Hormone- and Light-Mediated Regulation of Heat-Induced Differential Petiole Growth in Arabidopsis

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Plants react quickly and profoundly to changes in their environment. A sudden increase in temperature, for example, induces differential petiole growth-driven upward leaf movement (hyponastic growth) in Arabidopsis (Arabidopsis thaliana). We show that accessions that face the strongest fluctuations in diurnal temperature in their natural habitat are least sensitive for heat-induced hyponastic growth. This indicates that heat-induced hyponastic growth is a trait subject to natural selection. The response is induced with kinetics remarkably similar to ethylene- and low light-induced hyponasty in several accessions. Using pharmacological assays, transcript analysis, and mutant analyses, we demonstrate that ethylene and the photoreceptor protein phytochrome B are negative regulators of heat-induced hyponastic growth and that low light, phytochrome A, auxin, polar auxin transport, and abscisic acid are positive regulators of heat-induced hyponastic growth. Furthermore, auxin, auxin polar transport, phytochrome A, phytochrome B, and cryptochromes are required for a fast induction of heat-induced hyponastic growth.

Temperature is an important environmental factor that varies over seasons but also profoundly during the day. Supraoptimal temperatures are among the most damaging abiotic factors in crop plants (Mittler, 2006; Barnabas et al., 2008), and minor changes in temperature can already have dramatic effects on plants, for example, on fine-tuning of several key processes in plant development such as germination (Toh et al., 2008), floral transition (Halliday et al., 2003; Balasubramanian et al., 2006), and circadian clock entrainment and compensation (Samach and Wigge, 2005; Gould et al., 2006; Penfield, 2008).

A study on the natural variation of leaf angles in Arabidopsis (Arabidopsis thaliana) accessions originating from different geographic origins revealed that leaves of accessions found at lower latitudes are more erect than those from northern accessions (Hopkins et al., 2008). A recent report by Koini and colleagues (2009) showed that a sudden increase in temperature induces an increase in leaf inclination (hyponastic growth), which is controlled by PHOT1 (PIF4).

We present evidence that ethylene and phytochrome B act as negative regulators and that low light, phytochrome A, auxin, polar auxin transport, and abscisic acid (ABA) are positive regulators of heat-induced hyponastic growth. Furthermore, auxin, auxin polar transport, phytochrome A, phytochrome B, and cryptochromes are required for a fast induction of heat-induced hyponastic growth.

Hyponastic growth is also associated with shade and submergence avoidance and brings leaves toward light and air, respectively (Ballare, 1999; Cox et al., 2003; Pierik et al., 2004; Millenaar et al., 2005, 2009). Accumulation of the gaseous hormone ethylene is the key trigger for the induction of hyponastic growth in submerged plants, including Arabidopsis (Millenaar et al., 2005). Interestingly, reduced light intensity (low light) triggers a hyponastic growth response with similar kinetics as ethylene (Millenaar et al., 2005). Both genetic and pharmacological data indicated that the auxin and polar auxin transport are involved in low light-induced, but not in ethylene-induced, hyponastic growth, whereas the response to low light is ethylene independent (Millenaar et al., 2009). Based on these data, we hypothesized that ethylene and low light induce hyponastic growth via largely independent routes but probably share functional components downstream, which explains the phenotypical resemblance.

In this report, we demonstrate that a rapid temperature shift (from 20°C to 38°C) induces hyponastic growth with highly similar kinetics to ethylene- and low light-induced hyponasty. Leaf angles of naturally occurring accessions that face the strongest fluctuations in diurnal temperature in their natural habitat are least sensitive to heat, suggesting that this trait is subject to natural selection. The regulation of this response was studied by a combination of pharmacological experiments, gene expression studies, and mutant analyses. We present evidence that ethylene and phytochrome B are negative regulators and that low light, phytochrome A, auxin, and abscisic acid (ABA) are positive regulators of heat-induced hyponastic growth.
RESULTS

Heat-Induced Leaf Inclination in Arabidopsis

To characterize the response of Arabidopsis petioles to elevated temperatures in detail, petiole angles were measured after 7 h of exposure to different temperatures, using the standard accessions Columbia-0 (Col-0), Landsberg erecta (Ler), and Wassilewskija-2 (Ws-2). A positive correlation for all accessions was found in the temperature range from approximately 16°C to approximately 38°C, demonstrating that Arabidopsis petioles actively react with a differential growth-driven upward leaf movement (heat-induced hyponastic growth) to changes in environmental temperature (Fig. 1A). Below 16°C, none of the tested accessions changed petiole angles, and above approximately 30°C, the response of Col-0 and Ler reached a plateau whereas Ws-2 leaf angles continued to increase.

From this experiment, it is not conclusive if petiole angles adjust to the absolute temperature, to the relative change in temperature, or to both. Therefore, petiole angles of Col-0 plants that were pregrown for 3 weeks at 10°C were measured. In agreement with the results of Hopkins et al. (2008), this cold (vernalization) pretreatment induced a significant increase ($P < 0.001$) in initial petiole angle ($35.9° ± 0.6°$) relative to plants grown at 20°C ($25.8° ± 0.4°$). The dose-response curve was similar regardless of the pregrowth temperature, but cold-pregrown plants lost the ability of heat-induced hyponasty at temperatures exceeding approximately 32°C (Fig. 1A). Moreover, as cold-pregrown plants showed higher initial angles, the absolute angles of cold-pregrown plants were significantly higher ($P < 0.001$) than in Col-0 grown at 20°C (Fig. 1B). These results seem to suggest that the degree of heat-induced hyponastic growth is determined by absolute temperature. Regarding the responses of all tested accessions, a shift from 20°C to 38°C induced a strong hyponastic growth response, and this is used throughout the experiments.

A time-lapse camera setup (Cox et al., 2003; Millenaar et al., 2005) was employed to monitor the kinetics of heat-induced hyponastic growth. In Col-0, the response starts within 1 h, the angle change per unit of time is maximal after approximately 3 h, and the maximal angle is reached around 7 h after start of the treatment (Fig. 1C). Heat-induced hyponastic growth is irreversible once induced, since placing the plants back to the control temperature after 3 or 6 h did not alter the maximum response angle (amplitude) at 7 h. However, plants placed back at the control temperature were less able to maintain high leaf angles after the response maximum throughout the experimental period, which resulted in a faster decline of leaf angles after reaching the maximum response.

Figure 1. Characterization of the hyponastic growth response to different temperatures. A, Dose-response relation of temperature and petiole angles after 7 h of treatment with different temperatures relative to the initial angle. Symbols are as follows: 20°C-grown (solid lines) Ws-2 (circles), Col-0 (diamonds), and Ler (triangles) and 10°C-pregrown Col-0 (squares, dashed line). Angles were pairwise subtracted, which gives the difference between the angles of treated and control plants at each time point (Benschop et al., 2007). B, Absolute (initial) petiole angles of Col-0 pregrown at 20°C (gray bars) or at 10°C (black bars) after 7 h of treatment at the indicated temperatures. C, Col-0 petiole angle kinetics upon exposure to heat (38°C) for 24 h (triangles) and of reversal from 38°C to 20°C after 3 h (circles) and 6 h (squares). Depicted angles are pairwise subtracted. Error bars represent SE; $n > 10$. *** $P < 0.001$. 
The Amplitude of Heat-Induced Hypnastic Growth Correlates with the Natural Diurnal Temperature Range of Arabidopsis Accessions

To compare heat-induced hypnastic growth with ethylene- and low light-induced hypnastic growth (Millenaar et al., 2005, 2009), we exposed five frequently used accessions to these three signals (Fig. 2). Strong similarities in responses were found in Col-0, Ler, and C24 (Fig. 2, A–C). Elevated temperature induced a much stronger response than exposure to ethylene or low light in Ws-2 (Fig. 2D). Finally, Cape Verde Islands-0 (Cvi-0) lacked a marked response to heat, ethylene, and low light relative to the air-control treatment (Fig. 2E).

To directly compare heat-induced hypnastic growth among the five accessions, a pairwise subtraction was performed to correct for diurnal and/or circadian petiole movements (Benschop et al., 2007; Fig. 2F). Cvi-0, which originates from near the equator, is a weak responder, whereas C24, originating from the Portuguese accession Coimbra (Schmid et al., 2006),
has an intermediate response. On the contrary, central European accessions (Ler, Col-0, andWs-2) showed strong responses. These data suggest a correlation between geographic origin and amplitude of heat-induced hyponastic growth. To test this, we measured the absolute leaf angle increase to 7 h of heat treatment in 21 selected accessions, together roughly covering the biogeographical latitudinal distribution range of Arabidopsis (Fig. 2G; Supplemental Table S1). Considerable variation was observed in the amplitude of the response after 7 h of treatment (less than 5° for Pak-1 to approximately 30° in Be-0). No significant correlations between the amplitude of heat-induced hyponastic growth with latitude, longitude, or altitude were found (Supplemental Fig. S1), although average temperature itself was strongly negatively correlated to latitude ($r^2 = -0.76$).

Subsequently, we analyzed if local environmental conditions at the collection sites correlated with the amplitude of heat-induced hyponastic growth (Supplemental Table S2). For this aim, mean annual climate data acquired over a 30-year period were examined (New et al., 1999; Supplemental Table S2). A small but significant negative correlation ($P = 0.032, r^2 = -0.22$) was observed between maximum response angles at 7 h after induction and diurnal temperature range (i.e., the differences between maximum and minimum temperature during the day; Fig. 2H). This might indicate that plants from a location where daily temperatures are highly fluctuating respond less to heat than plants from relatively temperature stable environments.

Ethylene Is a Negative Regulator of Heat-Induced Hyponastic Growth

Because ethylene-induced hyponastic growth phenocopies heat-induced hyponastic growth (Fig. 2, A–C), we hypothesized that ethylene may be a downstream component of heat-induced hyponasty. To test this, we first measured ethylene release upon heat treatment in Col-0. In the first hours after treatment, ethylene release tended to decrease relative to the ethylene release during control temperatures, and this became significant ($P < 0.05$) 3 to 6 h after the start of the heat treatment (Fig. 3A).

Transcription of the ethylene biosynthesis gene 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE4 (ACO4) and the ethylene receptor ETHYLENE RESPONSE SENSOR2 (ERS2) increases in the presence of ethylene (Wilkinson et al., 1995; Hua and Meyerowitz, 1998; Vriezen et al., 1999). We tested the expression of these ethylene marker genes in plants subjected to heat. Quantitative reverse transcription (RT)-PCR analysis (Fig. 3, B and C) did not reveal increases in transcript levels of these ethylene marker genes, whereas the heat-inducible positive control marker (Busch et al., 2005) HEAT SHOCK TRANSCRIPTION FACTOR A2 (HSFA2) was strongly up-regulated (Fig. 3D).

As a third approach to test ethylene involvement in heat-induced hyponasty, ethylene-related mutants were assayed. The ethylene-insensitive lines ethylene insensitive4 (ein4) and ethylene response1 (etr1) showed enhanced heat-induced hyponastic growth (Fig. 3, E and F). Enhancement was not observed in ein2 (Fig. 3G). A mutant with constitutive ethylene response, constitutive triple response1 (ctr1), the triple loss-of-function mutant with a strong constitutive ethylene response phenotype, etr1 ein4 etr2, and ethylene over-producer1 (eto1; 2.6× more ethylene release than in the wild type) all had marked decreases in amplitude of the response to heat treatment (Fig. 3, H–J). Consistent with a role for ethylene as a negative modulator in heat-induced hyponastic growth, pretreatment with the ethylene receptor antagonist 1-methylcyclopropene (1-MCP) resulted in an enhanced hyponastic growth response to heat (Fig. 3K). Finally, application of ethylene and heat simultaneously resulted in a reduced hyponastic growth response compared with heat treatment alone. Particularly, these plants were less able to maintain high leaf angles after the response maximum (Fig. 3K).

Overall, these data consistently indicate that ethylene is a negative regulator of heat-induced hyponastic growth.

Low Light Enhances Heat-Induced Hyponastic Growth

Plant responses to high temperature and low red to far-red (R:FR) ratio, mimicking natural canopy signals, are highly similar (Gray et al., 1998; Halliday et al., 2003; Halliday and Whitelam, 2003; Penfield, 2008). This is perhaps best supported by Koini et al. (2009), who showed that responses to high temperature are mediated by PIF4. Complementing low R:FR/spectral shade, we tested here for possible interactions between spectral neutral low light and heat signals in hyponastic growth. We applied low light and heat simultaneously to Col-0 and Ler. In both accessions, the combined treatment (heat + low light) resulted in a strong enhancement of hyponastic growth amplitude as compared with plants subjected to either treatment alone (Fig. 4, A and B).

The roles of various photoreceptors in heat-induced hyponastic growth were studied with photoreceptor mutants in the Ler genetic background. The phytochrome A (phyA) mutant showed delayed induction of the response but exhibited a wild-type amplitude after 6 h (Fig. 4C). Interestingly, the response of phyB was delayed, but we observed enhanced petiole angles compared with the wild type for the remainder of the experimental period (up to 24 h; Fig. 4D). The phyA phyB double mutant was initially delayed but was indistinguishable from the wild type after 24 h (Fig. 4E). Thus, phyA appears to antagonize the enhanced response of phyB in heat-induced hyponastic growth. To further test if PhyB is a negative regulator of heat-induced hyponastic growth, we assayed phyB9 in Col-0. Interestingly, this mutant had an enhanced response to
heat throughout the 24-h experimental period (Fig. 4F). The response of the leaky chromophore-deficient mutant long hypocotyl2-genome uncoupled3, which has reduced levels of all five phytochromes (phyA to phyE), was similar to that of the phyA phyB double mutant (Fig. 4G). This suggests that phyC and phyD do not play prominent roles in the response, although their involvement cannot be completely ruled out. Heat-induced hyponastic growth was slightly delayed in the cryptochrome1 cryptochrome2 (cry1 cry2) double mutant (Fig. 4H) compared with the Ler wild type, suggesting that these proteins are required for a fast induction of heat-induced hyponastic growth.

In summary, our results demonstrate that PhyA, PhyB, and Cry photoreceptor proteins are required for proper induction of heat-induced hyponastic growth in Ler. PhyB act as a negative regulator of the response amplitude in both the Col-0 and Ler backgrounds, and loss-of function phyA rescues this effect, at least in Ler. Notably, the heat-induced hyponastic growth response of phyB in both Col-0 and Ler mimicked the response of the combination treatment of heat and low light in the respective wild types.

The contrasting effects of ethylene and low light on heat-induced hyponastic growth allowed studying prioritization of the signals. The enhanced hyponastic growth response as the result of simultaneous application of heat and low light could be repressed by additional ethylene in Col-0 and Ler (Fig. 5, A and B). In agreement, the combination of heat plus ethylene repressed the enhanced amplitude of heat-treated phyB in both Col-0 and Ler to a level similar to a single ethylene treatment (Fig. 5, C and D).

In summary, our results demonstrate that PhyA, PhyB, and Cry photoreceptor proteins are required for proper induction of heat-induced hyponastic growth in Ler. PhyB act as a negative regulator of the response
tutive ethylene signaling mutant ctr1 (Fig. 5G) lacked any additive effect of simultaneous application of low light and heat. Together, these data suggest that ethylene is a dominant negative signal with respect to heat-induced hyponastic growth.

Auxin Signaling and Polar Auxin Transport Are Required for Heat-Induced Hyponastic Growth

Auxin and polar auxin transport (PAT) are required for low light-induced hyponastic growth in Arabidopsis (Vandenbussche et al., 2003; Millenaar et al., 2009), and auxin plays a role in submergence-induced hyponasty in Rumex palustris (Cox et al., 2004). Moreover, auxin has been associated with responses to high temperature (Gray et al., 1998; Koini et al., 2009).

To test the involvement of auxin in heat-induced hyponasty, we pharmacologically inhibited the auxin efflux carriers with 2,3,5-triiodobenzoic acid (TIBA) and naphthylphthalamic acid (NPA). These treatments reduced and abolished heat-induced hyponasty, respectively (Fig. 6A). This indicates that auxin and PAT are required for heat-induced hyponastic growth. We examined the expression of the auxin activity marker IAA19/MSG2 (Tatematsu et al., 2004) by quantitative RT-PCR. IAA19/MSG2 mRNA levels did not change dramatically (less than two times for each time point), although expression was enhanced 3 to 6 h after initiation of heat treatment compared with controls (Fig. 6B), suggesting that our heat treatment might enhance auxin activity.

Auxin is perceived by the F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1) and AUXIN SIGNALING F-BOX (AFB) proteins (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser 2005). The tir1 mutant showed a delayed response and a slightly reduced amplitude (Fig. 6C), whereas the quadruple auxin receptor mutant tir1 afb1 afb2 afb3 exhibited a similar delay but also a strongly reduced hyponastic growth response amplitude (Fig. 6D). This suggests that redundancy exists among the TIR/AFB proteins in heat signaling with respect to hyponastic growth. In agreement with the pharmacological data, auxin efflux carrier mutants pin-formed7 (pin7) and pin3 showed delayed and abolished responses, respectively (Fig. 6, E and F).

Together, these data suggest that auxin signaling and PAT are required for a fast induction and maximum amplitude of heat-induced hyponastic growth.

ABA Is a Positive Regulator of Heat-Induced Hyponastic Growth

ABA is a negative regulator of ethylene-induced hyponastic growth in R. palustris and Arabidopsis (Cox et al., 2004; Benschop et al., 2007). Application of exogenous ABA did not change the hyponastic growth response of the petiole to heat (Fig. 7A). Application of
the ABA biosynthesis inhibitor fluoridon repressed the amplitude of the response. This suggests that ABA is a positive regulator of heat-induced hyponastic growth and that endogenous ABA levels may saturate the response.

ABA-INSENSITIVE1 (ABI1) is negative regulator of ABA signaling (Leung et al., 1997; Hoth et al., 2002). We found that \( \text{ABI} \) expression was modestly enhanced (approximately two times at 3 and 6 h) under heat treatment (Fig. 7B), suggesting that heat may desensitize ABA signaling by enhanced ABI1-1 expression. In agreement, the constitutive ABA-hypersensitive \( \text{era1}-2 \) mutant showed enhanced heat-induced hyponastic growth response (Fig. 7C), and several ABA biosynthesis and ABA-insensitive mutants (Fig. 7, D and G–I) showed repressed and delayed heat-induced hyponasty. In contrast, \( \text{aba2} \) (Fig. 7E) exhibited a wild-type response, and the amplitude of \( \text{aba3} \) (Fig. 7F) was slightly enhanced. This apparent contradiction may be explained by the observation that specifically these two ABA-related mutants have increased levels of ethylene release (LeNoble et al., 2004), which may interfere with heat-induced hyponastic growth (Fig. 3). In accordance, we found a strongly significant \( (P < 0.001) \) increase in ethylene release in \( \text{aba2}-1 \) and \( \text{aba3}-1 \) (0.63 ± 0.7 and 0.62 ± 0.05 nL g\(^{-1}\) fresh mass [FM] h\(^{-1}\), respectively, compared with 0.36 ± 0.02 nL g\(^{-1}\) FM h\(^{-1}\) in wild-type Col-0) in the adult vegetative tissues that were used in this study, whereas all other tested ABA-related mutants did not have significant differences in ethylene release (data not shown). Overall, our results indicate that ABA and ABA signaling are positive regulators of heat-induced hyponastic growth.

### DISCUSSION

**Heat-Induced Hyponastic Growth as an Adaptive Response**

Petiole hyponasty is a resource-directed reorientation of plant organs to escape from diminished growth conditions. The response is associated with escape from complete submergence and reduced light intensities as well as canopy signals (Cox et al., 2003; Millenaar et al., 2005; Mullen et al., 2006). Here, we studied the kinetics of elevated temperature-induced hyponastic growth in Arabidopsis and showed that petiole angles reversibly adjust, in a dose-dependent manner, to changes in their growth temperature in a range of physiologically relevant temperatures. This is in agreement with Millenaar et al. (2005), who found a modest increase in leaf angle when plants were transferred from 20°C to 30°C, and with the observations of Koini et al. (2009). Notably, the strongest response was observed at approximately 38°C, after which the responses attenuated. This is in accordance with Millenaar et al. (2005), who found a modest increase in leaf angle when plants were transferred from 20°C to 30°C, and with the observations of Koini et al. (2009). Notably, the strongest response was observed at approximately 38°C, after which the responses attenuated. This is in accordance with results of McCabe and Leaver (2000), who showed that a temperature of 38°C is just sublethal for Arabidopsis plants in which no acquired thermotolerance was induced before.

Strong natural variation was observed for the amplitude of the heat-induced hyponastic growth response among natural accessions (Fig. 2). A latitudinalcline in initial leaf angles was described previously (Hopkins et al., 2008). Yet, the observed high-temperature response amplitude was not correlated with latitude. However, heat response amplitude negatively correlated to diurnal temperature range at the accession...
collection sites. Thus, accessions that on average face large daily temperature fluctuations in their natural habitat showed the weakest hyponastic growth response to heat. Tempering hyponastic growth may be adaptive to save resources in a strong fluctuating daily environment, while responding to temperature changes in an otherwise stable temperature environment may potentially bring fitness benefits. Leaf (re)positioning can be explained by functional arguments that steep leaf angles have efficient solar light capture in the morning and winter, whereas it may optimize photosynthesis by avoiding overirradiation, excess heat flux, and extensive water loss at midday and during summer (King, 1997; Falster and Westoby, 2003). In agreement, Fu and Ehleringer (1989) showed that heliotropic leaf movements in common bean (Phaseolus vulgaris) are controlled by air temperature and that leaves are positioned such that photosynthesis is close to optimal. Moreover, a positive correlation between leaf angles and air temperature has been observed in P. vulgaris and Phaseolus acutifolius (Yu and Berg, 1994). Analogously, Gray et al. (1998) suggested that in Arabidopsis, heat-induced growth is employed to reposition the photosynthesizing tissues away from heated soil and consequently allow better access to evaporative cooling by moving air. Thus, heat-induced hyponastic growth in Arabidopsis may also be employed for optimization of photosynthesis.

Light Signaling Interacts with Heat in Hyponastic Growth

Light and temperature signaling are tightly connected (Mazzella et al., 2000; Blázquez et al., 2003; Penfield, 2008; Koini et al., 2009). For example, early flowering of phyB mutants is repressed at low growth temperatures (16°C). In contrast, PhyA, PhyD, and PhyE, acquire a more prominent functional role at 16°C (Halliday et al., 2003; Halliday and Whitelam, 2003). This cross talk is response specific, as the typical, elongated phyB phenotype is temperature independent (Halliday et al., 2003). Moreover, phototropic curvature of Arabidopsis seedlings was delayed by increased temperatures (Orbović and Poff, 2007). Accordingly, our study established extensive cross talk between spectral neutral low light and heat signaling with respect to hyponastic growth. Low light intensity enhanced the amplitude of heat-induced hyponastic growth (Fig. 4), and this response was mimicked in phyB mutants. This is in agreement with the work of Larkindale and Knight (2002), who showed that heat-induced oxidative damage in Arabidopsis was prevented in the dark. This indicates that oxidative damage to membranes resulted from photoinhibition of the electron transport chain. Strikingly, a similar inhibition of the photosynthesis-related electron transport chain using 3-(3,4-dichlorophenyl)-1,1-dimethylurea induced hyponastic growth in normal

Figure 6. Roles of auxin and polar auxin transport in heat-induced hyponastic growth. A, Effects of heat treatment on Col-0 petiole angles in the presence of TIBA (gray triangles) and NPA (white squares; both 50 μmol) compared with mock-treated wild-type Col-0 (black circles). B, Quantitative RT-PCR analysis of IAA19/MSG2 in air controls (gray circles) and heat treatment (black squares). Expression values are normalized to 1 at time 0 (n > 3). C to F, Effects of heat on petiole angles of auxin signaling and PAT mutants (black circles) compared with the wild-type (wt) response (solid lines). Angles are the result of pairwise subtraction. Error bars represent SE; n > 10.
Figure 7. Role of ABA in heat-induced hyponastic growth. A, Effects of heat treatment on petiole angles of Col-0 treated with 20 μM ABA (gray triangles) or soil drained with 100 μM fluoridon (gray diamonds) compared with their respective mock treatments (ABA, black circles; fluoridon, black squares). B, Quantitative RT-PCR analysis of ABI1 in control plants in air (gray circles) and heat treatment (black squares). Expression values are normalized to 1 at time 0 (n > 3). C to I, Effects of heat on petiole angles of ABA-related mutants (black circles) compared with the wild-type (wt) response (solid lines). Angles resulted from pairwise subtraction. Error bars represent SE; n > 10.

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(noninducing) light conditions (Millenaar et al., 2009). This supports the notions that heat-induced hyponastic growth may indeed function to optimize photosynthesis and that the photosynthesis-associated electron transport chain may be a potential integration point for both signals.

Altogether, our data show that low light sensitizes the plant to heat, resulting in an enhanced hyponastic response, or alternatively, that heat desensitizes the plant for light (i.e. sensitizes it for low light) via the photoreceptor proteins. However, we cannot conclusively distinguish between these options.

Hormonal Control of Heat-Induced Hyponastic Growth

Ethylene action protects against, and facilitates in the repair of, heat-induced oxidative damage and predominantly functions in basal thermotolerance in Arabidopsis. In this respect, ethylene-insensitive mutants were more susceptible to heat-induced oxidative damage (Larkindale and Knight, 2002; Larkindale et al., 2005). In agreement with this, our data on ethylene production and insensitive and hypersensitive/overproducing mutants, together with pharmacological data (Fig. 3), strongly suggest that ethylene is a negative regulator of heat-induced hyponastic growth. In contrast to other ethylene-insensitive mutants, ein2 did not show an enhanced hyponastic phenotype (Fig. 3). Perhaps altered ABA levels, as observed in ein2 (Ghassemian et al., 2000), interfere in an unknown manner with the response. Yet, we found that ABA is a positive modulator of heat-induced hyponastic growth (Fig. 7).

Ethylene is the pivotal trigger of submergence-induced hyponastic petiole growth in Arabidopsis and other species (Cox et al., 2003; Millenaar et al., 2005; Benschop et al., 2007), but it was not required for low light-induced hyponastic growth (Millenaar et al., 2009). The combined (triple) ethylene + low light + heat treatment repressed the cumulative effect of heat and low light on hyponastic growth (Fig. 5). The combination treatment of low light and ethylene did
not result in an altered hyponastic growth response (Millenaar et al., 2005). Therefore, we conclude that the inhibitory effect of ethylene in the triple combination treatment (heat + low light + ethylene; Fig. 5) is due to repression of specifically heat signaling components rather than low light-specific components.

Altogether, the data suggest that ethylene is a general antagonist of heat effects in Arabidopsis. However, temperature increase (20°C–29°C) induced hypocotyl elongation independent of ethylene action (Gray et al., 1998). This might indicate that the specific regulation of heat-induced hyponastic growth in hypocotyls differs from the regulation in vegetative adult plants (this study) and seedlings (Larkindale and Knight, 2002; Larkindale et al., 2005) or, alternatively, that ethylene is not required per se in the response to relatively mild temperature increases used by Gray et al. (1998).

Remarkably few studies have described the role of auxin in temperature-related processes, whereas the roles of auxin and polar auxin transport are relatively well understood in differential growth responses, including hyponasty (Vandenbussche et al., 2003; Cox et al., 2004; Millenaar et al., 2009). Enhanced levels of free auxin have been observed in high temperature-treated Arabidopsis hypocotyls (Gray et al., 1998). Furthermore, auxin was required for high temperature-induced hypocotyl elongation. This is in agreement with our pharmacological and mutant analyses showing that auxin is required for heat-induced hyponastic growth. IAA19/MSG2 marker gene expression in petiole tissue tended to increase during 38°C heat treatment, although differences were small. This is consistent with the observations made by Gray et al. (1998) and Koini et al. (2009), who showed increased expression of the auxin-responsive genes IAA4 and IAA29 in hypocotyls upon heat treatment (29°C and 28°C, respectively) and suggested that heat may sensitize the petioles to auxin.

The auxin-mediated regulation of heat-induced hyponastic growth shows remarkable parallels with low light-induced hyponastic growth that was also attenuated by TIBA and abolished by NPA treatment (Millenaar et al., 2009). Moreover, similar to heat, a full induction of low light-induced hyponasty required AFB1, AFB2, AFB3, TIR1, PIN3, and PIN7 (Millenaar et al., 2009).

ABA is involved in the induction of acquired thermotolerance in bromegrass (Bromus inermis), maize (Zea mays), creeping bentgrass (Agrostis stolonifera), and Arabidopsis (Larkindale and Huang, 1994; Robertson et al., 1994; Gong et al., 1998; Larkindale and Knight, 2002). Moreover, heat stimulates ABA synthesis in Arabidopsis seeds, which prevents germination at nonoptimal temperatures (Toh et al., 2008). In contrast, ABA does not seem to be involved in high temperature-induced hypocotyl elongation (Gray et al., 1998).

ABA is a negative regulator of ethylene-induced hyponastic growth in Arabidopsis (Benschop et al., 2007) and submergence-induced hyponasty in R. palaustris (Cox et al., 2004). In sharp contrast, our data showed that ABA is a positive regulator of heat-induced hyponastic growth (Fig. 7). However, not all tested ABA-related mutants exhibited reduced heat-induced hyponastic growth. aba2-1 and aba3-1 showed normal, or even slightly exaggerated, heat-induced responses, which might be due to their leaky nature (Léon-Kloosterziel et al., 1996). Nonetheless, these lines also showed enhanced ethylene release (LeNoble et al., 2004). Ethylene is a negative regulator of heat-induced hyponastic growth (Fig. 3), but may explain why aba2 and aba3 had a close to wild-type/exaggerated response, in an unknown manner. In agreement, aba2 and aba3 showed only moderate effects on acquired and basal thermotolerance relative to aba1-1 and aba2-1 (Larkindale et al., 2005). However, the same was true for aba1 and aba3 lines that did show a markedly reduced heat-induced hyponastic growth response. Additionally, aba2-1 and aba3-1 were affected in ethylene-induced hyponastic growth (Benschop et al., 2007).

Vice versa, aba1-1 and aba2-1 did not show an altered response to ethylene but did show a clearly reduced amplitude upon heat treatment, and aba1-1, aba1-1, aba3-1, and aba2-1 exhibited decreased hyponastic responses to heat and were unaffected (aba1-1 and aba2-1) or enhanced (abi1-1 and abi2-1) in ethylene (Benschop et al., 2007). Overall, these data suggest that the net effects of ABA on ethylene- and heat-induced hyponastic growth are opposite and that the ABA-related genetic components employed in the regulation of the response differ between the treatments.

ABI1 is a negative regulator of ABA signaling and ethylene-induced hyponastic growth (Benschop et al., 2007). ABI expression was enhanced by ethylene (De Paepe et al., 2004; Benschop et al., 2007) and ABA (Leung et al., 1997; Hoth et al., 2002) application. During heat treatment, ABI1 acts as a positive regulator of hyponastic growth. Yet, ABI1 transcription was enhanced during heat treatment (Fig. 7B). This suggests that heat modulates (desensitizes) petioles for hyponastic growth partly via transcriptional regulation of ABI1.

CONCLUSION

Regulation of hyponastic growth is complex, and different environmental stimuli are integrated in the control of this differential petiole growth response in Arabidopsis. We demonstrated that ethylene act as dominant negative regulator, and auxin and ABA as positive regulators, of heat-induced hyponastic growth. Heat and low light act additively on hyponastic growth. Ethylene-induced hyponastic growth is independent from auxin signaling (Millenaar et al., 2009), and ABA is a negative regulator of ethylene-induced hyponasty (Benschop et al., 2007). In contrast, low light-induced hyponasty depends on auxin and is independent of ethylene signaling (Millenaar et al., 2009).
2009). Thus, factors required for heat-induced hyponastic growth are both overlapping and different from low light signaling toward hyponastic growth, and the same might be true for factors controlling ethylene-induced hyponastic growth.

Nevertheless, the similarities in kinetics suggest that the signaling routes may converge and affect a similar set of functional components (Millenaar et al., 2009). Recently, Koini et al. (2009) demonstrated that heat-induced hyponasty was abolished in the pif4 mutant. PIF4 is required for induction of shade-avoidance phenotypes. Active PhyB physically interacts with PIF4 and targets the protein for degradation and subsequently inhibits cell elongation (Huq and Quail, 2002; Lorraín et al., 2008). Accordingly, phyB constitutive shade-avoidance phenotypes are attenuated in phyb pif4 double mutants. Heat induces a transient increase in PIF4 transcript levels (Koini et al., 2009). Together, these data facilitate a model in which PhyB-dependent low light signaling (Millenaar et al., 2009) and heat signaling toward hyponastic growth converge on PIF4. Perhaps also, cross talk of these signals with ethylene occurs via PIF proteins, as at least PIF5 affects ethylene levels (Khanna et al., 2007). Our results suggest that loss of phyA counteracted the enhanced hyponastic growth phenotype of phyB (Fig. 4). In agreement, phyA responses do not involve PIF4 action and PhyA may be dominant over PhyB in this response (Huq and Quail, 2002). Therefore, we cannot exclude that PIF-independent pathways (in addition) control heat-induced hyponastic growth.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) seeds were from the Nottingham Arabidopsis Stock Centre (with accession numbers in parentheses) or were gifts of the authors who described the mutant. All accessesions are described in Supplemental Table S1. Ethylene mutants are all in Col-0: etr1 (N8075; Kieber et al., 1993), ein2-1 (N3071; Guzman and Ecker, 1990), ein2-1 (N8053; Roman et al., 1995), eto1-1 (N3072; Guzman and Ecker, 1990), etr1-4 (Chang et al., 1993), etr1-6 etr4-2 etr3-3 (Hua and Meyerowitz, 1998). Photoreceptor mutants are in Ler, unless stated otherwise: cry1 cry2 (fly fluina; Yanovsky et al., 2003), hic2 (N68; Koornneef et al., 1980; Kohchi et al., 2001), phyA-201 (N6219; Nagatani et al., 1993), phyA-201 phyB-5 (N6224; Reed et al., 1994), phyB-9 in Col (Reed et al., 1993). Auxin mutants are in the Col-0 background unless stated otherwise: pin3-4 (N9365; Friml et al., 2003), pin1-1 (N9363; Friml et al., 2002), pin1-1 (N9364; Friml et al., 2003), tir1-1 (N5798; Ruegger et al., 1997), tir1-1 afb1-1 afb2-1 afb3-1 in Col/WS (Dharmasiri et al., 2005b). ABa-related mutants are in the Col or Ler background: abi2-1 (N156; León-Kloosterziel et al., 1996), abi3-1 (N157; León-Kloosterziel et al., 1996), crd2-1 (Cutler et al., 1996), abf1-1 (N21; Koornneef et al., 1982), abf1-1 (N22; Koornneef et al., 1984; Leung et al., 1994), abf2-1 (N23; Koornneef et al., 1984; Leung et al., 1997), abf3-1 (N24; Koornneef et al., 1982).

Plants were grown on a fertilized mixture of potting soil and perlite (1:2, v/v) relative humidity, 9-h photoperiod of 200 μmol m⁻² s⁻¹ (photosynthetic active radiation) by covering the plants with specially neutral cloth, which did not influence light quality as checked with a LICOR-1800 spectroradiometer (LI-COR).

Treatments

Plants in developmental stage 3.9 (Boyce et al., 2001) were used for all experiments and were transferred to the experimental setups 1 d before treatment to allow acclimatization. Treatments always started (time 0) 1.5 h after photoperiod start to minimize diurnal and/or circadian effects. Plants used for cold pretreatment experiments were transferred to a low-temperature (10°C) cabinet 3 weeks before treatment, with other conditions similar to those described above.

Temperature increase was accomplished by modifying the program of the growth cabinet; 30°C was reached after approximately 22 min, and 38°C was reached after approximately 45 min.

Low light was induced by reduction of the light intensities to 15 to 20 μmol m⁻² s⁻¹ (photosynthetic active radiation) by covering the plants with specially neutral cloth, which did not influence light quality as checked with a LICOR-1800 spectroradiometer (LI-COR).

Ethylene was applied in continuous flow-through (75 L h⁻¹) in glass cuvettes containing one plant each as described by Millenaar et al. (2005) and Benschop et al. (2007). Ethylene (Hook, Leos) and air (70% [v/v] relative humidity) were mixed using flow meters to generate a concentration of 5 μL L⁻¹, which is saturating for the hyponastic response (Millenaar et al., 2005). Ethylene concentrations were checked regularly on a gas chromatograph (GC955; Synspec) and remained constant for the duration of the experiment. Control cuvettes were flushed with air (70% [v/v] relative humidity) at the same flow rate.

Pharmacological Treatments

Gaseous 1-MCP (1 μL L⁻¹; Ethylocel Floralife) was applied 3 h prior to treatment as described by Millenaar et al. (2005) in the same cuvettes used for ethylene treatment. TIBA (Duchefa), NPA (Duchefa), and ABA (Sigma-Aldrich) were dissolved in MilliQ containing 0.1% Tween and 0.1% dimethyl sulfoxide (ABA) or 0.1% ethanol (NPA and TIBA), to a concentration of 50 μM (TIBA, NPA) and 20 μM (ABA). These solutions were sprayed on the plants. Fluoridone (Riedel-de Haën) was dissolved in MilliQ containing 0.1% Tween and 0.07% acetonitrile to 100 μM and was applied to the soil, until saturation, to plants saved from watering for 2 d.

All pretreatments took place 66, 42, and 18 h before the start of the experiments. Control plants (mock) were treated similarly with a pretreatment solution lacking the active components.

Computerized Image Analysis of Angle Kinetics and Calculations

Hyponastic growth kinetics experiments were conducted using an automated time-lapse photography setup (Cox et al., 2003; Millenaar et al., 2005; Benschop et al., 2007). Plants were placed singly in glass cuvettes with petioles of control plants. NewSE values were calculated by taking the square root gives the angle difference between treated and control plants at each time point of control plants. New SE values were calculated by taking the square root from the sum of the two squared SE values.

Plants used for single-time-point angle measurements were manually photographed. Angles were measured using ImageJ (http://rsb.info.nih.gov/ij). For all replicate plants, angles of two petioles were averaged prior to further analysis.

Geographic Climate Data

Geographic parameters of the collection sites of individual accessions were taken from the Natural Variation in Arabidopsis Web site (http://dbgap.versailles.inra.fr/vnat/) or from the authors describing the accessions (Supplemental Table S1). Environmental data of the collection sites (0.5° latitude × 0.5° longitude surface land area plots) of the used accessions were extracted from the Climate Baseline Data of the Intergovernmental Panel on Climate
Ethylene Release

Ethylene release from rosettes was measured as described by Milenaar et al. (2005). Whole rosettes of approximately 300 mg were placed in a syringe. Ethylene was allowed to accumulate in the syringe for 15 to 20 min and subsequently analyzed on a gas chromatograph (GC955; Superspec). This short time frame prevented wound-induced ethylene production, which commenced only after 25 min (data not shown).

Real-Time RT-PCR

Col-0 petioles were snap frozen in liquid nitrogen. RNA was isolated from with the RNAeasy extraction kit (Qiagen). Genomic DNA removal, cDNA synthesis, and real-time RT-PCR were performed as described by Milenaar et al. (2005, 2009). Real-time RT-PCR data were calculated with the comparative CΔΔT method (Livak and Schmittgen, 2001) expressing mRNA values relative to β-Tubulin-6. Primers used are listed in Supplemental Table S3.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Correlation between amplitude of heat-induced hyponastic growth and geographic parameters.

Supplemental Table S1. Natural variation in response to heat in Arabidopsis accessions.

Supplemental Table S2. Correlations between heat-induced hyponastic petiole growth and climate data at the collection sites of the used Arabidopsis accessions.

Supplemental Table S3. Primers for quantitative RT-PCR analysis used in this study.

Supplemental Literature S1.

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LITERATURE CITED


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