Hormonal regulation of temperature-induced growth in Arabidopsis.

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Summary

Successful plant survival depends upon the proper integration of information from the environment with endogenous cues to regulate growth and development. We have investigated the interplay between ambient temperature and hormone action during the regulation of hypocotyl elongation, and we have found that gibberellins (GA) and auxin are quickly and independently recruited by temperature to modulate growth rate, whereas activity of brassinosteroids (BR) seems to be required later on. Impairment of GA biosynthesis blocked the increased elongation caused at higher temperatures, but hypocotyls of pentuple *DELLA* knockout mutants still reduced their response to higher temperatures when BR synthesis or auxin polar transport were blocked. Expression of several key genes involved in the biosynthesis of GA and auxin was regulated by temperature, which indirectly resulted in coherent variations in the levels of nuclear RGA-GFP accumulation and in the activity of the *DR5* reporter. DNA microarray and genetic analyses allowed the identification of the transcription factor PIF4 as a major target in the promotion of growth at higher temperature. These results suggest that temperature regulates hypocotyl growth by individually impinging on several elements of a preexisting network of signaling pathways involving auxin, BR, GA and PIF4.

Introduction

As sessile organisms, plants have to cope with ever changing environmental conditions. In the wild, plants are exposed to a variety of temperatures ranging from values below 0ºC to values above 40ºC, and they have developed the ability to continuously sense this environmental variable to modulate their growth and development accordingly. Plants are able to tolerate extreme low and high temperatures by triggering cold acclimation and thermo tolerance, respectively (Penfield, 2008). These processes involve massive changes in the physiology of the plant directed to avoid the potential damaging effects of extreme temperatures. Between these extremes there is a range of temperatures that is optimal for plants' life. For instance, exposure to low temperatures is critical to initiate several developmental programs, such as flowering or breaking bud dormancy (Dennis and Peacock, 2007; Hovath et al., 2003). This way plants living in temperate regions guarantee that these important developmental transitions occur in spring rather than during the less favorable months of winter. Plants respond in different ways to changes within the optimal, physiological range of temperatures. On the one hand, they are able to buffer these changes in temperature to avoid any unwanted effect on their basic physiology, which is the case, for example, of the temperature compensation of the circadian clock (Hotta et al., 2007). On the other hand, they actively respond and take advantage of moderate changes in ambient temperature to, for example, modulate flowering time (Blázquez et al., 2003), plant architecture (Halliday and Whitelam, 2003; Mazzella et al., 2000) or cell expansion (Gray et al., 1998). These responses illustrate well the high degree of plasticity in plant development, even under small changes in an environmental variable (Casal et al., 2004).

A good example of the plastic integration of multiple cues into a particular developmental decision is the interplay between endogenous and environmental signals in the control of hypocotyl elongation (Alabadí and Blázquez, 2009). In this process, as in other

stages of development, hormones have been placed at the center of the regulatory network, often mediating the effect of different external cues. For instance, hormone action has been proposed as an output target of the circadian clock during the regulation of daily hypocotyl growth (Covington and Harmer, 2007; Michael et al., 2008). In a similar way, light targets gibberellin (GA) synthesis to repress hypocotyl elongation when photomorphogenesis is triggered (Achard et al., 2007; Alabadí et al., 2008). And the promotion of hypocotyl growth by increasing temperatures requires the concurrence of at least auxin and brassinosteroids (BR) (Gray et al., 1998). Besides, there is evidence for the relevance of GA integrating information from the ambient temperature to regulate elongation in species other than Arabidopsis, such as pea (Stavang et al., 2007), apple (Steffens and Hedden, 1992), wheat (Tonkinson et al., 1997), and citrus (Vidal et al., 2003).

Therefore, the study of the molecular interactions that occur during hypocotyl growth provides a possibility to elucidate the molecular basis for the plasticity, and investigate whether it relies on the interplay between multiple signaling pathways. More in particular, does temperature recruit the activity of different hormones independently, or is the involvement of multiple hormones a consequence of mutual interactions between them? Does temperature activate any hormone-independent mechanism to modulate growth? In this work we address these issues with a combination of genetic and microarray analyses in hormone mutant backgrounds at different temperatures.

Results

The activity of the gibberellin pathway is necessary for hypocotyl elongation in response to high temperature

Analysis of temperature-induced hypocotyl growth in the weak, GA-deficient mutant *ga4* led to the conclusion that the GA pathway was not involved in promoting growth at high

temperatures in Arabidopsis (Gray et al., 1998). To unambiguously demonstrate if the GA pathway is necessary for hypocotyl elongation in response to an increase in the ambient temperature in this species, we studied how this response was affected by severe inhibition of the GA biosynthesis, both pharmacologically and genetically. As shown in Figure 1a, increasing amounts of the GA biosynthesis inhibitor paclobutrazol (PAC) in the medium progressively reduced the elongation response to temperature, being negligible at 1 μM PAC. Consequently, the response was not affected when medium was simultaneously supplemented with PAC and GA (Figure 1b). The blockage of the response at the highest PAC concentration was similar to the effect observed in the severe GA-deficient mutant *ga1-3* (Figure 1c). These results indicate that GA are necessary for hypocotyl elongation in response to high temperature. Genetic analyses with several GA receptor mutants (Griffiths et al., 2006), confirmed the involvement of this pathway and indicated that the response is almost fully dependent upon de activity of GID1a and GID1c (Figure 1d).

Hypocotyl and stem growth responses to GA are largely regulated by the DELLA proteins GAI and RGA (Dill and Sun, 2001; King et al., 2001). To investigate whether these two proteins are also key regulators in temperature-induced growth, we studied this response in plants carrying semi-dominant (*gai-1* and *rga-Δ17*; referred to as *gai-D* and *rga-D* in Figure 1e) and null alleles (*gai-t6 rga-24*) of these genes (Dill et al., 2001; Dill and Sun, 2001; King et al., 2001; Peng et al., 1997). *gai-1* and *rga-Δ17* seedlings responded like the wild-type (Figure 1e), suggesting that the block in GA signaling caused by single dominant DELLA alleles can be overcome by temperature. Consistent with this idea, null mutations of both genes in the triple mutant *ga1-3 gai-t6 rga-24* were not enough to completely bypass the need of extra GA to properly respond to the higher temperature, in spite that they restored the wild-type hypocotyl length at 20ºC (Figure 1c; King et al., 2001), indicating that other DELLAs have to be inactivated to complete extra growth under this condition. In fact,

removal of GAI and RGA in the *GA1* genetic background (*gai-t6 rga-24*) did not affect the response (Figure 1e), whereas seedlings deficient in four (*rga-t2 gai-t6 rgl1-1 rgl2-1*; Achard et al., 2006) or in all five *DELLA* genes (*rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1*; Feng et al., 2008), in which the GA pathway is fully active, showed a partial response (Figure 1f and 2). All these results indicate that activity of the GA pathway is needed to promote hypocotyl growth when ambient temperature rises, albeit it is not sufficient, since pentuple *della* mutant seedlings still show a partial response.

The auxin and BR pathways control temperature-induced growth independently of the activity of the DELLA *proteins*

Seedlings need intact auxin, BR, or GA pathways to be able to increase hypocotyl length in response to temperature (Figure 1; Gray et al., 1998). In certain developmental processes there exists cross-regulation between these hormone pathways, for example, auxin and BR jointly control hypocotyl growth and the expression of many genes (Nemhauser et al., 2004; Vert et al., 2008), and the control that auxin exerts on either root growth and apical hook maintenance requires proper GA signaling (Achard et al., 2003; Fu and Harberd, 2003). Therefore, we tried to establish if there is a hierarchy in the action of these three hormone pathways, or they act independently of each other to control this growth response. For example, we reasoned that if part of auxin activity to promote hypocotyl growth is mediated by GA, mutant seedlings with fully active GA pathway should be resistant, or partially resistant, to chemical blockage of the auxin pathway. The same rationale was applied to investigate cross-regulation between BR and GA. Pentuple *della* mutant seedlings, which have a fully active GA pathway, responded similarly to the wild-type to treatment with an inhibitor of the polar auxin transport 1-naphthylphthalamic acid (NPA) and to treatment with the BR biosynthesis inhibitor brassinazole (Figure 2a). The growth restraint effect of both

inhibitors, therefore, is not mediated by the growth repressive activity of the DELLA proteins. These results suggest that auxin and BR regulation of the hypocotyl growth in response to temperature is not mediated by GA, but through parallel pathways.

Triple *gsk3* mutants, which lack three of the ten GSK3, BIN2-like kinases that negatively regulate BR signaling and that therefore show a very active BR pathway (Vert and Chory, 2006), were more sensitive than the wild-type to temperature, but they responded like the wild-type to the NPA-treatment (Figure 2b). This is consistent with the current molecular mechanism for auxin and BR synergistic interaction: the highly active BR pathway sensitizes the seedling to the temperature-stimulated augment in the endogenous auxin levels, and this is abolished when auxin transport is inhibited (Nemhauser et al., 2004; Vert et al., 2008). Triple *gsk3* mutants were not resistant to the inhibitory effect of PAC on temperature-induced growth (Figure 2b). In the absence of any molecular data that support a possible interaction, the most straightforward explanation is that GA activity is not mediated by an activation of the BR pathway to properly promote growth in response to temperature.

Expression of hormone metabolism genes is modulated by ambient temperature

Hypocotyls of seedlings growing at 29ºC have increased levels of both free and conjugated IAA when compared to hypocotyls of seedlings growing at 20ºC, suggesting that temperature adjusts IAA levels to modulate growth (Gray et al., 1998). In order to gauge the possibility that temperature also targets the GA and/or BR metabolism to promote hypocotyl growth, we analyzed the expression of genes encoding key enzymes in both pathways (Yamaguchi, 2008; Fujioka and Yokota, 2003) as well as those coding for enzymes involved in IAA biosynthesis (Benjamins and Scheres, 2008; Stepanova et al., 2008; Tao et al., 2008), in response to a temperature shift. For this purpose we made use of four-day-old seedlings grown at 20ºC and transferred to 29ºC for different times; importantly, seedlings of this age

were competent to respond to the temperature shift concerning promotion of hypocotyl growth (Figure S1). In addition, to have an idea of where in the seedling changes in gene expression take place we analyzed separately cotyledons and hypocotyls. The most remarkable changes in GA metabolism gene expression were observed in the hypocotyl, where expression of two major biosynthetic genes in the pathway, *AtGA20ox1* and *AtGA3ox1*, was rapidly upregulated after the transfer to the higher temperature (Figure 3). On the contrary, transcript levels of the gene encoding a major GA-inactivating enzyme, *AtGA2ox1*, decreased soon after the transfer, being 10-fold lower than in the controls 4 h later. In cotyledons, same trends were observed for the expression of both *AtGA3ox1* and *AtGA2ox1* genes, although changes were smaller in the later case. Conversely, transcript levels of *AtGA20ox1* did not significantly change over the time course in this organ. The localization of the expression of these genes shown by the qRT-PCR analysis was further supported by the expression pattern of a transcriptional fusion of GUS under the control of the *AtGA3ox1* promoter (Mitchum et al., 2006), which increased all over the cotyledons and in the expansion zone of the hypocotyl, 2 h after the temperature shift (Figure 4a). The other members of the GA20ox family (*AtGA20ox2-5*) either did not change or showed little changes during the time course (Figure S2a). Interestingly, transcript levels of other members of the GA2ox family tended to accumulate late in the time-course in both organs, whereas that of *AtGA3ox2* steadily decreased in hypocotyls after the temperature rise (Figure S2a), which could be a consequence of the feed-forward and feed-back regulatory mechanisms, respectively, that operate to control GA homeostasis (Hedden and Phillips, 2000). The increased expression of *AtGA2ox4* may alternatively respond to a protective mechanism to avoid flowing of GA to the shoot apical meristem (Jasinsky et al., 2005). To assess if these changes in gene expression result in the activation of the GA signaling pathway, we analyzed the accumulation pattern of the DELLA protein RGA in the elongation zone of hypocotyls of four-day-old seedlings

transferred from 20 to 29ºC. For that purpose, we made use of *pRGA:GFP-RGA* seedlings that express a GFP-RGA fusion that faithfully recapitulates the activity of the endogenous RGA protein (Silverstone et al., 2001). Confocal imaging showed that the nuclear fluorescence due to GFP-RGA accumulation strongly decreased in the elongation zone of hypocotyls 4 h after temperature rose, being barely detectable 8 h after the shift (Figure 5). The decrease in GFP-RGA levels was observed across several optical sections in the hypocotyls of seedlings transferred to 29ºC (see average projections of image Z-stacks in Figure S2b, as well as series of the individual focal planes from the same images in Movies S1-S4 in the Supporting Information available with the online version of this article). Importantly, GFP-RGA accumulation was not modulated by temperature in hypocotyls of the GA deficient mutant *ga1-3*, suggesting that changes observed in the wild-type, *GA1* genetic background are a consequence of alterations in GA levels triggered by the temperature shift (Figure 5). On the other hand, variations in temperature did not affect the endogenous or the transgenic *RGA* transcript level (data not shown). All together, these results suggest the GA pathway is more active at 29ºC than at 20ºC, most probably as a result of increased GA levels.

A less complex scenario seems to occur in the case of BR metabolism genes, since the expression of only two genes changed in response to the temperature shift: *CPD* and *DWF4* were moderately upregulated in cotyledons and in hypocotyls, respectively (Figure S3a), suggesting a possible increase in the biosynthesis of BR. No significant changes were observed for other biosynthesis genes in the pathway, such as *DET2*, *ROT3*, and *BR6ox1*, whereas expression of *BAS1*, which encodes a BR inactivating enzyme, slightly increased late in the time course most likely as a consequence of the feed-forward mechanism that regulates BR homeostasis (Figure S3a). To test whether these changes in gene expression result in an activation of the BR pathway in hypocotyls, we investigated the phosphorylation status of the positive BR-signaling elements BZR1 and BES1 in response to an increase in temperature

(Vert and Chory, 2006). Interestingly, the ratio of dephosphorylated (active) vs phosphorylated (inactive) versions of BZR1 and BES1 was higher in hypocotyls of five-day-old seedlings continuously growing at 29ºC than at 20ºC (Figure 4b and S3b); however, BR signaling does not seem to be a primary target for temperature since we could not detect changes in this ratio during the first 8 h after a shift from 20ºC to 29ºC (Figure 4b and S3b). Besides, part of the long-term effect of temperature upon BR signaling was exerted at the level of BZR1 protein accumulation, because it was higher in seedlings growing at 29ºC compared to those at 20ºC (Figure 4c and S3b). An increase in ambient temperature did not affect, however, the *BES1* or *BZR1* transcript levels (data not shown).

As mentioned above, a moderate temperature increase promotes IAA accumulation in hypocotyls (Gray et al., 1998). Genetic analyses indicated that this process involves at least two of the Trp-dependent IAA biosynthesis branches, the one defined by the genes *CYP79B2* and *CYP79B3* (Zhao et al., 2002), and the one defined by *TAA1* (Stepanova et al., 2008; Tao et al., 2008). To estimate if the other Trp-dependent pathway, namely the one defined by the *YUCCA* (*YUC*) family (Zhao et al., 2001), could be involved in the increase of IAA biosynthesis at higher temperature, we analyzed expression of all members of the family after a temperature shift (Figure 3). The larger changes were observed in cotyledons. In this organ, *YUC8* and *YUC9* showed a strong increase in their expression levels, whereas they were only marginally higher in the hypocotyl of the shifted seedlings. To the best of our knowledge, there is no experimental evidence showing that these two genes participate in IAA biosynthesis. However, the strong sequence homology with *YUC5* (Cheng et al., 2006), a bona fide IAA biosynthesis gene (Woodward et al., 2005), suggests they may also be actively involved in this pathway. The expression of all other members of the family, as well as that of *TAA1* and one of its homologues, *TAR2*, either did not change or slightly changed after the temperature shift (Figure S4); expression of *TAR1*, the other *TAA1* homologue gene, was

below detection limit (data not shown). Up-regulation of *YUC8* and *YUC9* genes in cotyledons in response to a moderate temperature increase is consistent with their participation in the promotion of IAA biosynthesis in this organ. In fact, the activity of the *DR5:GUS* reporter increased specifically in cotyledons shortly after the shift, which is likely the result of the local accumulation of newly synthesized IAA (Figure 4a).

In summary, these results suggest that the activity of auxin and GA pathways is quickly upregulated in response to an increase in the ambient temperature and that it may be the result of de novo accumulation of hormone pools, promoting therefore hypocotyl elongation.

Global transcriptomic changes in response to differences in temperature

In an effort to identify primary targets of temperature regulation and study their connection with the hormonal network described above, we analyzed the transcriptome of seedlings that have been subjected to a short-term increase in temperature, by using 70-mer oligonucleotide arrays that represent the majority of the Arabidopsis genes (http://www.ag.arizona.edu/microarray). Three biological replicates were used for the analysis following the same experimental design described in the previous section, although samples were harvested from whole seedlings and only 2 h after the shift. A 2-fold cutoff value allowed us to identify 113 genes that were differentially expressed in the shifted samples, 100 of which were upregulated, while 13 of them were downregulated (Table S1). Subsequently, we sought to identify any Gene Ontology term (GO; Ashburner et al., 2000) over- or under-represented in the list of differentially expressed genes compared to the rest of genes included in the array by using the FatiGO algorithm (Al-Shahrour et al., 2005). Several biological processes, one of the three GO categories, were over-represented among the genes upregulated in response to the temperature shift (Table 1). Four of the biological processes differentially represented, namely response to heat, response to light intensity, response to

reactive oxygen species, and protein folding mostly shared the same genes, encoding several heat shock proteins (Table S2). In spite that 29ºC may lay within the physiological range of growth temperatures, a step transfer from 20ºC to 29ºC may also activate the heat stress response, which shares signaling components with other abiotic stress pathways, such as light intensity or oxidative stresses (Kotak et al., 2007; Nishizawa et al., 2006; Rizhsky et al., 2004). More importantly, two biological processes related to growth were identified as over-represented (Table 1 and Table S2): response to auxin stimulus and response to red or far-red light. The over-representation of auxin-regulated genes, though striking, may be a direct manifestation of auxin accumulation after the temperature shift. Seven out of eight auxin-regulated genes belong to the *SAUR* family (McClure and Guilfoyle, 1987). As far as we know, no direct role in growth promotion for any *SAUR* gene has been demonstrated. Nonetheless, expression of these genes is usually associated to elongating tissues, for instance expression of two *SAUR* genes is transiently induced by simulated shade, a condition that also promotes hypocotyl elongation (Roig-Vilanova et al., 2007). On the other hand, over-representation of genes related to light signaling among those upregulated by the temperature shift pointed to an otherwise expected cross-regulation between the two pathways, which oppositely regulate hypocotyl elongation. Two genes encoding transcription factors whose activity promotes elongation, *PIF4* and *ATHB-2* (Huq and Quail, 2002; Steindler et al., 1999), were identified in this group.

PIF4 activity is critical for temperature-induced hypocotyl growth

PIF4 activity has been demonstrated to be important to regulate elongating growth under several physiological contexts, acting usually in concert with other PIF proteins (Alabadí et al., 2008; de Lucas et al., 2008; Huq and Quail, 2002; Koini et al., 2009; Leivar et al., 2008a, b; Lorrain et al., 2008; Nozue et al., 2007). To investigate if the expression of other *PIF* genes

involved in growth promotion, i.e. *PIF1* (Shen et al., 2005), *PIF3* (Kim et al., 2003), and *PIF5* (Nozue et al., 2007), were also targets for temperature regulation, we analyzed their expression by qRT-PCR in dissected seedlings subject to a temperature shift. As shown in Figure 6a, the induction of *PIF4* expression at high temperature was confirmed, and detected in both cotyledons and hypocotyls, while expression of *PIF5* was similarly increased only in hypocotyls. On the contrary, transcript levels of *PIF1* and *PIF3* were not altered in either organ. Interestingly, expression of *PIF4* was maintained at higher levels in hypocotyls of seedlings continuously growing at 29ºC compared to those growing at 20ºC (Figure S5a). We assayed PIF4-HA protein levels from hypocotyls of plants constitutively over-expressing the fusion protein (Nozue et al., 2007) grown at either 20ºC or 29ºC in order to test whether there is any effect of temperature on PIF's protein amount at the post-transcriptional level. The amount of PIF4-HA was similar at the two temperatures (Figure 6b), suggesting that post-translational regulation of PIF4 levels by temperature is negligible.

To investigate the physiological relevance of changes induced by temperature in *PIF4* and *PIF5* expression, we measured hypocotyl lengths at 20 and 29ºC in seedlings carrying null alleles in *PIF* genes. This analysis confirmed that the stimulation of hypocotyl growth by temperature is largely mediated by PIF4 activity, since *pif4* null mutant seedlings hardly responded to temperature (Figure 7). Response of *pif1*, *pif3*, and *pif5* null mutants was very similar to that of the wild-type (Figure 7a,b), while the double *pif4 pif5* behaved like *pif4* single mutant (Figure 7a). Hence, genetic analysis clearly points to PIF4 as a critical element in the regulation of growth by temperature. The discrepancy between the lack of a defect in the response of *pif5* mutants and the induction of *PIF5* expression in hypocotyls may be due to redundancy with PIF activities other than PIF4, for instance PIF7, since seedlings over-expressing PIF5, like those that over-express PIF4, showed reduced elongation response suggesting the growth pathway is partially saturated at the lower temperature (Figure 7c).

Discussion

The ability of plants to cope with different, ever changing environmental conditions is key to survive, and this is most significant at seedling stage. Temperature is ubiquitous among the environmental factors that a plant can perceive on a daily basis, and one remarkable effect is that not only the basic physiological processes like metabolism, carbon partitioning, or photosynthesis are affected, but also developmental decisions, such as growth, that ultimately govern the architecture of the plant, are modulated according to small variations in ambient temperature (Penfield, 2008). Elucidating the molecular events that underlie this regulation is key to understand the fundaments of plasticity.

The results presented here show that temperature regulates growth by modulating the activity of a preexisting network that involves auxin, BR, GA and the transcription factor PIF4 –known to be part of this network through its regulatory interaction with GA-regulated DELLA proteins (de Lucas et al., 2008), and shown to act as a growth promoter in several developmental stages (Alabadí et al., 2008; Huq and Quail, 2002; Koini et al., 2009; Leivar et al., 2008a, b; Lorrain et al., 2008; Nozue et al., 2007).

Interestingly, the mechanism by which changes in ambient temperature enhance or downplay the network relies mostly on transcriptional regulation. Although transcriptional control is usually associated with long-term regulation during development and might seem difficult to conciliate with the plastic, reversible nature of the regulation of growth by temperature, two arguments support this transcriptional model. First, the changes in the expression level of GA and auxin metabolism genes, as well as *PIF4*, occur very fast after the seedlings undergo a temperature shift (Figure 3 and 6). And second, it has been shown in pea plants that moderate drops in ambient temperature rapidly and reversibly stimulate the expression of *PsGA2ox2* gene that parallels the reversible decrease in the growth rate

(Stavang et al., 2007). This growth promoting mechanism seems to be widely conserved in plants, since the GA pathway is also targeted by temperature to promote growth in apple (Steffens and Hedden, 1992), wheat (Tonkinson et al., 1997) and citrus (Vidal et al., 2003). Besides, our results show the expression of several HSP genes, such as the ones encoding the chaperones HSP70 and HSP101, is clearly affected by temperature changes within the range that modulates growth (i.e. between 20 and 29ºC in this study). These genes are direct targets for the HSF family of transcription factors (Busch et al., 2005), and the consensus cis-elements recognized by these proteins are also present in the promoters of some of the GA-, BR- and auxin metabolism genes identified here (data not shown), so it is likely that they are directly regulated as part of the general response to temperature.

All of the above indicates that articulating rapid changes in the transcriptional activity of key metabolic genes in certain hormone pathways may be a general strategy by which temperature modulates hypocotyl or stem growth rate. More importantly, the spatial localization of the transcriptional control of hormone genes occurs in the regions known to be natural sources for hormone synthesis (i.e. auxin in the cotyledons) or the hormone-responsive tissue where active expansion takes place, such as the top third of the hypocotyl (Figure 3-6).

The framework for the control of growth by temperature that emerges now presents two additional features characteristic of an adaptive response: plasticity and robustness. An important element that provides plasticity to the network is that each individual pathway is recruited by temperature and contributes to the precise response to environmental changes. And, moreover, certain degree of cross-regulation is likely to contribute to this property of the network in wild-type plants, as proposed for auxin and BR (Figure 2; Nemhauser et al., 2004; Vert et al., 2008), and for auxin and GA (Frigerio et al., 2006). Furthermore, this regulatory mechanism includes a module that appears to be very robust: on the one side, temperature

promotes PIF4 activity at the transcriptional level as an early and direct response to this environmental cue, independently of hormonal homeostasis of the seedling (Figure S5b). On the other side, temperature might act at the post-translational level through the transcriptional activation of the GA pathway, as a result of releasing the inhibitory effect of the DELLA proteins on PIF4's DNA binding activity (de Lucas et al., 2008). The similar phenotype of pentuple *della* mutants and PIF4 overexpressors, highlights a prominent feature of the strategy to control growth by temperature, which is the independent effect on at least two pathways (namely GA and PIF4) that eventually merge and reinforce the response (Figure S6). According to this view, a simple explanation for the apparently paradoxical observation that pentuple *della* mutants still respond to an increase in temperature (Figure 1 and 2; Koini et al., 2009) is that the absolute level of PIF4 protein is higher at 29ºC; while the observation that PIF4 overexpressors are taller at 29ºC than at 20ºC (Figure 7) would rely on the diminished negative interaction between PIF4 and DELLA proteins at the higher temperature. Moreover, other pathways are also triggered in these mutants, *i.e.* auxin and BR signaling, which would also participate in the concurrent regulation of growth by temperature. The same rationale might be applied to explain why *yucca* mutant seedlings still respond to temperature (Nemhauser et al., 2004). It is likely that the mechanisms through which GA and auxin/BR may control growth in response to temperature is different, based on the comparison between triple *gsk3* and pentuple *della* mutants or the PIF4 over-expressing lines (Figure 1, 2 and 7). A model that accommodates these observation would be that GA regulate the downstream "growth genes" mainly through the activity of PIF4 (de Lucas et al., 2008), whereas auxin and BR pathways regulate target "growth genes" directly through the activity of ARFs and BES1/BZR1 transcription factors, respectively (Benjamins and Scheres, 2008; He et al., 2005; Li et al., 2008; Yin et al., 2005), and/or modulate each other's activity to jointly undertake this task (Vert et al., 2008).

In natural environments ambient temperature usually fluctuates on a daily basis, with warm days and cool nights. The ability of seedlings to grow also oscillates diurnally, being highest at the end of the night due to the joint action of light signaling pathways and the circadian clock that phases PIF4' and PIF5's growth promoting activities towards the end of the dark period (Nozue et al., 2007). Then, when is the growth-stimulating effect of temperature physiologically relevant? One can envision at least two possibilities: in certain natural niches this regulation might be relevant during the day, to take advantage of the warmer temperatures and thus to counteract the negative effect that light exerts on both PIF4 stability and on active GA levels (de Lucas et al., 2008; Nozue et al., 2007; Zhao et al., 2007); alternatively, in certain natural environments it could be relevant during the night, to enhance growth rate in the dark. Several lines of evidence support the first hypothesis: first, pea plants grow taller under a regime of warmer days than under warmer nights, due to decreased GA-inactivation rate in warm days (Grindal et al., 1998; Stavang et al., 2005); second, temperature drop treatments are more effective to reduce growth rate in pea plants when applied in the middle of the light period rather than in the middle of the night, and this correlates with diminished levels of active GA (Stavang et al., 2007). Light and temperature signals regulate the same set of GA metabolism genes in opposite ways to control hypocotyl elongation in Arabidopsis (Figure 3; Alabadí et al., 2008), suggesting that the GA pathway may be indeed key to integrate both environmental cues during the day and to accordingly modulate growth rate (Franklin, 2008). Lastly, increase cell expansion at high temperatures may allow the seedling to bring cotyledons and apical meristem away from the heated soil in certain landscapes (Gray et al., 1998).

In any case, it is worth mentioning that our microarray analysis provides additional support to the view that growth modulation is only a subset of the responses triggered by mild changes in ambient temperature, and the observation that genes like *ASN1* encoding

asparagine synthetase (Lam et al., 2003) or *APRR7*, encoding a pseudo-response regulator, are induced at higher temperatures may provide clues to understand at the molecular level the regulation of carbon partitioning by temperature, and temperature compensation of the circadian clock (Salome and McClung, 2005), respectively.

In summary, our results indicate that ambient temperature influences hypocotyl elongation through the rapid modulation of auxin and GA hormone pathways, and of PIF4 activity, while the BR pathway seems to be required at later stages of growth. Remarkably, seedling's growth response to temperature partly relies on a dual feed-forward mechanism in which temperature robustly enhances elongation by simultaneously promoting PIF4's activity at the transcriptional and post-translational levels, the latter mediated by the GA pathway.

Experimental Procedures

Plant material, growth conditions and hypocotyl length measurements

Arabidopsis thaliana accessions Col-0 and La*er* were used as wild-type. *pRGA:GFP-RGA ga1-3* line was obtained by genetic crosses and isolated from an F3 population. All seeds were surface sterilized for 5 min in 70% (v/v) ethanol and 0.01% (v/v) Triton X-100, followed by 5 min in 96% (v/v) ethanol. Seeds were sown on plates of 1/2 MS medium (Duchefa, http://www.duchefa.com), 0.8% (w/v) agar, 1% (w/v) sucrose, and stratified at 4°C for 6 days in darkness. Stratification of *ga1-3*, *ga1-3 rga-24 gai-t6* and *pRGA:GFP-RGA ga1-3* seeds was done in water containing 10 μM GA_3 (Duchefa) and then rinsed several times with water before sowing. Germination was induced by placing plates for 24 h under white fluorescent light (90-100 μmol m^{-2} sec^{-1}) at 20ºC in a Percival E-30B (http://www.percival-scientific.com). In those experiments involving growth at two temperatures, one group of plates was kept at 20ºC and the replica was placed at 29ºC; in both cases the lighting regime was set to continuous white fluorescent light (20-30 μmol m^{-2} sec^{-1}) and seedlings were grown

for a total of 5 or 7 days. In experiments involving a temperature rise, germination was induced as above and plates were kept at 20ºC and continuous white fluorescent light (20-30 μmol m^{-2} sec^{-1}) for 3 additional days before transfer to 29ºC for several hours; the lighting regime was kept constant.

In experiments involving pharmacological treatments, seeds were sown on sterile filter papers, placed on 1/2 MS plates and stratified as above. Filter papers harboring seeds were transferred to control or treatment plates by the end of the 24 h period of induction of germination. PAC-treatments were 0.01 μM to 1 μM (Duchefa) and the corresponding control plates contained 0.01% acetone (v/v, final concentration). GA_3-treatments were 50 μM and the control plates contained 0.014% ethanol (v/v, final concentration). BRZ-treatments were 3 μM and the control plates contained 0.01% DMSO (v/v, final concentration). NPA-treatments were 100 μM (Sigma, http://www.sigmaaldrich.com) and the control plates contained 0.2% DMSO (v/v, final concentration).

In all cases plates were placed horizontally in the growing cabinets. To measure the hypocotyl length, seedlings were scanned and length was measured with the ImageJ software (http://rsb.info.nih.gov/ij/).

Gene expression analysis by "real-time" quantitative RT-PCR

Total RNA was isolated from either whole seedlings or separately from hypocotyls and cotyledons by using the E.Z.N.A.™ Plant RNA Mini Kit (Omega Bio-tek, http://www.omegabiotek.com). cDNA synthesis and quantitative PCR as well as primer sequences for amplification of GA metabolism genes, as well as *PIF1*, *PIF3*, *PIF4* and *EF1–α* genes has been described (Alabadí et al., 2008; Frigerio *et al.*, 2006). Sequences of other primers used in this study can be found in Table S3.

Gene expression analysis by long oligonucleotide microarrays

Wild-type Col-0 seeds were sterilized, sown, stratified, and germinated as described above. All plates were kept for three additional days at 20ºC under constant, white fluorescent light (20-30 μmol m^{-2} s^{-1}). One set of plates was transferred to 29ºC under continuous, white fluorescent light (20-30 μmol m^{-2} s^{-1}) for 2 h; control plates were kept at 20ºC under the same lighting regime. Three independent biological replicates were used for the analysis. Total RNA from whole seedlings was extracted as above. RNA amplification, labeling, and hybridization of microarray slides were carried out as described in Bueso et al. (2007). Microarray slides were scanned with a GenePix 4000B scanner (Axon Molecular Devices, http://www.moleculardevices.com). Spot intensities were quantified using Genepix Pro 6.0 software (Axon Molecular Devices) and those with a net intensity in both channels lower than the median signal background plus twice standard deviations were removed as low signal spots. Data were normalized by median global intensity and with LOWESS correction (Yang et al., 2001) with Genepix Pro 6.0 and Acuity 4.0 software (Axon Molecular Devices), respectively. Microarray raw data have been deposited in the NCBI's GEO database under accession GSE13822.

GUS staining

GUS staining was performed as described in Frigerio et al. (2006).

Protein extraction and western blots

Total proteins from hypocotyls of *35S:BES1-GFP* (Yin et al., 2002), *pBZR1:BZR1-CFP* (Wang et al., 2002) and *35S:PIF4-HA* (Nozue et al, 2007) transgenic lines were extracted by homogenizing ground, frozen tissue in 1 volume of 62 mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, and DTT (20 mg/ml), boiled for 5

min and centrifuged at maximum speed for 10 min at room temperature. Protein concentration in supernatants was quantified using the *RC DC* Protein Assay (Bio-Rad, http://www.bio-rad.com). 20-40 µg of denatured proteins were separated in Precise™ 8% Tris-HEPES-SDS gel (Pierce, http://www.piercenet.com) and transferred onto PVDF membranes (Bio-Rad). Signal from bound antibodies was detected using the ECL Advance Western Blotting Detection Kit (GE Healthcare, http://www1.gelifesciences.com). GFP and CFP fusion proteins were detected using an anti-GFP (clone JL-8, Clontech, http://www.clontech.com), and PIF4-HA was detected using an anti-HA-peroxidase (clone 3F10, Roche, https://www.roche-applied-science.com). Antibodies against RPT5 were used as loading control (Yu et al., 2008). Quantification of intensity of protein bands was carried out using a Luminescence Image Analyzer LAS-3000 (Fujifilm, http://www.fujifilmlifescienceusa.com).

Confocal imaging

Fluorescence from the GFP-RGA fusion protein was detected using a Leica TCS SL confocal microscopy (Leica Microsystems, http//:www.leica-microsystems.com). The excitation wavelength was 488 nm and GFP emission was detected between 505-530 nm. To discriminate fluorescence emitted by GFP from that emitted by chloroplasts, which is detected in the same window, chloroplasts were further visualized by detecting red fluorescence between 610-660 nm. Superimposition of both images shows orange chloroplasts, due to auto-fluorescence, and green nuclei due to GFP. The same settings were used to detect fluorescence from all samples. Images shown in Figure S2b depict 30-60 µm Z-stacks and correspond to the sum of individual optical sections whose step size is 1 µm.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Temporal window of temperature-induced hypocotyl growth.

Figure S2. Effect of temperature on both the expression of several GA metabolism genes and GFP-RGA nuclear accumulation.

Figure S3. Effect of temperature on both the expression of several BR metabolism genes and BES1-GFP and BZR1-CFP phosphorylation status.

Figure S4. Temperature does not affect or slightly affects expression of several auxin-metabolism genes.

Figure S5. Induction of *PIF4* expression by temperature is mostly independent on the hormonal status of the plant, and it is maintained high in seedlings acclimated to 29ºC for several days.

Figure S6. Model explaining how auxin, BR, and GA hormone pathways modulate growth in response to changes in ambient temperature.

Movies S1 to S4. Modulation of GFP-RGA abundance across the hypocotyl in response to temperature.

Table S1. List of temperature-regulated genes in wild-type seedlings.

Table S2. Lists of genes belonging to the GO terms over-represented in the temperature-induced genes in the wild-type.

Table S3. Sequence of primers used in this study.

Table 1 Non-redundant GO categories over-represented in the set of genes induced by temperature

GO term	GO level	Dif. expressed genes (%)	Total genes (%)	Corrected *P* value
Response to heat	5	16.98	0.86	1.9 e-7
Response to auxin stimulus	5	15.09	2.20	1.8 e-3
Response to light intensity	6	20.00	0.45	9.9 e-9
Red or far-red light signalling pathway	6	12.50	1.13	1.3 e-2
Protein folding	6	15.00	2.27	2.9 e-2
Response to hydrogen peroxide	7	22.58	0.33	1.1 e-8

Figure Legends

Figure 1. The activity of the GA pathway is needed to elongate the hypocotyl in response to temperature.

(a,b) Hypocotyl length of 7-day-old Col-0 wild-type seedlings grown in continuous light in the presence of increasing concentrations of paclobutrazol (a) (P; 0.01 to 1 μM), or in the presence of 1 μM paclobutrazol (P), 1 μM paclobutrazol plus 50 μM GA_3 (P+GA), or mock solution (b) at the indicated temperatures. Meaning of bar colors is indicated in inset of Figure 1a and applies for all graphs in the figure.

(c-f) Hypocotyl length of 7-day-old L*er* (c, e, f) or Col-0 (d) wild-type and mutant seedlings grown in continuous light at the indicated temperatures.

Experiments were repeated twice with similar results. Data from one representative experiment is shown. Error bars represent standard error of the mean (n=15-20).

Figure 2. The auxin and BR pathways control temperature-induced growth independently of the activity of the DELLA proteins.

(a,b) Hypocotyl length of 7-day-old L*er* (a) or Col-0 (b) wild-type and mutant seedlings grown in continuous light in mock media, or in media supplemented with 100 μM NPA, 3 μM brassinazole (BRZ) or 1 μM PAC (PAC) at the indicated temperatures. Error bars represent standard error of the mean (n=15-20). Experiments were repeated twice with similar results. Data from one representative experiment is shown.

Figure 3. Temperature modulates the expression of genes encoding key metabolic enzymes of the auxin and GA pathways.

Four-day-old wild-type Col-0 seedlings were grown at 20ºC under continuous light, as described in Experimental Procedures, and transferred to 29ºC for the indicated times, in hours (h). Transcript levels in cotyledons and hypocotyls were determined by qRT-PCR. Expression level at time 0 for each gene was set to 1 and level in the other samples was

calculated relative to the corresponding time 0 value. Data represent mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

Figure 4. Spatial activation of the auxin and GA pathways. Activity of the BR signaling pathway.

(a) Seedlings were grown in continuous light for 4 days at 20ºC and then kept at the same temperature or transferred to 29ºC for 2 h. Seedlings were fixed and stained for ß-glucuronidase activity as described in Experimental Procedures. Representative seedlings are shown.

(b,c) Five-day-old *35S:BES1-GFP* and *pBZR1:BZR1-CFP* seedlings were grown in continuous light at 20ºC and transferred to 29ºC for several hours (4 h and 8 h) or were grown continuously at either 20ºC or 29ºC (5 d). Ratio of non-phosphorylated vs. phosphorylated versions of BZR1 and BES1 (b), and the total amount of each protein (c) is shown. In (c), protein amount at time points 4 h and 8 h in the time-course was normalized to RPT5 and then to the amount at time 0; protein amount from seedlings continuously growing at 29ºC was normalized to RPT5 and then to the amount in seedlings grown at 20ºC. Total proteins were extracted from hypocotyls and the transgenic proteins detected by Western-blotting using an anti-GFP antibody. RPT5 levels were used as loading control. Quantification was carried out as described in Experimental Procedures. Results are mean of two independent biological repeats and error bars allude to standard deviation.

Figure 5. GFP-RGA disappears from nuclei of the hypocotyl elongation zone after ambient temperature increases.

Four-day-old *pRGA:GFP-RGA* and *ga1-3 pRGA:GFP-RGA* seedlings were grown at 20ºC under continuous light, transferred to 29ºC for the indicated times in hours (h), and fluorescence of GFP-RGA and chloroplasts in the upper, apical third of the hypocotyls

detected by confocal imaging as described in Experimental Procedures. Arrow indicates nuclear GFP-RGA fluorescence. Images are representative of two independent biological repeats including 10-15 seedlings per time point and per genotype each.

Figure 6. Temperature upregulates *PIF4* expression.

(a) Four-day-old wild-type Col-0 seedlings were grown at 20ºC under continuous light and transferred to 29ºC for the indicated times, in hours (h). Transcript levels in cotyledons and hypocotyls were analyzed by qRT-PCR. Expression level at time 0 for each gene was set to 1 and level in the other samples was calculated relative to the corresponding time 0 value. Data represent mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

(b) Five-day-old *35S:PIF4-HA* (Nozue et al, 2007) seedlings were grown at either 20ºC or 29ºC in continuous light. Total proteins were extracted from hypocotyls and the transgenic proteins detected by Western-blotting using an anti-HA-peroxidase antibody. As non-transgenic control, total proteins from hypocotyls of 5-day-old wild-type Col-0 seedlings grown at 20ºC were used. Loading was checked by Ponceau staining.

Figure 7. PIF4 activity is crucial for temperature-stimulated hypocotyl elongation.

(a-c) Hypocotyl length of 7-day-old Col-0 wild-type and mutant seedlings grown in continuous light at the indicated temperatures.

Experiments were repeated twice with similar results. Error bars represent standard error of the mean (n=15-20).

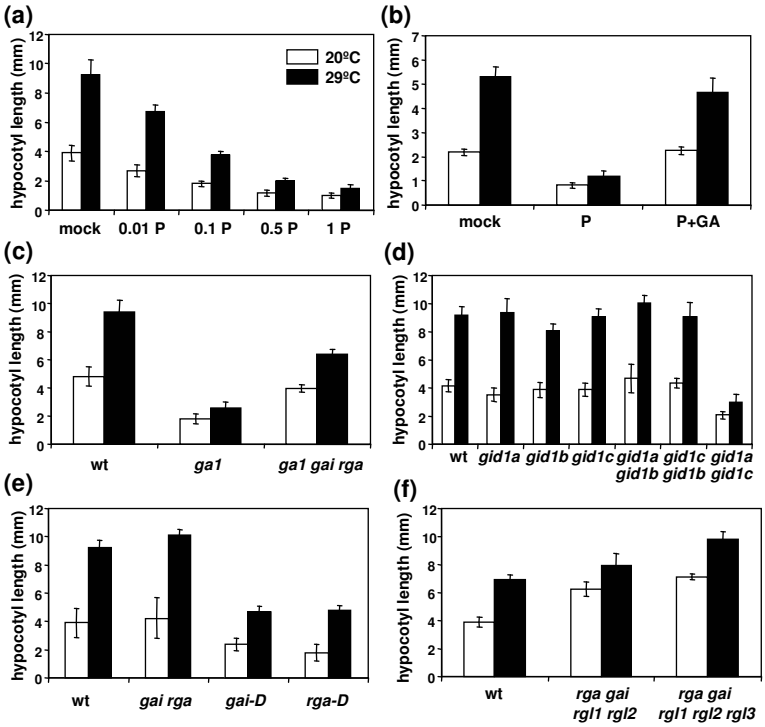

(a)
hypocotyl length (mm)
20ºC
29ºC
mock
0.01 P
0.1 P
0.5 P
1 P
(b)
hypocotyl length (mm)
mock
P
P+GA
(c)
hypocotyl length (mm)
wt
ga1
ga1 gai rga
(d)
hypocotyl length (mm)
wt
gid1a
gid1b
gid1c
gid1a gid1b
gid1c gid1b
gid1a gid1c
(e)
hypocotyl length (mm)
wt
gai rga
gai-D
rga-D
(f)
hypocotyl length (mm)
wt
rga gai rgl1 rgl2
rga gai rgl1 rgl2 rgl3

Figure 1

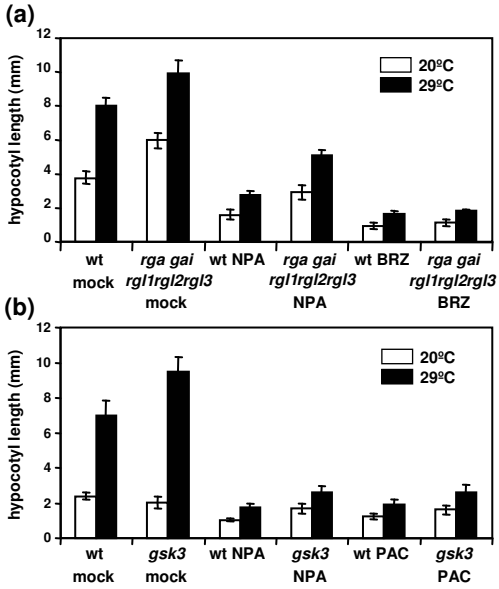

(a)
hypocotyl length (mm)
20ºC
29ºC
wt mock
rga gai rgl1rgl2rgl3 mock
wt NPA
rga gai rgl1rgl2rgl3 NPA
wt BRZ
rga gai rgl1rgl2rgl3 BRZ
(b)
hypocotyl length (mm)
20ºC
29ºC
wt mock
gsk3 mock
wt NPA
gsk3 NPA
wt PAC
gsk3 PAC

Figure 2

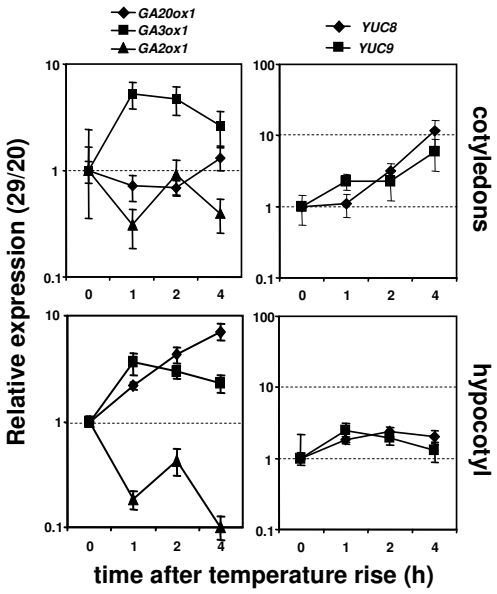

GA20ox1
GA3ox1
GA2ox1
YUC8
YUC9
Relative expression (29/20)
cotyledons
hypocotyl
time after temperature rise (h)
10
1
0.1
100
0
1
2
4

Figure 3

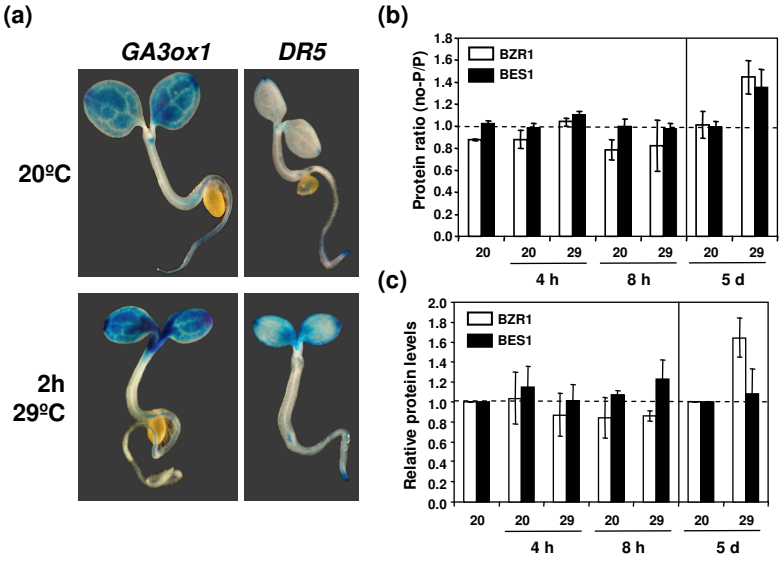

(a)
GA3ox1
DR5
20ºC
2h
29ºC
(b)
BZR1
BES1
Protein ratio (no-P/P)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
20
29
20
29
20
29
4 h
8 h
5 d
(c)
BZR1
BES1
Relative protein levels
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
20
29
20
29
20
29
4 h
8 h
5 d

Figure 4

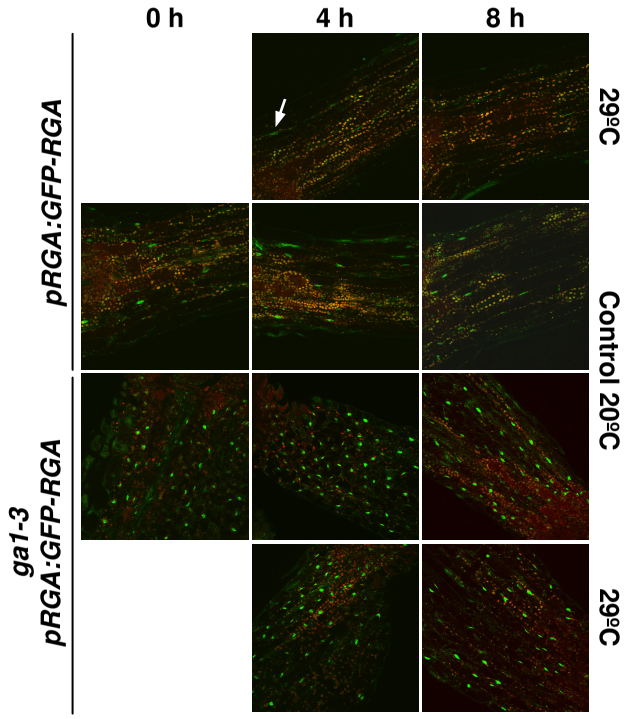


Figure 5

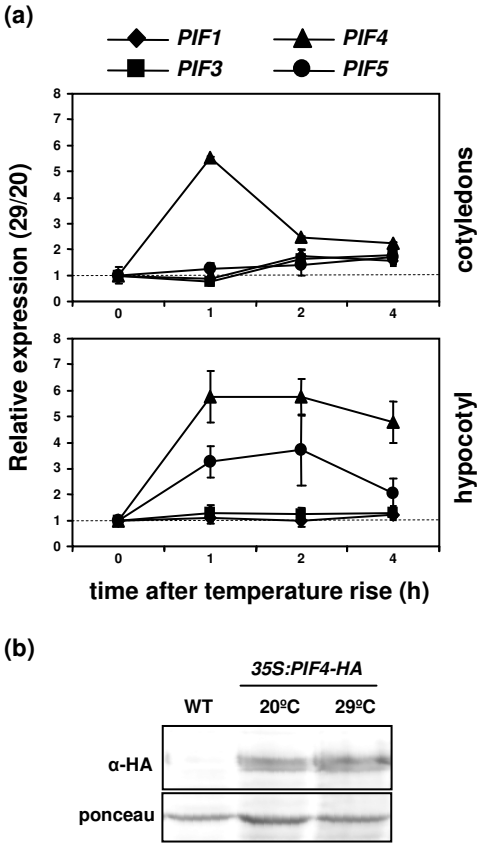

(a)
PIF1
PIF4
PIF3
PIF5
Relative expression (29/20)
cotyledons
hypocotyl
time after temperature rise (h)
(b)
35S:PIF4-HA
WT
20ºC
29ºC
α-HA
ponceau

Figure 6

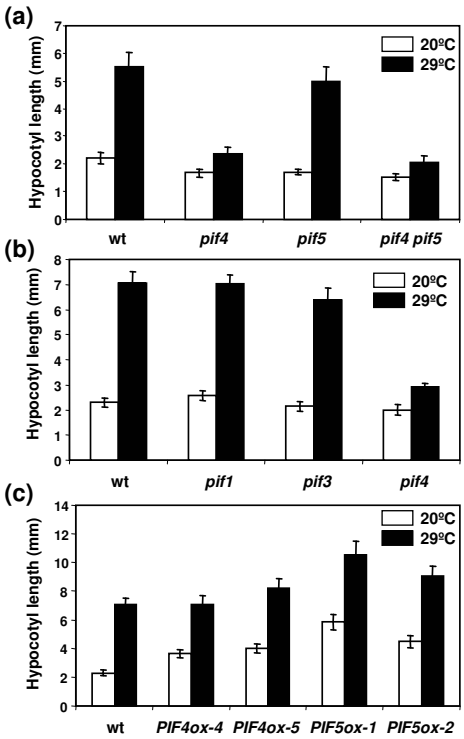

(a)
Hypocotyl length (mm)
20ºC
29ºC
wt
pif4
pif5
pif4 pif5
(b)
Hypocotyl length (mm)
20ºC
29ºC
wt
pif1
pif3
pif4
(c)
Hypocotyl length (mm)
20ºC
29ºC
wt
PIF4ox-4
PIF4ox-5
PIF5ox-1
PIF5ox-2

Figure 7

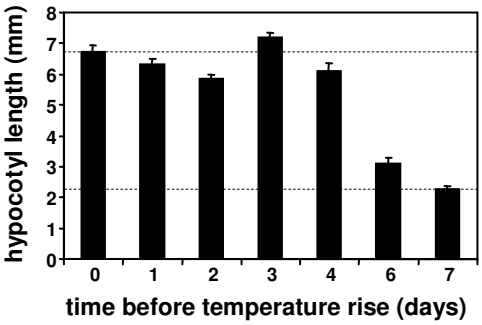
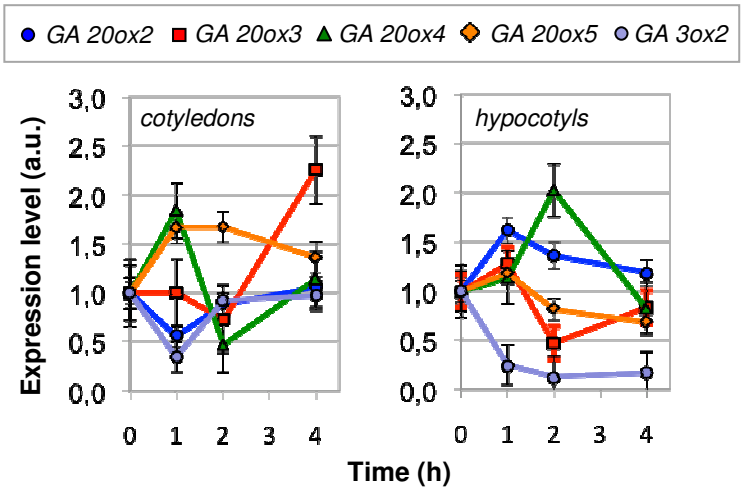
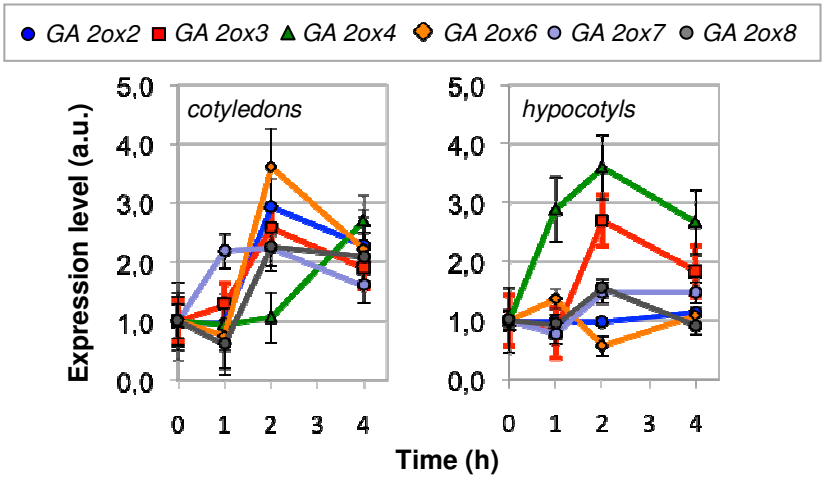

hypocotyl length (mm)
8
7
6
5
4
3
2
1
0
0
1
2
3
4
6
7
time before temperature rise (days)

Figure S1

(a)


GA 20ox2
GA 20ox3
GA 20ox4
GA 20ox5
GA 3ox2
cotyledons
hypocotyls
Expression level (a.u.)
Time (h)


GA 2ox2
GA 2ox3
GA 2ox4
GA 2ox6
GA 2ox7
GA 2ox8
cotyledons
hypocotyls
Expression level (a.u.)
Time (h)

(b)

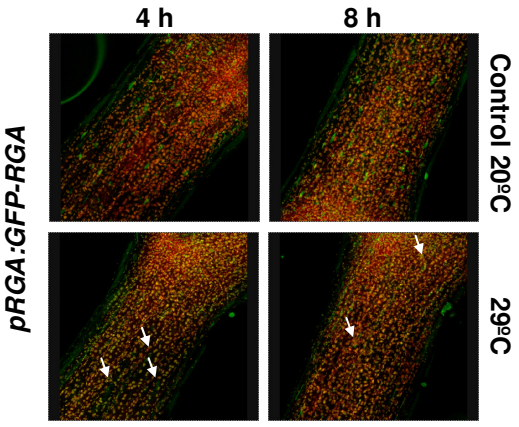
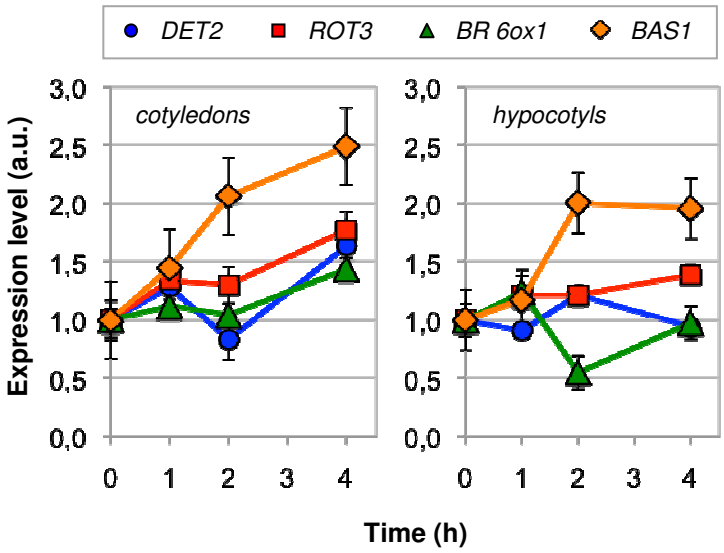

4 h
8 h
pRGA:GFP-RGA
Control 20ºC
29ºC

Figure S2

(a)


DET2
ROT3
BR 6ox1
BAS1
cotyledons
hypocotyls
Expression level (a.u.)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
1
2
3
4
Time (h)

(b)

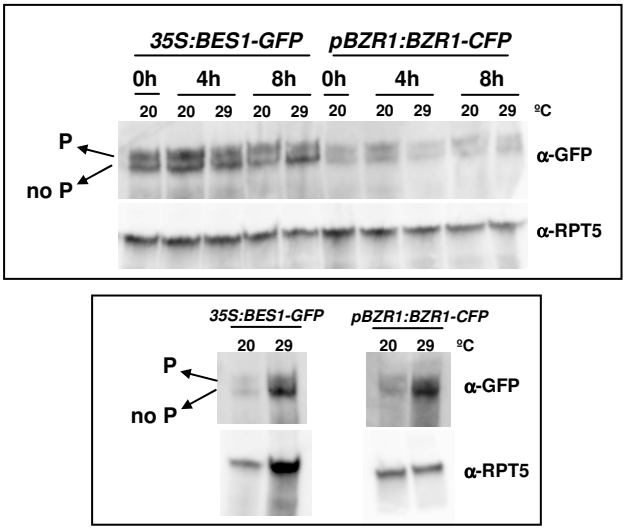

35S:BES1-GFP
pBZR1:BZR1-CFP
0h
4h
8h
0h
4h
8h
20
20
29
20
29
20
20
29
20
29
ºC
P
no P
α-GFP
α-RPT5
35S:BES1-GFP
pBZR1:BZR1-CFP
20
29
20
29
ºC
P
no P
α-GFP
α-RPT5

Figure S3

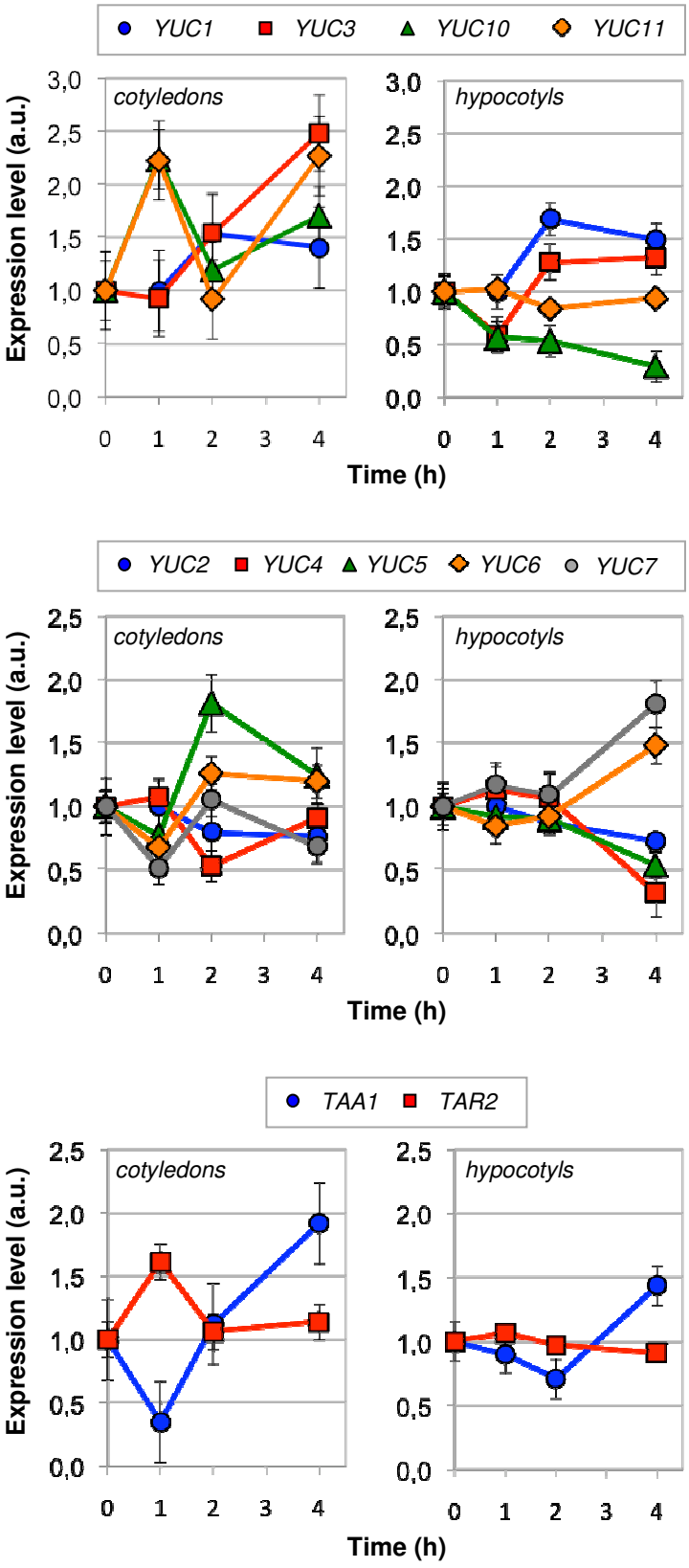
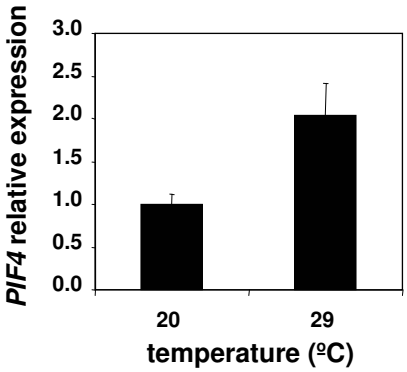

YUC1
YUC3
YUC10
YUC11
cotyledons
hypocotyls
Expression level (a.u.)
Time (h)
YUC2
YUC4
YUC5
YUC6
YUC7
cotyledons
hypocotyls
Expression level (a.u.)
Time (h)
TAA1
TAR2
cotyledons
hypocotyls
Expression level (a.u.)
Time (h)

Figure S4

(a)


PIF4 relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
20
29
temperature (ºC)

(b)

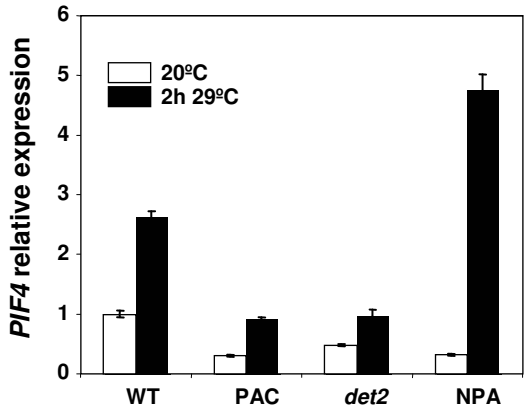

PIF4 relative expression
6
5
4
3
2
1
0
20ºC
2h 29ºC
WT
PAC
det2
NPA

Figure S5

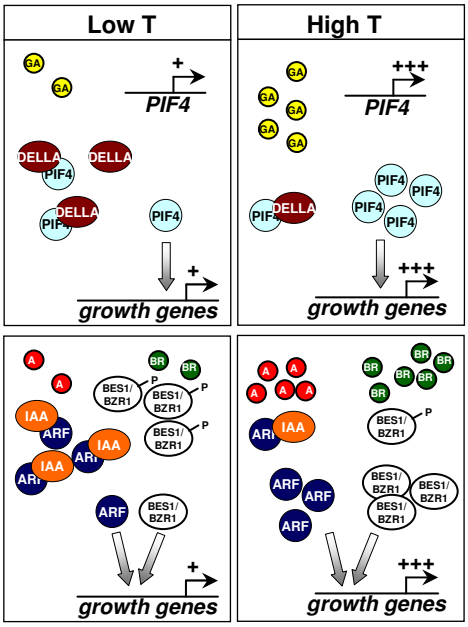


Figure S6