a final petiole angle of almost 80 degrees (Figure 2, Table 1). Since the accumulation of enough ethylene inside plant tissues of submerged plants takes time (approximately 1 h; Banga *et al.*, 1996) it was not expected that these plants would have a shorter lag-phase than ethylene-treated plants. Particularly so, because ethylene concentrations in the cuvettes for ethylene treatment reached hyponasty-saturating concentrations within a few minutes after onset of ethylene treatment (data not shown), thus probably exposing the cells within minutes to saturating ethylene levels. This result suggests that, next to ethylene, also other factors contribute to hyponastic growth under water. This hypothesis is consistent with the observation that hyponastic growth was not completely abolished when submerged plants were treated with 1-MCP (Cox *et al.*, 2004).

The present study shows that if the petiole angle is manipulated to below zero degrees (-10 degrees) hyponastic growth starts immediately in ethylene-treated plants, but interestingly also in non-ethylene exposed control plants. This might indicate that this phase in hyponastic growth is ethylene independent (Figure 4A). Alternatively, the very low control levels of endogenous ethylene may suffice to allow this upward petiole movement. After 2-3 h, ethylene-induced hyponastic growth occurred, characterised by a higher rate in ethylene-exposed plants. Also control plants kept increasing their angles until 6 h, but it is yet unknown if this involves ethylene or not.

The hyponastic growth in control plants with an artificially reduced petiole angle of -10 degrees is presumably a restoration of the leaf angle to the so-called gravitropic set-point angle (GSA; Digby and Fern, 1995). GSA is the angle to the gravity vector that organs maintain as determined by developmental stage and environmental conditions (Digby and Fern, 1995). How this is initiated is unknown, but one of the possibilities may be that plants sense the position of tissues and organs by means of the sedimentation of starch grains (statoliths) (Fukaki *et al.*,

1998) and act upon that to restore their original position with respect to the horizontal. The GSA in *R. palustris* is seasonally regulated and changes even in stable growth conditions, i.e. a climate room (Cox *et al.*, 2003). The mechanistic cause of this variation is not understood so far.

Cell length

Measuring the cell lengths of epidermal cells on both the abaxial and adaxial side of petioles treated for 4 h with ethylene revealed a slight increase of cell length on the basal abaxial side in cell number 20 to 80 (Figure 3, Table 2). The accumulated increase of cell lengths in this row of 60 cells is sufficient to explain the observed change in angle (Table 2).

Earlier work on submerged plants demonstrated that hyponastic growth is caused by a length increase of the 40 most basal epidermal cells at the abaxial side of the petiole (Cox *et al.*, 2004). Since great care was taken that the plants for both data sets (ethylene and submergence) were grown under identical growth chamber conditions and had reached a similar developmental phase at the start of the experiments we conclude that the precise cellular location of hyponastic growth depends on the treatment. Submergence results in a more basal location of hyponastic growth than ethylene application.

Enhanced petiole growth

Cox *et al.* (2003) showed that the initiation of submergence-induced enhanced petiole elongation requires a minimal petiole angle of 50 degrees. Also ethylene-induced petiole elongation depends on the petiole angle. This is based on observations showing a decrease in lag phase of the induction of elongation growth by ethylene from 2.8 ± 0.1 h in petioles starting at a 40 ± 1.5 degrees initial angle to 1.9 ± 0.3 h in petioles with an initial angle higher than 70 degrees (Table 1). Further evidence came from linear displacement transducer experiments, where petioles that were kept at very low angles did not elongate upon ethylene exposure (Figure 6B).

Fast ethylene-induced petiole elongation was observed in vertically positioned petioles (Figure 6A). Elongation growth expressed a diurnal rhythm with the highest growth rates during day time. Vreeburg *et al.* (2005) showed a comparable pattern of enhanced elongation growth in submerged *R. palustris* plants.

Obviously, during submergence a variety of processes are affected of which those regulated by ethylene are only a subset. Not only the build-up of high ethylene concentrations, but also the reduction of oxygen levels in some tissues will induce processes that are not induced during ethylene treatment alone. Furthermore, submergence results, in contrast to an ethylene treatment, in a slight upward change of the petiole angle (approximately 10 degrees) due to buoyancy (Cox *et al.*, 2003). It is possible that this slight change in angle affected the kinetics of hyponastic growth and elongation of the entire petiole.

Despite the small differences between submergence- and ethylene-induced hyponastic growth and petiole elongation we conclude that ethylene explains a substantial part of the morphological submergence response in *R. palustris*. This conclusion justifies the use of ethylene treatments to mimic submergence in our search for genes that regulate hyponastic growth and petiole elongation in *R. palustris*.

Materials & Methods

Plant material and growth condition

R. palustris plants were grown as described by Cox *et al.* (2003). The third petiole of 27 d old plants (after sowing) was used in all experiments, since this petiole exhibits clear hyponastic growth and elongation (Banga *et al.*, 1997). For all experiments at least four replicates were used.

Ethylene treatment

Plants were treated with ethylene in air tight cuvettes in a flow-through system and the growth response was photographed every 10 min One day before the experiment plants were placed in the cuvettes under the following conditions; 20°C, 70% Relative Humidity (RH) and 16 h light (200 µmol m^{-2} s^{-1} photon flux density). A growth saturating ethylene concentration of 5 µL L^{-1} was used in all ethylene experiments (Voesenek and Blom, 1989). To obtain this concentration, ethylene (100 µL L^{-1}; Hoekloos BV, the Netherlands) and air were mixed using flow meters (Brooks Instruments BV, the Netherlands). The concentration of ethylene (5 µL L^{-1}) inside the cuvettes was reached in less than 10 min (data not shown).

Angle manipulation

To study the interaction between hyponastic growth and petiole elongation in response to ethylene and to compare this to submergence experiments, we manipulated the initial angles of the petioles. To enable manipulation of the angle of petioles, a freely revolving ring, in which the plants could be placed, was attached inside the cuvettes.

Computerised digital camera system and image analysis

A computerised digital camera set up was used to measure the changes in the angle of the third petiole during control, ethylene and submergence treatments. Individual plants were photographed every 10 min until 16 h after treatment. To avoid confounding effects of diurnal rhythms, all the experiments started at 9 am. The angle and the length of the third petiole were measured with image analysis software as described by Cox *et al.* (2003). The data of hyponastic growth and petiole elongation of individual plants was fitted using a logistic function (Cox *et al.*, 2003).

Petiole growth measurement

To measure the rate of the increase in length of the petiole, linear variable displacement transducers were applied (Voesenek *et al.*, 2003b). To monitor the elongation rate of the petiole, we designed a clamp that was attached to the third petiole in either a horizontal or a vertical position (see Figure 5). Fitting lines through 20 min intervals were used for growth rates calculation. Ethylene (5 $\mu L\ L^{-1}$) was applied one hour after the measurement started.

Cell length measurement

To measure the length of the epidermal cells at the abaxial and adaxial side of the third petiole, imprints were made according to the procedure described in Cox *et al.* (2004). To calculate if the difference between the abaxial and adaxial side of the petiole could modify the angle of the petiole as observed in ethylene treated plants, we adopted the calculation method described in Cox *et al.* (2004). In our set up we compared the imprint slides of abaxial and adaxial side of the petioles after 4 h of treatment (air and ethylene).

Data fitting

To describe and compare the kinetics of hyponastic growth or petiole elongation (Y), we used a logistic function program (Cox *et al.*, 2003) as explained below.

$$Y= \{(A1-A2)/ (1+ [time/X0] \hat{}p)\} +A2$$

Where A1 is the starting value, A2 is the final value, X0 is the point of inflection, and p is the factor determining shape and steepness of the curve.

Fitting was performed by minimising the sum of squares of the difference between the measured and the predicted data using the solver function (standard settings) in Microsoft Excel 2000 (Microsoft, Redmond, WA).

The X0 value, obtained from the fitting program, was used to divide the measured data into two subsets. First, the subset of data below X0 was used to determine the lag phase of the start of hyponastic growth or that of stimulated petiole elongation. Second, the subset of data above X0 was used to determine the time in which hyponastic growth was completed. Determination of the lag phase (Xlag) took place by fitting two linear lines (Y1 and Y2) through a subset of measured data in such a way that the point of discontinuity is equal to the interception of the two lines:

$$Y1= a+b*x \qquad x<Xlag$$

$$Y2= (a+b*Xlag)+d*(x-Xlag) \qquad x>Xlag$$

Chapter 3

Transcriptional profiling by cDNA-AFLP, cDNA-subtraction and micro-array analysis revealed novel genes putatively involved in ethylene-induced hyponastic growth and petiole elongation in *Rumex palustris*

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Abstract

Flooding is one of the most severe abiotic stresses worldwide, both in natural and man-made ecosystems. *Rumex palustris* has the capacity to survive, when subjected to complete submergence, by changing the orientation of its rosette leaves to almost vertical (hyponastic growth) and subsequently by elongating petioles (elongation growth) to reach the water surface and restore gas exchange. Ethylene mimics largely this morphological submergence response in *R. palustris* and therefore we used ethylene treatments to mimic submergence in our search for genes that regulate hyponastic growth and petiole elongation in *R. palustris*. By applying cDNA subtractive hybridisation and cDNA-AFLP (RNA fingerprinting) we identified 119 differentially expressed genes during ethylene-induced hyponastic growth and petiole elongation. From those, 21 have very good homology with Arabidopsis genes. For most of the putative Arabidopsis orthologues of the identified *R. palustris* genes a positive function in cell growth and development was found in literature. Furthermore, we applied whole genome profiling by hybridising mRNA from *R. palustris* to a CATMA spotted array containing Arabidopsis gene specific tags and this resulted in more than 5000 genes that were differentially regulated in one or both traits. Furthermore, we examined the presence of transcription factor binding motifs in the promoters of the differentially expressed genes and demonstrated an over-representation of Abscisic acid responsive elements.

Introduction

Plants need mechanisms to respond to their environment, since, in the event of a (hostile) change, they cannot move to more appropriate conditions. Flooding is among one of the most severe abiotic stresses worldwide in both natural and man-made ecosystems. Most terrestrial plant species are unable to survive a prolonged exposure to such a stress. The flood tolerant terrestrial plant *Rumex palustris* has the capacity, when subjected to complete submergence, to change the orientation of its rosette leaves to almost vertical and subsequently elongate the petioles to reach the water surface and restore gas exchange. During flooding stress, plants face a decrease of the internal oxygen concentration and an accumulation of ethylene due to a severely reduced rate of gas diffusion in water compared to air (Blom and Voesenek, 1996). Extensive studies were performed to elucidate the mechanisms underlying the tolerance to submergence in *R. palustris*. It was shown that several plant hormones, more specific auxin, gibberellin, ethylene and abscisic acid regulate parts of the adaptive response to this stress (Benschop *et al.* 2005; 2006; Cox *et al.*, 2003; 2004; 2006; Vreeburg *et al.*, 2005). In all investigations to date it was shown that accumulation of ethylene is the primary signal that triggers the induction of upward leaf movement (hyponastic growth) and the elongation of young petioles during submergence (Cox *et al.*, 2003; 2004; 2006; Vreeburg *et al.*, 2005; Voesenek *et al.*, 1997; 2006). However, most downstream molecular components of the signal transduction pathway involved in these ethylene-driven growth responses are as yet unidentified.

Changing the shape or position of a tissue or an organ occurs when some cells or groups of cells divide or expand at a different rate than adjacent cells or cells nearby (Jesuthasan and Green, 1989). Differential growth produces alteration of form described as rolling or curling. If the direction of growth is determined by the organ itself rather than by the position of the external stimulus, the differential growth is defined as being nastic. Upward curvature resulting from differential enhancement of growth of the lower side of the petiole is called hyponastic growth (Hitchcock and Zimmerman, 1935).

In various dicotyledonous species ethylene promotes elongation growth at the adaxial side of the petiole causing downward curvature resulting in a so-called epinastic response (Jackson and Campable, 1976; Tonneijck *et al.*, 2000; Ursin and Bradford, 1989). However, in most (semi)aquatic plants and plants exhibiting a shade avoidance response, an increase in the exogenous ethylene concentration can induce individual leaves to grow differentially to a more vertical position (e.g. *Arabidopsis thaliana*) that in some cases may be followed by enhanced petiole or stem elongation like in *Rumex palustris* and *Nicotiana tabacum*, respectively (Pierik *et al.*, 2004; 2005; Voesenek *et al.*, 1997; 2006).

The plant hormone ethylene has vast-ranging and dramatic effects on growth, development and stress responses of plants throughout their life. The consequences of this hormone for growth differ between plant species and organs. In shoots,

ethylene is usually associated with inhibition of stem elongation in both monocotyledonous and dicotyledonous plants, however enhancement of growth has also been observed. Promotion of seedling and shoot growth by ethylene was reported for barley, oat and rice (Abeles, 1992; Ku *et al.*, 1970a; 1970b; Pierik *et al.*, 2006; 2007; Voesenek *et al.*, 2006). In Arabidopsis, ethylene induced hypocotyl elongation in light grown seedlings and inhibited hypocotyl elongation in dark grown seedlings (Smalle *et al.*, 1997). Application of 100 µM of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene biosynthesis, enhanced ethylene production by barley seedlings and stimulated shoot growth (Locke *et al.*, 2000). In *R. palustris* complete submergence leads to upward petiole growth followed by enhanced elongation of petioles. This phenomenon could be mimicked by exposing plants to exogenous ethylene (Cox *et al.*, 2004; Voesenek *et al.*, 1989, 2006).

Identification of signal transduction components involved in ethylene-induced elongation in *R. palustris* not only clarifies the pathway of events in this species, but might also increase our insight in the lack of these responses in other species.

An important approach to obtain insight into ethylene-induced hyponastic growth and petiole elongation in *R. palustris* is the identification of genes that are differentially regulated during these responses and analysis of the possible functions of their products. To identify these genes we applied cDNA subtractive hybridisation and cDNA-AFLP (RNA fingerprinting) with transcripts isolated from ethylene treated and air treated petioles. With these methods we were able to identify 119 differentially expressed genes. Furthermore, we applied whole genome profiling by hybridising mRNA from *R. palustris* to a CATMA spotted array containing 24,411 Arabidopsis gene-specific tags, which resulted in and more than 5000 genes that were differentially regulated in one or both traits.

Results

Background

The identification of differentially expressed genes by cDNA-subtraction, cDNA-AFLP and micro-array required the development of specific experimental designs for each method. Besides genes that were specific for ethylene-induced hyponastic and/or enhanced elongation growth we were also interested in excluding genes, whenever possible, that were either the general result of the ethylene treatment (ethylene effect genes) or angle manipulation of the plants (position effect genes).

For the cDNA-subtraction method total RNA isolated from ethylene treated plants with (normal) initial angle of 30-40 degrees (30E) was subtracted from total RNA isolated from ethylene treated plants with a manipulated petiole angle of 70 degrees (70E) and *vice versa.* The ethylene treatment lasted 2 h for both experimental groups. Since hyponastic growth only took place in 30E plants and not in

70E plants (Chapter 2) we assumed that the isolated genes were specific for hyponastic growth.

Contrary to the subtraction method, the number of analyses is not a limiting factor for cDNA-AFLP. Therefore, we analysed RNA samples of 8 different treatments (no manipulation, petiole manipulated to 70 degrees and petioles fixed at 0 and 90 degrees) to detect genes putatively involved in ethylene-induced hyponastic growth and enhanced petiole elongation (Figure 1). Besides using plants with an initial angle of 30-40 (30E/30C) degrees we included a treatment of plants with a fixed petiole angle of 0 (0E/0C) degrees as an extra control. This treatment was of particular interest since no ethylene-enhanced petiole elongation occurred during this treatment (Chapter 2). Plants with petioles manipulated to and above 70 degrees do not show hyponastic growth. However, in these treatments elongation of the entire petiole starts after 2 h in response to ethylene (Chapter 2). Furthermore, since we measured the highest growth rate in plants with 90 degrees fixed petiole at 2-3 (in short term) and 26-28 (long term) h after ethylene treatment, we include 90 degrees ethylene and air treatment in our set up to identify genes involved in ethylene-induced short term and long term enhanced elongation. To exclude ethylene-induced genes, not specific for differential growth, gene fragments present in the 30E and 0E treatments were compared to expressed genes in ethylene treated (2 h) samples from plants in which the petiole was manipulated to 70 degrees and those with a fixed petiole at 90 degrees (70E, 90E). Comparing 70E and 90E (treated for 2h with ethylene) with air grown plants (70C, 90C) revealed genes involved in short term elongation growth (Figure 1). To exclude ethylene-induced genes not specific for growth, gene fragments present in the 70E and 90E

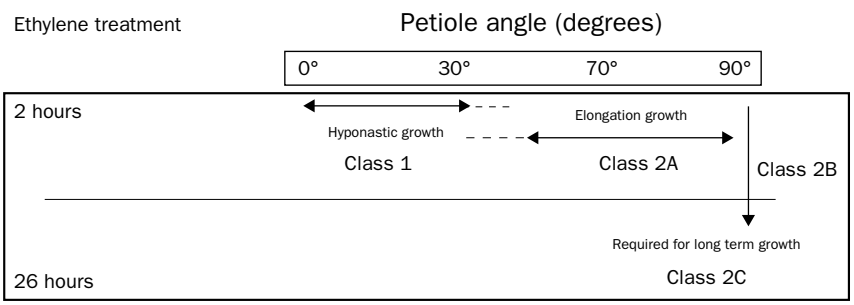

Figure 1. Schematic representation of changes in petiole angles in responses to ethylene in relation to the initial petiole angle. The arrows depict the presumed activity of genes, as observed during cDNA-AFLP analysis, involved in either hyponastic or enhanced elongation growth. The dotted lines indicate that 'hyponastic genes' may be active during elongation growth and the other way around. Gene expression observed during cDNA-AFLP analysis determines the classes to which genes are assigned to.

treatments were compared to 0E and 30E. Genes involved in long term ethylene-induced elongation growth could be identified by comparing RNA samples from plants with a fixed petiole angle of 90 degrees and treated with ethylene for 26 h (90E) with air grown plants for 26 h with the same fixation treatment (Figure 1).

By treating plants for 2 h, genes involved in early ethylene-induced (hyponastic) growth could be identified by cDNA-AFLP and cDNA subtraction, but to be able to detect genes expressed slightly later during (hyponastic) growth, and to be able to compare the results with previous micro-array analyses (Millenaar *et al.* submitted), we treated plants with ethylene for 3 h and analysed the expressed genes with micro-array technology. In these analyses 30E was compared with 30C and 70E with 70C (see material and methods).

Identification of genes by cDNA subtraction

The target cDNA was isolated from the third petiole with a natural initial angle of 30-40 degrees exposed to 5 $\mu L\ L^{-1}$ of ethylene (30E). This treatment results in hyponastic growth after a lag phase of approximately 2 h (Chapter 2). The driver cDNA was isolated from petiole tissue that was manipulated to an angle of 70 degrees, exhibiting no hyponastic growth in response to ethylene (Chapter 2) (70E). A total of 7 cDNA fragments, ranging from 155 to 1300 bp, listed in Table 1, were isolated after forward and reverse subtraction. Six individual cDNA fragments from the forward subtraction and one from the reverse subtraction were cloned. As an initial check, the inserts in the plasmids were amplified by PCR using the forward and reverse nested primers that were initially used to isolate them. The majority of the clones analysed had inserts between 200 and 500 bp. To identify putative functions of these genes, sequences were compared with sequences in the available databases (Table 1). Furthermore, based on the nucleotide sequence, gene specific primers were designed and used on *R. palustris* cDNA and genomic DNA. Each of the primer pairs amplified a band in either one or both of the *R. palustris* DNA pools, thus confirming the origin of the sequence (data not shown).

Identification of genes by cDNA-AFLP

cDNA-AFLP analysis was performed to identify genes related to differential and non-differential ethylene-induced petiole growth in *R. palustris* (Figure 1). By using 128 primer combinations, 112 cDNA fragments were found to be differentially expressed indicating possible involvement in ethylene-induced hyponastic growth or petiole elongation as explained above and in Figure 1. These *R. palustris ethylene-induced hyponastic growth* gene (*RpEIHG*s) fragments and *R. palustris ethylene-induced enhanced elongation growth* gene (*RpEIEGs*) fragments varied in length from 60 to 500 bp. The bands were excised from the gels, re-amplified by PCR, cloned and sequenced. Only in two cases different cDNA fragments could be traced back to the same transcript. These fragments were not characterised further. All the sequence information obtained was compared to sequences present in the

Table 1. List of *Rumex palustris* expressed sequence tag's (ESTs) isolated by subtractive hybridisation. Sequences were used to search the appropriate databases to identify relevant orthologues genes. *RpEIH(E)G: Rumex palustris ethylene-induced hyponastic (elongation) growth* gene fragments. They are classified based on the expression profile in cDNA-subtraction and functional categories.

Clone number (class)	Length (bp)	Orthologous to	E-value	organism	Expression pattern	Functional group
RpEIHG1 (1)	1327	DNA polymerase III, epsilon subunit	1e-81	*Rhodopseudomonas palustris*	30°, 2h ethylene treatment	DNA or RNA metabolism
RpEIHG2 (1)	826	Homo sapiens muscleblind-like	0.0	*Homo sapiens*	30°, 2h ethylene treatment	DNA or RNA metabolism
RpEIHG3 (1)	664	glioblastoma amplified sequence	0.0	*Homo sapiens*	30°, 2h ethylene treatment	Plasma Membrane and wall associate kinase protein
RpEIHG4 (1)	475	ADP-ribosylation factor guanine nucleotide-exchange	0.0	*Homo sapiens*	30°, 2h ethylene treatment	Intercellular protein and cation trafficking
RpEIHG5 (1)	279	Methylmalonyl-CoA mutase	9e-33	*Thermoanaerobacter tengcongensis*	30°, 2h ethylene treatment	Electron transport and energy pathway
RpEIHG6 (1)	284	oxygenase	8e-04	*Streptomyces antibioticus*	30°, 2h ethylene treatment	Oxido reductase activity
RpEIEG8	155	DNA clone	1e-07	*Mesorhizobium loti*	70°, 2h ethylene treatment	-

Genbank databases (data not shown). Of the total of 112 *RpEIEG* and *RpEIHG* gene fragments sequenced, 17.9% (20) showed significant homology to genes with a known or putative function. *RpEIEG74* (0.9%) was homologous to uncharacterised genes (ESTs and unknown proteins) (Table 2). The remaining 91 gene fragments (112-21; 81.2%) showed no significant matches or the match was only with genomic clones without allocated function. From the 112 genes identified, 21 gene fragments had a known putative orthologue in *Arabidopsis thaliana.* These were further analysed (Table 2).

Classification of ethylene-induced hyponastic growth and petiole elongation genes

Of the 112 and 7 *RpEIEG* and *RpEIHG* gene fragments isolated by cDNA-AFLP and cDNA subtraction methods, respectively, 28 fragments (21+7) revealed homology to genes with a known or putative function. Based on this homology we grouped them into several functional categories (Table 1, 2). Furthermore, based on their expression patterns, we were able to arrange them in two major classes (1 and 2) and four expression groups (1, 2A, 2B and 2C; Figure 1). Below, we describe the

Table 2. List of *Rumex palustris* expressed sequence tag's (ESTs) isolated by cDNA-AFLP. Sequences were used to search appropriate databases to identify relevant orthologues genes. *RpEIHG: ethylene-induced hyponastic growth genes* and *RpEIEG: ethylene-induced elongation* growth genes in *Rumex palustris*. They are classified based on the expression profile in cDNA-AFLP and functional categories.

Clone number (class)	Length (bp)	Orthologous to	E-value	organism	Expression pattern	Functional group
RpEIEG15 (2B)	315	Putative VAMP protein	2e-26	*Arabidopsis thaliana Oryza sativa*	30°, 2h; 9°, 2 and 26 h ethylene treatment	Intercellular, protein and cation trafficking
RpEIEG27 (2B)	99	Putative beta-n-acetylhexosaminid Glycosyl hydrolase	5e-9 4e-05	*Arabidopsis thaliana*	70°, 2h ethylene treatment	Hydrolase activity
RpEIHG35 (1)	244	40s ribosomal protein S13	3e-24	*Arabidopsis thaliana*	30°, 0°, 2h ethylene treatment	Protein metabolism
RpEIHG42 (1)	205	Glutathione transportersGT1 isp4 like protein	6e-18 5e-18	*Brassica juncia Arabidopsis thaliana*	30°, 0°, 2h ethylene treatment	Intercellular, protein and cation trafficking
RpEIEG45 (2B)	140	Auxin-induced protein similar to 10A5 or 6B	9e-05	*Arabidopsis thaliana*	90°, 2 and 26h ethylene treatment	Signal transduction activity
RpEIEG47 (2A)	68	Stomatal cytokinesis defective	6e-05	*Arabidopsis thaliana*	30°, 70°, 90° 2h and 90°, 26h ethylene treatment	Intercellular, protein and cation trafficking
RpEIHG48 (1)	71	Apical meristematic formation protein	1e-03	*Arabidopsis thaliana*	30°, 0°, 2h ethylene treatment	Transcription factor activity (auxin related)
RpEIEG49 (2A)	39	EIF4A ATP dependent helicase	7e-05	*Arabidopsis thaliana*	30°, 70°, 90° 2h and 90°, 26h ethylene treatment	Protein metabolism
RpEIEG57 (2B)	68	BTB and TAZ domain protein	1e-04	*Oryza sativa Arabidopsis thaliana*	90° ,26h ethylene treatment	Transcriptional regulating
RpEIEG58 (2B)	99	2-oxoglutarate-dependent dioxygenase, 1-ACO	6e-04 6e-04	*Arabidopsis thaliana Phaseolus vulgaris*	30°, 90° 2h and 90°, 26h ethylene treatment	Oxido reductase activity
RpEIEG59 (2A)	68	Wall associated kinase protein	7e-04	*Arabidopsis thaliana*	90°, 30°and 70°, 2h ethylene treatment	Membrane and wall associate kinase protein
RpEIHG69 (1)	219	Sodium proton exchange	8e-14	*Arabidopsis thaliana*	0°, 2h ethylene treatment	Intercellular, protein and cation trafficking
RpEIEG72 (2C)	124	Root hair defective 3 GTP-binding protein	5e-17	*Arabidopsis thaliana*	90°, 26h ethylene treatment	Protein metabolism
RpEIEG74	157	Expressed protein	1e-08	*Arabidopsis thaliana*	30° and 70°, 2h ethylene treatment	-
RpEIEG79 (2C)	108	BTB/POZ domain-containing protein	1e-03	*Arabidopsis thaliana*	90°, 26h ethylene treatment	Transcriptional regulating

Table 2. Continued

Clone number (class)	Length (bp)	Orthologous to	E-value	organism	Expression pattern	Functional group
RpEIEG91 (2C)	119	Putative Auxin responsive factor	4e-14 2e-15	*Oryza sativa* *Arabidopsis thaliana*	90°, 26h ethylene treatment	Transcription factor activity
RpEIEG92 (2C)	140	Photosystem I reaction centre subunit VI	6e-05	*Arabidopsis thaliana*	90°, 26h ethylene treatment	Oxido reductase activity
RpEIEG93 (2C)	83	Glyceraldehydes-3-phosphate dehydro-genase (GAPDH)	2e-04	*Atriplex nummularia*	90°, 26h ethylene treatment	Oxido reductase activity
RpEIEG96 (2C)	103	Ferroxin- dependent glutamate synthase	2e-12	*Arabidopsis thaliana*	90°, 26h ethylene treatment	Oxido reductase activity
RpEIHG99 (1)	300	Wall associated kinase 4 protein	8e-06	*Arabidopsis thaliana* *Oryza sativa*	30°, 0°, 2h ethylene treatment	Membrane and wall associate kinase protein
RpEIEG104 (2C)	84	Elongation factor 1-alpha	8e-25 1e-06	*Arabidopsis thaliana* *Cichorium intybus*	90°, 26h ethylene treatment	Protein metabolism

isolated genes based on their expression patterns and functional categories. Gene functions are suggested, based on homology of the gene fragments with genes in the databases. The function during hyponastic growth or petiole elongation remains to be elucidated.

Class 1 genes

The *RpEIHG* gene fragments in this group represent genes that are present in samples with a petiole angle of below 30 degrees and 2 h ethylene treatment (0E, 30E) and not in air control samples (0C, 30C) and not in ethylene and air control samples of petioles with an angle of 70 degrees or higher (70E, 70C, 90E, 90C). Based on this specific expression pattern these genes are putatively involved in hyponastic growth (Chapter 2, Figure 1) and they can be classified into several biological and molecular functional groups (Table 2).

Several of the putative orthologues of the differentially expressed genes in class 1 are possibly associated with changes in the plasma membrane and cell wall. In addition, genes involved in cell trafficking either from Golgi to plasma membrane or changes in the electric charge of the cell by transferring cations in or out of the cell are regulated during hyponastic growth. For most of the putative Arabidopsis orthologues of the identified *R. palustris* genes a positive function in cell growth and development was found in literature (Supplementary data, appendix A).

Class 2 Genes

These *RpEIEG* gene fragments represent genes that are present in samples that show enhanced petiole elongation and are therefore considered to be putatively involved in this process. Beside differentially expressed genes showing involvement in cell growth/elongation, the group of class 2 involves genes with a putative involvement in transcriptional/(post)-translational regulation, metabolism and photosynthesis. The data suggests, similar to the hyponastic response related genes, that genes responsible for altering the membrane electric charge or the pH of the cell are regulated during elongation growth. This class could be divided in 3 subclasses.

Class 2A genes

The *RpEIEG* gene fragments in this group expressed in samples with a petiole angle of 30 degrees or higher (70 and 90 degrees) after 2 h of ethylene treatment (Figure 1) (30E, 70E, 90E). These genes are not expressed in the 0E treatment, that shows no elongation growth. This suggests that they are specific for ethylene-induced elongation growth. The three genes that were identified in this group can be assigned to three different functional groups (Table 2). Overall, we conclude that the genes in this group fit into the same functional categories as the genes in class 1.

Class 2B genes

The *RpEIEG* gene fragments in this group were expressed only in samples with petioles angle higher than 70 degrees (70 and 90 degrees) after 2 and 26 hrs of ethylene treatment. This group may contain genes that induce enhanced petiole elongation or genes involved in the maintenance of long term enhanced petiole elongation (Figure 1). The four genes identified in this class were assigned to 4 different functional groups (Table 2).

Class 2C genes

The *RpEIEG* gene fragments in this class were expressed only in samples with a petiole angle of 90 degrees after 26 hrs of ethylene treatments (90E). These genes might be involved in the maintenance of long term enhanced petiole elongation. They can be divided in 4 functional classes (Table 2).

Analysis of micro-array data

Transcriptome analysis of non-model species like *R. palustris* is not straightforward since sequence information is scarce and expressed sequence tags (ESTs) are not available. To confirm results from cDNA-AFLP analysis and to extend our knowledge about differentially expressed genes during ethylene-induced hyponastic and elongation growth we used the CATMA *Arabidopsis thaliana* full genome micro-array. We hybridised this array with cDNA from *R. palustris* plants treated for 3 h with ethylene. We isolated RNA from petioles with an initial angle of 30

degrees and treated with ethylene (30E) and compared this sample to 30 degrees control (30C). This comparison might reveal relevant genes associated with hyponastic growth. However, we cannot exclude the regulation of early ethylene-induced elongation genes at this point in time. Furthermore, we compared 70 degrees ethylene (70E) with 70 degrees control (70C). We hypothesise that regulated genes in this comparison might be relevant for petiole elongation as hyponastic growth is lacking in 70 degrees petioles.

To exclude genes just responding to ethylene and not related to hyponastic/elongation growth and to exclude genes that were differentially expressed as a result of the position change of the petiole (from 30 degrees to 70 degrees) transcripts of 30E and 70E were compared and those of 30C and 70C, respectively.

Since the transcripts of Rumex were applied on an Arabidopsis array (heterologous hybridisation) not all the transcripts hybridised with all the oligo's on the array. The total number of genes that could be detected, irrespective of the treatments was about 19,700 (80%). This is a surprisingly high number considering the assumed large genetic distance between the species. To avoid false positive results we only considered those genes with an expression higher than the cut-of value (see material and methods). This resulted in 4,894 genes that were differentially expressed higher/lower than 1.4 fold (cut-of threshold) in one of the relevant comparisons as explained above. These genes were considered as putative ethylene-induced (differential) growth genes (Figure 2).

For genes likely to be involved in hyponastic growth (30C and 30E compared) we observed 1,299 (792+248+259) genes to be downregulated and

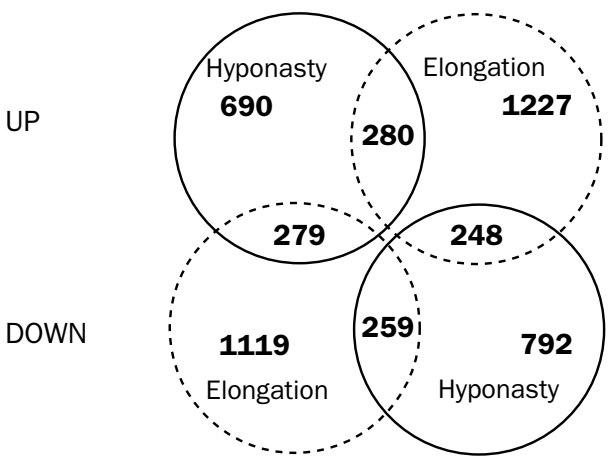

Figure 2. Total number of genes differentially expressed 1.4-fold during ethylene-induced hyponastic growth and enhanced petiole elongation as identified by micro-array analysis. Moreover, common genes up or downregulated during hyponastic growth and enhanced petiole growth are depicted.

1,249 (280+279+690) upregulated above the significance threshold of 1.4-fold (Figure 2). When we compared 70C and 70E, for genes involved in petiole elongation we observed 1,657 (1119+259+279) genes to be downregulated and 1,755 (248+1,227+280) genes to be upregulated at least 1.4 fold. Some of the genes were expressed differentially in both traits, either in the same direction or opposite. From those, 279 genes were upregulated during hyponastic growth and downregulated during elongation, for 248 genes it was the other way around. Furthermore, 280 genes were upregulated and 259 were downregulated in both comparisons (Figure 2).

Of the 120 putative Arabidopsis orthologues of 21 *R. palustris* genes that were identified in the cDNA-AFLP experiment (blast search based data); we

Table 3. List of differentially expressed genes during ethylene-induced (differential) growth identified by cDNA-AFLP and micro-array analysis. The first column represent the number of ESTs clones that were isolated from *Rumex palustris* during ethylene-induced hyponastic growth or enhanced petiole elongation by applying cDNA-AFLP. The second column shows their putative Arabidopsis orthologues that were detected by BLAST searches and micro-array analysis. The expressed values of orthologous genes during ethylene-induced (hyponastic) growth were calculated by dividing the intensity of the individual spot through the average intensity of all presented spots and they represent the mean value of 4 biological and 4 experimental replicates. The cut-off value is 1.4 fold change compared to the background. Putative function and localisation of the gene products taken from data available in the *Arabidopsis* database (TAIR).

***R. palustris* EST**	**Putative Arabidopsis orthologue**	**Direction of regulation**		**Putative function**	**Localisation**
		Hyponastic fold change	**Elongation** fold change		
RpEIHG42	At4g10770	No effect	Down (-1.45)	oligopeptide transporter	membrane
RpEIHG42	At4g16370	Down (-1.6)	Down (-1.8)	oligopeptide transporter	membrane
RpEIHG42	At5g53510	Up (1.4)	Up (1.8)	oligopeptide transporter	membrane
RpEIHG42	At5g53520	Up (2.9)	No effect	oligopeptide transporter	membrane
RpEIEG47	At1g49040	Up (2.6)	Up (1.62)	SCD1 (cytokinin defective protein)	cytoplasm
RpEIEG47	At4g14620	Down (-2.3)	Down (-12.6)	unknown protein	
RpEIEG49	At1g54270	No effect	Up (1.6)	member of eIF4A - eukaryotic initiation factor 4A	

Table 3. Continued

R. palustris EST	Putative Arabidopsis orthologue	Direction of regulation		Putative function	Localisation
		Hyponastic fold change	**Elongation** fold change		
RpEIEG57	At3g48360	Up (1.6)	No effect	BT2 protein; component of the TAC1-mediated telomerase activation pathway , transcription factor activity	nucleus
RpEIEG59	At1g07550	No effect	Up (1.6)	leucine-rich repeat protein kinase	endomembrane
RpEIEG59	At1g48480	Down (-1.6)	Down (-1.7)	receptor-like protein kinase (RKL1) gene	
RpEIEG59	At1g55610	No effect	Down (-4.3)	LRR Receptor Kinase	endomembrane
RpEIEG59	At2g19190	No effect	Down (-2.1)	receptor-like protein kinase. Involved in early defense signaling	endomembrane
RpEIEG59	At2g19230	No effect	Down (-2.7)	similar to FRK1 (FLG22-I induced-receptor like kinase 1), similar to light repressible receptor protein kinase	endomembrane
RpEIEG59	At2g45910	No effect	Down (-1.7)	protein kinase family protein / U-box domain-containing ubiquitin ligase complex	
RpEIEG59	At3g26940	Down (-1.7)	No effect	receptor-like cytoplasmic kinase, RLCKVII subfamily	cytoplasm
RpEIEG59	At4g23250	Up (1.7)	Up (1.5)	embryo defective kinase	endomembrane
RpEIEG59	At5g25930	No effect	Up (3.5)	leucine-rich repeat protein kinase, similar to receptor-like protein kinase 5	endomembrane
RpEIEG74	At3g07350	No effect	Down (-1.9)	unknown protein	
RpEIEG79	At3g24270	Down (-1.4)	No effect	RNA binding protein	endomembrane
RpEIEG79	At4g01160	Up (1.8)	No effect	BTB/POZ domain-containing protein	
RpEIEG91	At5g37020	No effect	Down (-1.6)	encodes a member of the auxin response factor family *ARF8*	nucleus
RpEIEG91	At1g30330	No effect	Up (1.5)	encodes a member of the auxin response factor family *ARF6*	
RpEIEG93	At1g79530	Down (-1.6)	No effect	Glyceraldehyde-3-phosphate dehydrogenase	plastid

Table 3. Continued

R. palustris EST	Putative Arabidopsis orthologue	Direction of regulation		Putative function	Localisation
		Hyponastic fold change	**Elongation** fold change		
RpElEG93	At3g04120	No effect	Up (1.7)	cytosolic GADPH (C subunit) involved in the glycolytic pathway but also interacts with H_2O_2 (putative ROS cascade)	mitochondrial envelope
Rp ElEG96	At3g26570	Down (-1.8)	Down (-1.8)	phosphate transporter activity	
Rp ElEG96	At3g26570	Down (-1.8)	Down(-1.8)	phosphate transporter activity	
RpElHG99	At1g06840	No effect	Down (-4.8)	leucine-rich repeat trans-membrane protein kinase	
RpElHG99	At1g07570	No effect	Down (-1.4 4)	Protein kinase capable of phosphorylating tyrosine, serine, and threonine residues APK1	
RpElHG99	At1g06840	No effect	Down (-4.8)	leucine-rich repeat trans-membrane protein kinase	
RpElHG99	At1g07570	No effect	Down (-1.44)	protein kinase capable of phosphorylating tyrosine, serine, and threonine residues APK1	
RpElHG99	At1g18390	Down (-1.7)	No effect	serine/threonine protein kinase family protein, similar to wall-associated kinase 4-like	endomembrane
RpElHG99	At1g27190	Up (2.4)	No effect	leucine-rich repeat trans-membrane protein kinase	endomembrane
RpElHG99	At1g51810	Up (3.35)	No effect	kinase protein	
RpElHG99	At1g66880	Down (-1.5)	No effect	serine/threonine protein kinase family protein, similar to wall-associated kinase 4-like	endomembrane
RpElHG99	At1g70460	Up (1.6)	No effect	kinase protein	membrane
RpElHG99	At2g01820	Up (1.6)	No effect	protein kinase family protein similar to Trance membrane kinase 1(1 (TMK1)	
RpElHG99	At2g04300	No effect	Up (1.74)	leucine-rich repeat protein kinase	endomembrane
RpElHG99	At2g19230	No effect	Down (-2.7)	similar to light repressible receptor protein kinase	endomembrane
RpElHG99	At2g26290	Up (3.2)	No effect	root specific kinase 1 (ARSK1)	

Table 3. Continued

***R. palustris* EST**	**Putative Arabidopsis orthologue**	**Direction of regulation**		**Putative function**	**Localisation**
		Hyponastic fold change	**Elongation** fold change		
RpEIHG99	At2g28940	Down (-1.5)	No effect	protein kinase family protein	endomembrane
RpEIHG99	At3g24540	No effect	Up (1.8)	protein kinase family protein; similar to praline extension-like receptor kinase 1 (ATPERK1)	
RpEIHG99	At3g46340	No effect	Up (1.4)	maternal effect embryo arrest 39 (MEE39)	endomembrane
RpEIHG99	At4g11530	Up (1.6)	No effect	protein kinase family protein similar to cysteine –rich RLK11 (CRK11)	endomembrane
RpEIHG99	At5g56460	No effect	Up (1.7)	protein kinase	
RpEIEG104	At1g35550	Down (-1.7)	No effect	elongation factor 1-alpha	

could confirm differential expression for 45 Arabidopsis genes that are putatively orthologues to 12 *R. palustris* genes. These genes were found as differentially expressed genes in the micro-array experiment and listed in Table 3.

Transcription factor binding motif analysis of genes regulated during hyponastic growth and petiole elongation

As a first step towards identifying transcription factors (TF) that may be involved in hyponastic growth and/or petiole elongation we examined the over-representation of possible TF binding motifs in the promoters of the differentially expressed genes from the micro-array analysis. To do this we used the ATHENA database and its search tool (O'Connor *et al.*, 2005; http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl). The developers of this tool have set the p-value threshold for significant enrichment at $p<10^{-4}$ and they used the Bonferroni correction since multiple hypotheses are being tested (up to 105 different TF binding sites) (O'Connor *et al.*, 2005). The results using their settings and criteria are listed in Table 4.

Interestingly, only during ethylene-induced elongation growth over-representation of possible TF binding motifs in the promoters of the differentially expressed genes were found. The results indicate an over-representation of promoters carrying ABA responsive elements (ABRE-like binding site motif) upregulated during

Table 4. Specification of cis-elements, transcription factor binding motifs, that were identified, using the ATHENA database and tool, to be over-represented among the genes differentially up or downregulated during enhanced petiole elongation in *Rumex palustris*, as identified by micro-array analysis.

Response during	TF binding site motif	P-value	Function	Reference
Elongation growth down-regulated	CACGTGMOTIF	$<10^{-4}$	Essential for expression of beta-phaseolin gene during embryogenesis in bean, tobacco, Arabidopsis; Tomato Pti4 (ERF) regulates defence-related geneexpression	Chandrasekharan *et al.*, 2003 Busk and Pagès 1998
Elongation growth down-regulated	ACGTABREMOTIFA2OSEM	$<10^{-4}$	The bZIP class Abscisic acid Responsive Element (ABRE)-binding factor regulate ABA-mediated transcription and in Arabidopsis ABRE are interdependent in the ABA-responsive during rd29A expression	Hattori *et al.*, 2002
Elongation growth upregulated	ABRE-like binding site motif	$<10^{-4}$	Response to ABA	Huang *et al.*, 2007

elongation growth (Table 4). Within this subset of 361 genes other ABA associated promoter motifs (ACGTABREMOTIFA20SEM (62%), CACGTGMOTIF (48%), ABRE binding site motif (21%) and a GA associated promoter motif (GADOWNAT (37%)) are over-represented. As an example, this means that of the 361 promoters in this subset 62% (227 promoters) not only have the ABRE-like binding site motif but also the ACGTABREMOTIF20SEM motif.

For the genes that are downregulated during elongation growth two over-represented promoter motifs were identified. Both of these motifs (CACGTGMOTIF and ACGTABREMOTIFA20SEM) are also associated with ABA action. Within these groups about 50% of the promoters have both motifs and the same motifs identified for the upregulated elongation growth genes subset are observed as being over-represented in this subset.

Our results suggest that regulation by ABA may play a role during elongation growth. Furthermore, it shows that the genes that were found to be enriched in ABA action associated genes carried additional motifs related to ABA and GA action.

Discussion

Differential expression tools unravelled novel genes involved in ethylene-induced (differential) growth of Rumex palustris

In this study cDNA-AFLP, cDNA-subtraction and micro-array analysis were employed to discover novel sets of genes putatively involved in hyponastic growth and petiole elongation, two growth responses induced by flooding and ethylene

treatment. cDNA-AFLP and cDNA-subtraction hybridisation are PCR-based transcript profiling methods with high specificities and the ability to detect rare transcript tags (Ahn *et al.*, 2002; De Paepe *et al.*, 2004; Reijans *et al.*, 2003). Furthermore, prior sequence information is not needed for these methods which make them very useful tools to identify novel genes in non-model organisms (Ditt *et al.*, 2001). In addition, PCR based transcript analysis techniques are more sensitive than hybridisation based techniques (De Paepe *et al.*, 2004). Arabidopsis micro-array analysis, a hybridisation-based assay, on the other hand authorises a set of data that can be compared with other studies.

To have controlled conditions with little side effects, *R. palustris* plants were treated with ethylene, which was shown to mimic submergence-induced hyponastic growth and petiole elongation in this plant to a large extent (Chapter 2). To detect genes that were early and late ethylene-induced during (hyponastic) growth we used 2 and 3 h of ethylene treatment and to identify genes involved in long term maintenance of elongation growth we used 26 h ethylene treatments.

When cDNA-AFLP and cDNA-subtraction were used, 119 expressed sequence tags (ESTs) were identified as putative genes regulated during either hyponastic growth, petiole elongation, or in both growth responses. Database searches with sequences of these ESTs, revealed homologies of 28 ESTs with Arabidopsis or other organisms (Table 1, 2). For 21 of the ESTs a total number of 120 putative orthologues gene were identified in Arabidopsis.

Little is known about the genome of *R. palustris*, therefore to obtain a wider spectrum of genes involved in hyponastic growth and petiole elongation, the expressed transcripts of ethylene-induced *R. palustris* plants were hybridised with a full genome Arabidopsis array (CATMA). In this assay 4894 genes were found to be differentially expressed beyond the 1.4 fold threshold in one or both comparisons. Forty-five genes were identified as differentially expressed in both cDNA-AFLP (~37% of 120 genes) and micro-array analysis (Table 3). A total number of 4894 genes were 44% of the total number of genes for which the expression was significantly altered in ethylene-induced (differential) growth. In general a good correlation between cDNA-AFLP and micro-array results has been observed for example in *Saccharomyces cerevisiae* (Reijans *et al.*, 2003). However, in our set up comparison between cDNA-AFLP and micro-array analysis revealed only 37% correlation between the cDNA-AFLP and the micro-array data. This low correlation may be caused by two facts. First, the micro-array analysis hybridisation was heterologous hybridisation (transcripts of *R. palustris* were hybridised to an Arabidopsis array) which causes reduction in the number of hybridised fragments. Secondly, from the 119 identified differentially expressed ESTs in cDNA-AFLP only 21 showed high homology with Arabidopsis and other plant species.

Still it is quite surprising that the pool of *R. palustris* transcripts hybridised to 80% of the Arabidopsis genes, demonstrating that CATMA arrays could be used as a tool to study the global changes in the expression of genes in this plant.

Involvement of identified genes in hyponastic growth and/or petiole elongation

Analysis of ethylene-induced growth in *R. palustris* strongly indicates that hyponastic growth and enhanced petiole elongation can be uncoupled by specific manipulations of the angle of the petiole (Chapter 2). Moreover, induction of growth by ethylene or submergence strongly depends on the petiole angle (Cox *et al.*, 2003; Chapter 2). The ethylene-induced petiole response was classified into three different aspects: ethylene-induced hyponastic growth, ethylene-induced early petiole growth, and ethylene-induced long term enhanced petiole growth (Figure 1). Although many similar genes were present in these groups, in some cases, dissimilarities were found. For instance, based on the expression pattern in cDNA-AFLP, *RpEIHG69* (Table 2) was classified as a gene putatively involved in hyponastic growth (the expression of the gene was higher at the early stage of ethylene treatment). However, in the micro-array analysis this gene was downregulated during elongation growth. The putative Arabidopsis orthologue of this gene (At3g05030) is involved in sodium:hydrogen antiport activity. This exchange of sodium and hydrogen can affect the pH of the cell (Padan *et al.*, 2001; Wiebe *et al.*, 2001). The role of apoplastic low pH in cell elongation is evident (Cleland *et al.*, 1991; Grebe, 2005; Li *et al.*, 2006; Qiu *et al.*, 2003; Vreeburg *et al.*, 2005). Therefore, it is possible that higher expression of this gene in early stages of ethylene treatment may be involved in the initiation of enhanced elongation growth. What downregulation of this gene in the next stage would mean is not yet clear.

Sometimes determination of involvement of the identified genes in ethylene-induced hyponastic growth or petiole elongation is difficult. This could be due to the tight relation and similarities between the two traits. Indeed, any change in the distribution of a particular hormone or the spatial balance of a plant growth regulator change normal growth (elongation) into differential growth (e.g. hyponastic) (Edelmann *et al.*, 2005; Friml *et al.*, 2002; Hays *et al.*, 1978). For instance, a local increase in auxin concentration was shown to result in a change in gene expression, which consequently led to alteration of the growth from absolute to differential (Esmon *et al.*, 2006). Furthermore, gravitropism and phototropism are consequences of an asymmetric distribution of auxin (Friml *et al.*, 2002; Vieten *et al.*, 2007). Indeed auxin-related genes were differentially expressed in our assay during ethylene induced (differential) growth. Furthermore, interaction between auxin and ethylene in differential growth is well established (Cox *et al.*, 2004; Friml *et al.*, 2002; Harper *et al.*, 2000; Lehman *et al.*, 1996; Luschnig *et al.*, 1998; Stepanova *et al.*, 2007). Therefore, there is a possibility that changes in the expression of auxin related genes are caused by changes in concentration or distribution of auxin during the time course of ethylene treatment which resulted in hyponastic growth during the first few hours and elongation growth later on.

While trying to identify homologous sequences we found that in some cases there were similarities between an *RpEST* and a conserved sequence in various

Arabidopsis genes (with either the same or different designated functions). For instance, products of all possible Arabidopsis orthologues of *RpEIHG42* are membrane-located and involved in oligopeptide transport; however, the genes showed different expression patterns during hyponastic growth or petiole elongation (Table 3). A putative orthologue of *RpEIHG42* (i.e. At4g16370) was downregulated, whilst another one (i.e. At5g53510) was upregulated (Table 3). Similarly, of the two putative orthologues of *RpEIEG91*, i.e. Arabidopsis auxin responsive factors *ARF8* and *ARF6* (Aida *et al.*, 2002; Berleth *et al.*, 2004) one was upregulated, the other downregulated. Differential expression of *ARF8* and *ARF6* in hyponastic growth and petiole elongation are likely to be due to the important role of proteins belonging to this family in both absolute and differential growth (Aida *et al.*, 2002; Remington *et al.*, 2004; Tian *et al.*, 2004). However, the differences could very well be caused by the dissimilarities in either the promoter or the function of genes in *R. palustris* and Arabidopsis.

From the data presented here we may conclude that there is a tight relation between ethylene-induced differential and enhanced petiole elongation. Since these are both growth responses it is likely that many genes will be expressed during both, albeit with temporal and positional differences.

Ethylene-induced hyponastic growth requires various physiological components

A small group of 10 genes was early-induced by ethylene in plants with an initial petiole angle of less than 30 degrees, suggesting their involvement in hyponastic growth (Chapter 2, Table 1, 2). These genes belong to different functional categories including proliferation and replication regulation (*RpEIHG1*), transcription factor activity (*RpEIHG48*), protein synthesis (*RpEIHG35*), protein kinase (*RpEIHG3*, *RpEIHG99*), energy production (*RpEIHG5*), and intracellular traffic and protein trafficking (*RpEIHG4*). These results indicate that ethylene-induced hyponastic growth is a complex growth response, during which diverse components with either regulatory or signalling function are regulated.

Cell trafficking might be an important event in ethylene-induced hyponastic growth and enhanced petiole elongation

The presence of an expressed stomatal cytokinesis-defective gene (*RpEIEG47*) and other cytokinesis related genes (Table 5), in both cDNA-AFLP and micro-array analysis, for At1g49040, a putative orthologue of *RpEIG47*, more than 2 fold increase in hyponastic growth and 1.6 in elongation growth was observed, suggest the possible involvement of changes in cytokinesis processes during (differential) growth (Table 3, 5). The Involvement of cell expansion in submergence-induced petiole elongation is well established (Voesenek *et al.*, 1999; Vreeburg *et al.*, 2005). In addition, abundant evidence clearly indicates that membrane trafficking is

Table 5. Overview of genes involved in ethylene-induced hyponastic growth and petiole elongation identified after micro-array analysis of transcripts of *R. palustris* on the *Arabidopsis thaliana* array. First column represents the putative Arabidopsis orthologues that were differentially expressed during hyponastic growth and/or petiole elongation during ethylene treatment. The expressed values of orthologue genes during ethylene-induced (hyponastic) growth are calculated by dividing the intensity of the individual spot through the average intensity of all presented spots and they are presenting the mean value of 4 biological and 4 experimental replicates. The cut-off value for gene expression is 1.4. Gene descriptions were taken from the Arabidopsis database (TAIR). Numbers in parenthesis were not significantly changed in expression.

AGI locus identifier	Gene description	Hyponastic fold change	Elongation fold change
At1g49040	stomatal cytokinesis defective/SCD1 protein	2.4	1.6
At1g02010	cytokinesis-related Sec1 protein	2.0	(1.1)
At5g55230	microtubule associated protein (MAP65/ASE1) family protein	-1.6	(1.3)
At5g54110	vesicle-associated membrane family protein/VAMP family protein	2.1	1.4
At2g45140	vesicle-associated membrane protein	(1.0)	2.0
At3g54300	synaptobrevin family protein similar to vesicle-associated membrane protein 7B	1.4	(1.1)
At1g04750	synaptobrevin family protein similar to Vesicle-associated membrane protein 722	(1.1)	1.4
At1g73430	sec34-like family protein similar to Vesicle docking protein SEC34 homolog [*Homo sapiens*]	(1.2)	-1.4
At4g39090	similar to cysteine proteinases, induced by desiccation but not abscisic acid, cysteine-type endopeptidase activity, response to desiccation	(-1.2)	-3.0
At1g21160	eukaryotic translation initiation factor 2 family protein/eIF-2 family protein similar to Elongation factor Tu domain 2	(1.2)	3.4
At1g66070	translation initiation factor-related similar to Eukaryotic translation initiation factor 3 subunit 1 (eIF-3 alpha)	2.3	1.7
At1g13950	eukaryotic translation initiation factor 5A-1/eIF-5A 1	2.1	-1.2
At1g76720	eukaryotic translation initiation factor 2 family protein/eIF-2	(1.1)	2.3
At2g40290	eukaryotic translation initiation factor 2 subunit 1, putative/eIF-2A	(-1.1)	1.9
At4g11420	eukaryotic translation initiation factor 3 subunit 10/eIF-3 theta/eIF3a (TIF3A1)	1.5	(1.1)
At2g39820	eukaryotic translation initiation factor 6, putative/eIF-6	1.5	(-1.2)
At3g02270	eIF4-gamma/eIF5/eIF2-epsilon	1.4	(1.1)
At3g56150	eukaryotic translation initiation factor 3 subunit 8	(-1.2)	1.6
At1g54270	eukaryotic translation initiation factor 4A-2/eIF-4A-2	(1.0)	1.6
At5g20920	eukaryotic translation initiation factor 2 subunit 2, putative/eIF-2-beta	(1.0)	1.5
At4g20980	eukaryotic translation initiation factor 3 subunit 7, putative/eIF-3 zeta	(-1.5)	(-1.1)

Table 5. Continued

AGI locus identifier	Gene description	Hyponastic fold change	Elongation fold change
At2g04520	eukaryotic translation initiation factor 1A	-1.5	1.3
At1g51380	eukaryotic translation initiation factor 4A, putative/ eIF-4A, putative	-2.1	(-1.1)
At4g33250	eukaryotic translation initiation factor 3 subunit 11/eIF-3	(-1.2)	-2.3
At1g04170	eukaryotic translation initiation factor 2 subunit 3	(-1.1)	-4.5
At1g72370	40S ribosomal protein SA (RPSaA) identical to laminin receptor-like protein	3.4	2.1
At2g17360	40S ribosomal protein S4 (RPS4A) contains ribosomal protein S4 signature from residues 8 to 22	(-1.2)	3.0
At2g41840	40S ribosomal protein S2 (RPS2S) protein	-1.1	2.2
At5g07090	40S ribosomal protein S4 (RPS4B)	(-1.3)	2.1
At5g41520	40S ribosomal protein S10 (RPS10B) contains similarity to 40S ribosomal protein S10	(1.1)	-4.0
At5g59240	40S ribosomal protein S8 (RPS8B) 40S ribosomal protein S8, *Prunus armeniaca*, EMBL:AF071889	-2.1	(1.0)
At1g29965	60S ribosomal protein L18A (RPL18aA) JRW	(1.1)	2.5
At3g48960	60S ribosomal protein L13	-2.3	2.3
At4g13170	60S ribosomal protein L13A	(-1.2)	2.1
At5g45775	60S ribosomal protein L11 (RPL11D)	(-1.1)	-22.6
At2g 01250	60S ribosomal protein L7 (RPL7B)	-1.6	-4.6
At4g14320	60S ribosomal protein L36a/L44 (RPL36aB)	(1.3)	-4.6
At1g14320	60S ribosomal protein L10 (RPL10A)	-2.0	-2.8
At2g33450	50S ribosomal protein L28, chloroplast (CL28)	-1.6	-1.4
At2g33310	auxin-responsive protein / indoleacetic acid-induced protein 13 (IAA13)	2.5	-
At1g04250	auxin-responsive protein / indoleacetic acid-induced protein 17 (IAA17)	1.6	(1.1)
At5g25890	auxin-responsive protein / indoleacetic acid-induced protein 28 (IAA28)	(1.1)	1.7
At1g04100	auxin-responsive protein / indoleacetic acid-induced protein 10 (IAA10)	-1.7	1.7
At3g23050	auxin-responsive protein / indoleacetic acid-induced protein 7 (IAA7)	-1.9	1.6
At1g51950	auxin-responsive protein / indoleacetic acid-induced protein 18 (IAA18)	-1.4	(1.3)
At3g15540	auxin-responsive protein / indoleacetic acid-induced protein 19 (IAA19)	-1.4	-1.7
At5g65670	auxin-responsive protein / indoleacetic acid-induced protein 9 (IAA9)	(1.1)	-3.7

Table 5. Continued

AGI locus identifier	Gene description	Hyponastic fold change	Elongation fold change
At2g46530	transcriptional factor B3 family protein / auxin-responsive factor AUX/IAA-related	(-1.3)	2.1
At2g01200	auxin-responsive AUX/IAA family protein contains Pfam profile: PF02309 AUX/IAA family	(-1.1)	1.8
At3g53250	auxin-responsive family protein similar to auxin-induced protein TGSAUR22	(-1.1)	6.5
At2g32410	auxin-resistance protein, putative strong similarity to SPIP42744 Auxin-resistance protein AXR1 {*Arabidopsis thaliana*}	3.4	(1.4)
At3g51200	auxin-responsive family protein similar to auxin-induced protein TGSAUR12 (GI:10185816) [*Tulipa gesneriana*]	(1.0)	4.2
At4g17270	similar to MO25 protein (early mouse development protein family), auxin-responsive family protein similar to auxin-induced protein AIR12 (GI:11357190) [*Arabidopsis thaliana*]	(1.0)	-12.1
At2g33860	auxin-responsive factor (ARF3) / ETTIN protein (ETT) identical to ETTIN GB:AF007788 from [*Arabidopsis thaliana*]	(-1.2)	-2.5
At2g04850	auxin-responsive protein-related related to auxin-induced protein AIR12 GI:11357190 [*Arabidopsis thaliana*]	-2.1	(-1.3)
At5g65980	auxin efflux carrier family protein K2A18	1.7	1.6

essential to the centrifugal development of the cell plate during plant cytokinesis (Minorsky, 2004). Furthermore, the involvement of endocytosis was proposed in gravitropism in Arabidopsis, which resulted in differential growth. The role of the protein encoded by stomatal cytokinesis-defective gene has been demonstrated in polarised vesicle trafficking during plant cytokinesis and cell expansion (Falbel *et al.*, 2003). Mutation of the gene in Arabidopsis, *cytokinesis defective 1* (*cyd1*), caused partial or missing cell walls in stomata and it was suggested that this mutation interfered with the execution of cytokinesis (Yang *et al.*, 1999). Identification of the cytokinesis-defective gene and many other genes involved in cell trafficking and cytokinesis (Table 2, 5) suggests that cell trafficking and changes in cytokinesis processes in order to change cell expansion may be important for ethylene-induced (differential) growth.

Ethylene-induced hyponastic growth and petiole elongation involve translationally and post-translationally regulation

The expression of genes encoding proteins involved in protein synthesis such as subunits of *Elongation Initiation Factors* (*EIF2, EIF3, EIF4, EIF5, EIF6*), were changed between 1.4-4.5 times in either ethylene-induced hyponastic growth or petiole elongation or in both responses (Table 2, 5). Furthermore, expression of genes encoding other components involved in the protein synthesis pathway, like

genes that encode *ATP dependent helicase*, *Elongation factor 1-alpha*, *Elongation factor Ts* and various ribosomal subunits and proteins (40S, 50S and 60S), were also differentially regulated between 1.4 - 22 times in either or both responses (Table 2, 5). Hutchins *et al.* (2004) presented the molecular mechanism linking cell proliferation with translational control through the cyclin-dependent kinases-Elongation initiation factor 4A (CDK-EIF4A) complex. Moreover, the role of Elongation factor 1-alpha in regulation of cytoskeletal rearrangement of the plant cell was demonstrated (Shiina *et al.*, 1994). In our data we identified a gene encoding *root hair defective3*, encoding a GTP binding protein. This protein is important in translation regulation (Table 2). GTP binding proteins are important in the perception of signals (Assmann *et al.*, 2002; Eckardt *et al.*, 2004; Hooley 1998) suggesting the involvement of these proteins in the regulation of many aspects of cell growth, differentiation, gene expression, and movement inside cells through the cytoskeleton. As a group, they form a universal mechanism that allows a cell to adapt to its environment (Assmann *et al.*, 2002; Hooley 1998).

Changes in the expression of genes encoding kinase proteins and glycosylation proteins are also observed in the cDNA-AFLP and micro-array experiments (Table 2, 3). These data suggest the effect of post-translational regulation in ethylene-induced responses through kinase- and glycosylation activity.

Identification of many differentially expressed genes involved in various aspects of protein synthesis by cDNA-AFLP and the micro-array experiment, suggest that changes in protein synthesis and protein activity through post–translational changes like phosphorylation and glycosylation are important for ethylene-induced (hyponastic) growth.

Redox signalling might be changed during ethylene-induced hyponastic growth and petiole elongation

Four genes putatively involved in redox signalling were differentially expressed in response to ethylene: subunit H of photosystem I reaction centre (PSI-H), ferodoxin dependent glutamate synthesis, glutathione transporters and GADPH (Table 2), (Buchanan and Balmer, 2005; Koh *et al.*, 2004; May *et al.*,1998; Schurmann 2003; Pfannschmidt *et al.*, 2001a; 2001b). Plants lacking PSI-H or PSI-L are highly deficient in state transitions and PSI-H is specifically associated with state transitions in PSI (Lunde *et al.*, 2000). State transitions are important in optimising photosynthesis when light is limited (Pfannschmidt *et al.*, 2001a; 2003). There is evidence that photosynthesis generates important signals to light control of gene expression by changes in the reduction/oxidation (redox) state of signalling molecules (Pfannschmidt *et al.*, 2001a; 2001b; 2003). Redox signalling mechanisms provide photosynthesis with the possibility to change the structure of the photosynthetic apparatus via a feedback control of photosynthesis gene expression (Fey *et al.*, 2005; Pfannschmidt *et al.*, 2001a).

Although no effect of photosynthesis on ethylene-induced (differential) growth is known, the expression of these genes suggest that the redox state of certain cells or tissues may be involved in this process.

Involvement of auxin in ethylene-induced stimulation of elongation growth

Amongst the ethylene-induced (hyponastic) growth regulated genes, several genes appeared to encode auxin related genes (Table 2, 3, 5). Auxin has been shown to be involved in hyponastic growth and the direction of stimulated growth (Hayes, 1978; Stowe-Evans *et al.*, 1998). Furthermore, auxin-induced proteins were shown to be important in auxin-mediated cell elongation in maize through post-transcriptional regulation (Knauss *et al.*, 2003). Moreover, Cox *et al.* (2006) and Voesenek *et al.* (2006) suggested that auxin is involved in the response of *R. palustris* to submergence. Moreover, our data, analysis of ethylene-induced hyponastic growth and petiole elongation genes, revealed several genes encoding *Indole-3-acetic acid-induced* proteins (IAA) (e.g. *IAA7, IAA10, IAA13*) (Table 5), small auxin-up RNA's (SAURs), (Table 2) and *auxin-responsive factors* (*ARF6 ARF8, ARF 3*) (Table 2, 5). These genes were differently expressed, sometimes more than 12 fold, during ethylene-induced (differential) growth. Furthermore, the expression of genes involved in auxin transport, e.g. auxin efflux, was also changed by ethylene-induced (hyponastic) growth (Table 5). These data suggest that ethylene-induced hyponastic growth and petiole elongation likely involves auxin transport/action.

Regulatory mechanisms

Regulation of gene expression is generally through transcription factor (TF) activity (Bussemaker *et al.*, 2006, Sun *et al.*, 2006). TFs bind to specific DNA sequences in the promoter region of genes thus altering expression of the genes. Alteration in the activity of certain TFs upon exposure to a stimulus modifies the proteins and enzymatic activity in individual plant cells.

Promoter analyses of differentially expressed genes during hyponastic and elongation growth by means of the ATHENA database and tool (O'Connor *et al.*, 2005) revealed a correlation between ethylene-induced genes and known TFs. There was an over-representation of certain motifs in the promoter region of the genes (Table 4), suggesting the presence of a global ethylene-dependent mechanism to control the expression of ethylene-induced genes. Two motifs could be recognised by ABA. The ACGT-core in the Abscisic acid Responsive Element (ABRE)-binding factor in rice and the CACGTG motif (another ABA motif, Hattori *et al.*, 2002, Yazaki *et al.*, 2004) were both enriched in genes downregulated during ethylene-induced elongation growth (Table 4). Interestingly, the ABRE-like binding motif was found to be enriched in genes that were upregulated in ethylene-induced enhanced growth (Table 4).

Genes containing ABRE and CACGTG motifs were shown to be upregulated when plants were treated with ABA (Yazaki *et al.*, 2004). Genes carrying the ABRE-like motif, however, were downregulated in response to ABA treatment (Hung *et al.*, 2007). These data suggest that there is interaction between ethylene and ABA in elongation growth induced by ethylene. This interaction was already suggested in submergence-induced growth in *R. palustris* (Benschop *et al.*, 2005) and is confirmed and extended by our data.

Materials & Methods

Plant materials and treatment

Seeds of *R. palustris* germinated on black polyethylene beads (EIf atochem, France) saturated with tap water. Before use the beads were heated at 95°C for one week to prevent ethylene release during the germination procedure. The container with the seeds was placed in a growth cabinet for 9 days (12 h light at 20°C, 70 µmol m^{-2} s^{-1} photon flux density, and 12 h of darkness at 10°C). Seedlings of identical size were transplanted to plastic pots (70 mL) filled with a mixture of potting soil and sand (2:1, v/v), enriched with 0.14 mg of MgOCaO per pot. Each pot was saturated with 20 mL of nutrient solution containing: 7.50 mM $(NH_4)_2SO_4$, 15.00 mM KH_2PO_4, 15.00 mM KNO_3, 86.35 µM Fe-EDTA, 4.27 µM $MnSO_4$, 1.81 µM $ZnSO_4$, 0.32 µM $CuSO_4$, 42.67 µM H_3BO_3, and 0.53 µM Na_2MoO_4. Plants were grown for 19 d in a growth chamber (20°C, 70% relative humidity, 16 h of light, 200 µmol m^{-2} s^{-1} photon flux density). Plants in pots were placed on irrigation mats (Maasmond-Westland, Utrecht, the Netherlands). The mats were automatically watered with tap water to saturation twice a day, and the excess water was drained.

Plants used for the isolation of differentially expressed genes with cDNA-subtraction were exposed to 2 different treatments:

1. Non-manipulated petioles, natural initial angle of 30-40 degrees; treated with ethylene for 2 hours.
2. Petiole angle manipulated to 70 degrees, treated with ethylene for 2 h.

Plants used for the isolation of differentially expressed genes with cDNA-AFLP were exposed to 8 different treatments:

1. Non-manipulated petioles, original start angle of petioles with natural initial angle of 30-40 degrees; treated with ethylene for 2 h.
2. Petiole angle manipulated to 70 degrees, treated with ethylene for 2.
3. Petiole angle manipulated to 0 degrees (horizontal), fixed angle, grown in air.
4. Petiole angle manipulated to 0 degrees (horizontal), fixed angle, exposed to ethylene for 2 h.

5. Petiole angle manipulated to 90 degrees (vertical), fixed angle, grown in air.
6. Petiole angle manipulated to 90 degrees (vertical), fixed angle, exposed to ethylene for 2 h.
7. Petiole angle manipulated to 90 degrees (vertical), fixed angle, grown in air for 26 h.
8. Petiole angle manipulated to 90 degrees (vertical), fixed angle, exposed to ethylene for 26 h.

Plants used for micro-array analysis were exposed to 4 different treatments:

1. Non-manipulated petioles, original start angle of petioles with natural initial angle of 30-40 degrees; treated with ethylene for 3 h.
2. Non-manipulated petioles, original start angle of petioles with natural initial angle of 30-40 degrees, grown in air for 3 h.
3. Petiole angle manipulated to 70 degrees, treated with ethylene for 3 h.
4. Petiole angle manipulated to 70 degrees, grown in air for 3 h.

Ethylene treatment

R. palustris plants were treated with 5 μL L^{-1} ethylene in a flow-through system as described in Chapter 2.

Total RNA Isolation

Total RNA was extracted from plants using a modified Kiefer *et al.* (2000) method. Nucleon Phytopure DNA extraction resin (50 μL) (GE Healthcare, Diegem, Belgium; cat. No. RPN 8510) was added to the samples after heating at 60˚C. The RNA concentration was determined spectrophotometrically and adjusted to a final concentration of 1 μg μL^{-1}. The integrity of the RNA was tested by agarose gel analysis.

cDNA synthesis and cDNA subtraction analysis

First and second cDNA strands were synthesised according to standard protocols of super smart cDNA PCR synthesis kit from Clontech (Westburg, Leusden, the Netherlands, cat. No.635000). To amplify the cDNA by Long Distance PCR, each sample was subjected to 27 cycles. To generate short, blunt-end double stranded cDNA fragments that are necessary for adaptor ligation, *Rsa*I digestion was applied. To subtract genes that are regulated in hyponastic growth we used two treatments: petioles with the initial angle of approximately 30 degrees were designated sample 1 and the plants for which the orientation of the pots, and therefore the petioles, was artificially set at 70 degrees was called sample 2. Both samples were treated with ethylene for 2 h. The second sample did not demonstrate hyponastic growth in response to ethylene. cDNA subtraction was performed using the Clontech PCR-Select cDNA subtraction kit (Westburg, Leusden, the Netherlands,

cat. No. 637401) according to the manufacturer's instructions. Twenty-seven cycles of primary PCR and 12 cycles of secondary PCR were performed using the Advantage cDNA polymerase mix. We generated forward subtracted cDNA pool 'F cDNA pool' with cDNA 1 as tester and cDNA 2 as driver and reverse-subtracted cDNA pool 'R cDNA pool' with cDNA 2 as tester and cDNA 1 as driver.

cDNA-AFLP analysis

The cDNA-AFLP-based transcript profiling procedure was performed according to a modified cDNA-AFLP protocol (Breyne *et al.*, 2002, Feron *et al.*, 2004). Double-stranded cDNA was made from 5 µg total RNA according to the protocol of the manufacturer (Roche, Germany, Cat. No. 11787896001) mRNA capture Kit). The restriction enzymes used to digest the cDNA for this plant species were *BstY*I and *Mse*I (New England Biolabs, Beverly, MA, USA). For pre-amplification, an MseI-primer without a selective nucleotide (Mse0) was chosen with a *BstY*I-primer containing a T or a C at the 3' end (BstC and BstT). The amplification mixtures obtained were diluted 600-fold and 5 µL was used for final selective amplifications. BstT or BstC with one selective nucleotide and an Mse2 primer with two selective nucleotides, respectively, were used for the cDNA-AFLP analysis. All 128 possible primer combinations labelled with [^{33}P]ATP-labelled (Amersham, y-^{33}P-ATP, specific activity 3000 Ci mmol^{-1}) were performed. Selective [^{33}P]ATP-labelled amplification products were separated on a 4.5 % polyacrylamide gel that was allowed to run until 4300 Vh using the 'sequigen system' (Bio Rad, Veenendaal, the Netherlands). Gels were taken from the glass plate and dried onto 3MM Whatman paper, and positionally marked before being exposed to Kodak Biomax film (GE Healthcare, Diegem, Belgium) for 3 d.

Isolation and identification of fragments

Films were aligned with markings on the gels. The bands of interest were marked, cut out with a razor blade, and incubated in 100 µL of water and boiled for 5 min. DNA was precipitated from the solution by cooling on ice before adding 10 µL 3 M NaAc (pH 5.2), 2 µl 20 mg mL^{-1} glycogen as co-precipitant and 200 µL 96% ethanol and incubated overnight at -20ºC, prior to centrifugation. The isolated DNA was re-amplified using the same PCR conditions and primer combinations as for the non-selective amplification. The re-amplified products from the AFLP experiments and the separated bands from two way subtraction experiments were ligated to the pGEM-T EASY vector (Promega, Leiden, the Netherlands) and clones were sequenced using T7 and SP6 primers (Macrogen, Korea, http//www.macrogen.com). Database searches were performed using BLAST algorithms (Altschul *et al.*, 1997) at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST) and TAIR (The Arabidopsis information resource; http://www.arabidopsis.org) network services.

Genome-wide expression profiling of Rumex palustris *to identify genes involved in ethylene-induced hyponastic growth and petiole elongation*

To identify genes involved in ethylene-induced hyponastic growth and petiole elongation of *R. palustris* we compared the transcript levels in the petioles of differently treated plants. We selected ethylene /control and initial petiole angle, 30 or 70 degrees, as variables. This resulted in 4 different RNA pools, control/30degrees (30C), ethylene/30degrees (30E), control/70degrees (70C) and ethylene/70degrees (70E). The following comparisons (30C/30E, 30E/70E, 70E/70C and 70C/30C) were made on an Arabidopsis full genome micro-array (CATMA). Because of the sequence difference between Arabidopsis and *R. palustris* we could only measure transcripts with sufficient homology. Since the transcript of Rumex was applied on Arabidopsis arrays (heterologous hybridisation), not all the transcripts hybridised with all the arrays. Around 5300 (5313) genes were present on at least half of the 12 slides whereas only ~1900 (1916) were present on 10 or more. The transcripts present on at least half of the slides were further studied.

CATMA micro-array analysis

Micro-array analysis was performed with CATMA version 2 arrays (complete Arabidopsis transcriptome micro-array; Allemeersch *et al.*, 2005; Hilson *et al.*, 2004) which contained 24,411 gene-specific tags (GSTs). The GSTs, which are between 150 and 500 bp in length and show no more than 70% identity with any other sequence in the Arabidopsis genome, were spotted on GAPSII glass slides (Corning Incorporated, Acton, MA, USA) using a BioRobotics Microgrid II TAS spotter (Genomic Solutions, Ann Arbor, MI, USA) and cross-linked for 4 h at 80°C. Detailed information about CATMA and database access can be found at htpp://www.catma.org (Crowe *et al.*, 2003) and http://genomics.bio.uu.nl/.

Micro-array labelling, hybridisation and scanning

R. palustris mRNA was amplified with the MessageAmp RNA kit (Ambion, Hoogeveenenweg, the Netherlands). Amplified mRNA (5 µg) was used as a template to synthesise modified cDNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random nonamers (Gene Link, Westchester County, NY, USA) for 2 h at 42°C with the incorporation of 5-(3-aminoallyl)-dUTP (Ambion; ratio dUTP/dTPP of 7/3), yielding approximately 2 µg cDNA. RNA template was removed by hydrolysis with 3 µL 2.5 M NaOH per 30 µL for 15 min at 37°C. The hydrolysis was stopped by neutralisation with 15 µL 2 M MOPS and put on ice. cDNA was purified using the MineLute PCR purification kit (Qiagen, Venlo, the Netherlands) and labelled with Cy3 or Cy5 mono-reactive dye (Amersham, Buckinghamshire, UK). The reaction was quenched after 60 min using 4.5 µL 4 M hydroxylamine (Sigma-Aldrich, Zwijndrecht, the Netherlands) and incubated in

the dark for 15 min The labelled cDNA was purified as described above, and incorporation of Cy3 or Cy5 was determined using an UVmini-1240 spectrophotometer at 550 or 650 nm, respectively. CATMA arrays were denatured in boiling demineralised water for 3 min and dipped in ethanol afterwards. Slides were spun dry for 3 min at 300 g in 50 mL tubes, covered with a LifterSlip (Erie Scientific Company, Portsmouth, NH, USA), and subsequently pre-hybridised at 55°C with 100 µL filtered pre-hybridisation solution containing 25% formamide (Merck, Whitehouse Station, NJ, USA), 5x SSC, 0.1% SDS and 1% BSA, fraction V, minimum 96% (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 15 min at 55°C.

Arrays were transferred to demineralised water, dipped five times and immediately submerged in isopropanol. Slides were spun dry as before, covered with a LifterSlip, and hybridised within 1 h. For each hybridisation, 50 µL filtered (0.2 µm) 2x hybridisation mix was made, containing 50% de-ionised formamide (Sigma-Aldrich, Zwijndrecht, the Netherlands), 10xSSC and 0.2% SDS. Herring-sperm DNA (1 µL of 11 mg mL^{-1} stock; Sigma-Aldrich, Zwijndrecht, the Netherlands) was added, and the mix was heated to 42°C to prevent precipitation of SDS. Each pair of *R. palustris* RNA was concentrated in a SpeedVac (type SC100; Savant Instruments, Holbrook, NY, USA) to a volume of 50 µL, and added to 50 µL 2x hybridisation mix. The probes were denatured for 5 min at 95 °C and centrifuged for 2 min at 15000 g in a standard tabletop centrifuge and immediately applied to the arrays. The arrays were hybridised in hybridisation chambers (Corning Incorporated, Acton, MA, USA) containing one drop of 20 µL water on each side, covered by foil, and placed for 16–20 h at 42°C in a water bath. After the hybridisation, the arrays were washed twice in a low-stringency wash solution containing 1x SSC, 0.2% SDS and 0.1 mM DTT for 4 min at 40°C. The arrays were subsequently washed in a high-stringency wash solution containing 0.1x SSC, 0.2% SDS and 0.1 mM DTT for 4 min at 40°C and in a final wash solution containing 0.1x SSC and 0.1 mM DTT each for 4 min at room temperature. The slides were dipped five times in demineralised water and immediately submerged in isopropanol.

The slides were spun dry as described above and scans of the arrays were made using a ScanArray Express HT (PerkinElmer, Wellesley, MA, USA). Spot intensities of the scans were determined by ImaGene software version 6.5.1 (BioDiscovery, El Segundo, CA, USA).

Micro-array statistics

Spot intensities from CATMA arrays were analysed by LIMMA (Smyth, 2004) version 1.7.2 and limmaGUI version 1.2.5 (Wettenhall and Smyth, 2004) software packages from Bioconductor (Gentleman *et al.*, 2004) running in R version 1.9.1 (CR Foundation, Vienna, Austria; Ihaka and Gentleman, 1996). The intensities were normalised by the print tip LOESS to correct for possible within array, dye and print-tip effects. Subsequently, all arrays were normalised between arrays by scaling to obtain the same median absolute deviation for each array,

thereby enhancing the comparison between them (Smyth and Speed, 2003). Spots were determined as present when their individual average intensity was above the average intensity of all spots. Since most spot per array were empty an average intensity of all spots will generate cut-off intensity just above the real back ground. Transcripts were treated as differently abundant between samples when the log2 ratio was > 0.5 or < -0.5. The standard error of the difference in log2 ratio due to slide variations (4 slides per treatment) was ±0.46.

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Chapter 4 **Expression analysis of genes putatively involved in ethylene-induced hyponastic growth and petiole elongation of *Rumex palustris*, and the functional analysis of orthologous candidate genes in *Arabidopsis thaliana***

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Abstract

The complexity of ethylene-driven growth in *Rumex palustris* upon submergence suggests the involvement of many genes under tight regulation. A comprehensive understanding of the changes in the expression of genes responsive to ethylene in *R. palustris* is crucial to elucidate the signal transduction networks that lead to hyponastic growth and under water elongation. *R. palustris*, a model for studying plant responses to flooding, was used to identify genes potentially responsible for elongation growth, either differential or non-differential, in response to ethylene. In this study, the expression kinetics of the identified genes of *R. palustris* was monitored in response to ethylene and submergence using Real Time RTPCR and the putative involvement of candidate genes in (differential) growth during ethylene and submergence treatment was confirmed. Clear differences in the patterns of gene expression during ethylene treatment and submergence were observed. Since elevated ethylene is just one of the changes during submergence, difference in gene expression between ethylene and submergence treatment are to be expected. In addition, investigation of the localisation of two candidate genes by *in situ* hybridisation, using sections of petioles of *R. palustris* exposed to ethylene and submergence, showed that, although different expression between the control and treatments were obvious, these genes were not differentially expressed between the abaxial and adaxial side of the petiole. The expression of both genes is restricted to the basal zones of the petioles.

Little information about the genome of *R. palustris* is available and *Arabidopsis thaliana* petioles, like *R. palustris*, respond to ethylene with hyponastic growth, therefore the functionality of the candidate ethylene-induced genes was studied in Arabidopsis. Analysing the function of putative *R. palustris* orthologous genes in Arabidopsis during ethylene-induced hyponastic growth revealed that a subset of these genes is likely to control the initial angle of the petiole since, mutations in these genes resulted in constitutive elevated initial petiole angles in Arabidopsis mutant plants compared to the wild type Columbia-0. Interestingly, some of the genes have an effect on either the angle of the petiole or leaf blade angle or both. Mutant analysis revealed a role for PSI-H subunit of photosystem I, glyceraldehyde-3-phosphate dehydrogenase and glutamate synthase in hyponastic growth of Arabidopsis. Additionally, the effect of auxin response factor *ARF8* on hyponastic growth was demonstrated.

Introduction

In plants, growth is the result of cell division and cell enlargement which is regulated by both internal and external stimuli, such as hormones or abiotic and biotic stress (den Boer and Murray, 2000; Francis and Sorrell 2001; Kirham *et al.*, 1972; Qi and John, 2007; Stoynova-Bakalova *et al.*, 2004). In contrast to growth of all cells, differential growth, growth to occur only in particular parts of an organ or tissue, will change the orientation of plant organs (Kang, 1979). This enables plants to anticipate to directional stresses induced by e.g. submergence or neighbouring plants (Cox *et al.*, 2003; 2004; 2006; Friml *et al.*, 2002; Millenaar *et al.*, 2005; Pierik *et al.*, 2003; Voesenek and Blom, 1989).

Flooding is a severe environmental stress in which a (fast) reaction of plants is required in order to survive. Differential growth of the abaxial side of the petiole (Chapter 2, Cox *et al.*, 2003) followed by non-differential elongation of the entire petiole occurs in *Rumex palustris* in response to flooding (Cox *et al.*, 2003; 2004; 2006; Voesenek and Blom, 1989). The primary role of ethylene in submergence-induced growth responses is indicated by observations that exogenous ethylene application without submergence largely mimics these growth responses (Chapter 2, Cox *et al.*, 2003). Fast accumulation of ethylene inside plant tissues under water, due to slow gas diffusion rates, can induce changes in growth via modifications in gene expression (Voesenek *et al.*, 1993; 2006).

In addition to ethylene, other plant hormones, i.e. abscisic acid and gibberellin also play important roles in the regulation of petiole elongation during submergence (Benschop *et al.*, 2006; Voesenek *et al.*, 2003a). More downstream, the up-regulation of cell wall loosening proteins (e.g. expansins) is associated with submergence-induced elongation (Vreeburg *et al.*, 2005; Vriezen *et al.*, 2000).

We already showed that hyponastic growth and petiole elongation in *R. palustris*, as a result of submergence or ethylene treatment, are very similar (Chapter 2). Interestingly, in *Arabidopsis thaliana* hyponastic growth of the petiole and enhanced growth of the hypocotyl can also be induced by ethylene (Millenaar *et al.*, 2005; Smalle *et al.*, 1997). In Arabidopsis, the interaction of ethylene, brassinosteroids and auxin controls elongation of the hypocotyl (De Grauwe *et al.*, 2005). Moreover, transcriptional profiling of ethylene-related genes and their effect in different responses, which were studied extensively over the past few years (Alonso *et al.*, 2003; Schenk *et al.*, 2000), demonstrated a regulatory effect of ethylene on many biological processes, from metabolism to signal transduction (Alonso *et al.*, 2003; Schenk *et al.*, 2000). The complexity of ethylene-driven growth enhancement in *R. palustris* upon submergence suggests the involvement of many genes under tight regulation. A comprehensive understanding of the changes in the expression of genes responsive to ethylene in *R. palustris* is crucial to elucidate the signal transduction networks that lead to hyponastic growth and under water elongation. *R. palustris*, a model for studying the plant responses to flooding, was used to identify genes potentially responsible for elongation growth in response to ethylene,

either differential or non-differential (Chapter 3). These genes were identified by employing three differential expression approaches i.e., cDNA-AFLP, cDNA subtraction and micro-array analysis of plants treated with ethylene.

In this study, the expression kinetics of the identified genes of *R. palustris* was monitored using Real Time RTPCR. In addition, the localisation of two of these candidate genes was investigated by *in situ* hybridisation, using sections of petioles of *R. palustris* exposed to both ethylene and submergence. Since little information about the genome of *R. palustris* is available and transformation is not yet possible, the functionality of the candidate ethylene-induced genes was studied in Arabidopsis. Since Arabidopsis petioles, like *R. palustris*, respond to ethylene with hyponastic growth (Millenaar *et al.*, 2005; Voesenek *et al.*, 2006), we hypothesise that the involved regulatory mechanism underlying this trait is very similar. Finally, an Arabidopsis mutant-based approach, using putative *R. palustris* orthologous genes in Arabidopsis, was used to analyse the function of these genes in ethylene-induced hyponastic growth in Arabidopsis.

Results

Patterns of gene expression and growth show similar kinetics during ethylene and submergence treatment in R. palustris

In Chapter 3 we identified by subtraction and cDNA-AFLP analysis 119 genes that are differentially expressed during ethylene-induced hyponastic growth and/or enhanced petiole elongation. From those, 21 show high homology with expressed genes in Arabidopsis or other plants species (Table 2 in Chapter 3). To analyse the kinetics of the transcription of those 21 genes, total RNA was isolated from the third petiole during treatments with air, ethylene and submergence at several time points (see material and methods). Almost all 21 genes showed significant differences in expression during ethylene treatment compared to control ($p<0.05$) at least in one time point (data not shown). *RpEIHG35* was the only gene that failed to amplify.

If expressions of the genes were plotted per treatment a slight peak of expression for most of the genes was observed during control conditions at t=2 h (data not shown). When the relative expression patterns of candidate genes (fold expression of treatment subtracted from fold expression of air grown plants) were considered during ethylene and submergence treatment, a striking difference in amplitude between the two treatments was observed (Figure 1, 2). Ethylene treatment induced the genes to a much higher level than submergence. Moreover, there is a shift in timing of expression since ethylene induced discrete peaks at 2.5 and 6 h (Figure 1), whereas a more diffuse pattern was observed during submergence (Figure 2).

Three distinguishable stages were observed in the growth responses of plants to ethylene and/or submergence. The first stage in ethylene-treated plants is the

Ethylene

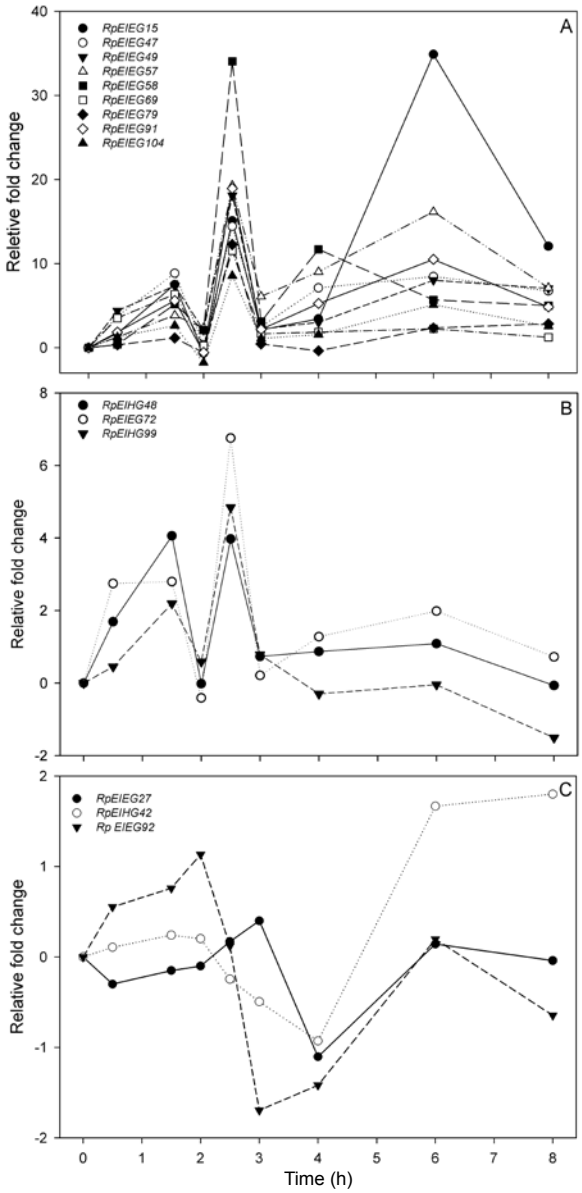

Figure 1. Ethylene-induced expression pattern of *Rumex palustris* genes selected by cDNA-AFLP and confirmed by RT-RT-PCR in ethylene treatment. A to C represent expression patterns of three individual clusters (1-3) from the hierarchical clustering. The level of expression of every individual gene was normalised with the expression value of 18S and expressed to the relative value of the air treatment. The expression value for gene *RpEIEG58* was divided by 15 to fit (graph A). The values are means of 4 independent replicates in which every replicate contained 3 individual petioles. The relative expression values represent the fold expression of treatment subtracted from the fold expression of air grown plants and the significant differences between treatment and control were calculated by Student test ($p<0.05$).

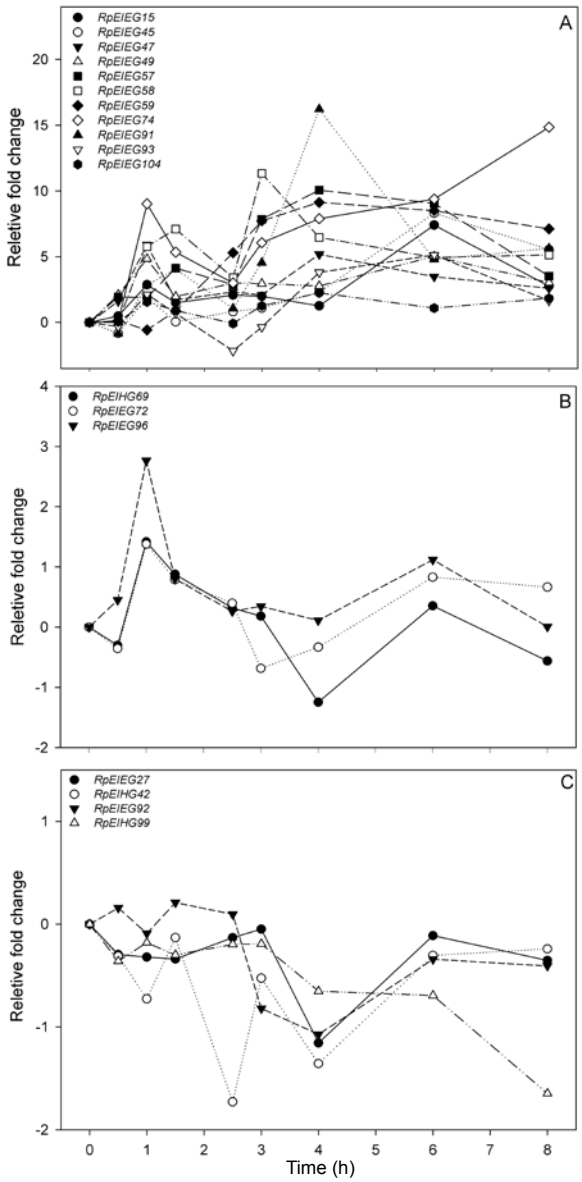

Figure 2. Submergence-induced expression pattern of *Rumex palustris* genes selected by cDNA-AFLP and confirmed by RT-RT-PCR in submergence treatment. A to C represent expression patterns of three individual clusters (1-3) from the hierarchical clustering. The level of expression of every individual gene was normalized with the expression value of 18S and expressed to the relative value of the air treatment. The expression value for gene *RpEIEG58* and *RpEIEG74* were divided by 10 to fit (graph A). The values are means of 4 independent replicates in which every replicate contained 3 individual petioles. The relative expression values represent the fold expression of treatment subtracted from fold expression of air grown plants and the significant differences between treatment and control were calculated by Student test ($p<0.05$).

so-called lag phase characterised by an unchanged phenotype. This period lasted 2 h and within this period an augmented expression of several genes, 1.5 h after the start of the treatment was observed (Figure 1 A, B).

A second phase was distinguished between the end of the lag phase for hyponastic growth (2 h) and the end of the lag phase for petiole elongation (3h) (Chapter 2, Figure 2). An increase in the expression of most genes studied upon ethylene application was recorded 2.5 h after the start of the treatment (Figure 1A, B).

The third phase, starting after 4 h, is characterised by maintenance of enhanced elongation growth and relatively high petiole angles. Hyponastic growth in ethylene treated plants stopped after the petiole angle reached approximately 70 degrees after 4-5 h, but elongation growth continues for days (Chapter 2). Also this phase is characterised by high expression levels of many of the investigated genes compared to control conditions (Figure 1A).

Submerged plants demonstrated all three phases; these were characterised by slightly shorter lag phases for both hyponastic growth and petiole elongation compared to ethylene treated plants (Chapter 2). These phases were correlated to an overall lower expression level in the studied genes during submergence compared to ethylene-exposed plants (Figure 2).

A hierarchical average linkage clustering of the relative expression of candidate genes for both ethylene and submergence treatments resulted in 3 main expression profiles during ethylene and submergence treatments.

During ethylene treatment cluster 1 contains a subset of genes that exhibit an early augmented expression (after one hour of treatment) that is maintained up to 8 h (Figure 1A). The second cluster contains three genes (*RpEIHG48*, *RpEIEG72*, *and RpEIHG99*) and exhibited a strong increase in expression (2.5- to 6-fold) during the first 3 h of ethylene treatment (Figure 1B). However, the genes *RpEIEG93* and *RpEIEG96*, which cluster to this group as well, did not show significant increase in their expression in first two hours and showed higher expression at a later stage (after 2.5 h). Based on this we did not consider them as early expressed gene in the 2nd cluster (data not shown).

The third cluster contains the gene fragments *RpEIEG27*, *RpEIHG42* and *RpEIEG92,* that showed a significant decrease in expression during the first 2 to 4h of ethylene (Figure 1C). However, a significant increase of expression after 6-8 h was observed for *RpEIHG42*. Out of these three clusters the expression profile of gene *RpEIEG59,* showed a significantly lower expression in ethylene compared to control in the first 3 h of the experiment. However, it shows higher levels of expression than control plants after 4 h of treatment and lasted until the end of the experiment (data not shown).

In the submergence treatment (Figure 2) the genes of the first cluster were highly expressed (2-200 fold) from 1 h after the start of the treatment; this pattern lasted until the end of the experiment (Figure 2A). The initial expression pattern of the second cluster (Figure 2B) is similar to the second group in ethylene treat-

ment: high expression level during the first 2 h of the submergence treatment. The third subset of genes including *RpEIEG27*, *RpEIEG92*, and *RpEIHG99* showed a decrease of expression in response to submergence compared to control, especially after 3 h (Figure 2C).

In situ *hybridisation*

Using RT-RTPCR we showed the expression of isolated genes in the petioles of ethylene- and submergence-induced plants. Based on the results observed in Chapter 2 with respect to the location of the elongation zone (Chapter 2, Figure 4), we hypothesise that genes differentially expressed during hyponastic growth

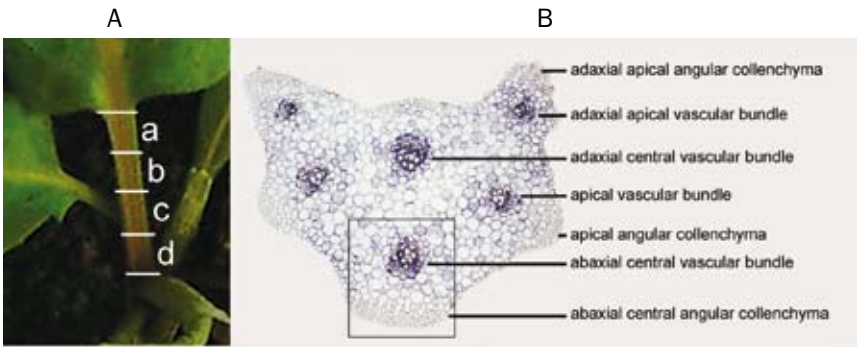

Figure 3. (A) *Rumex palustris* petiole depicting the zones, from apex to base (a-d) from which cross sections were made and (B) Cross section of a petiole defining the localisation of tissues. The square represents the abaxial part of the petiole in a cross section of the petiole of *R. palustris*. Only this subsection is shown in subsequent pictures (Figure 5, 6 and 7)

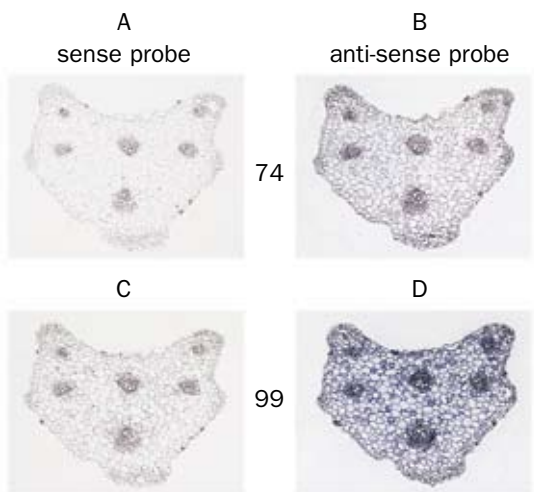

Figure 4. *In situ* hybridisation of petiole cross sections of *Rumex palustris* with the sense probes of *RpEIEG74* (A) and *RpEIHG99* (C) and the antisense probes of *RpEIEG74* (B) and *RpEIHG99* (D).

are likely to be localised mainly in cells at the basal part of the petiole, specifically at the abaxial side (Chapter 2). For genes involved in elongation growth we expect to find equal expression on both sides of the petiole. To test this hypothesis RNA *in situ* hybridisation was used to localise the expression of two differentially expressed genes, one possibly related to hyponastic growth *RpEIHG99*, a putative receptor kinase protein, and one that might be related to elongation growth *RpEIEG74* (no clear function).

Petioles were first divided transversally into 4 pieces (Figure 3A). Subsequently, *in situ* expression analysis was performed on cross sections (Figure 3B) at 4 time points during the 8 h treatment (1 h, 2 h, 3 h, and 8 h). Data of 2 h and 8 h are

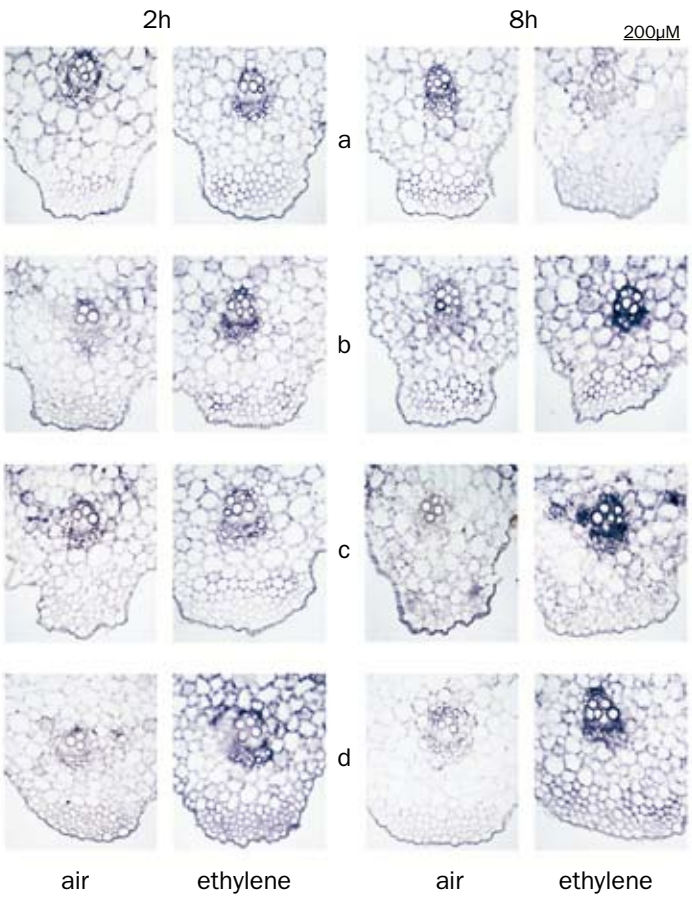

Figure 5. *In situ* expression pattern of *RpEIEG74* mRNA during control conditions (air) and 2 and 8 h ethylene treatment. *In situ* hybridisation with *RpEIEG74* antisense probe was performed on the cross sections of abaxial part of the petiole of *Rumex palustris*. Petioles were divided into 4 parts from apex to base (a-d). Scale bar: 200 µm.

presented. For both genes the anti-sense probe showed higher intensity compared to the sense probe (Figure 4), indicating a good signal-to-noise ratio.

A general elevated expression of both genes in ethylene-induced petioles was observed in the first two sections from the base (Figure 5 section c/d and 6 section c/d compare air and ethylene 2 h treatment). In submerged plants the intensity of the expression of *RpEIHG99* was only higher than the control in the most basal section of the petiole (Figure 7 section d). Although a higher expression at the abaxial side of the petiole for *RpEIHG99*, a gene that seems to be involved in hyponastic growth (Chapter 3), was expected, no dissimilarity in expression was observed at the abaxial side compared to the adaxial side of the petiole (data not shown).

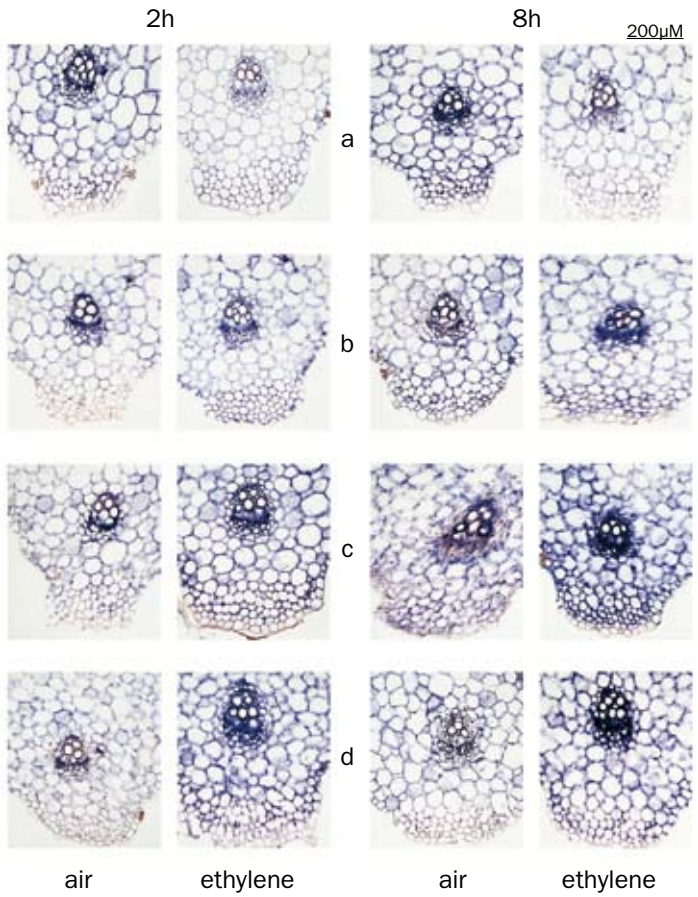

Figure 6. *In situ* expression pattern of *RpEIHG99* mRNA during control conditions (air) and 2 h and 8 h ethylene treatment. *In situ* hybridisation with *RpEIHG99* antisense probe was performed on the cross sections of abaxial part of the petiole of *Rumex palustris*. Petioles were divided into 4 parts from apex to base (a-d). Scale bar: 200 µm.

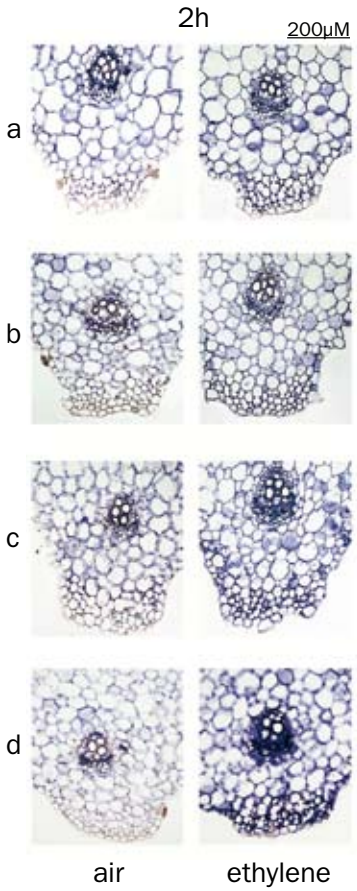

Figure 7. *In situ* expression pattern of *RpElHG99* mRNA during control conditions (air) and 2 h submergence. *In situ* hybridisation with *RpElHG99* antisense probe was performed on the cross sections of abaxial part of the petiole of *Rumex palustris*. Petioles were divided into four parts from apex to base (a-d). Scale bar: 200 µm.

Comparison of the expression of both genes after 2 h and 8 h during both ethylene and submergence treatments with the expression of the genes in control revealed intense labelling in treated tissue compared to control (Figure 5, 6). These results indicate enhanced expression after start of the treatment until the end of the treatment.

Analysis of Arabidopsis thaliana *lines carrying T-DNA insertions in putative orthologous genes differentially expressed upon ethylene treatment in* R. palustris

To investigate the functional significance of the expressed genes during hyponastic growth, mutants carrying T-DNA insertions in the putative orthologues of *R. palustris* genes in Arabidopsis were analysed for ethylene-induced hyponastic growth (Table 1). A quantitative measurement on the angles of petioles and leaves

was performed during 24 h of ethylene treatment. The angles of the petioles and leaf blades were measured relative to the horizontal line (Figure 8) and the response to ethylene was measured by subtracting the angle of the petiole or leaf blade during ethylene treatment from the angle of air control treatment. Subsequently, this was compared to the response of Col-0 wild type plants (since all the mutants provided by the Arabidopsis stock center, NASC, were in a Col-0 background).

The mutant analyses revealed the involvement of three classes of Arabidopsis genes in ethylene-induced hyponastic growth. The first group of genes only affected petiole movement and was therefore designated as petiole specific. This group consisted of one gene, At1g19220 a putative orthologue of *RpEIEG91,* coding for *ARF19* (an auxin responsive factor) in Arabidopsis (Table 1). Insertion mutation of this gene resulted in a stronger hyponastic response to ethylene after 13 h of treatment, suggesting a role in maintaining hyponastic growth in the Arabidopsis petiole (data not shown).

The second group of mutants was affected in leaf blade movement and is therefore putatively leaf blade specific (Table 1) (Figure 9, 10). In this group, only the mutation in gene At3g16140 (a putative orthologue of *RpEIEG92*) caused a transient decrease in response to ethylene; other mutations resulted in a temporary increase in the response of the plants to ethylene treatment (Figure 9, 10). Moreover, two distinct patterns of response were observed in this group of mutants. The first pattern consisted of mutants exhibiting a higher hyponastic response after 2 h of treatment, lasting until the end of the treatment i.e. At5g04140 (putative orthologue of *RpEIEG96*) and At1g14390 (putative orthologue of *RpEIEG45*) (Figure 9, Table 1). The second pattern of response comprised of mutants showing a higher hyponastic response only in the first few hours of ethylene treatment; i.e. mutation in At1g04380 (a putative orthologue of *RpEIEG58*) showed a response from the start of the experiment and lasted 7 h after treatment. Mutation in At5g64410 (a putative orthologue of *RpEIHG42*) showed an increase in response between 3 h until 10 h of treatment (Figure 10).

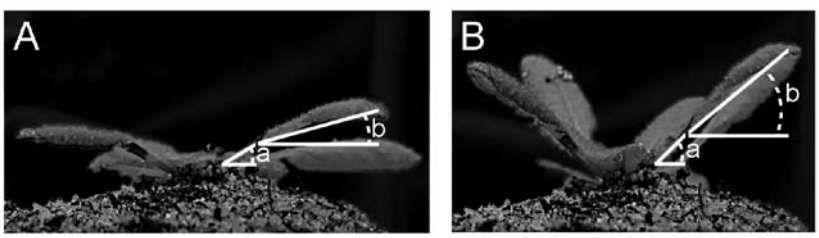

Figure 8. Example of Arabidopsis Colombia-0 treated with air (A) and 2µL L^{-1} ethylene (B). The angle of the petiole (a) and the leaf blade (b) is indicated in both photographs.

Table 1. List of the gene fragments from *Rumex palustris*. T-DNA insertion mutants corresponding to the orthologous genes in Arabidopsis as provided by the European Arabidopsis Stock Center (NASC) and their effect on ethylene-induced growth.

Gene	AGI number	NASC number	SALK , SAIL or Wisconsin number	Putative involvement in petiole and/or leaf blade ethylene- induced hyponastic response based on mutant analysis
RpElEG15	At3g11660	N567117	SALK_067117	No significant effect on ethylene response
RpElEG15	At3g52470	N608012	SALK_108012	No significant effect on ethylene response
RpElEG27	At1g65590	N653424	SALK_108078	No significant effect on hyponastic growth
RpElHG42	At5g64410	N567314	SALK_067314	Leaf specific gene
RpElEG45	At5g27780	N25025	SALK_014108	No significant effect on hyponastic growth
RpElEG45	At1g14390	N578409	SALK_078409	Leaf blade specific gene
RpElEG47	At1g49040	N531730	SALK_031730	No significant effect on hyponastic growth
RpElEG49	At3g19760	N808417	SAIL_174_A01	Petiole and leaf blade specific gene+ constitutive hyponastic gene
RpElEG49	At1g72730	N595627	SALK_095627	No significant effect on hyponastic growth
RpElEG57	At5g63160	N852478	WiscDsLox354H05	Constitutive hyponastic gene
RpElEG58	At1g04380	N529816	SALK_029816	Leaf blade specific gene
RpElHG69	At3g05030	N859454	SALK_036114	No significant effect on hyponastic growth
RpElHG69	At5g27150	N859470	SALK_065623	Leaf blade specific gene
RpElEG72	At5g45160	N629180	SALK_129180	Petiole and leaf blade specific gene
RpElEG74	At4g14620	N618439	SALK_118439	No significant effect on hyponastic growth
RpElEG74	At3g07350	N565101	SALK_065101	Petiole and leaf blade specific gene
RpElEG79	At3g61600	N600118	SALK_100118	No significant effect on hyponastic growth
RpElEG91	At5g37020	N527136	SALK_027136	Petiole and leaf blade specific gene+ constitutive hyponastic gene
RpElEG91	At1g19220	N521481	SALK_021481	Petiole specific gene
RpElEG92	At3g16140	N833597	SAIL_752_E05	Leaf blade specific gene+ constitutive hyponastic gene
RpElEG92	At1g52230	N523988	SALK_023988	Petiole and leaf blade specific gene+ constitutive hyponastic gene
RpElEG93	At1g79530	N653315	SALK_080245	Petiole and leaf blade specific gene
RpElEG96	At5g04140	N511035	SALK_011035	Leaf blade specific gene
RpElEG96	At4g36870	N505398	SALK_005398	Petiole and leaf blade specific gene
RpElEG104	At1g07920	N579753	SALK_079753	Petiole and leaf blade specific gene

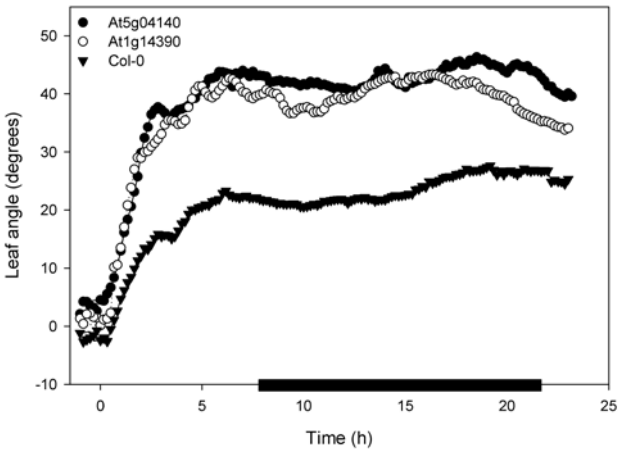

Figure 9. Leaf blade angles in response to ethylene (the response to ethylene was measured by subtracting the angle of the petiole or leaf blade during ethylene treatment from the angle of air control treatment), in *Arabidopsis thaliana* Col-0 accession and insertion T-DNA mutants in At5g04140 (putative orthologue of *RpEIEG96*) and At1g14390 (putative orthologue of *RpEIEG45*) based on the sequence of differentially expressed *Rumex palustris* genes. The values are means of 16 independent replicates (average SE for Col-0= 2.5 and for mutants = 3.0). Plants were in continuous light during the experiment and the black bar represents the period of the normal night. The treatment started at t=0.

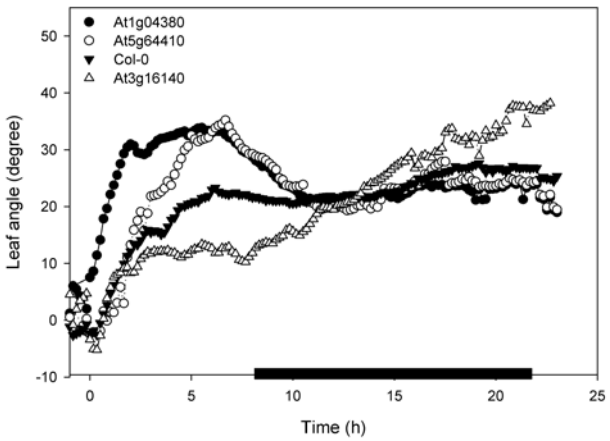

Figure 10. Leaf blade angles in response to ethylene (the response to ethylene was measured by subtracting the angle of the petiole or leaf blade during ethylene treatment from the angle of air control treatment)of *Arabidopsis thaliana* Col-0 accession and insertion T-DNA mutants in putative orthologous Arabidopsis genes At1g04380 putative orthologue of *RpEIEG58*, At5g64410 putative orthologue of *RpEIHG42* and At3g16140 putative orthologue of *RpEIEG92*, based on the sequence of differentially expressed *R. palustris* genes. The values are means of 16 independent replicates (average SE for Col-0= 2.5 and for mutants = 3.5). Plants were in continuous light during the experiment and the black bar represent the period of the normal night. The treatment started at t=0.

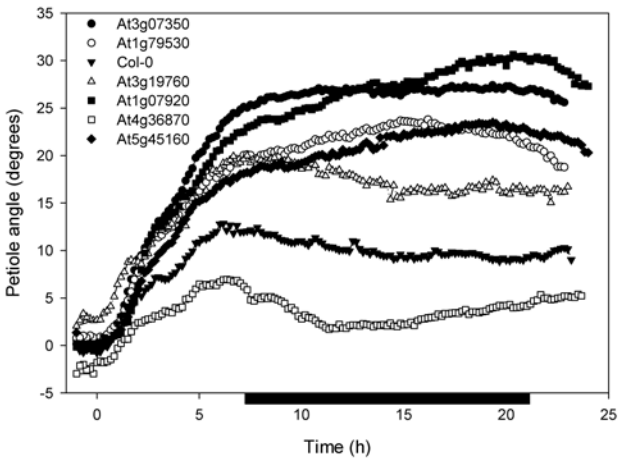

Figure 11. Petiole angles in response to ethylene of *Arabidopsis thaliana* Col-0 accession and insertion T-DNA mutants in putative orthologous Arabidopsis genes (At3g07350 putative orthologue of *RpEIEG74*, At1g79530 putative orthologue of *RpEIEG93*, At3g19760 putative orthologue of *RpEIEG49*, At1g07920 putative orthlogue of *RpEIEG104*, At4g36870 putative orthologue of *RpEIEG96* and At5g45160 putative orthologue of *RpEIEG72*), based on the sequence of differentially expressed *Rumex palustris* genes. The values are means of 16 independent replicates (average SE for Col-0 = 2.5 and for mutants = 3.0). Plants were in continuous light during the experiment and the black bar represent the period of the normal night. The treatment started at t=0.

The third group included genes that are possible involved in both petiole and leaf blade movement (Figure 11, only changes in the angle of petiole are shown; Table 1). The mutants in this group follow two patterns of response: (i) either a higher hyponastic response from the start of the treatment lasting only 8 h (i.e. mutation in At1g52230, a putative orthologue of *RpEIEG92*; data not shown), (ii) a higher hyponastic response from start of treatment which lasted to the end of treatment (i.e. mutation in At1g79530, putative orthologue of *RpEIEG93*; Figure 11). In contrast, a mutation in At4g36870 (a putative orthologue of *RpEIEG96*) led to a decrease in ethylene-induced hyponastic growth (Figure 11). The other interesting gene in this group is *ARF8* (*auxin responsive factor 8*), a putative orthologue of gene fragment *RpEIEG91*. T-DNA insertion lines, *arf8-1* in the Wassilewskija (Ws) background (Tian *et al.*, 2004), the SALK line provided by NASC (N527136) in Columbia background and the transgenic overexpression line *ARF8OX* in Ws background (Tian *et al,*. 2004), all revealed the effect of this gene in hyponastic growth in both leaves and petiole (Figure 12, only petiole responses are shown). Auxin is required for the initiation of hyponastic growth and maintenance of a high petiole angle in submerged *R. palustris* (Cox *et al.*, 2004). The T-DNA insertion line N527136 shows a severe increase in the hyponastic response of petioles and leaf blades (data not shown) to ethylene from 1 h until 13 h of treatment and the mutation in *arf8-1* causes an increased response to ethylene in mutant plants

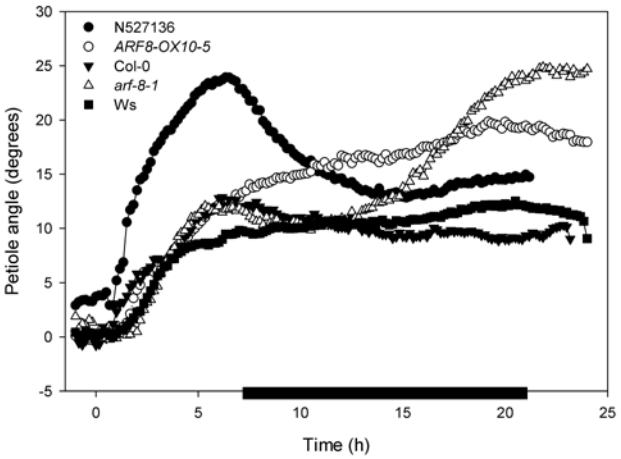

Figure 12. Petiole angle of *Arabidopsis thaliana* Col-0 and Ws accession, one of the T-DNA insertion mutants in the *ARF8* gene (*arf8-1*) and *ARF8OX* are in the Ws background (Tian *et al.,* 2004). The values are means of 16 independent replicates (average SE for Col-0 = 2.5 and for WS = 3.0 and mutants = 3.0). Plants were in continuous light during the experiment and the black bar represent the period of the normal night. The treatment started at t=0.

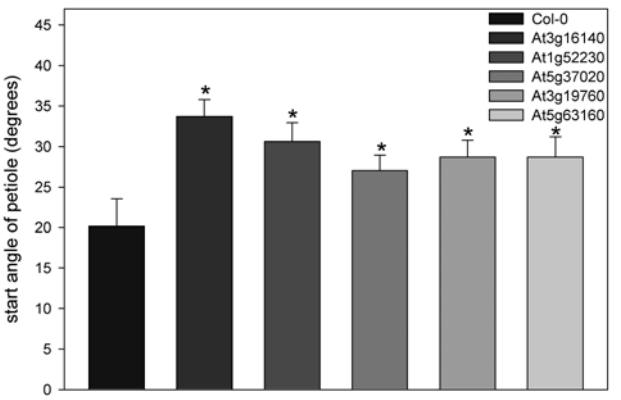

Figure 13. Initial petiole angle in Col-0 and T-DNA insertion mutants in putative orthologous Arabidopsis genes (At3g16140; putative orthologue of *RpEIEG92*, At1g52230; putative orthologue of *RpEIEG92*, At5g37020; putative orthologue of *RpEIEG91*, At3g19760; putative orthologue of *RpEIEG49* and At5g63160; putative orthologue of *RpEIEG57*) based on the sequence of differentially expressed *R. palustris* genes. The data present mutants with significantly different petiole starting angle as compared to Col-0 ($p<0.05$,*).

compared to the Col-0 after 13h of treatment, lasting until the end of the treatment. In contrast, the over expressor *ARF8OX 7-5* showed no different phenotype compared to the control (data not shown), the over expressor of *ARF8OX 10-5* caused a higher response to ethylene (Figure 12).

Additionally, some of the T-DNA insertion lines showed significantly higher initial petiole angles (Figure 13). Therefore, we consider them as mutations causing a constitutive hyponastic response: i.e. mutation in gene At3g16140 and At1g52230, putative orthologues of *RpEIEG92* and the SALK line mutant of At5g37020 causing a mutation in *ARF8* (Table 1) also showed higher initial petiole angles.

Discussion

Hyponastic growth and petiole elongation are two responses upon submergence in *Rumex palustris* that help to survive this severe stress. In Chapter 2 we justified the use of ethylene application to mimic submergence in our search for genes that regulate (hyponastic) growth in *R. palustris*. By doing so we identified several genes potentially involved in hyponastic growth and petiole elongation (Chapter 3). The differential expression of these novel ethylene-induced hyponastic (*RpEIHG*) and elongation growth (*RpEIEG*) genes was studied using quantitative RT-PCR (Figure 1). Furthermore, the expression was also studied during submergence (Figure 2). In general, higher expression was observed for the genes when exposed to ethylene compared to submerged plants. There was only one gene, *RpEIEG74* that was expressed to a higher level during submergence compared to ethylene (Figure 2A). The function of *Arabidopsis thaliana* and other plant species orthologues of *RpEIEG74* is unknown, which makes it difficult to speculate about the cause of the higher expression of this gene during submergence.

RpEIEG58 showed a 500 fold increased expression during ethylene treatment (Figure 1A). This gene shows homology to a dioxygenase gene in Arabidopsis and tomato (Chapter 3, Table 2) and is designated 1-aminocyclopropane 1-carboxylic acid (ACC) oxidase (ACO) in tomato (Yamada *et al.*, 2003). This suggests that also in *R. palustris* it may act as an ACO in ethylene biosynthesis. ACC is converted to ethylene by ACO proteins (Yang and Hoffman 1984). The expression of *RpEIEG58* was also increased by submergence (Figure 2A). Vriezen *et al.* (1999) showed an increase in the expression of *RpACO1* and in the activity of RpACO1 at the protein level during submergence of *R. palustris* plants. However, *RpEIEG58* does not show homology to this *RpACO1* oxidase gene that was identified by Vriezen *et al.* (1999; Genbank accession number AF041479). These data suggest that there are more genes in *R. palustris* that code for ACO proteins or proteins that posess ACO activity.

Genes belonging to cluster 1 are characterised by differential expression peaks at 1.5, 2.5 and 6 h (Figure 1A) upon ethylene or submergence treatments. This suggests that genes belonging to this group are involved in both hyponastic growth and enhanced petiole elongation. The second group of genes is characterised by high

temporary expression during the first three hours of the treatments, suggesting that these genes might be related to hyponastic growth (Figure 1B). However, we cannot exclude their involvement in elongation growth (initiation), because fast elongation growth starts already after a 3 h lag phase (Chapter 2). The 1.5-2 fold decreased expression after 2-4 h of ethylene apllication in the third group of genes (Figure 1C) suggests a repressive role of these genes on ethylene-induced growth.

Clear differences were observed in the patterns of gene expression during ethylene treatment and submergence. Since elevated ethylene is just one of the changes during submergence, differences in gene expression between ethylene and submergence were to be expected.

In situ *transcript localisation of* RpEIHG99 *and* RpEIEG74 *in* R. palustris *petioles*

In order to localise the transcripts of genes *RpEIHG99* and *RpEIEG74*, *in situ* hybridisation was applied to petioles of *R. palustris* plants exposed to ethylene and submergence. Transcripts of both genes were upregulated and were mainly localised in the first two basal sections of the petiole during ethylene treatment (Figure 5, 6 sections c, and d, 2h treatment).

Cox *et al.* (2004) showed that submergence-induced hyponastic growth is localised in the first 30 cells of the cell files on the abaxial side of the petiole. Ethylene-induced hyponastic growth is also localised in the lower half of the petiole (Chapter 2). However, no differences in expression of both genes were observed between the abaxial and adaxial parts of the petiole (data not shown), but comparing the localisation of the genes at an early and a late time point (2 and 8 h, respectively) showed an increased labelling in the ethylene and submerged petioles compared to the respective controls (Figure 5, 6). This suggests that the elevated expression of both genes starts from the beginning of the treatment and lasts up to the end of the treatment. Based on these observations we conclude that these genes are probably not target genes that determine differential growth. Interestingly, the expression of both genes is restricted to the basal zones of the petioles. Non-differential ethylene-induced elongation growth takes place over the entire length of the petiole. We therefore cannot exclude that the products of these genes play a role in hyponastic growth that is mainly regulated in the most basal part of the petiole.

Arabidopsis thaliana *orthologues of* Rumex palustris *ethylene-induced (differential) growth genes exhibit alteration in hyponastic growth when mutated*

From the orthologous genes we identified in Arabidopsis based on the original *R. palustris* sequences we identified several T-DNA insertion lines in the Arabidopsis database (www.arabidopsis.org). We studied the phenotype of these insertion mutants and found that several of them to be involved in hyponastic growth. Since Arabidopsis does not show ethylene- or submergence-induced petiole elon-

gation we were not able to test this in these lines (Millenaar *et al.*, 2005). The data revealed that a subset of these genes are likely to control the initial angle of the petiole (Figure 13) as mutations in these genes resulted in constitutively elevated petiole angles in Arabidopsis mutant plants compared to Columbia-0 (Col-0). Interestingly, some of the genes have an effect on either the angle of the petiole (Table 1) or the angle of the leaf blade or both (Figure 10, 11, 12 and Table 1). Most Arabidopsis insertion lines (Table 1) showed an increased response to applied ethylene (Figure 10, 11, 12). These data suggest that they are most likely to be involved in growth responses to ethylene in Arabidopsis. However, mutations in some putative orthologous Arabidopsis genes of *R. palustris* i.e. At3g19760, At5g63160 putative Arabidopsis orthologues of *RpEIEG49,* 57, respectively, have no correlation with the expression pattern in *R. palustris*. These genes were highly expressed in *R. palustris* during ethylene treatment and submergence. However, mutations in putative Arabidopsis orthologues increased the ethylene-induced hyponastic response instead of the expected decrease.

Mutant analysis reveals a role for PSI-H subunit of photosystem I in hyponastic growth

At1g52230 and At3g16140 encode putative PSI-H subunits of photosystem I (PSI) which are essential for state transitions in plant photosynthesis (Lunde *et al.*, 2000). Arabidopsis T-DNA insertion lines of two orthologues of *RpEIEG92* (At1g52230 and At3g16140) showed petioles with relatively high initial angles (Figure 13). Furthermore, these mutants had a higher hyponastic response during the first hours of ethylene exposure compared to the Col-0 plants (Figure 10). The reduced expression of *R. palustris* gene *RpEIEG92* after 2 to 4 h of both ethylene and submergence treatments (Figure 1C), suggests a negative role for this gene in ethylene-induced hyponastic growth. Furthermore, alteration in light intensity leads to hyponastic growth in *Arabidopsis thaliana* and *R. palustris* (Millenaar *et al.*, 2005; Pierik *et al.*, 2005). Changes in light intensity lead to state transition in the photosystem I reaction center, which in turn controls the expression of genes through alteration in the reduction/oxidation (redox) state of signalling molecules (Pfannschmidt *et al.*, 2001b). We hypothesise that the product of *RpEIEG92* might function downstream of ethylene-induced hyponastic growth in *R. palustris* since disruption of the Arabidopsis orthologous gene causes a changed initial angle of the petiole and a changed hyponastic response in Arabidopsis.

A novel function for glyceraldehyde-3-phosphate dehydrogenase and glutamate synthase in hyponastic growth of Arabidopsis

Arabidopsis lines with a T-DNA insertion in At1g79530, the putative Arabidopsis orthologue of *RpEIEG93*, showed an increased hyponastic growth when exposed to ethylene compared to the wild type. This mutant showed a stimulated

response in both petiole and leaf blade (Figure 11, Table 1). This gene encodes a glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of *RpEIEG93* in *R. palustris* was significantly lower than control in both ethylene and submergence treatment during the first 2 h of treatment. The results of mutant and gene expression studies suggest a negative role of *RpEIEG93* in ethylene- and submergence-induced hyponastic growth. GAPDH is involved in the Calvin cycle and is strongly regulated by light/dark conditions (Marri *et al.*, 2005; Trost *et al.*, 2006). The expression of GAPDH may also be regulated through the redox status (Buchanan and Balmer, 2005).

At5g04140 is a putative orthologue of *RpEIEG96*. It encodes a chloroplast related gene that responds to light stimuli and photorespiration (Coschigano *et al.*, 1998; Dai *et al.*, 2000; 2007; Feraud *et al.*, 2005; Suzuki and Rothstein 1997). The Arabidopsis T-DNA insertion line that putatively disrupts this gene, exhibited an increased hyponastic response of the leaf blade induced by ethylene (Figure 10, Table 1). The expression of this putative orthologue in *R. palustris* decreased, especially during the submergence treatment, within 3 h of treatment (Figure 2B). Furthermore, in Chapter 3 several genes were identified by cDNA-AFLP and micro-array experiments to be involved in redox signalling in chloroplasts. Based on this we proposed that changes in redox signalling occur during ethylene-induced (differential) growth in *R. palustris*. In addition, mutations in putative orthologues of *R. palustris* genes identified by cDNA-AFLP and putatively involved in redox signalling, increased ethylene-induced hyponastic growth in Arabidopsis. Taken together, we hypothesise that redox signalling is reduced by applying ethylene and submergence treatment, which affects GAPDH and probably other Calvin cycle enzymes resulting in a positive effect on hyponastic growth.

The effect of auxin response factor ARF-8 *on hyponastic growth*

The importance of auxin for hyponastic growth in *R. palustris* is well known (Cox *et al.*, 2004, 2006), but the exact mechanism is not clear. In this plant species the initiation of hyponastic growth and maintenance of the maximum petiole angle are regulated by ethylene, ABA and auxin (Cox *et al.*, 2004; Peeters *et al.*, 2002). In ethylene-induced hyponastic growth we identified differentially expressed auxin responsive/induced genes from the cDNA-AFLP and the micro-array assays (Chapter 3). Two Arabidopsis lines with T-DNA insertions in putative orthologues of *RpEIEG45* (At5g27780 and At1g14390), both auxin induced genes, were tested in our system. Disruption of At5g27780 showed no differences in ethylene-induced hyponastic growth compared to wild type plants, but mutation in At1g14390 caused an increase in the ethylene-induced hyponastic response.

At5g37020, another auxin related gene, is a putative orthologue of *RpEIEG*91and encodes an auxin response factor, *ARF8*. Several Arabidopsis lines with mutations in and over expressing this gene were tested. The genetic difference

between two T-DNA insertion lines is the location of the disruption in the gene and the genetic background. In the SALK line N527136 (Col-0) the T-DNA insertion landed in the last exon and in *arf8-1* (Ws) in the third exon of the gene. The results revealed different effects of these mutations on ethylene-induced hyponastic growth. The mutant *arf8-1* caused higher response at the end of the treatment and corresponds with auxin being involved in maintaining a high petiole angle. The expression of the gene *RpEIEG91* is highly increased within 3h of ethylene and submergence treatments (Figure 1 and 2). Increased hyponastic response in *ARF8OX10-5*, a construct over expressing *ARF8*, was in agreement with the expression pattern of the gene in *R. palustris*. However, *arf8-1* did not show a decrease in hyponastic response in first few hours of the treatment, which may be explained by the fact that *arf8-1* is not a complete knockout mutant (Tian *et al.*, 2004). The increase in response to ethylene in N527136, in which *ARF8* is disrupted by a T-DNA insertion, could very well be caused by other hormones since an interaction of brassinosteroid (BR) and auxin acts via ARF8 in Arabidopsis (Nemhauser *et al.*, 2004). These authors showed that BR negatively regulated ARF8.

We conclude that *ARF8* is likely to be an important gene in the regulation of hyponastic growth induced by ethylene, although the true nature and the suggested interaction with BR needs to be proven.

General conclusion

In this paper we confirmed the expression of candidate ethylene-induced growth genes (Table 1) during ethylene and submergence treatment by monitoring the kinetics of their expression. By studying the localisation of two differentially expressed genes we showed that although different expression between the control and treatments (ethylene and submergence) were obvious, these genes were not differentially expressed between the abaxial and adaxial side of the petiole. In addition, mutant analysis in Arabidopsis revealed that mutations in Arabidopsis orthologues of *R. palustris* genes altered the hyponastic response to ethylene.

Materials & Methods

Plant materials and treatments

Rumex palustris plants were grown as described in Chapter 3. Rosette plants with an age of 27 days were treated either with ethylene (5 $\mu L\ L^{-1}$) as described in Chapter 2 or submerged. For submergence, cuvettes were placed in a growth cabinet, and filled with tap water a night before the start of the experiment. Plants were placed in these water-filled cuvettes and completely submerged for 8 h at a temperature of 20ºC. Petiole number 3 (see Cox *et al.*, 2003) was used in all analyses. Four biological replicates each containing 3 petioles from either air control, submerged or ethylene-treated plants were harvested at different time points including 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h after start of the treatment.

Table 2. Sequences and lengths of primers designed for the RT-RTPCR reaction and the length of the amplicon. Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3_www.cgi).

Gene name	Forward primer	Reverse primer	Amplicon size (bp)
RpEIEG15	CCTCCATCCCTTCCACCTAT	CCATCTCACCCGTCCATCTA	167
RpEIEG27	CGTGGATATCTCTCAGCGG	GGTGCATTGTAAGCAACCAG	96
REIHG42	CATTCCAGCCCATCCATATC	TCAAGGAGCACGTCATCATC	182
RpEIEG45	CTTAGAATGGCAGAGGAGTTCG	TGCGTCTTTGAATGATGGAG	103
RpEIEG47	TGGCATCCCAGAGCCTGAG	TGAGAGGAAGAGGATTTGTAG	57
RpEIHG48	GGCTCCACCAATAGATTTGAT	TTGTAGTACCATCTCCAGTAC	60
RpEIEG49	AGTGATGGCAAGAGTCTCGTAG	TCTTGGTGAAGCGTGATGAG	119
RpEIEG57	TGTTGAGAAGACAGAGGCCT	TCAAGTTCAAGCCAGGGATC	60
RpEIEG58	CGAGCCGCTTGTGGACAC	CCAAAGACTCGGACACGAG	90
RpEIEG59	CAAGGACATCGTCGAGGAC	GAGCAACACCACCCCGAAA	58
RpEIHG69	TCTTCATTGGTCAGGCTGTTC	CCTACATGGACCACGCACTA	160
RpEIEG72	TGATGACTTTCCAGATTTGCTG	GATTCTACCACTCAATAGCACCAG	105
RpEIEG74	GCGTCGACGATAGTACCACA	CGCCAGATCGGATACAATG	112
RpEIEG79	CGTGATCGATACGGTTCTATC	AAGTGCGACGTCGCCCATC	93
RpEIEG91	AATTCCTGGACTGGTGGTTG	GCGATACGAGCACTCATGG	100
RpEIEG92	CCCAAATCCTCCAATTCAAA	CTCAGCTAGTCGCCACACC	108
RpEIEG93	AAAAGGAACTCAACGCCAATG	CATGGGGTCAGACCGGTGC	70
RpEIEG96	CCATGGAATAGAAGTCATTATACCG	GGATGCACTGACAGCTCTTG	100
RpEIHG99	ACTTGACTCGGGAGTTGACG	CTGAACTCCAAGAAGTCACCAA	150
RpEIEG104	CCAGCATCTCCATTCTTCAAG	TAGACCAAGATCGACAGGCG	74
18S	CCGTTGCTCTGATGATTCATGA	GTTGATAGGGCAGAAATTTGAATGAT	92

Gene Expression and Quantitative Real-Time (QRT-PCR)

RNA was extracted from 4 replicates each containing 3 petioles from 3 plants as described in Chapter 3. Double-stranded cDNA was synthesised from 1 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen, Breda, the Netherlands) and random-hexamer primers (Superscript Choice; Invitrogen, Breda, the Netherlands). The primers (Table 2) for the candidate genes (Table 1) were designed with the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and used in a cocktail containing: 10 µL SYBR Green Supermix fluorescein (Bio-Rad Laboratories, Veenendaal, the Netherlands), 2 µL of each primer (9 µM), 5 µL cDNA (3 ng) and 1µL MQ.

Real-time PCR reactions were performed using an MyiQ Single-Color Real-Time PCR (Bio-Rad Laboratories, Veenendaal, the Netherlands) detection system with the following thermal program: 5 min 95°C and 40 cycles of: 30 s 95°C, 30 s 60°C, and 60 s 72°C. The data were analysed with MyiQ software (Bio-Rad Laboratories, Veenendaal, the Netherlands).

Data analysis

To be able to compare the expression of the different genes, an 18S (ribosome small subunit) gene from *R. palustris* was used as an endogenous control to normalise the changes caused by differences in the quantities of cDNA (Colmer *et al.*, 2004). Post-QPCR calculations to analyse relative gene expression were performed according to the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). To exclude any non-specific products, the melting curve profiles of all reactions were checked for each PCR run. Means and standard deviation from 4 biological replicates were calculated and the significance levels between treatment and control were calculated by student test ($P < 0.05$). The relative value for expression data were calculated by subtracting the $2^{-\Delta\Delta Ct}$ value of treatment from the $2^{-\Delta\Delta Ct}$ value of air controls.

Hierarchical clusters were calculated and drawn using the R software provided by departement of Statistics and Mathematics of Wisconsin University, (Wisconsin, USA; http://www.r-project.org/ R-2.5.1).

In situ *hybridisation*

For *in situ* hybridisation (ISH) petiole 3 was excised, divided transversally into 4 equal parts (Figure 3A), and immediately fixed in a solution containing 75% ethanol and 25% acetic acid. The fixed tissues were stored at 4°C. Embedding was done in Paraplast Plus (EMS, Washington, PA, USA), according to Drews (1998). Xylene-Substitute (EMS, Catalog #23410-01, Washington, PA, USA) was used instead of xylene. Sections were cut on a Reichert-Jung microtome 2030 to the thickness of 14 µm, floated on distilled water on Superfrost-plus slides (Esco scientific company, USA), and baked onto the slides for 72 h at 42°C.

Sections were then deparaffinised and rehydrated according to Drea *et al.*, (2005). Before hybridisation, sections were treated with 2 mg L^{-1} proteinase K for 30 min at 37°C and subsequently treated for 10 min with 0.1 M Triethanolamine and 0.05 M Acetic anhydride.

To visualise the expression of selected genes, *RpEIEG74* and *RpEIHG99,* the corresponding EST clones were used to produce probes with Digoxygenin-11-UTP (DIG) (Roche Diagnostics Nederland, Almere, the Netherlands) as a label. The plasmid clones (pGEM-T easy, Promega, Leiden, the Netherlands) containing the inserts of these two genes were linearised with the restriction enzymes *Nco*I (anti-sense, SP6) and *Spe*I (sense, T7) for *RpEIEG74*, and with *Nco*I (sense, SP6) and *Sal*I (anti-sense, T7) for *RpEIHG99*. Restriction enzymes were used according to

the manufacturer (Fermentas, St. Leon-Rot, Germany).

Run-off transcripts were prepared with a digoxigenin (DIG) RNA Labelling Kit (SP6/T7) (Roche Diagnostics Nederland, Almere, the Netherlands). The labeled probes were tested by gel-electrophoresis and semi-quantified with DIG Quantification Test strips (Roche Diagnostics Nederland, Almere, the Netherlands). Hybridisation was done overnight at 50°C with 100-300 ng mL^{-1} probes. Sections were treated with either anti-sense probe to visualise the transcripts or sense probe as a control.

Post-hybridisation washes were performed in low stringency 1 x SSC-solutions (NaCl 150 mM, Sodium Citrate 15 mM) at 50°C. Visualisation of the digoxygenin was established by applying an anti-digoxigenin antibody from sheep, conjugated with alkaline phosphatase (anti-digoxygenin-AP Fab fragments; 1:1500 diluted) (Roche Diagnostics Nederland, Almere, the Netherlands). A 100X dilution of Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) Stock Solution (Roche Diagnostics Nederland, Almere, the Netherlands) was applied to detect the alkaline phosphatase, which uses BCIP as a substrate and produces an insoluble NBT Diformazan end product. Precipitation of this blue colored end product was allowed to take place 5 and 9 days for genes *RpEIHG99* and *RpEIEG74*, respectively. Color development was visually monitored on an Olympus BX50 WI microscope, and sections were photographed with an Olympus DP 70 digital camera.

Functional analysis

To unravel the function of the identified genes in *R. palustris*, ethylene-induced hyponastic growth was studied in T-DNA insertion mutants of the relevant genes in *Arabidopsis thaliana*, generated at the SALK (La Jolla, California, USA), SAIL (Syngenta Arabidopsis Insertion Library) institute and Wisconsin University (University of Wisconsin-Madison http://www.biotech.wisc.edu/ Arabidosis). T-DNA insertion mutants (hereafter referred to as the SALK, SAIL and Wisconsin lines, Table 1) were provided by the European Arabidopsis Stock Centre (NASC, University of Nottingham, UK, http://arabidopsis.info/) in an Arabidopsis Columbia-0 background. To identify homozygous lines, the SALK lines were screened on selective media containing kanamycin (Duchefa Biochemie BV, Haarlem, the Netherlands); and SAIL and Wisconsin lines were sprayed with glufosinate-ammonium (Basta) solution. For these two lines, the soil was sprayed with 10,000 times diluted Basta from 150 g L^{-1} solution after three days and two weeks of sowing. Homozygous lines were produced through described screening and self pollination of heterozygous lines. Either wild type accession Columbia-0 or homozygous mutant lines were sown and treated with ethylene as described by Millenaar *et al.* (2005).

To measure the changes in petiole angle in wild type and mutant Arabidopsis plants, 36 days old plants were placed in a custom-built computerised digital camera system and photographed every 10 min for 24 h (Millenaar *et al.*,

2005). The data were analysed as described by Millenaar *et al.* (2005). For every individual line between 12 to 16 replicates were tested for both air and ethylene treatments.

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Chapter 5 Protein kinases are differentially regulated during hyponastic growth and petiole elongation in response to ethylene in *Rumex palustris*

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Abstract

Reversible protein phosphorylation is a fundamental strategy used by eukaryotes to regulate basic cellular function. Upon a variety of stimuli, kinases phosphorylate downstream kinases usually until the target transcription factor is phosphorylated. Moreover, for many metabolic processes reversible phosphorylation of proteins is a key regulatory mechanism. *Rumex palustris* is a flooding tolerant plant species that has the capacity to respond to complete submergence and applied ethylene with hyponastic (upward) movement of the leaves followed by stimulation of petiole elongation. We examined changes in the kinome profile of *R. palustris* in response to enhanced levels of ethylene and we discovered differential activity of many kinases. From these experiments, however, we cannot conclude the exact protein kinases involved in ethylene-induced (differential) growth since the design of the chip was not based on plant specific protein kinase consensus and a consensus can often be phosphorylated by different kinases.

The identity of the substrates, short peptide motifs, of these differentially (de)activated kinases led to many putative target genes in the *Arabidopsis thaliana* genome and thus orthologous genes in *R. palustris* that may be potentially differentially phosphorylated. Our results do show, however, that the kinase regulatory system is strongly regulated during ethylene enriched conditions that induce hyponastic growth and petiole elongation in *R. palustris*. To identify the exact kinases and *R. palustris* target genes, the activity of putative kinases should be examined in response to ethylene.

Introduction

Plant cells continuously monitor their environment and thus sense changes in this outside world. Since plants are sessile organisms they need to adjust appropriately when changes in the environment are detected. This leads to a continuous flow of information that is mediated by factors outside and inside the cells. Among the most abundant mediators of information inside eukaryotic cells are protein kinases that are encoded by 1 to 4 percent of the functional eukaryotic genes (Champion *et al.*, 2004; Stone and Walker, 1995). Protein kinases transfer the phosphate group of ATP (sometimes GTP) to a specific serine, threonine, histidine or tyrosine residue of a protein. These additions may change the conformation of proteins and thus the activity. Phosphorylation is usually reversible and phosphate groups can be removed by phosphatase proteins (for a review see Reményi *et al.*, 2006). Reversible protein phosphorylation is a fundamental strategy used by eukaryotes to regulate basic cellular functions (Bögre *et al.*, 2003). Important in this respect is the phosphorylation and thus activation or inactivation of transcription factors (TF) that give rise to changed gene expression patterns. Upon a variety of stimuli, kinases can phosphorylate downstream kinases until the target TF is phosphorylated. Furthermore, for many metabolic and protein degradation processes reversible phosphorylation of proteins is a key regulatory mechanism (Perales *et al.*, 2006)

Although signalling cascades have been conserved in evolution, the best studied plant model, *Arabidopsis thaliana*, does not possess genes encoding important animal signalling components like Hedgehog, JAK/STAT, Notch, Wingless, and Receptor tyrosine kinase-Ras pathways (Champion *et al.*, 2004). Alternatively, the Arabidopsis genome contains many protein serine/threonine receptor kinases. Moreover, plants possess two-component histidine kinases (Champion *et al.*, 2004) used to detect growth regulators such as ethylene and cytokinin (Urao *et al.*, 2001).

Submergence is an abiotic stress that has a severe impact on growth and production of natural and agricultural plant communities (Voesenek *et al.*, 2006). Excess of water can result in low oxygen conditions in plants within just a few hours (Agarwal and Grover 2006; Ito *et al.*, 1999; Santosa *et al.*, 2007). *Rumex palustris*, a member of the Polygonacaea family, grows in flood-prone environments and possesses various adaptations that allow this species to survive during flooding. One of the first visible processes induced by flooding is differential growth of petioles of *R. palustris* in such a way that the rosette leaves change their orientation from almost horizontal to near vertical, followed by strongly enhanced elongation of the entire petiole. As a result the water layer may be out-grown and contact with the atmosphere re-established (Armstrong *et al.*, 1994; Blom and Voesenek, 1996). It was shown that ethylene plays a pivotal role in this process (for a review see Voesenek *et al.* 2006).

Ethylene signal transduction is associated with protein kinases and two ethylene receptors, ETHYLENE RESPONSE2 (ETR2) and ETHYLENE RE-

SPONSE SENSOR2 (ERS2), have conserved histidine kinase domains (Li and Guo, 2007). Ethylene receptors interact physically with CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) protein; a negative regulator of ethylene responses. The involvement of mitogen-activated protein kinases (MAPKs) cascade in subsequent steps in ethylene signalling was strongly suggested but still is in doubt (Clark *et al.*, 1998; Li and Guo 2007). MAPKs have so far been reported to integrate multiple environmental stresses upon exposure to biotic and abiotic stimulation (Ludwig *et al.*, 2005). Classification of protein kinases is usually based on similarity in the catalytic amino acid sequence domain (Hanks *et al.*, 1988; Hanks, 2003). This property has proven to be a good indicator of, among others, substrate specificity (Hanks, 2003). Kinase activity can be monitored by so-called PepChip kinase arrays that are successfully applied in mammalian systems (Diks *et al.*, 2004; Lowenberg *et al.*, 2006). Recently, it was shown that these arrays can also be applied using *Arabidopsis thaliana* tissues (Ritsema *et al.*, 2007).

In a previous study (Chapter 3) we found that some kinase genes were differentially expressed during ethylene-induced hyponastic growth and petiole elongation in *R. palustris*. These findings might indicate the involvement of kinase proteins in these two growth processes. Furthermore, we identified differentially expressed genes that can be phosphorylated by specific kinases. Here we use the PepChip kinase arrays to create a differential phosphorylation profile in *R. palustris*, identifying several kinases specifically involved in ethylene-induced hyponastic growth and enhanced petiole elongation. Using this array we observed many differentially (de)activated kinases in *R. palustris* during ethylene-induced (differential) growth.

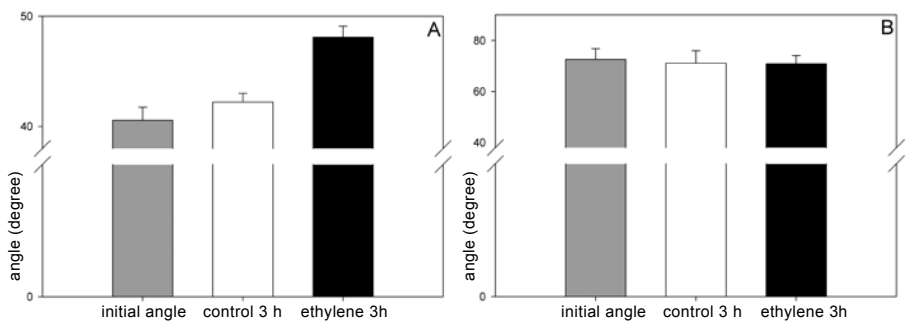

Figure 1. Ethylene-induced hyponastic growth in *Rumex palustris*. A) Natural initial angle of the petioles (approximately 40 degrees, grey bar) and after 3h air (white bar) or ethylene treatment (black bar). B) Artificially manipulated petioles higher than 70 degrees (grey bar) and during 3 hours in air (white bar) or treated with ethylene (black bar). Manipulations were applied by changing the position of the pots with a ring holding the plant pots. The averages presented are calculated from means of 4 biological replicates (± se).

Results and discussion

Background

Petioles of *R. palustris* plants with a natural initial petiole angle of 30-40 degrees demonstrate an increase in petiole angle (hyponastic growth) when exposed to elevated levels of ethylene (Figure 1A). These petioles also show an enhanced elongation rate starting 3 h after ethylene application (data not shown). These two responses can be uncoupled through petiole angle manipulation (Chapter 2). When the third petiole is manipulated to an angle of 70 degrees no hyponastic growth was observed upon ethylene treatment (Figure 1B). Ethylene-induced enhanced petiole elongation in these plants started already after 2 h of treatment (data not shown).

To study protein kinases that were differentially (de)activated during hyponastic growth and enhanced petiole elongation, we compared the following treatments: 30 degrees controls (30C) with 30 degrees ethylene (30E) and 70 degrees controls (70C) with 70 degrees ethylene (70E), respectively. To exclude kinases that respond to ethylene, but are not related to the growth response we compared the kinases of 30E with those of 70E and to exclude kinases that were differentially (de)activated as a result of just the manipulation of the petiole to a more vertical angle we compared kinases of 30C with those of 70C (Figure 2). In this respect, if kinases were differentially (de)activated only in the comparison between 30C with 30E or in 70C with 70E and not in 30E with 70E, they were considered as

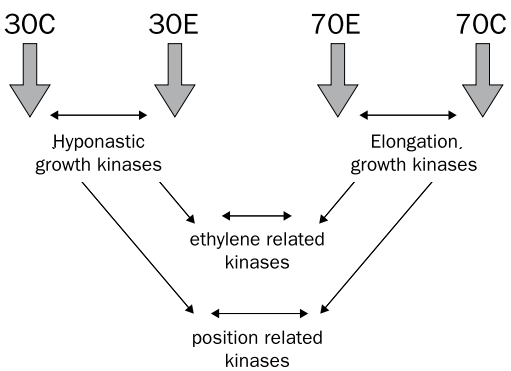

Figure 2. Schematic representation of the experimental design to identify differentially (de)activated protein kinases during ethylene-induced hyponastic growth and enhanced petiole elongation in *Rumex palustris*. We compared petioles with a natural initial angle of 30-40 degrees in air (30C) with plants in ethylene (30E) and plants with artificially manipulated petiole angles of 70 degrees in air (70C) with plants in ethylene (70E) respectively. If the kinases were differentially (de)activated only in the comparison between 30C with 30E or in 70C with 70E and not in 30E with 70E they were considered to be only ethylene response kinases not related to enhanced (differential) growth. Moreover, if kinases were differentially (de)activated in both comparisons (30E to 70E and 30C to 70C) they were regarded as kinases that were differentially (de)activated as a result of manipulation of the petiole.

only ethylene response kinases not related to (differential) growth. Moreover, if kinases were differentially (de)activated in the comparisons 30E to 70E and 30C to 70C they were considered to be kinases differentially (de)activated as a result of manipulation of the petiole angle (Figure 2). Having performed these controls we assume that these subtractions in the 30C-30E dataset result in kinases involved in ethylene-induced hyponastic growth. However, because petioles were not fixed at the angle of 30 degrees we can not exclude the detection of protein kinases that were involved in early ethylene-induced elongation growth. Similarly, the 70C-70E data set represents kinases involved in ethylene-induced elongation growth. This group did not contain ethylene-induced hyponastic kinases since at 70 degrees no hyponastic growth was observed (Figure 1B).

Differentially (de)activated kinase proteins during ethylene-induced hyponastic growth

To identify kinases that were differentially (de)activated during hyponastic growth we isolated proteins from the third petiole with a natural initial angle of 30-40 degrees and treated for 3 h with ethylene (30E). The results were compared to the controls (30C) (Figure 3A and B).

Of the 1,176 peptides present on the chip, 71 showed significantly higher phosphorylation activity during ethylene treatment, whereas only 3 showed a decrease in phosphorylation. Eighteen peptides were phosphorylated by unknown kinases (data not shown). The remaining 56 peptides are shown in Table 1. A total number of 19, 14, and 1 different peptide motifs were annotated to be phosphorylated by PKA (cAMP-dependent protein kinase A), PKC (phospholipid dependent protein kinase C), and PKG (cGMP-dependent protein kinase G), respectively (Table 1). These peptides all belong to the AGC kinase family protein (PKA/PKC/PKG). The higher phosphorylation of these 34 peptides (19+14+1) suggests that the activity of several kinases of the cyclic nucleotide-dependent kinases, AGC kinase family proteins, might be important during hyponastic growth. Furthermore, one peptide that is phosphorylated CaM-II protein kinase (calcium/calmodulin-dependent) and one peptide that phosphorylated with AMP-PK (adenosinemonophosphate protein kinase), both members of the CaMk class (calcium/calmodulin-dependent protein kinases; Stone and Walker, 1995). These were more phosphorylated during ethylene treatment compared to control (Table 1). From the class of CMGC kinases (CDK, MAPK, GSK-3 and CK families), 2 CDC2 kinases (one CDK , cyclin-dependent kinase and one p34CDC2, p34cyclin-dependent kinase), 12 CKs (casein kinase), 9 CKII (casein kinase 2), 2 CKI (casein kinase I), 1 not annotated CK and 1 MAPK (mitogen activated protein kinase) proteins were detected with higher activity during ethylene treatment (Table 1). The other highly phosphorylated substrate peptides during ethylene treatment belonged to the G-protein-coupled Ser/Thr kinase class (Palczewski, 1997), Rhodopsin kinase protein (Rhk) (1) and Myosin heavy chain kinase protein (1) (Table 1). Although the activity of Tyrosine protein kinases

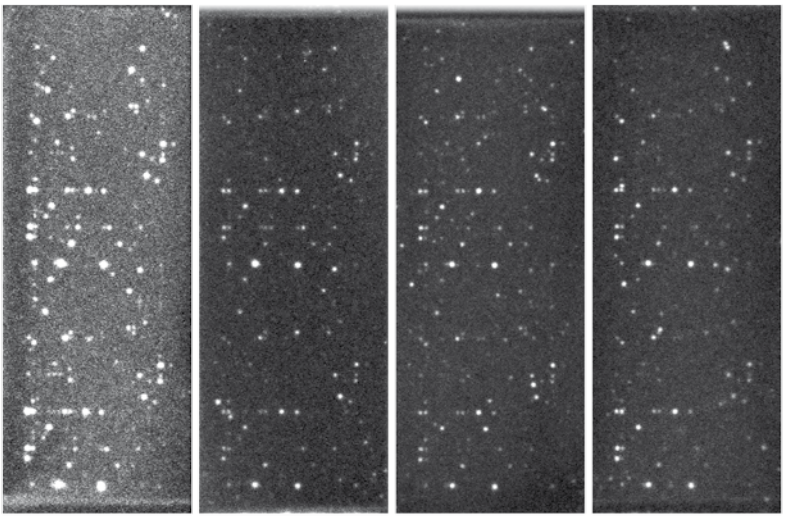

Figure 3. Typical example of PepScan slides used for kinome profiling. Samples used were 30E (A) 30C (B) 70 E (C) 70C (D) after 3h of ethylene treatment. The full slide contains 1,176 peptides, in two experimental replicates. Autoradiography cassette covered with a phosphor-imaging screen for 8 days. Radioactivity was analysed with a Molecular Imager FX and subsequently quantified with Bio-Rad Phopho-imager Quantity One software.

are mostly reported in mammalian cells, we found that some of those peptides that are annotated to be phosphorylated by Tyrosine kinases, were highly phosphorylated during hyponastic growth. For instance one EGFR (epidermal growth factor receptor) substrate and one V-Fps protein kinase substrate, indicating some Tyrosine kinases in *R. palustris* plants can phosphorylate these peptides. Moreover, we also detected 1 autophosphorylation of a kinase protein involved in hyponastic growth (Table 1).

The majority of the peptides that are differentially phosphorylated during ethylene-induced hyponastic growth indicate an increased kinase activity. What this means in terms of concentration and activity remains to be elucidated. Moreover, the vast majority of these peptides indicate an increase in the activity of kinases that belong to two major kinase families (AGC and GMGC).

Differentially (de)activated kinase proteins during ethylene-induced elongation growth

To assess the capacity of kinase proteins during ethylene-induced enhanced elongation growth we compared the kinome profiles of 70E with 70C after 3 h of ethylene treatment. Analysis of the peptides present on the chips revealed 60 peptides differentially phosphorylated in ethylene versus control and thus potentially

Table 1. The motif sequences phoshorylated with kinases and targets annotated to the differentially phosphorylated sequences provided by the PepScan Company. All presented sequences showed higher phosphorylation during hyponastic growth of *Rumex palustris*. The average of phoshorylation values of motifs present on the PepScan array are calculated from means of 3 biological and 2 experimental replicates (± se) in treatments (E30) and controls (C30). Significance levels were calculated by Student test ($P < 0.05$). Kinases shown in italics can most likely phosphorylate the sequence, and were identified through motif blast in the prosite database (Database of protein domains, http://www.expasy.org/prosite/).

Motif Sequence	Kinase Protein (PepScan)	Pep Chip targets	C30	E30	P value
RRRQSVLNL	PKA	CFTR, HUMAN	0.80 (0.02)	1.02 (0.01)	0.01
GSRGSGSSV	PKA	DESM, CHICKEN	0.80 (0.03)	0.98 (0.01)	0.01
ARRSTTDAG	PKA	CGHB, HUMAN	0.82 (0.02)	0.97 (0.03)	0.04
PMRRSVSEA	PKA	LIPS, BOVIN	0.77 (0.04)	0.93 (0.08)	0.04
QRRHSLEPP	PKA	SRC, RSVP	0.70 (0.07)	0.93 (0.03)	0.03
LKRASLG	PKA	LIVER PYRUVATE KINASE	0.78 (0.01)	0.91 (0.01)	0.01
RRRPTPATL	PKA	IPP1, RABIT	0.79 (0.01)	0.98 (0.01)	0.01
LRRNSI	*PKA*	CFTR, HUMAN	0.82 (0.03)	0.97 (0.03)	0.04
KLRRSSSVG	*PKA*	ACHD,TORCA	0.90 (0.04)	1.11 (0.02)	0.03
LRRWSLG	*PKA*	LIVER PYRUVATE KINASE	0.69 (0.04)	0.92 (0.05)	0.04
LRRASLDG	*PKA*	LIVER PYRUVATE KINASE	0.88 (0.09)	0.74 (0.05)	0.04
KRNSSPPPS	PKA	ATCP, HUMAN	1.06 (0.08)	0.69 (0.04)	0.04
FPRASFGSR	PKA	DESM, CHICK	0.80 (0.02)	1.02 (0.01)	0.01
TRKVSLAPQ	PKA	MBP, BOVIN	0.80 (0.04)	0.91 (0.02)	0.02
QRHGSKYLA	PKA	MBP, HUMAN	0.70 (0.7)	0.93 (0.05)	0.04
KRNSSPPPS	PKA	ATCP, HUMAN	0.69 (0.1)	1.06 (0.08)	0.04
AVRRSDRAY	PKA	TRIC, RABIT	0. 74 (0.02)	0.94 (0.04)	0.03
RRRS	*PKA*	TRIC, RABIT	0.83 (0.02)	1.03 (0.05)	0.04
LRRASGG	PKA	LIVER PYRUVATE KINASE	0.69 (0.04)	0.92 (0.06)	0.04
SSLKSRKRA	*PKC*	STP1, SHEEP	0.94 (0.003)	1.12 (0.03)	0.03
ASGSFKL	PKC	H11, BOVIN	0.74 (0.02)	0.88 (0.01)	0.01
VPTLSTFRT	PKC	DESM, CHICKEN	0.87 (0.04)	0.98 (0.07)	0.04
RSKRSGSV	*PKC*	KPBB,RABIT	0.76 (0.06)	1.01 (0.02)	0.04
RVRKTKGKY	PKC	GLR1, RAT	0.81 (0.01)	0.97 (0.001)	0.01
KQGSGRGL	*PKC*	PHS2, HUMAN	0.69 (0.03)	0.87 (0.01)	0.02
DPLLTYRFP	PKC	LAMA, HUMAN	0.64 (0.07)	0.87 (0.05)	0.03
EILNSPEKA	*PKC*	143B, BOVIN	0.77 (0.03)	0.91 (0.001)	0.01
KQGSGRGL	PKC	PHS2, HUMAN	0.69 (0.03)	0.87 (0.01)	0.02
GSLKSRKRA	*PKC*	STP1, PIG	0.749 (0.05)	0.92 (0.07)	0.04

Table 1. continued

Motif Sequence	Kinase Protein (PepScan)	Pep Chip targets	C30	E30	P value
EGTHSTKRG	PKC	FIBA, HUMAN	0.78 (0.1)	0.91 (0.06)	0.04
RVRKTKGKY	PKC	GLR1, RAT	0.81 (0.02)	0.97 (0.03)	0.01
NRLQTMKEE	PKC	LAMC, MOUSE	0.79 (0.05)	0.93 (0.02)	0.02
RVLESFRAA	PKC	MINK, RAT	0.77 (0.01)	0.89 (0.1)	0.03
LPVPSTHIG	PKG	KGPA, BOVIN	0.89 (0.1)	0.96 (0.01)	0.01
GRLSSMAMI	CaM-II	KMLC, CHICK	0.84 (0.02)	1.02 (0.04)	0.04
SRTLSVSSL	AMP-PK	UGS1, RABIT	0.70 (0.06)	0.86 (0.009)	0.00
GGGTSPVFP	CDK, cdc2	DESM, CHICK	0.65 (0.09)	0.90 (0.02)	0.01
NIYISPLKS	P34CDC2	RB, HUMAN	0.95 (0.01)	1.05 (0.01)	0.04
KKKGSGEDD	CK2	IF43, YEAST	0.83 (0.01)	0.92 (0.02)	0.03
PSSTSSSSI	CK2	TOP2,YEAST	0.97 (0.04)	1.10 (0.02)	0.03
SNPEYLSAS	CK2	INSR, HUMAN	0.80 (0.05)	1.02 (0.02)	0.04
TSSSSIFDI	CK2	TOP2, YEAST	0.66 (0.03)	0.93 (0.05)	0.02
EEDLSDENI	CK2	BAF1, YEAST	0.80 (0.03)	0.98 (0.02)	0.01
GSDVSFNEE	CK2	TOP2, YEAST	0.082 (0.01)	0.91 (0.003)	0.03
DAGASPVEK	*CK2*	MACS, BOVIN	0.87 (0.1)	0.96 (0.06)	0.04
SNPEYLSAS	*CK2*	INSR, HUMAN	0.80 (0.1)	0.1.02 (0.05)	0.04
KRKVSSAEG	*PKA,CK2*	HG14, BOVIN	0.69 (0.04)	0.86 (0.07)	0.03
EHVSSSEES	CKI	CAS2, BOVIN	0.76 (0.07)	0.95 (0.05)	0.04
PLSRTL	CKI	glycogen (starch) synthase, muscle	0.88 (0.01)	1.00 (0.04)	0.01
YHTTSHPGT	CK	B3AT, HUMAN	0.61 (0.1)	0.97 (0.06)	0.02
KVPQTPLHT	MAPK	MKK2, RABIT	0.80 (0.01)	0.90 (0.02)	0.01
TVSKTETSQ	RhK	OPSD, BOVIN	0.73 (0.01)	0.92 (0.03)	0.03
AGTTYAL	myosin I heavy chain kinase	myosin heavy chain IA	0.70 (0.02)	0.95 (0.001)	0.04
EEQEYVQTV	EGFR	ANX1, HUMAN	0.83 (0.01)	0.97 (0.02)	0.04
KQPIYIVME	V-Fps	FPS, AVISP	0.94 (0.01)	1.10 (0.03)	0.01
ESRISLPLP	Autophos-phorylation-dependent	VIME, MOUSE	0.74 (0.05)	0.96 (0.05)	0.04

involved in elongation growth. Interestingly, 11 peptides showed enhanced phosphorylation during ethylene treatment, whereas 49 peptides showed decreased phosphorylation. Nineteen peptides were differentially phosphorylated in ethylene-induced elongation growth with unknown kinase proteins (data not shown). The remaining 41 are presented in Table 2. In this assay we identified 13 different peptides that can be phosphorylated by PKA, 1 by PKG and 8 by PKC. All of them belong to the AGC protein kinase family (Stone and Walker, 1995). From the CMGC protein kinase class we found 11 different peptides that can be phosphorylated by CKII, 1 by CDK (CDC28), 1 by GSK-3 and 1 by MAPK1 (Homo sapiens), that is orthologous to AtMAPK2 in Arabidopsis (Hoffmann and Valencia, 2004) (Table 2). Furthermore, 2 peptides indicate phosphorylase kinase (PhK) activity, 2 peptides indicate insulin receptor protein kinase (INSR) activity and 1 peptide annotated to be phosphorylated by Myosin kinase are differentially phosphorylated during enhanced elongation growth (Table 2).

Contrary to the results for hyponastic growth, enhanced elongation was accompanied by a decreased phosphorylation of most peptides indicating a decreased phosphorylation capacity/activity of protein kinases. Interestingly, most of the protein kinases that were found to be differentially phosphorylated during elongation growth and hyponastic growth belong to similar kinase families (AGC and GMGC).

Protein kinase regulation during hyponastic growth and enhanced petiole elongation

As a first step towards understanding the post translational phosphorylation mechanism in ethylene-induced (differential) growth responses in plants, proteins extracted from ethylene-treated and control plants were tested on the kinome array chip. This revealed kinases (Table 1, 2) that were differentially activated or inactivated during these growth processes.

More than 15 different types of protein kinases were detected to be potentially involved in hyponastic growth and petiole elongation, most of them belonging to the AGC group of protein kinases (Stone and Walker, 1995; Bögre *et al.*, 2003). Members of this protein kinase family are downstream of the essential intracellular second messenger's cAMP, cGMP or phospholipids and Ca2+ in animals and yeast (Bögre *et al.*, 2003). These kinases play crucial roles in regulating protein synthesis, gene transcription, cell growth, apoptosis and cytoskeletal remodelling in animals and yeast (Bögre *et al.*, 2003). Functional analysis of the Arabidopsis AGC kinase protein mutants revealed similar important roles for these kinases in Arabidopsis (Bögre *et al.*, 2003). During hyponastic growth induced by ethylene, we identified a differentially activated adenosine mono phosphate protein kinase (AMP-PK) and a calmodulin-dependent protein kinase (CDPK) that both belonged to the super family of CDPKS–SnRK kinase family proteins (Chevalier and Walker 2005; Stone and Walker, 1995). This protein kinase family is known to be plant-

Table 2. The motif sequences phoshorylated with kinases and targets annotated to the differentially phosphorylated sequences provided by the PepScan Company. Sequences in bold showed higher phosphorylation in elongation growth in *Rumex palustris*. The averages presented are calculated from means of 3 biological and 2 experimental replicates (± se) in treatments (E70) and controls (C70). The level of significance were calculated by Student test ($P < 0.05$). Kinases shown in italics can most likely phosphorylate the sequence and were found through motif blast in prosite database (Database of protein domains, http://www.expasy.org/prosite/).

Motif Sequence	Kinase Protein (PepScan)	Pep Chip targets	C30	E30	P value
KRKRSRKES	PKA	H2B_HUMAN	1.43 (0.1)	1.82 (0.1)	0.04
TTRRSASKT	PKA	FIBA, HUMAN	0.80 (0.01)	0.89 (0.02)	0.04
KRRLSFSET	*PKA,CK2*	IL1A_MOUSE	1.40 (0.1)	2.45 (0.2)	0.03
KRPSNRAKA	*PKA,PKC*	MBP, BOVIN	1.01 (0.1)	1.45 (0.09)	0.04
PPEKTEEEE	*PKA, PKC*	SSR3, RAT	0.92 (0.04)	0.60 (0.01)	0.01
GSRPSESNG	PKA	MIP, BOVIN	1.01 (0.02)	0.95 (0.03)	0.03
TRSVSSSSY	PKA	VIME, MOUSE	0.92 (0.04)	0.65 (0.09)	0.03
RRSSSVGYI	PKA	ACHD, TORCA	0.91 (0.04)	0.66 (0.04)	0.02
QRRRSLEPP	PKA	SRC_CHIC	0.90 (0.07)	0.55 (0.08)	0.03
KRKVSSAEG	PKA, *CK2*	HG14_BOVIN	0.93 (0.04)	0.66 (0.06)	0.03
PKKGSKKAV	PKA	H2B, HUMAN	1.07 (0.02)	0.80 (0.02)	0.00
RVRISADAM	PKA	TRIC, RABIT	0.98 (0.05)	0.070 (0.1)	0.02
SRKGSGFGH	PKA	CXB1, RAT	1.01 (0.006)	0.97 (0.03)	0.04
SARLSAKPA	PKG	HG14, BOVIN	0.99 (0.02)	1.35 (0.07)	0.02
KQISVR	*PKC*	PHS2, HUMAN	0.93 (0.03)	1.11 (0.05)	0.02
KRRRSSKDT	*PKC*	NMZ1, HUMAN	1.04 (0.06)	0.96 (0.1)	0.04
ARTKRSGSV	PKC	KPBB, RABIT	1.14 (0.05)	0.88 (0.09)	0.04
DRLVSARSV	PKC	MET, HUMAN	0.93 (0.03)	0.070 (0.09)	0.04
YSLGSALRP	PKC	VIME, MOUSE	0.92 (0.06)	0.073 (0.08)	0.04
PRRVSRRRR	PKC	PRT1, CLUPA	0.97 (0.02)	0.082 (0.04)	0.01
KKRFSFKKS	PKC	MACS, BOVIN	0.91 (0.07)	0.66 (0.1)	0.04
ARTKRSGSV	*PKC*	KPBB, RABIT	1.14 (0.05)	0.88 (0.12)	0.04
LDPLSEPED	CK2	THA, CHICK	1.11 (0.07)	0.85 (0.1)	0.01
QLNDSSEEE	CK2	VE7, HPV16	0.93 (0.05)	0.73 (0.11)	0.04
TLSDSDDED	CK2	MYCN, HUMAN	1.01 (0.02)	0.83 (0.09)	0.01
SSSESGAPE	CK2	CLCB, BOVIN	0.97 (0.03)	0.81 (0.08)	0.04
FFSSSESGA	CK2	CLCB, BOVIN	0.97 (0.01)	0.81 (0.1)	0.04
GVDTYVEMR	*CK2*	KFMS, HUMAN	0.95 (0.04)	0.71 (0.1)	0.03
GLLRSWNDP	*CK2*	PRL, BOVIN	1.01 (0.009)	0.79 (0.09)	0.01

Table 2. continued

Motif Sequence	Kinase Protein (PepScan)	Pep Chip targets	C30	E30	P value
EDAESEDEE	*CK2*	NPM, HUMAN	0.94 (0.03)	0.79 (0.08)	0.04
GDSESGEEE	*CK2*	SRF, HUMAN	0.88 (0.07)	0.60 (0.1)	0.04
AGPTSARDG	*CK2*	RRPP, HRSVL	0.99 (0.001)	0.84 (0.05)	0.00
VIKRSPRKR	CDC28, DEPENDENT	SWI5, YEAST	1.04 (0.03)	0.97 (0.09)	0.04
NAPVSALGE	GSK-3	MYB, CHICK	1.04 (0.01)	0.82 (0.1)	0.04
KNIVTPRTP	MAPK1	MBP, BOVIN	0.98 (0.03)	0.83 (0.06)	0.03
GRALSTRAQ	PhK	TRIC, RABIT	0.98 (0.02)	0.83 (0.05)	0.01
GVERSVRPT	PhK	KPB1, RABIT	0.95 (0.05)	0.72 (0.1)	0.03
TDDGYMPMS	INSR	IRS1, RAT	0.90 (0.05)	0.64 (0.08)	0.03
YGNGYSSNS	INSR	B2AR, MESAU	0.87 (0.06)	0.62 (0.1)	0.04
RGRSSVYSA	MYOSIN	MYSC, ACACA	0.91 (0.06)	0.65 (0.08)	0.03
SAYRSVDEV	BRANCHED, *CK2*	ODBA, RAT	0.74 (0.1)	0.80 (0.1)	0.04

specific (Hrabak *et al.*, 2003). They are involved in growth, development, stomatal movement and abiotic stress (Cheng *et al.*, 2002; Ludwig *et al.*, 2005). From the CDPKS–SnRK protein family OsRK1 protein kinase was found in rice by Min-Ju *et al.* (2007). This protein is induced by dehydration stress and phosphorylates the abscisic acid-responsive element (ABRE) binding factor (Min-Ju *et al.*, 2007). These authors suggest that the OsRK1 protein kinase interacts with ABRE and this interaction probably represents an important part of a cross-talk mechanism in stress signalling networks in plants (Min-Ju *et al.*, 2007). Interestingly, genes containing ABRE or ABRE-like motifs were significantly over-represented in promoters of differentially expressed genes during enhanced elongation growth identified by our micro-array analysis (Chapter 3). It is possible that a similar interaction takes place between the AMP-PK and calmodulin protein kinase and ABA transcription factor in ethylene-induced hyponastic growth to adjust to submergence stress. However, it remains to be tested if the CDPKS–SnRK protein kinase identified here has similar functions.

Another protein kinase that was found in our assay is a Glycogen synthase kinase 3 (GSK3) or SHAGGY protein kinase. The peptide annotated to be phosphorylated by this protein was less phosphorylated during ethylene-induced petiole elongation. It is predicted that 10 GSK3 encoding genes are present in the genome of Arabidopsis (Chevalier and Walker, 2005), and that they are involved in plant stress responses, growth and hormone interactions (Li and Nam 2002; Piao *et al.*, 1999).

Unfortunately, we cannot conclude from our experiments what the exact identity of the shaggy protein kinase is that was regulated during ethylene-induced elongation growth. The same goes for the other kinases as the design of the chip was not based on plant-specific protein kinase consensus and moreover, a certain consensus can often be phosphorylated by different kinases (Ritsema *et al.*, 2007). Our results do show, however, that the kinase regulatory system is strongly regulated during induction of hyponastic growth and petiole elongation by ethylene in *R. palustris*. To identify the exact *R. palustris* kinases responsible for peptide phosphorylation in our assay, the activity of putative kinases should be examined in response to ethylene.

What functional pathways are possibly involved in ethylene induced-hyponastic growth and/or petiole elongation?

The activities of the kinases on the array are characterised by only a limited number of amino acids as substrates. To identify the most likely proteins that contain the peptide motifs present on the chip we analysed the peptide sequence that showed significant differences ($p \leq 0.05$) during ethylene treatment compared to control with a tool from the Arabidopsis website (The Arabidopsis Information Resource; TAIR). Using the pattern matching tool (Patmatch) we identified putative differentially phosphorylated targets in Arabidopsis. This ultimately leads to an overestimation of the number of proteins that are potentially regulated during ethylene-induced (differential) growth, since not all proteins carrying the substrate motif will be involved in the response.

The putative candidate target genes for the kinases identified, based on the sequence present on the chip, are listed in supplemental data (Appendix B, Supplementary Table 1, 2). This is all based on the Arabidopsis database since hardly any genomic data for *R. palustris* are available. We assume that orthologous genes in *R. palustris* may play similar roles (see also Chapter 3 and 4), but that remains to be elucidated.

Reversible protein phosphorylation is a fundamental strategy used in eukaryotes to regulate the basic cellular functions (Bögre *et al.*, 2003). All putative kinase substrates that were identified to be differentially phosphorylated during ethylene-induced hyponastic growth and petiole elongation were classified according to their functional categories to 8 and 9 subgroups, respectively (Figure 4). This classification was done by using the gene ontology based on their function in the literature. However, the activities of the kinases on the array are characterised by only a limited number of amino acids as substrates for these kinases.

Most of the functional groups appear in both hyponastic growth and (enhanced) petiole elongation, except proteins involved in the formation of cytoskeleton and plant cell movement i.e., microtubule, actin, myosin and tubulin family proteins, which were only found during ethylene-induced elongation growth (Figure 4).

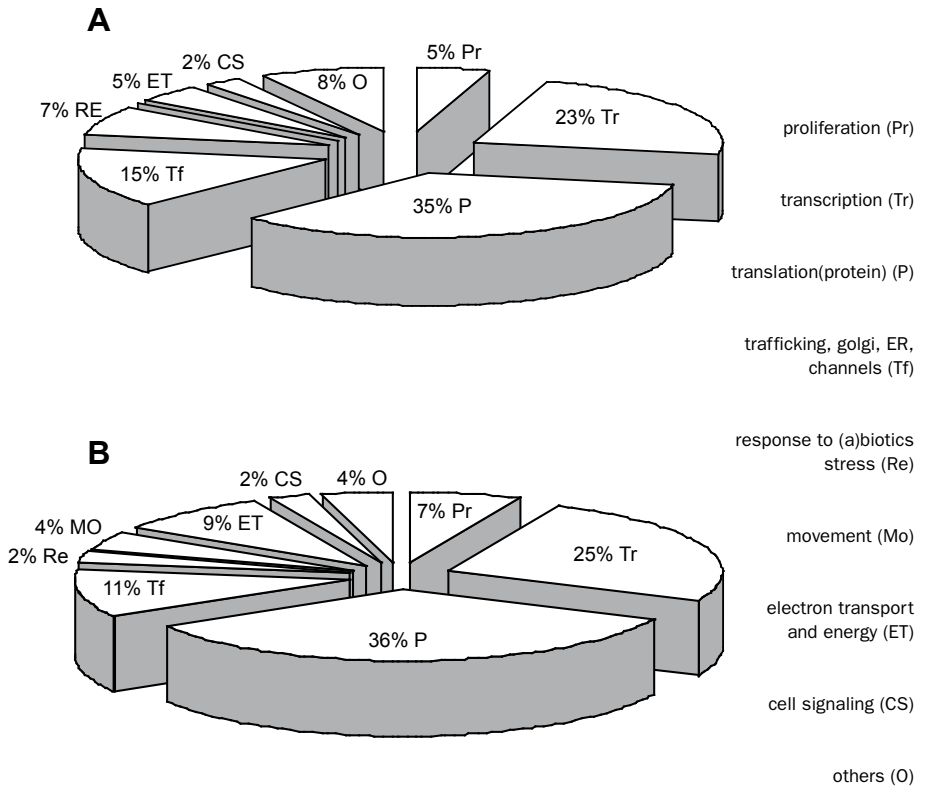

Figure 4. Functional analysis of putative substrates for kinase proteins differentially phosphorylated during ethylene-induced hyponastic growth (A) and petiole elongation (B) in *Rumex palustris* analysed with the kinome array. Distribution of the putative substrate carrying the motifs differentially phosphorylated on PepScan array in Arabidopsis by applying the Patmach tool over the functional categories, according to the function describes in the literature. Since the activities of the kinases on the array are characterised by only a limited number of amino acids as substrates for these kinases, we used the Patmatch tool to identify putative phosphorylated targets in Arabidopsis. This ultimately leads to an overestimation of the number of genes that are potentially regulated during ethylene-induced (differential) growth, since not all genes carrying the substrate motif will be involved in the response.

Activation of gene expression is often under the control of transcription factors (TFs) that may be regulated by reversible phosphorylation. Several transcription factors were identified in our assay and some are regulated during both ethylene-induced hyponastic and enhanced elongation growth. Examples of these are AtMYB11 and AtMYB70 that belong to the MYB TF family and At1g44830, At3g16280 encoding proteins belonging to the ERF/AP2 (DREB) TF family. At1g75520 belonging to Lateral Root Primordium (LRP) TFs, was found only

during hyponastic growth, whereas At2g34830 and At4g26440, members of the WRKY box TFs, were found only during ethylene-induced elongation growth (Appendix B, Supplementary Table 1, 2)

The MYB TF family is involved in responses to plant hormones and stress signalling (Chen *et al.*, 2005). More specific, involvement of MYB77 was suggested in auxin signalling in Arabidopsis (Shin *et al.*, 2007). The ERF/AP2 (ethylene response factor) transcription factor family was shown to be important in tolerance to biotic and abiotic stress (Agarwal *et al.*, 2006; Xu *et al.*, 2006). A member of this family, BOLITA, is suggested to be involved in cell growth through changing the expression of expansins and the actin remodelling factor ADF5 in Arabidopsis (Marsch-Martinez *et al.*, 2006). Lateral Root Primordium (LRP) TF is a member of the SHI gene family in Arabidopsis. The members of this family are partially redundant in function and synergistically promote gynoecium, stamen and leaf development in Arabidopsis (Kuuks *et al.*, 2006).

Another differentially regulated putative kinase substrate motif that was identified in both ethylene-induced hyponastic growth and enhanced petiole elongation is a pentatricopeptide (PPR) repeat-containing protein family that is involved in RNA modification (Appendix B, Supplementary Table 1, 2). The motif contains 35 amino acids and is found in a few animal and fungal proteins, but the family has greatly expanded in higher plants (Liu *et al.*, 2002; Mili *et al.*, 2003; Tsuchiya *et al.* 2002). For example, the Arabidopsis genome contains at least 441 members with mostly unknown functions. Some of these, however, seem to be important in controlling gene expression in a highly specific manner (Koizuka *et al.*, 2003).

Alanine aminotransferase 2 (At1g72330), (Appendix B, Supplementary Table 1), which is important for carbon fixation and in glutamate, alanine and aspartate metabolism, was identified as a putative substrate for kinases differentially (de)activated in hyponastic growth. This protein is involved in anoxia tolerance in *Medicago truncatula* (Ricoult *et al.*, 2005). Furthermore, At3g26810, an auxin signalling F-box 2 (AFB2) and At3g62980 which encodes an auxin receptor protein (TIR1) were also possibly (de)activated during hyponastic growth. The auxin receptor was shown to mediate auxin-regulated transcription (Dharmasiri *et al.*, 2005b). This protein contains a leucine-rich repeat domain and an F-box domain which interacts with a SCF ubiquitin ligase complex (Dharmasiri *et al.*, 2005a). Other proteins that were found to be potentially differentially phosphorylated in ethylene-induced hyponastic growth are Chanel a Potassium (KT2) which is a potassium transporter (Cherel *et al.*, 2002; Elumalai *et al.*, 2002; Grabov 2007). Mutations in KT2/KUP2 potassium transporter genes in Arabidopsis affect shoot cell expansion and mostly cause a decrease in cell expansion in the shoot (Elumalai *et al.*, 2002).

Mitogen-activated protein kinase (MAPK) and wall-associated kinases (WAK) are two important protein families that were predominantly identified as

putative substrates for kinases differentially (de)activated in ethylene-induced elongation growth (Appendix B, Supplementary Table 2). Moreover, D-3-phosphoglycerate dehydrogenase/3 (PGDH), acetyl-CoA, acyltransferase and malate oxidoreductase are important in energy production and glycolysis (Appendix B, Supplementary Table 2) and At2g32610, encoding a cellulose synthase protein, were other important proteins that were found to be possibly (de)activated during ethylene-induced elongation (Appendix B, Supplementary Table 2). Cell growth and synthesis of new cell walls require energy and synthesis and insertion of new cell wall material (Minorsky, 2006). The high energy status and reducing power of NADH and NADPH, collectively [NAD(P)H], make it main players in biosynthetic reactions and ATP production in plant cells (Minorsky, 2006). The orientation of cellulose fibrils is usually perpendicular to the axis of cellular expansion in growing tissue, a characteristic that has been suggested to facilitate directional, or anisotropic, cell growth (Paredez *et al.*, 2006). For instance a mutation in a gene encoding *CesA6*, a cellulose synthase protein in Arabidopsis, reduces cellulose synthesis and severely inhibits elongation growth (Mackinnon *et al.*, 2006). These results suggest that in *R. palustris* similar changes in the activity of proteins involved in energy cycle and cellulose synthesis may facilitate enhanced elongation in response to ethylene.

From these results we can conclude that many important putative substrates i.e. different transcription factors, cytoskeleton and cellulose synthesis proteins, auxin signalling proteins, potassium transporters, and proteins involved in energy metabolism are possibly phosphorylated with kinases that we found differentially (de)activated in one or both of the studied responses. However, to prove their involvement and to identify the exact kinase that phosphorylates these proteins it is necessary to conduct further kinase assay analysis. Furthermore, it is important to investigate the pattern of expression of these protein kinase substrate genes and analyse insertion mutants of specific kinases and their substrates.

Materials & Methods

Plant material and treatments

Rumex palustris plants were grown for 27 d as described in Chapter 3. To mimic submergence, plants were treated with ethylene for three hours as described in Chapter 2. Four different treatments were used to identify (de)activated kinase proteins in the third petiole of *R. palustris*:

1- Plants with a petiole angle around 30º, normal air control (30C)
2- Plants with an artificially manipulated angle to 70º, normal air control (70C)
3- Plants with a petiole angle around 30º, 3h ethylene treatment (30E)
4- Plants with an artificially manipulated angle to 70º, 3 h ethylene treatmen (70E)

Per treatment three biological replicates were used. For protein extraction 10 petioles were pooled per treatment and per biological replica.

Protein Extraction

Every biological sample contained 10 individual petioles. To extract proteins 100 mg frozen petiole tissue was ground in 200 µL lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$ (sodium pyrophosphate), 1 mM ß-glycerophosphate, 1 mM Na_3VO_4 (sodium orthovanadate), 1 mM NaF, 1 µg mL^{-1} leupeptin, 1 µg mL^{-1} aprotinin, 1 mM phenyl methane sulfonyl fluoride (PMSF). The suspension was incubated on ice for 5 min, centrifuged (10 min, 14000 rpm at 4ºC) and the supernatants filtered (0.2 µm) and centrifuged again (2 min, 700 rpm at 4ºC) for complete filtration. This cell lysate was used for hybridisation with PepChip kinase arrays.

Hybridisation of the lysates on kinase chips

Pepchip Kinase Trial slides (Cat. nr. PCKT00010, Pepscan Systems, Lelystad, the Netherlands), containing 192 potential substrates for kinases of 8 or 9 amino acids in length and the PepChip Kinase Full slide (Cat. nr. PCKF00020) with 1176 potential substrates were incubated with 50 µL cell lysate that was activated with 12.25 µL activation buffer (10 mL 50% glycerol, 0.15 mL 100 mM ATP, 0.6 mL 1 M MgCl2, 0.1 mL 3% Brij-35, 0.3 ml 5 mg mL^{-1} BSA) and labelled with 3 µL ^{33}P-γ-ATP (3 µCi, specific activity 1000-3000 Ci $mmol^{-1}$, GE Healthcare/Amersham, Diegem, Belgium). Slides were incubated at 30ºC for 2 h in a humidified stove and washed twice in Phosphate Buffered Saline (PBS) solution containing 0.05% tween 20, twice in 0.5 M NaCl and twice in H_2O.

Dried slides were placed in an autoradiography cassette covered with a phosphor-imaging screen (Kodak, Bio-Rad, Veenendaal, the Netherlands) for 8 d. Radioactivity was analysed with a Molecular Imager FX (Bio-Rad, Veenendaal, the Netherlands) and subsequently quantified with Bio-Rad Phospho-imager Quantity One software (Bio-Rad, Veenendaal, the Netherlands).

Verification and optimisation of the method

Since the kinome chip is a relatively new technique and its use with plants was limited to *Arabidopsis thaliana* (Ritsema *et al.*, 2007), optimisation and verification of the method using another plant species was desirable. To determine the optimal concentration of cell lysate for *R. palustris*, we incubated the Pepchip Kinase Trial slide with *R. palustris* lysate and ^{33}P-labelled ATP to visualise phosphorylation events.

For Arabidopsis, it was shown that 4x diluted cell lysates produced less noise than undiluted ones (Ritsema *et al.*, 2007). Testing *R. palustris* revealed that the best results were obtained using undiluted cell lysate.

To determine the efficacy of this array to study kinase activities in *R. palustris*

in response to ethylene, we calculated the correlation coefficients (r) and variance for two technical replicates and three biological replicates, each containing 10 petioles from 10 individual plants. The results showed that higher/lower kinase activity in some treatments was not due to background or inter-arrays variances.

Data retrieval and statistical analysis of the Pepchip arrays

The Pepchip Kinase Trial chip contains 192 peptides in two sets (experimental replicate) which, we used to optimise the experimental set up for *R. palustris*. The full slide contains 1,176 peptides, in two technical replicates, on a 25x75 mm glass slide. A full slide contains 4x6 sub-arrays in 7x7 spots of which the top right spot of all sub arrays contains a rhodamine labelled peptide for quality control. Spots were analysed using the ScanAlyse program from the Eisen group (http://rana.lbl.gov/EisenSoftware.htm).

Quantification of spots started at the top left spot and data were normalised by dividing the intensity of individual spot through the average spot intensity. For every treatment 3 biological replicates were used and together with two technical replicates on the chip in total 6 replicates were used. The intensity of every individual spot was divided by the average intensity of all spots and averages of two experimental replicates were calculated. Means and standard errors from 3 biological replicates were calculated. Every treatment was compared to control. To identify hyponastic response kinases, we compared 30C with 30E and for elongation growth we compared 70C with 70E (Chapter 2, 3). To filter out only ethylene responsive kinases which are not related to the hyponastic or elongation process the 30E and 70E were compared. The kinases, identified in the first two comparisons (30C with 30 E and 70C with 70E) but that are not differentially phosphorylated in 30E compared to 70E were removed, as only ethylene responsive kinases. To filter out kinases that were differentially phosphorylated in response to petiole angle manipulation, groups of 30C and 70C were compared and the similar kinases were removed. Student tests were performed for pair-wise comparison for three independent replicates in four comparisons (30C with 30E, 70C and 70E, 30C and 70C, 70E and 30E) to detect the significantly different phosphorylated peptides (P value < 0.05). To identify kinases that were capable to phosphorylate a certain peptide substrate we used three sources: (1) the annotation provided by the manufacturer of the chip, (2) ExPaSy ProSite (http://www.expasy.org/prosite/) search for consensus sites and (3) literature on plant kinase consensus sites. Only the last search is plant specific.

To identify the most likely protein that contains the peptide present on the chip we analysed the sequence that showed significant difference ($p \leq 0.05$) in treatment compared to control with The Arabidopsis Information Resource (TAIR) pattern matching tool (Patmatch, http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl). With this program we chose the AGI Proteins that contain can-

didate substrates of 11 amino acids differentially phosphorylated with a maximum of 2 replacements. The validity of conservative domains was decided based on chemical characteristics, and no deletions or insertions were allowed since kinases are generally sensitive to the distance of a particular amino acid from the phosphorylated residue (Ritsema *et al.*, 2007).

Chapter 6 General discussion

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Due to their sessile lifestyle plants are continuously threatened by all kinds of biotic attackers and abiotic stresses. Since plants hardly move to different environments, they have to adjust frequently and appropriately to the environmental changes. One of the more severe abiotic stresses that plants may encounter is flooding. Flooding has a dramatic effect on plant growth and yield (Voesenek *et al.*, 2003a, 2006). Upon flooding, plant growth is in most cases arrested and senescence processes are switched on. In submerged conditions, most plant species die within a few days; however, some species have adapted to flooding and are able to survive by employing a "quiescence" strategy (Bailey-Serres and Voesenek, 2008). Alternatively, some plant species, particularly with semi-aquatic origin i.e. *Rumex palustris*, have the capacity to escape from flooding stress. These species are extensively used to study the morphological, physiological and underlying molecular mechanisms by which they can resist flooding (Peeters *et al.*, 2002; Pierik *et al.*, 2005; Voesenek *et al.*, 2003a; 2006). In response to flooding, two distinct phases of growth could be observed in *Rumex palustris* (Cox *et al.*, 2003; Voesenek *et al.*, 1990). The first response consists of enhanced growth of the cells at the abaxial side of the petiole which establishes a lift of the leaf to a more upright position (hyponastic growth). The second response occurs as enhanced growth of the entire petiole (and to a much lesser extent of the leaf blade). These two processes help the plant to bring leaf tips above the water level, and thus to restore gas exchange between the plant and the air.

Exogenous application of ethylene results in hyponastic and petiole elongation responses of Rumex palustris

It has been demonstrated that ethylene plays a key role in initiating the submergence responses in *R. palustris* (Cox *et al.*, 2003; 2004; Peeters *et al.*, 2002; Voesenek *et al.*, 2006; Chapter 2) and exogenous application of ethylene largely mimics the growth responses observed during submergence.

Hyponastic growth and petiole elongation were very similar in both ethylene and submergence treatments, although some minor differences were observed with respect to timing (Chapter 2). Compared to submergence, ethylene-treated plants demonstrated a longer lag phase before any visible change in the petiole angle occurred. Differential growth in petioles of ethylene-treated plants occurred over the first 20-80 cells at the abaxial side, which was about half of the petiole length (Chapter 2). This differential growth was responsible for the increase of the petiole angle. Under submerged conditions, however, enhanced growth of only 30-40 cells at the abaxial side of the basal cells of submerged *R. palustris* petioles was sufficient for hyponastic growth (Cox *et al.*, 2004).

Moreover, *in situ* localisation of messenger RNA of two induced genes putatively involved in ethylene-induced hyponastic growth and petiole elongation of *R. palustris*, *RpEIHG99* and *RpEIEG74* respectively, revealed an overall increase

in the first two sections from the base of the petiole (approximately equal to the length of 60-80 cells). In submerged plants the expressions of both genes were higher just at the most basal section of the petiole (Chapter 4).

The growth data and expression results of the mentioned genes suggest that ethylene- and submergence-induced hyponastic growth and petiole elongation may be regulated similarly, but affect different regions of the petiole during hyponastic growth.

Furthermore, we showed that petiole hyponasty and petiole elongation could be uncoupled with specific manipulations of petiole angles (Chapter 2). To increase our insight into the underlying molecular processes of hyponastic growth and petiole elongation separately, we manipulated petioles and identified genes specifically involved in one of these processes.

Discovery of novel elements (genes) responsible for ethylene-induced hyponastic growth and petiole elongation in Rumex palustris

To date, 19 expansin A genes were identified in *R. palustris* (Colmer *et al.*, 2004; Vriezen *et al.*, 2000) and from those only *EXPANSIN A1* (*RpEXPA1*) was regulated during submergence-induced elongation growth and linked to the process of cell wall loosening (Vreeburg *et al.*, 2005; Vriezen *et al.*, 2000). Furthermore 11, 9-cis-epoxycarotenoid dioxygenase (NCED) genes were identified in *R. palustris* (Benschop *et al.*, 2005). These genes encode an enzyme that is assumed to be rate-limiting in ABA biosynthesis (Schwartz *et al.*, 1997). It was shown that submergence-induced elongation growth is accompanied by suppressed expression of *RpNCED*s, thus decreasing ABA biosynthesis (Benschop *et al.*, 2005). Furthermore, application of ABA inhibits ethylene-induced elongation growth. In addition to the NCED genes regulation of *1-aminocyclopropane-1-carboxylate oxidase* (*RpACO1*) gene and *Ethylene-Response Sensor* 1 (*RpERS1*) during submergence of *R. palustris* has been demonstrated (Vriezen *et al.*, 1997, 1999). The products of these genes play a role in ethylene biosynthesis and perception, respectively.

In this study we extended the search for genes involved in ethylene- or submergence-induced (differential) elongation growth by employing unbiased methods like cDNA-AFLP, cDNA-subtraction and micro-array analysis. By doing so we discovered a novel set of genes differentially expressed and thus putatively involved in hyponastic growth and/or petiole elongation (Chapter 3). More than 119 expressed sequence tags (ESTs) were identified by cDNA-AFLP and cDNA-subtraction hybridisation as genes putatively involved in either hyponastic growth, petiole elongation, or in both processes. Basic Local Alignment Sequence Tools (BLAST) searches using the sequenced ESTs, revealed homology of 27 ESTs with genes with known functions in other organisms. For 21 of them one or more putative orthologues could be identified in *Arabidopsis thaliana* ultimately adding up to a total number of 120 putative orthologous genes in Arabidopsis. The ethylene

growth response ESTs were classified into four different groups which were, based on their pattern of expression, either putatively involved in ethylene-induced hyponastic growth and/or involved in ethylene-induced early petiole elongation, and/or ethylene-induced long term enhanced petiole growth. Functional annotation of these genes revealed various functional categories including genes involved in 'DNA or RNA metabolism', plasma membrane and cell wall-associated kinase proteins, electron transport and energy pathways, protein metabolism, transcription factor activity, oxidoreductase activity and auxin related signal transduction activity.

The expression kinetics in *R. palustris* of each of the 21 ethylene-responsive genes differentially-expressed during hyponastic growth and/or petiole elongation was tested using quantitative real-time PCR (Chapter 4). Our results showed that growth kinetics of hyponastic growth and petiole elongation corresponds to the expression kinetics of genes putatively involved in ethylene-induced (differential) growth. In general, the expression levels of most differentially expressed genes were higher during ethylene exposure compared to the submergence treatment.

It is difficult, however, to functionally divide the identified genes over the two processes: hyponastic growth and petiole elongation. This may be due to the tight relation and similarities between the two growth responses. It is known that petiole elongation depends on a threshold angle of approximately 50 degrees reached via the process of hyponastic growth (Cox *et al.*, 2003, Chapter 2). Furthermore, both processes are presumably driven by turgor-driven cell elongation. In this respect it is not a surprise that similar genes are involved in both petiole elongation and hyponastic growth. It is tempting to speculate that small differences in trigger, place and timing provide the observed hyponastic or elongation growth.

Little is known about the genome of *R. palustris*, therefore to have a wider spectrum of genes and molecular mechanisms involved in hyponastic growth and petiole elongation we exploited *Arabidopsis thaliana*. Arabidopsis, like *R. palustris*, responds to ethylene with a hyponastic growth curvature (Millenaar *et al.*, 2005; Peeters *et al.*, 2002; Voesenek *et al.*, 2006).

Analysis of Rumex palustris *transcripts on CATMA Arabidopsis full genome arrays, and functional analysis of putative* R. palustris *orthologous genes in* Arabidopsis thaliana *mutants, in response to ethylene*

The expressed transcripts of *R. palustris* induced by ethylene were hybridised to a full genome Arabidopsis array (CATMA). In this assay 4894 genes were differentially expressed higher/lower than 1.4- fold (cut-off value) during one or both responses, indicating the change of expression of many genes during ethylene-induced (differential) growth. The *R. palustris* transcripts hybridised to 80% of the Arabidopsis genes present on the array, thus demonstrating that CATMA arrays are a suitable tool to study global changes in the expression of genes in this plant species.

Expression analysis of *R. palustris* genes, and mutant analysis of putative Arabidopis orthologous genes based on differentially expressed *R. palustris* genes showed that ethylene-induced hyponastic growth and petiole elongation is not only regulated at the gene expression level but may also be regulated at the level of protein synthesis. Apart from changes in expression of many genes involved in protein synthesis (Chapter 3), mutant analysis of the putative Arabidopsis orthologues of *RpEIEG104* (elongation factor 1-alpha), *RpEIEG49* (EIF4A ATP dependent helicase) and *RpEIEG72* (Root hair defective 3 GTP-binding proteins), all three involved in different processes of protein synthesis, caused increase in hyponastic response of Arabidopsis petioles in mutated plants in response to ethylene (Chapter 4). Our finding is in agreement with the described role for identified genes in cell growth and plant development in Tobacco, Maize and Arabidopsis (Cordin *et al.*, 2006; Gungabissoon *et al.*, 2000; Ursin *et al.*, 1991; Wang *et al.*, 1997).

Furthermore, we suggest that redox signalling may be reduced during ethylene-induced hyponastic growth since expressions of several genes involved in redox signalling were changed during ethylene-induced growth (Chapter 3). In addition, mutations in four putative orthologues of redox signalling related genes in Arabidopsis (At1g52230, At3g16140, At1g79530 and At5g04140) all connected to the redox regulatory system in the chloroplast increase the ethylene-induced hyponastic response in Arabidopsis (Chapter 4). Therefore, we hypothesise that ethylene induces a reduction in redox signalling that may have a positive effect on hyponastic growth (Chapter 3, 4).

It is known that the Arabidopsis ethylene receptor ETR1 mediates H_2O_2 signalling in stomatal guard cells and that it might serve as a sensor for H_2O_2 in plant cells (Desikan *et al.*, 2005). Additionally, ABA and redox signalling interact with each other downstream of ABI1 and ABI2 in the ABA signalling cascade (Baier *et al.*, 2004). In addition, hydrogen peroxide induced reversible inactivation of ABI1 and ABI2 proteins *in vitro* (Schweighofer *et al.*, 2004). ABI1 is a protein phosphatase2C (PP2C) (Zhang *et al.*, 2004) and the regulation of PP2C through the balance of cellular redox was proposed by Schweighofer *et al.* (2004). Benschop *et al.* (2005; 2007) and Cox *et al.*, (2004; 2006) demonstrated a negative effect of ABA on ethylene-induced hyponastic growth and petiole elongation in *R. palustris* and ethylene-induced hyponastic growth in Arabidopsis (Benschop *et al.*, 2007). Since ABA-insensitive mutants (*abi1-1*) showed an increase in hyponastic response to ethylene in Arabidopsis (Benschop *et al.*, 2007) we suggest that the effect of ethylene on redox signalling may be directed through alteration of ABA signalling, since no decrease in ABA concentration in Arabidopsis (Benbschop *et al.*, 2007), as opposed to *R. palustris* (Benschop *et al.*, 2005), could be observed. This suggestion is in agreement with the model that was proposed with respect to the suppression of ABA signalling cascade through redox signalling (Baier *et al.*, 2004).

Regulatory mechanisms underlying ethylene-induced hyponastic growth and petiole elongation

In order to induce gene expression in general, the presence of transcription factor binding motifs (TFBM) in promoters is essential. To address the question whether there is an over-representation of certain TFBM in the promoters of the differentially expressed genes that are putatively involved in hyponastic growth and/or petiole elongation; we examined TFBMs in the promoters of differentially expressed genes from the micro-array analysis (Chapter 3). Using the ATHENA search tool (O'Connor *et al.*, 2005) over-representation of TFBMs in the promoters of the differentially expressed genes during ethylene-induced elongation and not during hyponastic growth was revealed. The results indicate an over-representation of ABA responsive elements (ABRE-like binding site motif) in the promoters of upregulated genes during elongation growth (Chapter 3). Within this subset of genes other ABA associated promoter motifs (ACGTABREMOTIFA20SEM, CACGTGMOTIF, ABRE binding site motif) and a GA associated promoter motif (GADOWNAT) were also over-represented. For the genes that are downregulated during elongation growth two over-represented promoter motifs were identified. Both of these motifs (CACGTGMOTIF and ACGTABREMOTIFA20SEM) are also associated with ABA action. Benschop *et al.* (2005) demonstrated a negative effect of ABA on ethylene-induced elongation in *R. palustris*. In order to elongate upon submergence, this species downregulates ABA upon ethylene exposure via a very fast drop of *RpNCED* expression and an increase in catabolism (Benschop *et al.*, 2005). The downregulation of ABA is a prerequisite for GA1 enhancement, which seems essential for fast under water elongation (Benschop *et al.*, 2006). Moreover, Baier *et al.* (2004) suggested that it is possible that an integration and suppression effect of redox signalling on ABA signalling cascade exists through ABRE-binding factors (Baier *et al.*, 2004). These lines of evidence leads us to suggest that regulation of ABA during accumulation of ethylene in submerged *R. palustris* plants occurs through TFBMs of genes that were induced during ethylene treatment.

The role of protein kinases in the regulation of hyponastic growth and petiole elongation in response to ethylene in Rumex palustris

One of the most abundant mediators of information inside eukaryotic cells are protein kinases, encoded by 1 to 4 percent of the functional eukaryotic genes (Champion *et al.*, 2004; Stone and Walker, 1995). Important in this respect is the phosphorylation and thus activation or inactivation of transcription factors (TF) that give rise to changes in gene expression. Ethylene signal transduction is associated with protein kinases (Li and Guo, 2007). In chapter 3, we found many protein kinase genes that were differentially expressed during ethylene-induced

hyponastic growth and petiole elongation in *R. palustris*. This result suggests a role for protein kinases in ethylene-induced hyponastic growth and petiole elongation in *R. palustris*.

To underpin this suggestion we used the PepChip kinase arrays to create a differential phosphorylation profile for ethylene-induced (hyponastic) growth in *R. palustris* (Chapter 5). Our result showed 134 different peptides present on the chip to be differentially phosphorylated during both or one of the growth responses upon ethylene treatment. Contrary to the results for hyponastic growth, enhanced elongation was accompanied by decreased phosphorylation capacity. Interestingly, most of the protein kinases found to be differentially phosphorylated during hyponastic growth and elongation growth belonged to the same protein kinase families; PKA, PKG, and PKC kinases (AGC) and CDK, MAPK, GSK3, and CLK kinases (GMGC). In addition, using the Patmatch tool (The Arabidopsis Information Service, www.arabidopsis.org), we identified putative differentially phosphorylated targets for putative kinases potentially regulated during ethylene-induced (differential) growth in Arabidopsis. This analysis revealed that a large number of different proteins involved at various molecular and cellular levels could be subject to phosphorylation by protein kinases during ethylene-induced hyponastic growth and petiole elongation (Chapter 5). From our results we conclude that the protein kinase regulatory system is important for ethylene-induced hyponastic growth and petiole elongation in *R. palustris*. However, to identify the exact kinases and *R. palustris* target genes, the activity of putative kinases should be examined in response to ethylene.

A possible working mechanism that facilitates ethylene-induced hyponastic and elongation growth

Genes encoding two subfamilies of wall-associated kinases (WAK) and wall-associated-like kinase proteins (WAKL) belong to the receptor like kinase protein family (RLKs) (Wanger and Kohorn, 2001) and were differentially expressed during ethylene-induced growth in *R. palustris* (Chapter 3, 4). *In situ* localisation of messenger RNA of a wall-associated kinase protein ethylene-induced gene (*RpEI-HG99*) revealed an overall increase during ethylene and submergence treatment (Chapter 4). Furthermore, proteins that belong to these subfamilies are recognised as potential substrates of protein kinases that are differentially phosphorylated during ethylene-induced growth (Chapter 5). Members of this family of proteins in Arabidopsis are involved in plant development and hormone perception (He *et al.*, 1996; 1998; Lally *et al.*, 2001; Sieburth, 2007; Wanger and Kohorn 2001). For instance, the ethylene receptor ETR1 is a member of the RLK family of proteins (Chang *et al.*, 1993; Gamble *et al.*, 1998). WAK proteins in Arabidopsis are plasma membrane proteins, playing a crucial role at the cell wall-plasmalemma interface (Verica and He, 2002) and they are responsible for architectural changes in the cell

wall in response to stimuli (Verica and He, 2002). The extracellular domains of this protein family contain several repeats homologous to the epidermal growth factor (EGF) in animals (Verica and He, 2002; Kohorn *et al.*, 1992). A gene homologous to EGF was identified in *R. palustris* using cDNA-subtraction hybridisation of ethylene-treated plants (Chapter 3). Moreover, an EGF receptor kinase protein was found to be highly activated in ethylene-induced hyponastic growth (Chapter 5).

Wall Associated Kinase (WAK) proteins interact with Kinase Associated Protein Phosphatase (KAPP) resembling a Protein Phosphatase 2C (PP2C) in the cytoplasm (Li *et al.*, 1999; Lohrmann *et al.*, 2004; Schweighofer *et al.*, 2004). Our results suggest that in *R. palustris* a homologue of a WAK protein may be phosphorylated by an Epidermal Growth Factor Receptor (EGFR) like protein kinase or a phospholipid dependent protein kinase C (PKC), since the differential phosphorylation of both kinases was detected during ethylene-induced (differential) growth and EGFR protein is phosphorylated by PKC in humans (Crotty *et al.*, 2006). The autophosphorylation eventually leads to the subsequent phosphorylation of PP2C which contributes to hyponastic growth (Chapter 5). More than 14 genes encoding PP2C proteins were differentially expressed during ethylene-induced growth (Chapter 3) and several of them were putative substrates for differentially phosphorylated protein kinases (Chapter 5). The substrate of PP2C is SIMK (stress-induced MAPK), and PP2C is a negative regulator of the MAPK cascade. Furthermore, PP2C was shown to act as a negative regulator of ABA signalling (Benschop *et al.*, 2007; Merlot *et al.*, 2001; Rodriguez, 1998; Sheen, 1998). This may suggest that ethylene benefits from PP2C in a sense that upon augmented expression of this protein the negative effect of ABA on growth stimulation will be removed. Taken together, the data can be fitted into a putative model that explains ethylene-induced hyponastic and elongation growth. In this model, as is shown in Figure 1, we propose that during ethylene treatment the EGF-like domain of wall associated protein kinases is phosphorylated by a protein kinase similar to epidermal growth factor receptor kinase (EGFRK) in human or by PKC. The auto-phosphorylation may lead to the subsequent phosphorylation of PP2C which inhibits both the MAP kinase and ABA signalling cascade. Inhibition of ABA signalling facilitates ethylene and GA1 mediated underwater hyponasty and elongation growth in *R. palustris*.

Involvement of hormonal networks in ethylene-induced hyponastic and elongation growth

Brassinosteroid (BR) is a steroid plant hormone that has an important role in, among other processes, the regulation of cellular expansion, differentiation, and proliferation (Karlova and de Vries, 2006; Nemhauser and Chory, 2004; Savaldi-Goldstein *et al.*, 2007). Furthermore, the interaction of ethylene, auxin and brassinosteroid was suggested in controlling the growth process of Arabidopsis hypocotyls (De Grauwe *et al.*, 2005). Brassinosteroid-insensitive1 (BRI1) is a ki-

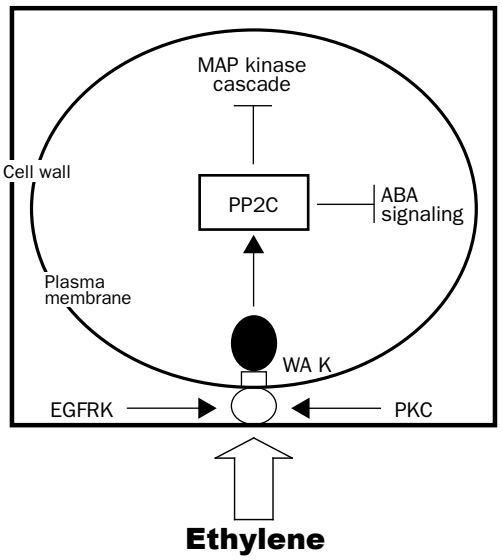

Figure 1. A model describing the involvement of Wall Associated Kinases (WAK) in ethylene-induced (differential) growth in *Rumex palustris*. In this model WAK is phosphorylated by EGFRK, PKC or –like proteins giving rise to subsequent signal transduction involving PP2C, ABA signaling pathway and, more downstream, MAPkinase pathway(s).

nase receptor in the brassinosteroid signal transduction pathway and is involved in BR perception. From our micro-array experiment we identified an Arabidopsis *BRI1-like* gene (At2g01950) (Caly and Nelson, 2002), and an Arabidopsis *BRI1-like* kinase gene (At1G74360) to be upregulated about 2-fold during ethylene-induced hyponastic growth. Downstream of BR perception, At1g19350 encoding BES1 (TAIR) and At4g33430 encoding BAK1 (BRI1-Associated receptor Kinase) (TAIR) were both 1.5 fold. At4g18890 encodes BES1/BZR1 Homologous protein 3 (BEH3) and the gene was more than 2.3 fold upregulated during elevated ethylene. The interaction of auxin and BR signalling was established in Arabidopsis hypocotyl elongation by Nemhauser *et al.* (2004). This interaction is through BES1/BRZ1 with *ARFs* (Halliday, 2004; Li and Deng, 2005). *ARF8* (At5g37020) was shown to be negatively regulated by BR and auxin (Nemhauser *et al.*, 2004). In our micro-array experiment and cDNA-AFLP analysis we found that *ARF8* was more than 1.5-fold downregulated during elongation growth (Chapter 3). Furthermore a mutation of *ARF8* caused stronger ethylene-induced hyponastic response in Arabidopsis (Chapter 4). Auxin and BR also interact through IAA19 (Nemhauser *et al.*, 2004), the gene encoding this protein is about 2-fold downregulated during both ethylene-induced growth responses (Chapter 3), and PIN4 (At2g01420) which gene was downregulated 1.5-fold (data not shown) during hyponastic growth. In addition, the interaction of auxin and ethylene has been reported to act through ARF19 (Li *et al.*, 2006; Rahman *et al.*, 2001). *ARF19* is about 2-fold downregulated during ethylene-induced growth responses.

Moreover, a mutation in *ARF19* caused a stronger ethylene-induced hyponastic response in Arabidopsis (Chapter 4). Based on the results of the micro-array analysis most of the genes involved in brassinosteroid signalling are differentially expressed during ethylene-induced hyponastic growth. Furthermore, auxin induced genes and auxin response factors, that are transcription factors involved in auxin signalling, were differentially expressed as were ethylene responsive genes. In addition, genes possessing ABA responsive elements were over-represented in ethylene-induced elongation genes (Chapter 3).

Therefore, we hypothesise that there is a regulatory network including ethylene, brassinosteroid, auxin and ABA during ethylene induced-hyponastic growth and petiole elongation. In Figure 2 we propose such a network for ethylene-induced hyponastic growth and petiole elongation.

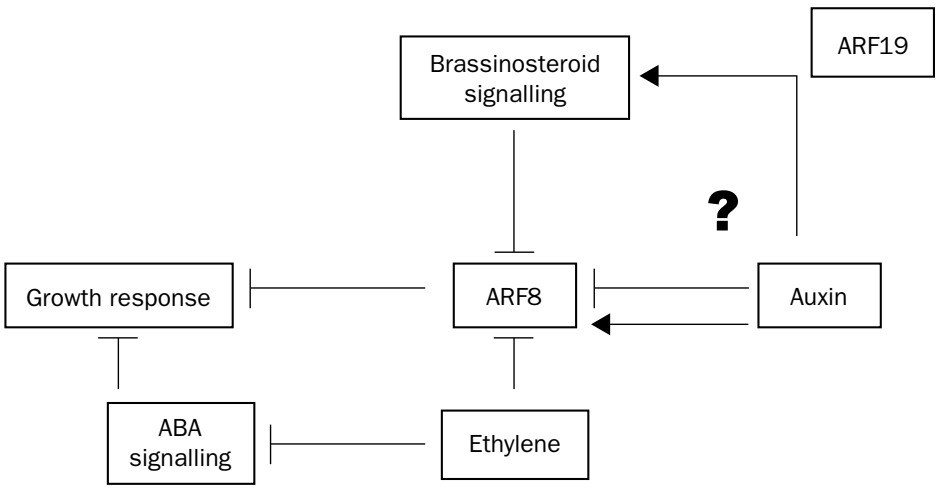

Figure 2. Putative network of plant hormones brassinosteroid, abscisic acid, ethylene and auxin regulating hyponastic and elongation growth of submerged *Rumex palustris plants*.

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Appendix A

Class 1 genes

The gene fragments described below represent genes that are, based on their expression patterns, grouped in class 1 and therefore probably involved in hyponastic growth.

RpEIHG1 is an orthologue of bacterial DNA polymerase III, which is a single-strand DNA-dependent ATPase. The gene family shows homology to clamp-loading proteins (involved in DNA replication) that are present in many organisms from phages to humans. There is a hypothetical protein in *Arabidopsis thaliana* identified that shows homology to this prokaryotic protein (Walker *et al.*, 2000).

RpEIHG2 represents an orthologue of the *Homo sapiens* gene called Muscleblind-like protein or Triplet-expansion RNA-binding protein (Swiss-Prot Q9NR56) and represents a double-stranded RNA binding protein that plays an important role in the regulation of disease related alternative splicing (e.g. insulin receptor) in humans (Ho *et al.*, 2004). Interestingly, no obvious putative orthologue of this gene in plants has been identified so far.

RpEIHG3 is a gene fragment that shows homology to a gene with kinase activity. This gene has a very high similarity with a *Homo sapiens* protein orthologue called, glioblastoma amplified sequence, a protein which contains the epidermal growth factor receptor gene (EGFR) and is amplified in several forms of human cancer. The amino acid sequence of this protein has an identifiable signal peptide and transmembrane motifs, as well as two tyrosine phosphorylation sites that suggest that the encoded protein may be a substrate for tyrosine kinases (Wang *et al.*, 1998). Another orthologue of this protein was reported in Arabidopsis which was a product of a cDNA encoding a 595-amino acid protein with at least two functional domains, one with similarity to the protein serine/threonine kinase family and the other contains an epidermal growth factor repeat. The identification of an EGF repeat in Arabidopsis indicates that the motif is conserved between the plant and animal kingdoms (Kohorn *et al.*, 1992). Kohorn and coworkers (1992) suggest a role for this protein in the assembly or regulation of a light-harvesting chlorophyll a/b-binding protein (LHCP).

RpEIHG4 is a putative orthologue of an ADP-ribosylation factor guanine nucleotide-exchange in *Homo sapiens*. The small ADP ribosylation factor (Arf) GTP-binding proteins are major regulators of vesicle biogenesis in intracellular traffic and protein trafficking (Pasqualato *et al.*, 2002). The Arabidopsis genome potentially encodes 19 proteins with sequence similarities to Arf's, indicating their presence in plants. Plant vesicle trafficking will serve similar functions to those seen in animals and yeast, as well as delivering noncellulosic polysaccharides to the cell wall (Gebbie *et al.*, 2005). The key developmental processes of cytokinesis and cell expansion require vesicle trafficking to deposit new wall material and increase plasma membrane area (Gebbie *et al.*, 2005). Involvement of two Arabidopsis orthologues of ADP-ribosylation factor

guanine nucleotide-exchange) in intracellular trafficking in plant cells as well, Arfl and Arf3, was proposed by Lee *et al.*, (2002).

RpEIHG5 represents a possible orthologue of Methylmalonyl-CoA mutase of *Thermoanaerobacter tengcongensis*. Orthologous genes coding for this enzyme were found in human and mouse (Narasimhan *et al.*, 1996). This enzyme (MCM, EC 5.4.99.2) is present in mitochondria and it is required to catalyse L-methylmalonyl-CoA to succinyl-CoA. This is an intermediate in the catabolism of some branched-chain amino acids, odd-chain fatty acids and cholesterol (Ledley *et al.*, 1988). Catabolism of branched-chain amino acids is shown in plants (Taylor *et al.*, 2004); the presence of Methylmalonyl-CoA mutase in plants remains obscure.

RpEIHG6 was shown to be a putative orthologue of an oxygenase protein of *Streptomyces antibioticus*, enzymes that catalyse the addition of oxygen to substances.

RpEIHG35 is a putative orthologue of an Arabidopsis 40S ribosomal protein S13 (*AtRPS13A*). Ribosomes are involved in protein biosynthesis in all living cells and depend on developmental stage and environmental conditions. As reported by Ito *et al.* (2000) AtRPS13A is essential for normal trichome development, vascular network development in early leaves and normal root growth. As the mutation in this gene in Arabidopsis caused aberrant leaf and trichome morphology in the early vegetative phase it was suggested that this protein has a greater contribution in growth and development in vegetative phase compared to the re productive phase (Ito *et al.*, 2000).

RpEIHG42 represents an orthologue of a *Zea mays* glutathione transporter. Glutathione is an important component of the ascorbate-glutathione cycle, which regulate hydrogen peroxide (H_2O_2) concentrations in plants and is known to minimize the lipid peroxidation of cellular membranes (Kocsy *et al.*, 2001). There is evidence that the tripeptide thiol glutathione (GSH) is involved in the regulation of cell division in the apical meristem of Arabidopsis roots (Sánchez-Fernández *et al.*, 1997).

Gene fragment ***RpEIHG48*** is a putative orthologue of an Arabidopsis gene called *CUP-SHAPED COTELEDON1 (CUC1)*. Encodes a transcription factor involved in shoot apical meristem (SAM) formation and auxin-mediated lateral root formation. The gene is thought not to be involved in stress responses (NaCl, auxins, ethylene) (Takada *et al.*, 2001; Hibara *et al.*, 2003). A close relative of this transcription factor family *NAM/ATAF/CUC1 (NAC1)* transduces auxin signals for lateral root formation (Guo *et al.*, 2004, 2005).

RpEIEG69 is a putative orthologue of an Arabidopsis protein with sodium:hydrogen antiport activity. The role of pH changes during cell elongation is evident (Cleland *et al.*, 1991; Grebe, 2005; Li *et al.*, 2006; Vreeburg *et al.*, 2005).

The cDNA fragment ***RpEIHG99*** represents a putative orthologue of an Arabidopsis wall associated kinase protein (WAK). Involvement of this communication protein between cell wall and cytoplasm in cell elongation and plant development has been shown in Arabidopsis (He *et al.*, 1996; Wagner and Kohorn, 2001; Verica and He, 2002, Verica *et al.*, 2003). WAK suppression results in inhibition of cell elongation and morphological alternations which are the result of substantial decrease of expansin transcripts (Lally *et al.*, 2001). Besides their role in cell expansion, the receptor-like wall associated kinase (RLK) gene familie plays an important role in pathogen resistance and heavy-metal stress tolerance in Arabidopsis (Zhang *et al.*, 2005).

Taken together, most of the putative orthologues of the differentially expressed genes in this class have a shown involvement in cell growth/elongation. These suggest a similar role for the *R. palustris* genes during ethylene induced (differential) growth. The exact natures of their involvement, however, need to be elucidated further.

Class 2A genes

The expression pattern of the gene fragments discussed in class 2 suggests a possible role in enhanced petiole elongation. The *RpEIEG* gene fragments in class 2A represent genes present in samples with a petiole angle of 30 degrees or higher after 2 h of ethylene treatment: We assume that this group contains genes that may induce enhanced petiole elongation, but we cannot exclude a possible role in hyponastic growth.

RpEIEG47 was shown to have homology to a gene encoding for a stomatal cytokinesis defective protein (*SCD1*) in Arabidopsis. This gene is required for cytokinesis and polarized cell expansion in Arabidopsis (Falbel *et al.*, 2003).

RpEIEG49 represents a possible Arabidopsis orthologue of the eukaryotic Initiation Factor called eIF4A (Metz *et al.*, 1992) which has an ATP dependent helicase activity and is known as a DEAD box protein (Tanner *et al.*, 2003). They include regions involved in ATP binding, ATP hydrolysis and RNA binding. The DEAD region is part of the ATPase domain (ATPase B motif). This group of proteins is involved in RNA unwinding in translation, ribosome biogenesis, splicing, and regulating processes such as spermatogenesis, oogenesis, cell growth and division (for a review see Cordin *et al.*, 2005).

RpEIEG59 represents a putative orthologue of an Arabidopsis wall associated kinase protein (WAK). WAK proteins are expressed throughout plant development and are essential for cell expansion. This protein family have a cytoplasmic protein kinase domain; across the plasma membrane, and an N terminus, binds to the cell wall therefore it is suggested as a protein to facilitate communication between the cell wall and cytosol (Wagner and Kohorn 2001). Some Wall-associated kinase like proteins, like AtWAK1, are transported to the cell surface which this is requires for correct cell-wall synthesis (Kohorn *et al.*, 2006).

Class 2B genes

The class 2B genes are differentially expressed in petioles with an angle higher than 70 degrees after 2 and 26 hrs of ethylene treatment and are most likely involved in petiole elongation.

RpEIEG15 is a gene fragment that shows homology to an Arabidopsis orthologue of the Vesicle-associated Membrane Protein (VAMP protein). VAMPs are anchored to membranes by a C-terminal transmembrane domain or by post-translational addition of lipids (Sanderfoot *et al.*, 2000). The possible function for this protein emerged from studies on AtVAMP-transformed yeast, and it was found to be involved in reduction of oxidation of lipids and plasma membrane proteins, which suggested improving membrane repair in yeast (Levine *et al.*, 2001). Moreover, there is evidence showing that orthologues of the mamalian *VAMP7* gene (Advani *et al.*, 1998) may be involved in endosomal/lysosomal trafficking in *Arabidopsis thaliana* (Sanderfoot *et al.*, 2000). This gene is important in vesicle trafficking; it delivers proteins to intracellular and extracellular compartments, cellulose synthase to the plasma membrane, and non-cellulosic polysaccharides to the cell wall.

RpEIEG27 is a putative orthologue of a glycosyl hydrolase protein in Arabidopsis. Glycosyl hydrolase family β-glucosidases catalyse hydrolysis of terminal, non-reducing beta-D-glucose residues with release of beta-D-glucose in inositol and cellulose biochemical pathways. These enzymes have been associated with many important physiological processes in plants; responses to biotic and abiotic stresses, defense against bacterial or herbivore attackers, activating phytohormones, lignification, and cell wall remodelling (Opassiri *et al.*, 2006).

RpEIEG45 shows homology to an auxin-induced protein in Arabidopsis which is similar to the auxin induced *Glycine max* gene 10a 6B (small auxin up RNAs, SAURs) (McClure *et*

al., 1989a; 1989b). The functions of the SAURs are unknown, but there are some indications that they encode short-lived nuclear proteins that are involved in auxin signaling by interacting with calmodulin (Knauss et al., 2003; Yang and Poovaiah, 2000). In Arabidopsis several auxin-response and gravity-response mutants exhibit decreased accumulation of the *SAUR-AC1* mRNAs in elongating etiolated seedlings (Gil *et al.*, 1994).

RpEIEG57 is a putative orthologue of a gene family in Arabidopsis (*AtBT1-5*) that have both a BTB (Drosophila proteins Broad-complex, Tramtrack and Bric-abrac) and a TAZ (transcriptional adapter zinc binding) domain and can bind calmodulin. The BTB domain is a protein-protein interaction module consisting of approximately 120 amino acids that is found in over 600 different proteins in many organisms. TAZ domains are zinc-binding motifs that are primarily involved in protein-protein recognition. Five proteins belong to this *AtBT* family, expressed in Arabidopsis and rice and it is assumed that they are plant specific. Proteins with BTB/TAZ are important in transcriptional regulation and may be induced/activated by second messengers like Ca^{2+} and H_2O_2 (Du and Poovaiah, 2005).

RpEIEG58 shows homology with a 2-oxoglutamate-dependent dioxigenase but the sequence also bares similarity to an ACC oxidase of tomato, suggesting a role in ethylene biosynthesis.

Class 2C genes

The *RpEIEG* gene fragments class 2C represent genes that are present only in samples with a petiole angle of 90 degrees and 26 hrs ethylene treatments: These genes are most likely to be involved in the maintenance of long term enhanced petiole elongation.

RpEIEG79 contain sequences homologous to a BTB/POZ domain. The BTB domain is a protein-protein interaction module consisting of approximately 120 amino acids that is found in over 600 different proteins in many organisms. The domain was first identified as a conserved sequence element in the developmentally regulated Drosophila proteins Broad-complex, Tramtrack and Bric-abrac (BTB). The BTB domain, also known as the POZ (poxvirus and zinc finger) domain, is often found at the N-termini of zinc finger transcription factors as well as some types of potassium channels. The BTB domain is associated with the regulation of gene expression through the local control of chromatin conformation (Dong *et al.*, 1996; Huynh and Bardwell, 1998; Tsukiyama *et al.*, 1994). The BTB domain can form dimers as well as mediating interactions with non-BTB domain containing proteins. Moreover, BTB proteins were fouind to act as substrate-specific adaptors of Cul-3 ligases form BTB E3s complex (Gingerich *et al.*, 2005). In Arabidopsis it was shown that leaf morphogenesis requires *BLADE-ON-PETIOLE1*, a gene possessing a BTB domain (Ha *et al.*, 2004).

RpEIEG91 represents a putative orthologue of Arabidopsis auxin responsive factor proteins (ARF) that have transcription factor activity. When the concentration of auxin is high in the cytoplasm the Aux/IAA proteins, which interact with ARFs at low auxin concentration are subjected for proteolysis. With Aux/IAA protein degradation by the proteasome ARFs are free to interact with promoters of auxin responsive genes thus activating transcription. Based on the structure of individual ARFs, they can either activate or suppress expression of auxin responsive genes (for a review see Berleth *et al.*, 2004).

Only the 3' end of fragment ***RpEIEG92*** shows high homology with a gene with known function. It may be orthologous to the subunit H of photosystem I. The PSI-H subunit of photosystem I is essential for state transitions in plant photosynthesis. Prolonged exposures to changes in illumination lead to changes in photosystem stoichiometry. In contrast, state transition is a dynamic mechanism that enables plants to respond rapidly to changes in illumination (Lunde *et al.*, 2000).

RpEIEG93 is similar to glyceraldehydes-3-phosphate dehydrogenase in the plant *Atriplex nummularia*. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) is an essential protein in glycolysis and gluconeogenesis. The glycolysis cycle takes place in the cytoplasm and during glycolysis process glucose is split into two pyruvate molecules or vice versa (gluconeogenesis).

RpEIEG96 is a putative homologue of Arabidopsis ferredoxin-dependent glutamate synthase. There are two genes in Arabidopsis encoding ferredoxin-dependent glutamate synthase. *GLU1* is predominantly expressed in the leaves and its expression is induced by light and sucrose, whereas *GLU2* is preferentially expressed in roots. Both have a chloroplast targeting signal. *GLU1* plays a major role in assimilating the large amount of ammonia released from photorespiration and is also involved in primary nitrogen assimilation in leaves. GLU2 may be involved in primary nitrogen assimilation in roots (Coschigano *et al.*, 1998).

RpEIEG104 and ***RpEIEG72*** are involved in protein translation, protein synthesis and protein regulation. ***RpEIEG72*** is a putative orthologue of an Arabidopsis protein called ROOT HAIR DEFECTIVE 3 (RDH3). It was shown by Wang and coworkers (1997) that the *RDH3* gene encodes a protein with GTP-binding motifs which are conserved during the evolution of plants species and it is likely to act downstream of the hormones auxin and ethylene. The RHD3 protein is a member of a new class of GTP-binding proteins that is present in many eukaryotes and is required for regulated cell enlargement. ***RpEIEG104*** represent a putative Arabidopsis orthologue of Elongation factor 1-alpha (EF-1 alpha). This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis (Swiss-Prot P53013). The expression pattern of EF-1 alpha is associated with high protein synthesis regions, like meristems, rapidly growing tissues, and developing gametophytes (Ursin *et al.*, 1991). In addition, EF-1 alpha expression was affected by changes in plants growth patterns including hormone treatments (Ursin *et al.*, 1991).

Appendix B

Supplementary table 1. Protein kinases annotated to phosphorylation sequences during ethylene-induced hyponastic growth.
The targets of certain kinases with known residues in different organisms were searched for most probable target or targets in Arabidopsis using the Pathmach search tool in the TAIR database.
All presented sequences showed higher phosphorylation during hyponastic growth.
Kinases shown in bold are most likely able to phosphorylate the target sequence, the ones not in bold are suggested by the chip manufacturer. This is in most cases not experimentally tested but through blast search in database of protein domains the motifs could be recognised (http://www.expasy.org/prosite/).
Note: Only the list of motifs which have similar known function targets in Arabidopsis using Pathmach search tool with maximum 2 replacements were listed in the table.

Sequence	Kinase protein	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
RRRQSVLNL	PKA	CFTR_HUMAN	RRRLSVLSL	At2g38770	Expressed protein
			RRRQSELPL	At3g18090	DNA-directed RNA polymerase family protein
SSLKSRKRA		STP1_SHEEP	SILKSRTRA	At1g06950	Involved in protein import into the chloroplast
			SSLKSRSRS	At1g13190	RNA recognition motif
			SSSKKRKRA	At1g59453	transcription factor-related
			SSLKARKRR	At3g49400	transduction family protein
GSRGSGSSV	PKA	DESM_CHICK	GGSGSGSSV	At2g28650	exocyst complex subunit
			GSSGSASSV	At4g02650	Calm-N N-Terminal Domain Of Clathrin Assembly Lymphoid Myeloid Leukaemia Protein
			GSTGSGISV	At4g35380	guanine nucleotide exchange family protein
			GSRGSGSVS	At5g59730	leucine zipper-containing protein
KKKGSGEDD	CK2	IF43_YEAST	KKSGSGEDW	At1g61100	disease resistance protein
			KKKSSDEDD	At3g14250	zinc finger protein-related
ASGSFKL	PKC	H11_BOVINE	ASGSFRL	At1g80530	similar to nodulin-like protein

Sequence	Kinase protein	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			ASGDFKL	At3g21600	senescence/dehydration-associated protein-related
			ALGSFKL	At3g27260	DNA-binding bromodomain-containing protein
			ASGSGKL	At3g51250	senescence/dehydration-associated protein-related
			ASHSFKL	At4g33860	glycosyl hydrolase family 10 protein
VPTLSTFRT	PKC	DESM_CHICK	VPVLSTFRK	At1g12170	F-box family protein
			VPVLSTRRT	At1g61210	similar to katanin p80 subunit
ARRSTTDAG	PKA	0.82(0.02)	ALRSITDAG	At3g58360	similar to ubiquitin-specific protease 12
KQPIYIVME	v-Fps	FPS_AVIS	KTKIYIVME	At2g34180	CBL-interacting protein kinase 13
			KTYIYIVME	At4g36070	CDPK family protein
			KRPIYIVGE	At5g41130	esterase/lipase/thioesterase family protein
AGTTYAL	myosin I heavy chain kinase	myosin heavy chain IA	IGTTYAL	At3g23550	efflux family protein, similar to ripening regulated protein
PMRRSVSEA	PKA	LIPS_BOVIN	PMRRSLSTA	At1g59870	ABC transporter family protein
			PLRRSVSET	At5g17980	C2 domain-containing protein
RSKRSGSV	PKC	KPBB_RABIT	RSKRSGST	At1g76740	weak similarity to fimbriae-associated protein Fap1
TEGQYQQQP		LCK_CHICK	TEVQEQQQP	At1g70320	ubiquitin-protein ligase-like protein
QRRHSLEPP	PKA	SRC_RSVP	YRRHSLEPS	At3g58620	tetratricopeptide repeat (TPR)-containing protein
SRTLSVSSL	AMP-PK	UGS1_RABIT	SRRLSSSSL	At1g56500	haloacid dehalogenase-like hydrolase family protein
			SSILSVSSL	At2g02960	zinc finger family protein
			SRPMSVSSL	At3g54350	nucleolar protein
			SRTVSSSSL	At3g62440	F-box family protein
			SLTRSVSSL	At5g27050	MADS-box family protein
			SRTLSVGRL	At5g45290	zinc finger family protein
PSSTSSSSI	CK2	TOP2_YEAST	SSSTSSSSI	At1g44830	ERF/AP2 transcription factor family
			PSSTSSSSS	At3g46800	DC1 domain-containing protein
LKRASLG	**PKA**	LIVER PYRUVATE KINASE	LKRASLG	At4g35030	protein kinase activity ATP binding kinas
RRRPTPATL	PKA	IPP1_RABIT	RRRPTLASL	At3g16000	matrix-localized MAR DNA-binding protein-related
			PRRPTPVTL	At5g58340	similar to myb family transcription factor Myb DNA-binding domain

Sequence	Kinase protein	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
LRRNSI	**PKA**	CFTR_HUMAN	LRRNS	At4g01570	pentatricopeptide (PPR) repeat-containing protein
			LRRNSI	At5g10470	kinesin motor protein-related
KLRRSSSVG	PKA	ACHD_TORCA	KLRRSASFG	At2g40140	Zinc finger
			KTRRSSSTG	At2g45580	cytochrome P450 family protein
			TLRTSSSVG	At4g00231	maternal effect embryo arrest 50
			KLRRSPSLG	At5g41730	protein kinase family protein
			KLRRFLSVG	At5g67240	similar to exonuclease family protein
ESRISLPLP	Autophosph. dependent	VIME_MOUSE	ENRISGPLP	At1g06840	leucine-rich repeat transmembrane protein kinase
			ENRISGPLP	At1g65300	MADS-box transcription factor protein
RVRKTKGKY	PKC	GLR1_RAT	RVKKTKGKY	At5g45250	disease resistance protein
SNPEYLSAS	**CK2**	INSR_HUMAN	SNPANLSAS	At3g46920	similar to MAP3K delta-1 protein kinase
AEPGSPTAA		MACS_BOVIN	AEPGSLTAL	At1g17840	similar to ABC transporter
TSSSSIFDI	CK2	TOP2_YEAST	ESSSSPFDI	At3g14140	oxidoreductase, 2OG-Fe(II) oxygenase family protein
			TSSYSIFSI	At4g36790	transporter-related protein
KQISVRGL		PHS2_HUMAN	KQESVRGN	At1g05640	ankyrin repeat family protein
			DQISNRGL	At1g15740	leucine-rich repeat family protein
			DQISVRGF	At1g71210	pentatricopeptide (PPR) repeat-containing protein
			KGISVRAL	At1g72330	alanine aminotransferase
			KAISVKGL	At2g37280	ABC transporter family protein
			KQLSRRGL	At2g39110	similar to protein kinase
			TQHSVRGL	At3g12670	CTP synthase
			KAISVEGL	At3g53480	ABC transporter family protein
			KEMSVRGL	At3g56030	pentatricopeptide (PPR) repeat-containing protein
			KAISVEGL	At4g15215	ABC transporter family protein
			KQIAVRYL	At4g22200	potassium channel protein 2
			KQITVRGV	At4g24120	oligopeptide transporter OPT family protein
EILNSPEKA	**PKC**	143B_BOVIN	EILNSPESA	At1g22300	14-3-3 protein GF14 epsilon

Sequence	Kinase protein	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			EILNSPERA	At1g34760	(GRF8)
			EILNSSEKA	At5g65430	14-3-3 protein GF14 epsilon in (GRF8)
LRRWSLG	**PKA**	LIVER PYRUVATE KINASE	LRRESLG	At1g18870	14-3-3 protein GF14 epsilon in (GRF8)
			LRRFSLG	At5g42940	protein with isochorismate synthase activity
TVSKTETSQ	RhK	OPSD_BOVIN	TVSKKETSD	At5g65510	zinc finger
KQGSGRGL	**PKC**	PHS2_HUMAN	KKGSGRGL	At2g32860	similar to ovule development protein
			KIGSGRGL	At2g45540	glycosyl hydrolase family 1 protein
LRRASLDG	**PKA**	LIVER PYRUVATE KINASE	LRRASRDI	At1g15440	WD-40 repeat family protein transducin family protein
			LRRLSLAG	At1g27190	leucine-rich repeat transmembrane protein kinase
			LRNNSLDG	At1g45616	leucine-rich repeat family proteinsimilar to disease resistance protein
			LRRASLVK	At1g67820	protein phosphatase 2C
			KRRISLDG	At1g80680	nucleoporin family protein
			LRRLYLDG	At2g15320	leucine-rich repeat family protein
			LRRASPVG	At3g21750	UDP-glucosyl transferase family protein
			LRRASRDI	At3g21800	UDP-glucosyl transferase family protein
			LRRLSLSG	At3g26810	AFB2 (auxin signalling F-box2)
			ERRASLDC	At3g54800	PH domain containing protein
			LRRLSLSG	At3g62980	transport inhibitor response 1
			LLNASLDG	At4g25600	similar to PBCV-1 prolyl 4-hydroxylase
			MRCASLDG	At5g18070	phosphoglucosamine mutase-related
			LKRASLDR	At5g24970	ABC1 family protein
			LRRASLTA	At5g54130	calcium-binding EF hand family protein
			LREASGG	At1g75520	lateral root primordium (LRP) protein-related
			KRRASGG	At2g23290	myb family transcription factor
			LRRYSGG	At5g18390	pentatricopeptide (PPR) repeat-containing protein
KRNSSPPPS	PKA	ATCP_HUMAN	KEFSSPPPS	At1g28420	homeobox transcription factor
			TANSSPPPS	At1g70640	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein

Sequence	Kinase protein	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			DDNSSPPPS	At2g39290	phosphatidylglycerolphosphate synthase
			KTNPSPPPS	At4g39753	kelch repeat-containing F-box family protein
			KASSSPPPS	At5g23120	photosystem II stability/assembly factor, chloroplast (HCF136
EHVSSSEES	CKI	CAS2_BOVIN	SSSTSSSSI	At1g44830	ERF/AP2 transcription factor family
			FSVNTPSPL	At2g22680	zinc finger
			SSSPTPSSL	At3g24240	leucine-rich repeat transmembrane protein kinase
			SSVPGPAPL	At3g44200	protein kinase family protein
			SCVPTQSPL	At3g46920	protein kinase family protein
			ASVPTPSPS	At4g26350	F-box family protein
			SSVPIPPPL	At5g67480	TAZ zinc finger family protein/ BTB/POZ domain-containing protein
			ESVSGSEES	At1g78540	similar to transcription factor-related
			ESESSSEES	At3g51180	zinc finge
			EKVSGSEES	At3g62300	agenet domain-containing protein
			EHSSSSTES	At5g23110	zinc finger
GSLKSRKRA	**PKC**	STP1_PIG	GSLKGRKKA	At1g08315	armadillo/beta-catenin repeat family protein
			GSKKSRKRK	At1g33390	similar to kurz protein
			GSLKSTLRA	At3g46370	F-box family protein
			GSLKSAKRL	At3g51530	F-box family protein
			GSIFSRKRA	At3g63530	zinc finger
			GSAKQRKRA	At4g25730	FtsJ-like methyltransferase family protein
EGGRTVGAG		EFTU_THETH	EGGRTVGAG	At4g02930	elongation factor Tu
			EGGKTVGAG	At4g20360	elongation factor Tu / EF-Tu
RVLESFRAA	PKC	MINK_RAT	RVLESERAA	At3g07700	ABC1 family protein
			RVLEKFRAL	At3g05470	FH2 domain-containing protein
			RVDESSRAA	At5g23150	PWWP domain-containing protein transcription factor (HUA2)

Supplementary table 2. Protein kinases annotated to phosphorylation sequences during ethylene-induced elongation growth.
The targets of certain kinases with known residues in different organisms were searched for most probable target or targets in Arabidopsis using the Pathmach search tool in the TAIR database.
Sequences presented in bold showed higher phosphorylation during elongation growth whereas the other sequences showed decreased phosphorylation.
Kinases shown in bold are most likely able to phosphorylate the sequence. This is in most cases not experimentally tested but through blast search in database of protein domains the motifs could be recognised (http://www.expasy.org/prosite/).
Note: Only the list of motifs which have similar known function targets in Arabidopsis using Pathmach search tool with maximum 2 replacements were listed in the table.

Sequence	Kinase Pepchip	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
KQISVR	**pkc**	PHS2_HUMAN	KQESVR	At1g05640	Ankyrin repeat family protein
			KQISRR	At1g07620	GTP-binding protein
			KSISVR	At1g17270	zinc finger (CCCH-type) family protein
			KSISVR	At1g68200	zinc finger (CCCH-type) family protein
			DQISVR	At1g71210	pentatricopeptide (PPR) repeat-containing
SARLSAKPA	PKG	HG14_BOVIN	SFRLSAKPK	At5g16390	biotin carboxyl carrier protein 1 (BCCP1)
SPRKSPKKS		H1_PARAN	SPSPSPKKS	At2g22100	RNA recognition motif (RRM)-containing protein
			SPVKSPKKA	At5g59870	histone H2A, protein
RPPGFTPFR		BRADYKININ	RVPGFTPTR	At1g64600	similar to Hypothetical 72.2 kDa protein in RPS27A-GPM1 intergenic region
SAYRSVDEV	Branched	ODBA_RAT	SAERSVKEV	At2g30710	RabGAP/TBC domain-containing protein
			SAYIEVDEV	At2g38420	pentatricopeptide (PPR) repeat-containing protein
KRRRSSKDT	**PKc**	NMZ1_HUMAN	FRRRSSKDV	At4g32650	identical to K+ inward rectifying channel protein KC1
			KRRRSSKVM	At4g36490	phosphoglyceride transfer protein
			KRRKSSKDN	At5g35338	methyl-CpG-binding domain-containing protein
KRRLSFSET	**PKA,ck2**	IL1A_MOUSE	SRRLSVSE	At3g02830	zinc finger (CCCH-type) family protein
MMTPYVVTR		JNK1_HUMAN	FMTEYVVTR	At1g01560	mitogen-activated protein kinase, putative
			IMTEYVVTR	At1g07880	MAPK, putative
			FMTEYVVTR	At1g10210	MPK1
			FMTEYVVTR	At1g59580	MPK2
			FMTEYVVTR	At2g18170	MPK7
			FMTEYVVTR	At2g43790	MPK6

Sequence	Kinase Pepchip	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			FMTEYVVTR	At2g46070	MPK12
			FMTEYVVTR	At3g45640	MPK3
			LMTEYVVTR	At3g59790	MPK10
			FMTEYVVTR	At4g01370	MPK4
			YMTEYVVTR	At4g11330	MPK5
			FMTEYVVTR	At4g36450	MPK14
LARASLG		LIVER PYRUVATE KINASE	LAAASLG	At1g11670	MATE efflux family proteinDDT-FR18 similar to ripening regulated protein
			LARASFG	At1g17060	cytochrome P450
			LARASLM	At1g21650	preprotein translocase secA family protein
			LAAASLG	At1g61890	MATE efflux family protein
			LARASLS	At3g07550	F-box family protein (FBL12)
			LAAASLG	At3g21690	MATE efflux family protein
			LARASLV	At3g48150	cell division cycle family protein / CDC family protein
			LARQSLG	At4g28520	12S seed storage protein, putative
			LARASLV	At4g35030	protein kinase family protein
			LAFASLG	At4g37460	Tetratricopeptide repeat (TPR)-containing protein
			LAVASLG	At5g22470	similar to poly (ADP-ribose) polymerase
			LALASLG	AtCg00340	Encodes the D1 subunit of photosystem I and II reaction centers
ARTKRSGSV	PKc	KPBB_RABIT	ARTLRSGLV	At1g52870	peroxisomal membrane protein-related
			AATKRSGFV	At5g02970	alpha/beta fold family protein
VGPDSD		P53_MOUSE	VGSDSD	At1g17745	D-3-phosphoglycerate dehydrogenase / 3-PGDH
			DGPDSD	At1g27770	-transporting ATPase 1, plasma membrane-type / Ca(2+)-ATPase
			VDPDSD	At1g56720	protein kinase family protein
			VVPDSD	At1g50610	leucine-rich repeat protein
			VPPDSD		early-responsive to dehydration protein
RKRTLRRL		EGFR_HUMAN	YWRTLRRL	At1g01190	cytochrome P450
			YKYTLRRL	At1g65950	ABC1 family protein

Sequence	Kinase Pepchip	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			DQRTLRRL	At1g6864	bZIP family transcription factor (PERIANTHIA)
			RKRTPRRQ	At1g77790	glycosyl hydrolase family 17 protein
			RSKTLRRL	At2g15320	leucine-rich repeat family protein
			EKRTLERL	At2g34210	KOW domain-containing transcription factor family protein
			RPRTLSRL	At3g02060	DEAD/DEAH box helicase, putative
			WKRTLPRL	At3g25510	disease resistance protein (TIR-NBS-LRR class
			SKRTRRRL	At5g04140	glutamate synthase (GLU1) / ferredoxin-dependent glutamate synthase
			LWRTLRRL	At5g10600	cytochrome P450 family protein
			LWRTLRRL	At5g10610	cytochrome P450 family protein
			RKRTLKRA	At5g24080	protein kinase family protein
			RKRRLRRS	At5g55630	outward rectifying potassium channel (KCO1)
			RKDTLERL	At5g67100	DNA-directed DNA polymerase alpha catalytic subunit
PPEKTEEEE	**pkA, pkc**	SSR3_RAT	PLEETEEEE	At1g09320	domain-containing protein
			PPSKGEEEE	At3g23670	phragmoplast-associated kinesin-related protein
			PPLTTEEEE	At4g26380	DC1 domain-containing protein
SRSRSPGRP	RS	LBR_CHICK	SRSRSPKRP	At1g16610	arginine/serine-rich protein
			SRSRSPRRP	At3g55460	SC35-like splicing factor
GSRPSESNG	PKA	MIP_BOVIN	GRRNSESNG	At2g03640	nuclear transport factor 2 (NTF2) family protein
			SSRRSESNG	At2g18450	succinate dehydrogenase (ubiquinone) flavoprotein subunit
			GSRPEESQG	At4g19190	zinc knuckle (CCHC-type) family protein
			GVSPSESNG	At4g32040	homeobox protein knotted-1 like 5
VIKRSPRKR	CDC28-dependent	SWI5_YEAST	FIKRSKRKR	At5g37450	leucine-rich repeat transmembrane protein kinase
LDPLSEPED	CK2	THA_CHICK	LDYGSEPED	At5g52530	dentin sialophosphoprotein-related
QLNDSSEEE	CK2	VE7_HPV16	ELNDSEEEE	At3g13225	similar to mKIAA1014 protein
GVDTYVEMR	ck2	KFMS_HUMAN	GVDTYVTWR	At1g23010	multi-copper oxidase type I family protein
			GVDTYVTWR	At1g71040	multi-copper oxidase type I family protein
QSPSSSPTH		CTPT_RAT	QSPSSSGTM	At1g43850	transcriptional co-regulator

Sequence	Kinase Pepchip	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			QLPSSSATH	At1g68510	LOB domain protein 42
			DSPSSSPTA	At1g70450	protein kinase family protein
			QSGSSSPTT	At2g28250	protein kinase family protein
			QSPSSSPQY	At3g08790	VHS domain-containing
			QSPSSSSTP	At3g23130	superman protein (SUP) / zinc finger (C2H2 type) family protein
			QSPISSPTK	At4g37450	arabinogalactan-protein (AGP18)
DRLVSARSV	PKC	MET_HUMAN	RRLVSATSV	At1g58180	carbonic anhydrase family protein
			DRLESARRV	At3g26540	pentatricopeptide (PPR) repeat-containing protein
			DRLQSTRSV	At5g17410	tubulin family protein
LAYESHESL		MGP_BOVIN	LAYVSRESL	At5g15410	cyclic nucleotide-regulated ion channel
KRKRSRKES	PKA	H2B_HUMAN	KRARSRKEN	At1g29630	exonuclease, putativsimilar to Swiss-Prot:P53695 exonuclease
			KRKREEKES	At1g31970	DEAH box helicase, putative
			KRKRSRKQL	At2g25170	chromatin remodeling factor CHD3
SRKGSGFGH	PKA	CXB1_RAT	SRKGSGYGS	At1g09550	Pectinacetylesterase putative
			SRKGSGFSD	At1g16120	wall-associated kinase
			KRKMSGFGH	At2g42790	citrate synthase, glyoxysomal putative
			KRKMSGFGH	At3g58750	citrate synthase, glyoxysomal putative
			ARKGSRFGH	At5g48230	acetyl-CoA C-acyltransferase, putative
LGSPLRRR		VIME_MOUSE	LESPLRRR	At2g37080	myosin heavy chain-related
			LGSSLRRR	At4g16950	disease resistance protein TIR-NBS-LRR class
TRSVSSSSY	PKA	VIME_MOUSE	TRSKSSSSN	At1g17000	alpha, alpha-trehalose-phosphate synthase2 domain
			TSSVSSSST	At1g27760	interferon-related developmental regulator family protein
			TRLSSSSSY	At2g04620	cation efflux family protein
			TRSSSSSSL	At2g34830	WRKY family transcription factor
			DRSVSSSSG	At2g48010	serine/threonine protein kinase
			TRSVSCSSI	At4g00230	subtilisin-like serine endopeptidase protein
			TRSVSSLDY	At5g27050	MADs-box family protein

Sequence	Kinase Pepchip	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			TASVSSSLY	At5g44290	protein kinase family protein
			TASVSSSSS	At5g67260	cyclin family protein, similar to cyclin D3.1
EASTTVSKT		OPSD_SHEEP	EAATTVVKT	At4g23510	disease resistance protein (TIR class), putative
YSLGSALRP	PKC	VIME_MOUSE	LSLPSALRP	At4g30360	cyclic nucleotide-regulated ion channel, putative
RKRSRAE	**pkA,ck2**	H2B_HUMAN	RKLSRAE	At1g62310	transcription factor jumonji (jmjC) domain-containing protein
			TKRSRAE	At4g11970	YT521-B-like family protein
			EKRSRAE	At4g24250	seven transmembrane MLO family protein
			RKRSKAE	At4g26440	WRKY family transcription factor
			RKRSRDE	At5g16780	SART-1 family protein
			RKWSRAE	At5g41220	glutathione S-transferase, putative
SSSESGAPE	CK2	CLCB_BOVIN	SSSESDAQE	At3g17360	kinesin motor protein-related
			SSSLSGAEE	At3g62610	myb family transcription factor
			SSSSSSAPE	At4g09040	recognition motif (RRM)-containing protein
			SSSSSGFPE	At4g37200	thioredoxin family protein
GRALSTRAQ	PhK	TRIC_RABIT	FRALSRRAQ	At4g02750	pentatricopeptide (PPR) repeat-containing protein
FFSSSESGA	CK2	CLCB_BOVIN	FFSSSASGY	At1g34010	myosin-related peroxisomal membrane protein-related
			FPSSSSSGA	At1g52870	peroxisomal membrane protein-related
			FFSSSTSGS	At2g39750	glucose-inhibited division family A protein
			KFISSESGA	At4g11160	translation initiation factor IF-2, mitochondrial
RRASR		KPYR_RAT	RRASR	At1g47056	F-box family protein
			RRASR	At3g21800	UDP-glucoronosyl/UDP-glucosyl transferase family protein
			RRASR	At4g26470	calcium-binding EF hand family protein oxidoreductase
			RRASR	At5g01780	2OG-Fe(II) oxygenase family protein
TLSDSDDED	CK2	MYCN_HUMAN	PRSVRRRR	At4g32600	zinc finger (C3HC4-type RING finger) family protein
			VLLDSDDED	At2g16390	SNF2 domain-containing protein
			GLVDSDDED	At3g24760	F-box family protein
			DLSWSDDED	At5g28250	protease family protein

Sequence	Kinase Pepchip	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
			TSSDSDDEL	At5g38190	myosin heavy chain-related protein
			VLSDSDDEN	At5g60410	Encodes a plant small ubiquitin-like modifie
PRRVSRRRR	PKC	PRT1_CLUPA	RRNSRRRR	At1g63170	Zinc finger contain protein
			PRVSMRRR	At2g20370	C3HC4-type RING finger family protein
			PRRSRRR	At2g20590	non-consensus AA donor splice siA
			PRRSRRR	At2g20590	reticulon family protein
			PRVSRLRR	At4g03520	thioredoxin M-type 2, chloroplast (TRX-M2)
			PRSVRRRR	At4g32600	zinc finger (C3HC4-type RING finger) family protein
KKRFSFKKS	PKC	MACS_BOVIN	KKRPSFKKV	At1g51500	ABC transporter family protein
GDSESGEEE	**ck2**	SRF_HUMAN	DDSESGEEE	At3g50690	leucine-rich repeat family protein
RRSSSVGYI	PKA	ACHD_TORCA	RRSKGVGYI	At5g09880	RNA recognition motif (RRM)-containing protein
			RDSSSVGYY	At5g54250	cyclic nucleotide-regulated ion channel
AARTPGGRR		MBP_BOVIN	RARTDGGRR	At1g67325	zinc finger (Ran-binding) family protein
			AAPTPGVRR	At2g31590	
			AARTHGSRR	At4g00570	malate oxidoreductase
RGRSSVYSA	myosin	MYSC_ACACA	RGRSSSYSP	At1g07350	transformerserine/arginine-rich
			RGRSSSYSS	At1g16610	arginine/serine-rich protein
LRHASLG		LIVER PYRUVATE KINASE	LRHASSG	At1g63170	zinc finger (C3HC4-type RING finger) family protein
			LRNASLG	At1g74200	leucine-rich repeat family protein
			LRIASLG	At2g16730	glycosyl hydrolase family 35 protein
			LRKASL	At4g37270	Gcadmium/zinc-transporting ATPase putative
			LRVASLG	At4g38590	glycosyl hydrolase family 35 protein
YGNGYSSNS	INSR	B2AR_MESAU	YGRGYSSSS	At3g13224	RNA recognition motif (RRM)-containing
			NGNGSSSNS	At5g67450	zinc finger (C2H2 type) protein 1
PKKGSKKAV	PKA	H2B_HUMAN	PKKGSKKKG	At2g33990	similar to calmodulin-binding family protein
			PKKGSVWAV	At2g35540	DNAJ heat shock N-terminal domain-containing
			PKKGSKKIL	At3g53970	proteasome inhibitor-related

Sequence	Kinase Pepchip	Pep Chip targets	Sequence of motif in Arabidopsis	Possible plant target	Target description
QRRRSLEPP	PKA	SRC_CHICK	SRRRSLETP	At5g27000	kinesin motor protein-related
EDAESEDEE	**ck2**	NPM_HUMAN	EEDESEDEE	At1g48920	nucleolin, putative, similar to nuM1 protein
			EDEESEEEE	At1g66620	seven in absentia (SINA) protein
			VAAESEDEE	At3g16280	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family
			SDEESEDEE	At3g47180	zinc finger (C3HC4-type RING finger) family protein
			EDEESDDEE	At3g51270	RIO1 family protein
			EYAESEDEA	At4g00755	F- box family protein
			EIDESEDEE	At5g11240	transducin family protein / WD-40 repeat family protein, contains 3 WD-40 repeats
			EDSESEDQE	At5g43310	COP1-interacting protein-related, contains similarity to COP1-Interacting Protein 7
			EDGESEDFE	At5g61330	rRNA processing protein-related

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Nederlandse samenvatting

Moeraszuring als modelsysteem

Moeraszuring (*Rumex palustris*) is een plantensoort die voornamelijk voorkomt op locaties die regelmatig overstromen, zoals in de uiterwaarden van rivieren. Dit is bijzonder want in het algemeen zijn maar weinig plantensoorten in staat om overstroming te overleven. Onder water staan betekent een sterke beperking van gasuitwisseling, omdat de diffusie van gassen in water zo'n 10.000 keer langzamer gaat dan in lucht. Dit heeft tot gevolg dat planten onder water te maken krijgen met een tekort aan zuurstof. Daarnaast kunnen andere gassen, zoals bijvoorbeeld het plantenhormoon ethyleen, de plant maar langzaam verlaten zodat deze gassen ophopen. Eén en ander heeft tot gevolg dat de plant zich aan moet passen om niet ten gevolge van zuurstof- en energietekort te sterven.

Planten hebben verschillende strategieën onwikkeld om met overstromingstress om te gaan. Sommige plantensoorten stoppen met al hun, op dat moment, overbodige activiteiten zoals bijvoorbeeld metabolisme en groei. Andere plantensoorten, waarvan moeraszuring er één is, gaan juist harder groeien om boven het water uit te komen en derhalve de gasuitwisseling met de atmosfeer te kunnen herstellen.

Het gaat bij de moeraszuring om een zeer specifieke vorm van groei. Deze vindt plaats aan de onderkant van de bladstelen en zorgt er voor dat bladeren min of meer loodrecht gaan staan. We noemen deze differentiële groei hyponastische groei. Vervolgens is er een versnelde verlenging van de hele bladstelen waardoor de punt van het blad richting wateroppervlak groeit.

Waarnemen van overstroming en regulatie van de versnelde groei

Uit eerder onderzoek is gebleken dat accumulatie van ethyleen een belangrijk signaal is om overstroming waar te nemen. De hele keten van signalen die als gevolg van de ophoping van dit plantenhormoon op gang komt leidt uiteindelijk tot de de versnelde (differentiële) groei.

Het was reeds bekend dat ethyleen zonder overstroming de groeireactie kan nabootsen. In **hoofdstuk 2** is in meer detail het effect van het hormoon ethyleen

op de groei bestudeerd. Dit onderzoek heeft aangetoond dat er minimale verschillen zijn tussen de reactie van de plant die behandeld is met ethyleen en een overstroomde plant. Onder invloed van ethyleen begint de plant wat later te reageren dan als gevolg van overstroming maar de maximale hoek en strekking worden op hetzelfde moment bij beide behandelingen bereikt. Indien planten bij behandeling met ethyleen kunstmatig op een lage bladhoek worden gehouden (<30º) heeft dat tot gevolg dat de plant wel hyponastische- maar geen lengtegroei vertoont. Als de hoek daarentegen heel hoog gehouden (>50º) wordt, vertoont de bladsteel uitsluitend lengtegroei.

Uit het bovenstaande blijkt dat hyponastische en lengtegroei door hoekmanipulatie te ontkoppelen zijn.

Welke genen spelen een rol gedurende door ethyleen geïnduceerde versnelde groei?

Doordat we in staat zijn om met toevoeging van ethyleen de groei van planten tijdens overstroming na te bootsen en doordat we door manipulatie van de hoek van de bladsteel de hyponastische groei kunnen ontkoppelen van de groei van de hele bladsteel, kunnen we op zoek gaan naar genen die bij elk van deze processen betrokken zijn. De keten van waarneming tot aan reactie is waarschijnlijk zeer complex en omvat zeer veel componenten. Onderzoek uit het verleden heeft al aangetoond dat er naast ethyleen ook nog andere plantenhormonen zoals auxine, abscisine zuur en gibberelline betrokken zijn. Tevens is aangetoond dat een aantal genen die betrokken zijn bij de biosynthese en afbraak van deze stoffen meer, danwel minder tot expressie komen. Helemaal aan het einde van de keten heeft men laten zien dat expansine RpEXPA1, een eiwit dat celwanden verweekt zodat ze verder uit kunnen rekken, zowel op transcriptie- als op eiwitniveau meer tot expressie komt.

In **hoofdstuk 3** wordt de isolatie en identificatie van genen die verschillend tot expressie komen gedurende door ethyleen geïnduceerde versnelde groei beschreven. Hierbij is gebruik gemaakt van drie moleculaire technieken om differentiële expressie van genen zichtbaar te maken. cDNA subtraction, waarbij in mRNA pools verrijkt wordt voor die genen die meer of minder tot expressie komen in één pool t.o.v. de andere. Tevens is gebruik gemaakt micro-arrays waarbij mRNA pools van *Rumex palustris* op *Arabidopsis thaliana* (zandraket) arrays zichtbaar gemaakt werden. De derde gebruikte methode was cDNA-AFLP waarbij tussen pools van mRNA de verschillen zichtbaar gemaakt werden. Bij alle methoden is gebruik gemaakt van manipulatie van de hoek van de bladsteel om hyponastische groei van de groei van de hele bladsteel te kunnen onderscheiden.

Met behulp van de eerste en derde methode waren we in staat om 119 genen die differentiëel tot expressie kwamen te isoleren en identificeren. Analyse met behulp van de GenBank databases bracht aan het licht dat 21 van deze 119 genen goede homologie vertonen met genen in *Arabidopsis thaliana* en andere planten-

soorten. Van veel van deze genen is bekend dat ze betrokken zijn bij groei en ontwikkeling. Micro-array analyse resulteerde in in meer dan 5000 genen die verschillend tot expressie kwamen tussen behandeling en controle. Van al deze genen is onderzocht of er een over-representatie van promotermotieven was. Het bleek dat DNA motieven die bekend staan als induceerbaar door abscisine zuur (ABA) in verhouding veel voorkwamen in de geïdentificeerde genen, hetgeen suggereert dat ABA bij deze processen betrokken is.

In **hoofdstuk 4** wordt de expressiekinetiek in de tijd van een aantal in hoofdstuk 3 beschreven genen geanalyseerd met behulp van Quantitative Reverse Transcriptase PCR (QPCR) tijdens normale groei in lucht, toevoeging van ethyleen en overstroming. Wat opvalt is dat de expressie van veel genen een patroon vertonen dat overeenkomt met het groeipatroon zoals dat te zien is in figuur 6 in hoofdstuk 2 waarbij verhoogde expressie waar te nemen is als de versnelde groei af gaat wijken van de controle groei. Opvallend is dat dezelfde genen ook tijdens groei in lucht een soortgelijk patroon vertonen maar minder hoog dan gedurende ethyleen behandeling of overstroming. Het is opvallend dat ethyleen behandeling de genen tot een hogere expressie brengt dan overstroming. Een reden hiervoor is niet duidelijk. De hogere expressie van de genen t.o.v. de controle suggereert dat in ieder geval een deel van de genen die bij normale groei betrokken zijn, ook opgereguleerd worden tijdens de twee behandelingen die versnelde groei veroorzaken.

Localisatiestudies van expressie door middel van *in situ* hybridisatie van twee van de beschreven genen uit hoofdstuk 3 op coupes van diverse delen van de bladsteel, resulteerde niet in een duidelijk verschil in localisatie tussen de boven- en onderkant van de bladsteel zoals verwacht voor één van de genen. Het resultaat laat zien dat beide genen gedurende overstroming en ethyleen behandeling hoger tot expressie komen en dan met name in het deel van de bladsteel dicht bij de aanhechting aan de plant, maar dat een verschil tussen boven- en onderkant van de bladsteel niet zichtbaar is.

De genen die hierboven beschreven staan zijn geïsoleerd uit *Rumex palustris*, een soort waar op DNA niveau niet veel van bekend is. Derhalve is de functionaliteit van deze genen onderzocht met behulp van *Arabidopsis thaliana*. De DNA basevolgorde van Arabidopsis is geheel bekend en geeft een vergelijkbare reaktie op ethyleen behandeling en overstroming, met uitzondering van verlenging van de bladsteel. Tevens zijn er van vrijwel elk gen mutanten beschikbaar die in het experimentele systeem, behandeling met ethyleen, getest kunnen worden. De mutanten werden geselecteerd op basis van homologie met de (korte) basevolgorde zoals die van de *Rumex palustris* genen geïdentificeerd is. Dit had tot gevolg dat voor één *Rumex palustris* gen meerdere Arabidopsis mutanten getest konden worden. Het was interessant om te zien dat de getestte mutanten uiteen vielen in 4 groepen. Eén groep had een continu verhoogde bladhoek ten opzichte van de controleplanten. De andere groepen hadden effect op de door ethyleen geïnduceerde hoek van de bladsteel, het blad of allebei. Deze mutantenanalyse liet verder zien dat genen van

het photosynthese apparaat, metabolisme en auxine betrokken zijn bij de hyponastische groei in Arabidopsis en mogelijk dus ook bij *Rumex palustris*.

Regulatie in cellen en weefsels vindt niet alleen plaats op het niveau van transcriptie (DNA) maar kan ook op het niveau van het transcript (mRNA) en eiwit plaatsvinden. In **hoofdstuk 5** staat beschreven welke veranderingen er gedetecteerd zijn op het niveau van fosforylering. Dit is een belangrijk mechanisme dat de activiteit van een eiwit reguleert. Met behulp van celextracten en een Pepchip is geïnventariseerd welke peptiden op de Pepchip meer of minder gefosforyleerd werden. Door enerzijds te analyseren welke kinases in staat waren die peptiden te fosforyleren en anderzijds de eiwitten (genen) te identificeren die dergelijke peptiden bezitten was het mogelijk om tot een ruwe inventarisatie te komen. Uit deze resultaten kunnen we concluderen dat veel mogelijke substraten voor de kinases zoals transcriptie factoren, cytoskelet en cellulose synthese eiwitten, auxine signalerings eiwitten, kalium transporters en eiwitten betrokken bij het energie metabolisme differentieel gefosforyleerd worden gedurende de behandeling ten opzichte van de controle. Om de precieze identiteit van zowel de kinases als hun substraat vast te stellen moet verder onderzoek verricht worden.

Concluderend uit de resultaten beschreven in dit proefschrift kan opgemerkt worden dat veel genen betrokken bij normale groei ook een rol spelen bij hyponastische en versnelde groei van de bladsteel onder invloed van ethyleen. De regulatie in tijd en plaats en het niveau van expressie maken waarschijnlijk de verschillen. Desondanks hebben we nu inzicht gekregen welke groepen van genen een mogelijke rol spelen.

In dit proefschrift worden verschillen in expressie en activiteiten van celwand gebonden kinases zowel op transcriptie- als op eiwitniveau gedetecteerd. Een mogelijk werkingsmechanisme kan zijn dat deze de signalering binnen een cel doorgeven wordt waarbij eventueel MAPKinases en abscisine zuur betrokken zijn met als uiteindelijk resultaat een toename van de cellengte (figuur 1, hoofdstuk 6).

Daarnaast zijn er aanwijzingen gevonden dat zowel auxine en brassinosteroïden een rol spelen. Een mogelijk mechanisme met betrekking tot deze twee plantenhormonen is beschreven in figuur 2 van hoofdstuk 6 waarbij waarschijnlijk ook een rol voor ABA en ethyleen bestaat.

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Curriculum Vitae

Zohreh was born on august 1972 in Tehran, Iran. After finishing high school in natural science, she moved to Esfahan University in 1991 and continued her study in plant biology till 1995. She became a high school teacher in biology and had a great fun and experience for couple of years. In 1998 she continued her study for the master degree in plant physiology at Shiraz University. She worked in the Agricultural biotechnology research institute in Karaj-Tehran for her master project under supervision of Drs H. Mohabatkar and B. Gharehyazi. She conducted a good research on Iranian Salicornia species and determined the genetic variation between and within the species using molecular markers, her work was evaluated as an excellent project. She was awarded a scholarship from the Ministry of Science, Research and Technology of Iran and Shiraz University to study plant molecular biology. She joined the department of Biology in Utrecht University, the Netherlands to work on transcriptome and kinome analysis during ethylene-induced growth in *Rumex palustris*. The results of this research are presented in this thesis. After her doctoral defence she will be appointed as assistant professor in the Shiraz university of Iran.