

INVITED REVIEW

## New Perspectives in Flooding Research: the Use of Shade Avoidance and *Arabidopsis thaliana*

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Received: 25 October 2004 Returned for revision: 20 January 2005 Accepted: 25 February 2005 Published electronically: 18 July 2005

• **Background** Complete submergence of *Rumex palustris* leads to hyponastic (upward) petiole growth followed by enhanced petiole elongation. Previous pharmacological experiments have provided insights into the signal transduction pathway leading to this combined 'escape' response. It will, however, be difficult to gain further knowledge using these methods. Consequently, new approaches are required.

• **Scope** Here we propose that different environmental signals resulting in similar phenotypes can help to understand better the submergence response. In this review, we show that both ethylene and shade induce similar growth responses in *R. palustris* and *Arabidopsis thaliana*. We illustrate how this can be exploited to unravel novel signalling components in submergence-induced elongation growth. Furthermore, we illustrate the potential of *Arabidopsis* as a useful model in submergence research based on similarities with submergence-tolerant species such as *R. palustris* and the molecular opportunities it presents. This is illustrated by examples of current work exploring this concept.

• **Conclusions** Incorporating different model systems, such as *Arabidopsis* and shade avoidance, into submergence research can be expected to create powerful tools to unravel signal transduction routes determining submergence tolerance.

**Key words:** *Rumex palustris*, *Arabidopsis thaliana*, submergence, shade avoidance, ethylene, microarray, light, QTL, growth, elongation, differential growth, hyponasty.

### INTRODUCTION

Transient flooding with fresh water is a world-wide phenomenon in river floodplains and wetlands as well as other terrestrial ecosystems. It frequently results in complete submergence of plants. In general, this excess of water has a negative impact on growth and survival of most terrestrial plants, especially when flooding occurs during the growing season (Blom and Voeselek, 1996; Colmer *et al.*, 1998). Well-adapted plants may overcome the effects of submergence by adopting an avoidance strategy in which individual leaves grow differentially to a more vertical position followed by enhanced elongation. Analysis of these processes at the molecular level will generate new insights into the complexity of plant–environment interactions and potentially help to improve submergence tolerance of agricultural crops. This review aims to highlight novel avenues of research to unravel molecular networks that are important for plant–environment interactions. We will show that, although rarely used in submergence research, experiments with *Arabidopsis thaliana* can be very useful. Furthermore, other signals such as shade, which are not related to submergence but that can induce phenotypically comparable responses, can help to identify signal transduction steps that determine plant responses to submergence.

### SUBMERGENCE: EFFECTS AND RESPONSES

Flooding of agricultural and natural ecosystems has a dramatic impact on the growth and occurrence of terrestrial plants in these systems (Blom and Voeselek, 1996; Lenssen *et al.*, 2004; Van Eck *et al.*, 2004; Voeselek *et al.*, 2004). The negative effects of flooding are strongly related to severely hampered diffusion of gases in water, which is 10 000 times slower than in air (Armstrong, 1975). As a result, the internal gas composition in plants changes, leading to oxygen and carbon dioxide shortage and thus energy deficits. Many plants, therefore, do not survive prolonged submergence (Vervuren *et al.*, 2003; Van Eck *et al.*, 2004). Consequently, variation in flooding tolerance among plant species strongly affects species composition and abundance in flood-prone areas (Lenssen *et al.*, 2004; Van Eck *et al.*, 2004). A further consequence of the selective death of plant species upon flooding stress is a marked reduction of the density of plants in flood-exposed vegetations, resulting in a reduced intensity of plant competition (Lenssen *et al.*, 2004). This is in contrast to the rarely flooded, highly productive grasslands at higher floodplain elevations where plant densities are high and competition is fierce. Consequently, a trade-off has been predicted between competitive ability and flooding tolerance (Lenssen *et al.*, 2004).

One way to ameliorate the effects of complete submergence on plant growth is an avoidance strategy in which plants grow out of the water and restore contact with the

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atmosphere. Rice is a crop species showing this adaptive response (Kende *et al.*, 1998; Almeida *et al.*, 2003), but species from more natural environments, including the wetland species *Rumex palustris*, also show this elongation response (Voeselek *et al.*, 2004). The gaseous plant hormone ethylene accumulates in submerged plants and is the primary signal inducing shoot elongation (Musgrave *et al.*, 1972; Kende *et al.*, 1998; Voeselek *et al.*, 2003a). Rather than inhibiting growth, which is the more commonly observed effect of high concentrations of ethylene (Abeles *et al.*, 1992), elevated ethylene levels stimulate shoot elongation in these wetland plants (Voeselek *et al.*, 2004).

Of course, this increased shoot elongation will position the leaf tips closer to and eventually above the water surface only when the orientation of the leaves is relatively close to vertical. This prerequisite is easily achieved in grass species such as rice that, by default, already possess an erect shoot morphology. In contrast, rosette species such as *R. palustris* have an initially horizontal orientation of the leaves, and faster elongation of leaves in this position would not result in renewed contact with the water surface. To avoid futile horizontal elongation, submergence-induced petiole extension in *R. palustris* is preceded by differential growth of the petiole at the abaxial side (hyponastic growth) leading to a more vertical position of these leaves (Cox *et al.*, 2003). Only when this more upright petiole orientation is reached does vigorous overall petiole elongation begin. Thus, approximately 6 h after submergence has started, hyponastic growth brings petiole inclination close to vertical (80°) (Cox *et al.*, 2003). In this position, petiole elongation rates achieve their maximum of more than three times that of non-submerged controls. Both this vertical leaf orientation and the high rate of petiole growth can be sustained for several days until the water surface is reached (Voeselek *et al.*, 2003b).

#### REGULATION OF SUBMERGENCE-INDUCED SHOOT ELONGATION

As stated above, submergence-avoidance growth responses in *R. palustris* are induced by ethylene accumulation in the shoot (Voeselek *et al.*, 1993, 2004; Peeters *et al.*, 2002), which may, in addition, be enhanced by reduced oxygen concentrations in the submerged shoot (Voeselek *et al.*, 1997). However, the hormonal regulation of this growth is more complex than this. Very quickly upon submergence of *R. palustris*, internal abscisic acid (ABA) levels drop to <20% of normal, and this change is ethylene mediated since pre-treatment with the ethylene perception inhibitor 1-methylcyclopropene (1-MCP) prevents this decline (Benschop, 2004). Furthermore, both the production of several gibberellins (GAs) and the tissue sensitivity to GA are increased upon submergence and ethylene treatment in *R. palustris* (Rijnders *et al.*, 1997; Benschop, 2004). This is significant since artificial reduction of GA biosynthesis with specific inhibitors such as paclobutrazol substantially represses the elongation response to submergence (Rijnders *et al.*, 1997). A similar mechanism regulates fast stem elongation in deepwater rice (Métraux and Kende, 1983),

where an ethylene-induced drop in ABA concentration seems to sensitize the tissue to GA (Hoffmann-Benning and Kende, 1992), positioning ABA as an intermediate between ethylene and GA (Kende *et al.*, 1998). Interestingly, the rapid decrease of ABA observed in *R. palustris* does not occur in the closely related, but submergence-intolerant species, *R. acetosa* that lacks the enhanced petiole elongation upon submergence. However, when internal ABA levels are artificially lowered by pre-treatment with fluridone, an inhibitor of ABA biosynthesis, *R. acetosa* does elongate upon submergence. This suggests that the downstream steps for enhanced shoot elongation are present in this species (Benschop, 2004). Although the involvement of auxin in submergence-induced petiole elongation in *R. palustris* has not yet been unambiguously determined (Rijnders *et al.*, 1996; Cox, 2004), it is likely to be a key player in differential petiole growth that reorients leaves by means of hyponasty. Removing the leaf lamina, a putative source of auxin in a plant, or using an auxin transport inhibitor delays this submergence-induced hyponastic readjustment of leaf angle in *R. palustris* for several hours (Cox *et al.*, 2004).

The submergence-induced signalling cascade is finally completed by increases in cell wall extensibility. This is believed to be the ultimate step controlling cell elongation (McQueen-Mason and Rochange, 1999). Proteins that loosen the cell wall include, among others, the so-called expansins (Cosgrove, 1998). To date 19  $\alpha$ -expansin genes have been isolated from *R. palustris* (Vriezen *et al.*, 2000; Colmer *et al.*, 2004). A fast upregulation of the  $\alpha$ -expansin gene *RpEXPA1* and increase of  $\alpha$ -expansin protein of the RpEXPA1 size class have been observed during submergence or ethylene exposure in petioles of *R. palustris* (Vriezen *et al.*, 2000; Vreeburg *et al.*, 2005). This response at the gene expression level was absent in the closely related species *R. acetosa* that fails to elongate more quickly when submerged (Vriezen *et al.*, 2000). A correlation between submergence-induced expansin gene expression and shoot elongation has also been shown in stems of deepwater rice (Lee and Kende, 2001).

#### RESPONSES TO SUBMERGENCE RESEMBLE RESPONSES TO SHADE

Phenotypically similar growth responses to those described for submergence can be observed when plants are shaded by proximate neighbours. When plants detect neighbours with canopy light signals, they display a suite of responses that are encompassed by the so-called shade avoidance syndrome. This includes enhanced shoot elongation and hyponastic growth (Ballaré and Scopel, 1997; Smith and Whitelam, 1997; Pierik *et al.*, 2003). Rather than bringing the leaf tips closer to the water surface, in dense canopies these responses bring the leaves closer to the light, thereby enhancing plant performance (Pierik *et al.*, 2003) and fitness (Schmitt *et al.*, 1995). The similarity between submergence- and shade-induced petiole elongation is illustrated in Fig. 1. *Rumex palustris* shows strongly stimulated petiole elongation when put in reduced light intensity with marked changes in the spectral composition (spectral shade). This

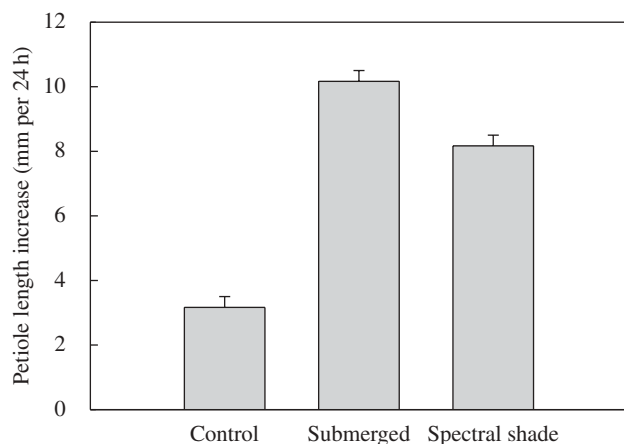


FIG. 1. Petiole elongation in 27-d-old *Rumex palustris* plants that were exposed to control growth conditions (Cox *et al.*, 2003), submerged or spectrally shaded [white light filtered through one layer of Fern Green filter (Lee Filters no. 122, Andover, UK), PAR =  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ , R : FR (655–665 nm : 725–735 nm) = 0.2]. Plants were treated for 24 h and petiole elongation was measured as the increase in length between the start and end of the experiment of the third emerged petiole. Data are means ( $n = 3$ )  $\pm$  s.e.

resembles a closed canopy light climate, and the elongation response is of similar magnitude to the elongation response to submergence (Fig. 1). These observations predict *R. palustris* to be responsive to proximate neighbours in a way that would facilitate its above-ground competitive ability. This suggests that, despite the suggested trade-off between submergence tolerance and competitive ability (Lenssen *et al.*, 2004), *R. palustris* combines both characteristics. This is strengthened further by the observation that spectral shade also induces a hyponastic growth response in *R. palustris*, as does submergence or ethylene (Fig. 2; Table 1). Canopy shade comprises a specific reduction in the photon fluence rates of red (R) and blue light, due to specific absorption by chlorophyll. As far-red (FR) light is not absorbed by chlorophyll, the canopy light climate is characterized by a low R:FR ratio and a reduced total light intensity. Of these two components, a low R:FR ratio induces a phenotype closely similar to that given by spectral shade treatment. In contrast, spectrally neutral shade, i.e. lower intensities with no change in the R:FR ratio, induce only a very weak hyponastic response in *R. palustris* (Fig. 2; Table 1). In this respect, *R. palustris* differs from tobacco and arabidopsis, which do show pronounced hyponasty in response to spectrally neutral shade (Pierik *et al.*, 2004b; Millenaar *et al.*, 2005).

The similarity between submergence- and shade-induced growth responses, as well as the generally low light intensities during natural flooding events in river floodplains (Vervuren *et al.*, 2003), might suggest responses to shade to be part of plant responses to submergence. However, recent experiments by Mommer *et al.* (2005) have already shown for photosynthetic acclimation responses that this is not the case. Furthermore, submergence-induced shoot elongation can take place when light intensities under water are relatively high (e.g.  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  in Figs 1 and 2). Finally, R:FR ratios are increased under water,

whereas a decrease is required to induce shade avoidance responses.

Interestingly, shade avoidance responses seem to involve hormonal interactions that bear a strong resemblance to those described for submergence avoidance. Upon detection of a reduced R : FR light ratio by the phytochrome family of photoreceptors (Smith, 2000), plant shoots are sensitized to GA (Reid *et al.*, 1990; Weller *et al.*, 1994; López-Juez *et al.*, 1995) or produce more GA (Beall *et al.*, 1996). This effect is comparable with the submergence responses of *R. palustris* and rice. Furthermore, ethylene production is controlled by the R : FR ratio (Finlayson *et al.*, 1999; Pierik *et al.*, 2004a) and was recently shown to be critical for hyponastic and stem elongation responses to neighbours (Pierik *et al.*, 2003) and shade (Pierik *et al.*, 2004b). Also, interactions between ethylene and GAs may occur during shade avoidance (Pierik *et al.*, 2004a). However, it remains uncertain if ABA is also involved in low R:FR-induced shoot elongation and/or hyponastic growth in the manner discovered in submerged plants. Furthermore, the molecular basis of these hormone interactions is as yet unknown. Recent work on GA signalling in *A. thaliana* has identified a potential point of integration of different hormone signalling pathways. This involves the so-called DELLA proteins, which inhibit growth and are targeted for breakdown by GA signalling (Harberd, 2003). Interestingly, the stability of these proteins can also be modified by ethylene and auxin (Achard *et al.*, 2003).

As for submergence studies, data for auxin involvement in shade avoidance are scarce. Nevertheless, regulated auxin transport has been suggested to be required for phytochrome-mediated hypocotyl elongation in arabidopsis (Morelli and Ruberti, 2000), and blue light-mediated phototropism (Friml *et al.*, 2002). Furthermore, the auxin-resistant *axr1-12 Arabidopsis* mutant has a severely attenuated hypocotyl elongation response to a low R:FR ratio (Steindler *et al.*, 1999). These data together suggest that auxin may be involved in shade avoidance responses, comparable with the submergence avoidance response.

#### EXPLOITING SHADE AVOIDANCE STUDIES TO IDENTIFY COMPONENTS OF SUBMERGENCE-INDUCED SIGNAL TRANSDUCTION

Because of close phenotypical (Figs 1, 2 and Table 1) and physiological similarities between submergence- and shade-induced hyponastic and elongation responses, shade avoidance can be used as a tool to gain further insight into the regulation of submergence- and ethylene-induced elongation responses. This approach exploits the similarities in various differential screens such as cDNA subtraction and cDNA-AFLP analyses applied to shaded vs. submerged/ethylene-treated plants. This idea is based on the hypothesis that downstream parts of shade- and submergence-induced signalling cascades demonstrate a high degree of similarity. It can, therefore, be expected that when comparing pools of mRNA from submergence- and shade-treated *R. palustris* plants, similar downstream messengers will show up in both treatments. This is because this latter part of the cascade is



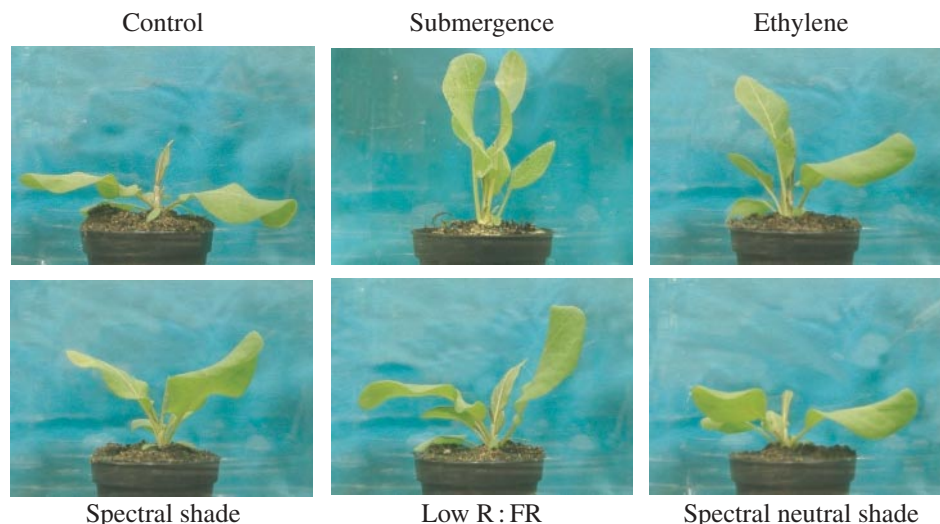


FIG. 2. *Rumex palustris* plants exposed to: air (control), submergence,  $3 \mu\text{L L}^{-1}$  ethylene (flow through at  $0.4 \text{ L min}^{-1}$ ), spectral shade, low red : far-red (R : FR) light and spectral neutral shade for 24 h. Growth conditions are described in Cox *et al.* (2003) and plants were 27 d old. All treatments, except neutral shade, resulted in rapid hyponastic growth of the petioles. See Table 1 for numerical details. Light conditions: control and ethylene treatment [white light: PAR =  $140 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , R : FR (655–665 nm : 725–735 nm) = 1.0], submergence treatment [PAR =  $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , R : FR = 1.4], spectral shade [white light filtered through one layer of Fern Green filter (Lee Filters no. 122), PAR =  $70 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , R : FR = 0.2], low R : FR [white light supplemented with far-red light (FR-emitting LEDs), PAR =  $140 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , R : FR = 0.2] and spectral neutral shade (white light filtered through neutral shade cloth, PAR =  $20 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , R : FR = 1.0).

TABLE 1. *Rumex palustris* petiole angles (third emerged petiole of 27-d-old plants) after 24 h of a given treatment and corrected for the control angle at the same time

Treatment	Differential angle	P
Submerged	45.5	<0.001
Ethylene	43.9	<0.001
Spectral shade	25.2	<0.001
Spectral neutral shade	7.0	<0.05
Low R : FR	27.6	<0.01

For treatment details, see Fig. 2. *P*-values indicate the significance of the difference between treatment and control values.

likely to be shared. If this notion is valid, a cDNA-AFLP analysis should allow selection of downstream genes to be identified that are regulated in the same way in shade- and submergence-treated plants. Studying these shared genes would then significantly reduce the chance of investigating genes that are pleiotropically induced by the treatment or are related to upstream steps in the upper unshared sections of the signal transduction pathway or are unrelated to the response studied.

Upstream components of either shade- or submergence-induced signalling cascades are thus expected to be differentially expressed to a much higher or lower extent in one or other of the two treatments. These components are specifically selected for in a cDNA subtraction analysis where all mRNAs that are expressed to a similar extent in the two treatments are removed. Such carefully designed differential screens may allow identification of upstream signalling intermediates that are involved in submergence-induced shoot elongation in *R. palustris*. Expression of those upstream genes could then be compared in *R. palustris*

and submergence-intolerant *R. acetosa*. This may ultimately provide the molecular explanation for the presence and absence of submergence-induced elongation in *R. palustris* and *R. acetosa*, respectively.

Of course, molecular analyses would be even more promising and effectively explored in species for which extensive genomic information is already available, such as rice and arabidopsis. However, little is known about potential shade avoidance in rice. Furthermore, only part of the submergence avoidance responses can be investigated since hyponastic growth is an irrelevant parameter in this species due to its inherently high leaf angles. Arabidopsis is therefore the model system of choice to investigate submergence-, ethylene- and shade-induced responses.

#### ARABIDOPSIS AS A SUITABLE MODEL SPECIES TO STUDY SUBMERGENCE AND SHADE AVOIDANCE

At first sight, *A. thaliana* would not be the ideal model species to study submergence-related responses since it is not particularly flooding tolerant (Pigliucci and Kolodynska, 2002). Yet, arabidopsis plants still show 50 % survival after approximately 1 week of complete submergence at  $20^\circ\text{C}$  in the dark. Moreover, strong natural variation can be observed between different natural accessions (D. Tholen and L. A. C. J. Voesenek, unpubl. res.). Furthermore, flooding survival has been reported to be increased in plants carrying a gene containing the senescence-specific SAG12 promoter fused to an *ipt* gene coding for isopentenyl transferase, a rate-limiting enzyme in cytokinin biosynthesis. After 2 weeks of submergence in the light, only 6 % of the wild-type plants survived, while up to 77 % of the transgenic plants survived (Zhang *et al.*, 2000).

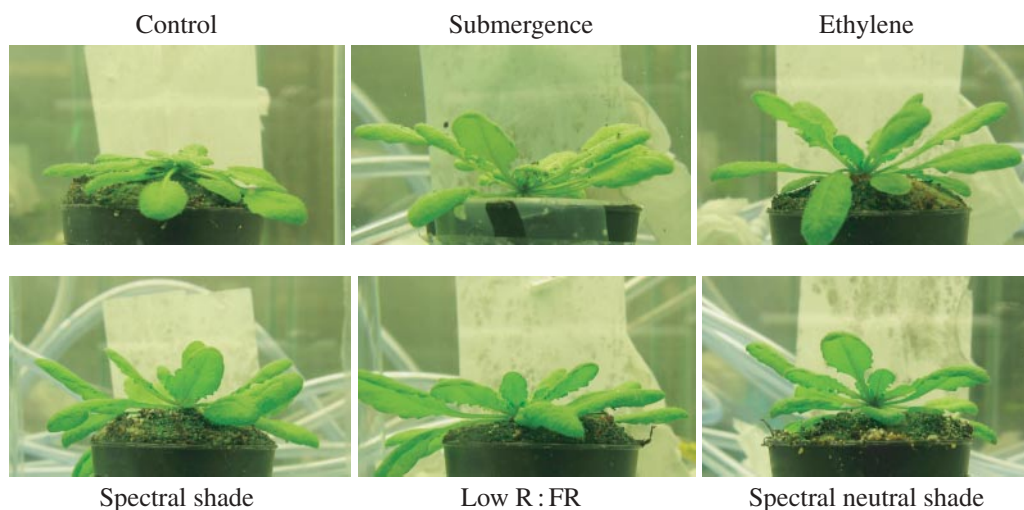


FIG. 3. *Arabidopsis thaliana* Columbia plants exposed to: air (control), submergence,  $3 \mu\text{L L}^{-1}$  ethylene (flow through at  $0.4 \text{L min}^{-1}$ ), spectral shade, low red: far-red (R:FR) light and spectral neutral shade for 6 h. Growth conditions are described in Millenaar *et al.* (2005). All treatments resulted in rapid hyponastic growth of the petioles. See Table 2 for numerical details. Light conditions: control, ethylene [white light: PAR =  $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ , R:FR (655–665 nm: 725–735 nm) = 1.0], submergence treatment [PAR =  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ , R:FR = 1.4], spectral shade [white light filtered through one layer of Fern Green filter (Lee Filters no. 122), PAR =  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ , R:FR = 0.2], low R:FR [white light supplemented with far-red light (FR-emitting LEDs), PAR =  $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ , R:FR = 0.2] and spectral neutral shade [white light filtered through neutral shade cloth, PAR =  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ , R:FR = 1.0].

One of the first responses to submergence of the submergence-tolerant species *R. palustris* is upward movement of the leaves. This hyponastic growth starts within 1.5 h and is saturated in approx. 6 h (Cox *et al.*, 2003). Both submergence and ethylene are also able to induce hyponastic growth in the arabidopsis accession Columbia (Fig. 3). In both *R. palustris* and arabidopsis, hyponastic growth takes place over a similar time interval (Cox *et al.*, 2004; Millenaar *et al.*, 2005). Therefore, we hypothesize that submergence- and ethylene-induced hyponastic growth can also be studied in arabidopsis. Exploiting this could speed up research, because of the extensive molecular tools available for arabidopsis research. One such tool is the arabidopsis ‘full’ genome microarray. This makes it relatively easy to profile virtually all transcripts in a plant. To identify new genes involved in ethylene-induced hyponastic growth, microarrays can readily analyse mRNA isolated from ethylene-treated plants compared with control.

Another advantage of arabidopsis is the large natural variation in this species, as found for many properties including survival after soil flooding (waterlogging) (Pigliucci and Kolodynska, 2002; Kolodynska and Pigliucci, 2003) and for hyponastic responses to ethylene (Millenaar *et al.*, 2005). The natural variation within a species can be explored, with the help of quantitative trait loci (QTLs). QTL analysis facilitates identification of genomic loci involved (either positively or negatively) in a certain trait. The basis of this technique is the presence of natural genetic variation for a given trait among accessions (natural variants) and within populations derived from crosses between accessions. The recombination present in the  $F_2$  progeny of a cross between two accessions is sufficient to identify QTLs. Usually more advanced populations, such as recombinant inbred lines (RILs; single seed descent for >8 generations of individual  $F_2$  lines), are used because they

TABLE 2. *Arabidopsis thaliana* ‘Columbia’ petiole angles after 6 h of a given treatment and corrected for the control angle at the same time

Treatment	Differential angle	P
Submerged	17.3	<0.001
Ethylene	16.0	<0.001
Spectral shade	13.8	<0.01
Spectral neutral shade	22.0	<0.001
Low R:FR	14.1	<0.001

For treatment details see Fig. 3. P-values indicate the significance of the difference between treatment and control values.

have the advantage of being homozygous and immortal. In arabidopsis, there are several populations of homozygous RILs available (Lister and Dean, 1993; Alonso-Blanco *et al.*, 1998; El-Lithy *et al.*, 2004). Therefore, it should be straightforward to determine loci on chromosomes that are essential for traits such as ethylene-induced hyponastic growth. Furthermore, by combining a QTL analysis with microarray data from individual RILs, it is possible to construct signal transduction pathways; a technique called genetical genomics (Jansen and Nap, 2001; Jansen 2003).

Not only does arabidopsis demonstrate hyponastic growth upon submergence and elevated ethylene levels, but it also displays this response to low light, to a reduction in the R:FR ratio or to a combination of low light intensity and reduced R:FR ratio (spectral shade) (Fig. 3; Table 2). These results are comparable with phytochrome-mediated hyponasty in *R. palustris* (Fig. 2). As already explained, it can be useful to use different signals which each result in the same phenotype as tools to unravel the signal transduction of hyponastic growth. The following two examples





## ACKNOWLEDGEMENTS

We thank R. A. M. Welschen and T. L. Pons for their help in building the spectral shade set-up, and L. B. Snoek for his unpublished QTL data. This work was supported by a PIONIER grant (800-84-470) of the Dutch Science Foundation (NWO) to L.A.C.J.V. and by a NIELS STENSEN grant to F.F.M.

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