

The brassinosteroid receptor BRL3 triggers acclimation to elevated temperature from phloem companion cells in *Arabidopsis*

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Abstract

Insights into plant receptor signaling are vital in mitigating the impact of climate change and plant disease on agriculture. The role of the BRASSINOSTEROID-INSENSITIVE 1 (BRI1) membrane receptor kinase in promoting growth and development in response to brassinosteroid (BR) hormones is well-established; however, the significance of vascular BRI1-LIKE 1 and 3 (BRL1/3) receptors remains largely overlooked. Here, in *Arabidopsis* (*Arabidopsis thaliana*), we reveal that loss of BRL3 causes defects in growth acclimation to elevated temperature. We establish that BRL3 activates the downstream signaling transcription factor BRI1-EMS-SUPPRESSOR 1 (BES1) and regulates the expression of several major growth pathways. We demonstrated via tissue-specific complementation experiments that BRL3 signaling in phloem companion cells is sufficient to regulate thermoresponsive growth. Transcriptome profiling revealed BRL3 regulates growth-related processes, including hormonal homeostasis and the switch to stress responses. Our research uncovers a previously unknown mechanism involving the BRL3 receptor that modulates growth adaptability under elevated temperature conditions, specifically from the phloem companion cells. This discovery challenges the conventional understanding of BR signaling and opens additional possibilities for developing climate-smart crops.

Introduction

The accelerating impact of climate change poses an imminent threat to agricultural and social sustainability worldwide (FAO 2023; Gupta et al. 2020). Indeed, elevated temperatures as a consequence of CO₂ emissions into the atmosphere have devastating effects on agricultural ecosystems, bringing episodes of climate instability, water scarcity, and more frequent outbreaks of pests and diseases, collectively resulting in dramatic crops losses (Cohen and Leach 2020; Arias et al. 2021).

Plants possess immense innate adaptive plasticity for responses to their environment. Thermomorphogenesis is the sum of adaptive growth responses of a plant exposed to warm ambient temperatures. Typical thermomorphogenesis responses include elongation of hypocotyls, petioles, and roots; leaf hyponasty; and acceleration of flowering (Casal and Balasubramanian 2019). A comprehensive understanding of the underlying molecular mechanisms is crucial to deliver crops adapted to high temperatures. Mechanistic understandings of how plants perceive and respond to warmth are sound, and in *Arabidopsis* (*Arabidopsis thaliana*), several thermosensory mechanisms are known, including the photoreceptor phytochrome B (PHYB), phototropins, a prion-like protein EARLY FLOWERING 3 (ELF3), an RNA thermo-switch, and an intrinsically disordered protein THERMO-WITH ABA-RESPONSE 1 (TWA1) (Jung et al. 2016, 2020; Fujii

et al. 2017; Chung et al. 2020; Bohn et al. 2024). Perception of warmth by a plant triggers molecular events such as activation of the PHYTOCHROME INTERACTING FACTOR 4 (PIF4)/PIF7 transcription factors, auxin biosynthesis, and cell-wall-modifying enzymes essential for thermomorphogenic growth adaptation (Koini et al. 2009; Sun et al. 2012; Delker et al. 2014; Legris et al. 2016; Fiorucci et al. 2020). Other factors including the chromatin modifier HISTONE DEACETYLASE 9 (HDA9) (van der Woude et al. 2019) and a plasma-membrane-localized kinase TARGET OF TEMPERATURE 3 (TOT3) (Vu et al. 2021) also control thermomorphogenesis. Most of these factors are also responsible for regulating several other growth and adaptation responses in plants. How changes in environmental cues alter growth patterns at the cell-specific level and how these are coordinated at the systems level have been studied. Identification of cell-specific components across different signaling pathways activated upon thermo-sensing will provide candidates for engineering climate-ready crops without yield penalties.

Receptor-like kinases (RLKs) initiate signaling cascades that control plant adaptation to environmental stress (Marshall et al. 2012; Smakowska-Luzan et al. 2018; Praat et al. 2021). Brassinosteroid ("BR") hormones directly bind to BR-INSENSITIVE 1 (BRI1) leucine-rich-repeat (LRR)-RLK family members on the plasma membrane (Li and Chory 1997; Wang et al. 2001; Kinoshita et al. 2005; Hothorn et al. 2011; She et al. 2011). Ligand

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perception triggers BRI1 to interact with the co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Li et al. 2002; Nam and Li 2002; Karlova et al. 2006) initiating a cascade of phosphorylation events that control gene expression mainly via the BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1) transcription factors (He et al. 2002; Wang et al. 2002; Yin et al. 2002). Most BR research centers on signal transduction via the ubiquitously expressed BRI1 receptor. Extreme dwarfism of *bri1* null alleles has established BRI1 as crucial for elongation growth. Besides BRI1, the *Arabidopsis* genome encodes 2 additional BRI1-like receptors, namely, BRI1-LIKE 1 (BRL1) and BRL3, which are predominantly expressed in the vasculature (Caño-Delgado et al. 2004) and are conserved across plant taxa. The functional relevance of BRLs has been largely overlooked due to their mutants apparently having wild-type phenotypes (Caño-Delgado et al. 2004; Hacham et al. 2011; Vragović et al. 2015; Kang et al. 2017) and vascular-development studies have reported redundancy in BRI1 and BRLs function. Expressing BRI1 alone in different cellular domains is often sufficient to restore developmental defects of BR-receptor null mutants, further supporting a model of BRLs being exchangeable with BRI1 and the ability of BR signaling acting noncell autonomously through the BRI1-dependent pathway (Hacham et al. 2011; Vragović et al. 2015; Kang et al. 2017; Graeff et al. 2020).

We previously found that overexpression of BRL3 confers drought resistance to *Arabidopsis* without penalizing growth by promoting the accumulation of osmoprotectant compounds (Fàbregas et al. 2018), suggesting a number of stress-specific roles of BRL3 protein still uncharacterized. This prompted us to investigate the possibility that BRLs in their native expression domain (i.e. the vasculature) modulate stress adaptation and are uncoupled from the ubiquitous BRI1 receptor (Planas-Riverola et al. 2019; Gupta et al. 2020). Here, we hypothesized that *Arabidopsis* uses a signaling pathway coordinated through BRL3 receptors to promote growth adaptation in response to abiotic environmental cues (Planas-Riverola et al. 2019; Gupta et al. 2020; Martignago et al. 2020).

Results

BRL3 activates brassinosteroid signaling required for thermomorphogenesis

Toward re-examining the function of *Arabidopsis* BRL3 in detail, 4 *brl3* T-DNA insertion-mutant seedlings were assessed and exhibited wild-type-like growth at the favorable temperature of 22 °C (Supplementary Fig. S1, A to D), in agreement with (Caño-Delgado et al. 2004). Shorter hypocotyls were however observed when *brl3* seedlings were exposed to elevated temperature (28 °C), owing to reduced cell length (Fig. 1, A to D). The *brl3* thermoresponsive hypocotyl-elongation defect (Fig. 1, C and D) was restored by expressing BRL3-GFP from its native promoter (BRL3_{pro}:BRL3-GFP) in the *brl3*-2 mutant background (hereafter *brl3*, Supplementary Fig. S1, E and F). By contrast, hypocotyls in a stable-transgenic BRL3-GFP overexpression line (35S_{pro}:BRL3-GFP; Col-0) (Fàbregas et al. 2018) had exaggerated elongation at 28 °C. Other hallmark thermomorphogenesis phenotypes such as petiole elongation and flowering time (Gray et al. 1998; Koini et al. 2009; Casal and Balasubramanian 2019) were also perturbed in mature *brl3* plants (Fig. 1, E and F, Supplementary Fig. S1, G and H). Akin to *brl3* mutants, downregulation of BRI1 signaling activity in *bri1* mutants or treatment of wild-type Col-0 plants with BR-synthesis inhibitor BRZ₂₂₀ also led to shorter hypocotyls, irrespective of temperature (Supplementary Fig. S2, A to C). To verify whether

brl3 thermomorphogenic hypocotyl-elongation defects are due to differential BRI1 expression, we assayed the transcript abundance of BRI1 and other BRLs in the *brl3* mutant background and found them to be at wild-type level (Supplementary Fig. S2D). These results indicate a functioning BR-mediated signaling cascade through the BRL3 receptor to modulate growth under elevated temperature conditions.

Upon elevated temperature, the core downstream BR signaling components BES1/BZR1 and BRASSINOSTEROID-INSENSITIVE 2 (BIN2) interact with the PHYTOCHROME INTERACTING FACTOR 4 (PIF4)-mediated thermoresponsive pathway to regulate genes involved in growth adaptation (Quint et al. 2016; Ibanez et al. 2018; Martínez et al. 2018). Toward confirming the role of downstream BR signaling components and BRL3, the *brl3* defective-hypocotyl phenotype at elevated temperature was rescued by the gain-of-function *bes1-d* mutation (Fig. 1, G and H). Further, *brl3* had weaker BES1 dephosphorylation at 28 °C (Fig. 1I). Exogenous application of the BIN2 inhibitor bikinin rescued *brl3* hypocotyl-elongation defects at 28 °C (Supplementary Fig. S2, E and F), supporting the hypothesis that BRL3 uses the canonical BRI1 downstream signaling pathway to promote adaptive growth at elevated temperatures.

BRL3 contributes to regulation of core thermomorphogenesis genes

To assay the role of BRL3 in thermomorphogenesis responses, we performed an RNA-seq analysis in *brl3* and WT shoots from 6-d-old seedlings grown at favorable temperature (22 °C) and elevated temperature (28 °C) conditions. A total of 1,247 genes were found to be deregulated in *brl3* vs Col-0 at 22 °C, while 891 were deregulated at 28 °C in the same comparison (Supplementary Fig. S3A). At 22 °C, BRL3-activated genes (those that are downregulated in *brl3* when compared to Col-0) were enriched in gene-ontology (GO) categories related to growth, development, homeostasis, light response and tyrosine metabolism and BRL3-repressed (those upregulated in *brl3* when compared to Col-0) genes were enriched in immune response, redox status and jasmonic-acid-mediated response ($|FC| > 1.5$, FDR < 0.05; Supplementary Fig. S3B, Supplementary Data S1). At 28 °C, BRL3-activated genes (those downregulated in *brl3* when compared to Col-0) were enriched in GO categories related to development and immunity, while BRL3-repressed genes (those upregulated in *brl3* when compared to Col-0) were enriched in abiotic stress responses, water transport and hydrogen peroxide responses (Supplementary Fig. S3C). Comparison of *brl3* transcriptome profiles at favorable versus elevated temperature (Fig. 2, A and B, Supplementary Data S2) revealed genes related to abiotic stress responses were not activated in the *brl3* mutant (i.e. an “under-response”, Fig. 2B). By contrast, negative regulators of ethylene, carboxylic-acid and carbon-catabolic processes responded stronger in the *brl3* mutant than in wild type (i.e. an “over-response”, Fig. 2B).

Among under-responsive genes (Fig. 2B), those known to promote thermotolerance such as TEMPERATURE-INDUCED LIPOCALIN (TIL), HEAT-SHOCK TRANSCRIPTION FACTOR A7A (HSFA7A) and MULTIPROTEIN BRIDGING FACTOR 1C (MBF1C) (Suzuki et al. 2005; Larkindale and Vierling 2008; Chi et al. 2009) and genes known to promote osmoprotectant biosynthesis such as GALACTINOL SYNTHASE 1 (GolS1) (Panikulangara et al. 2004) were identified (Fig. 2C). These trends agree with our previous finding with BRL3 overexpression lines (Fàbregas et al. 2018). Core thermomorphogenesis genes such as those annotated as direct targets of BES1/BZR1, PIF4, AUXIN-RESPONSE FACTOR 6 (ARF6)

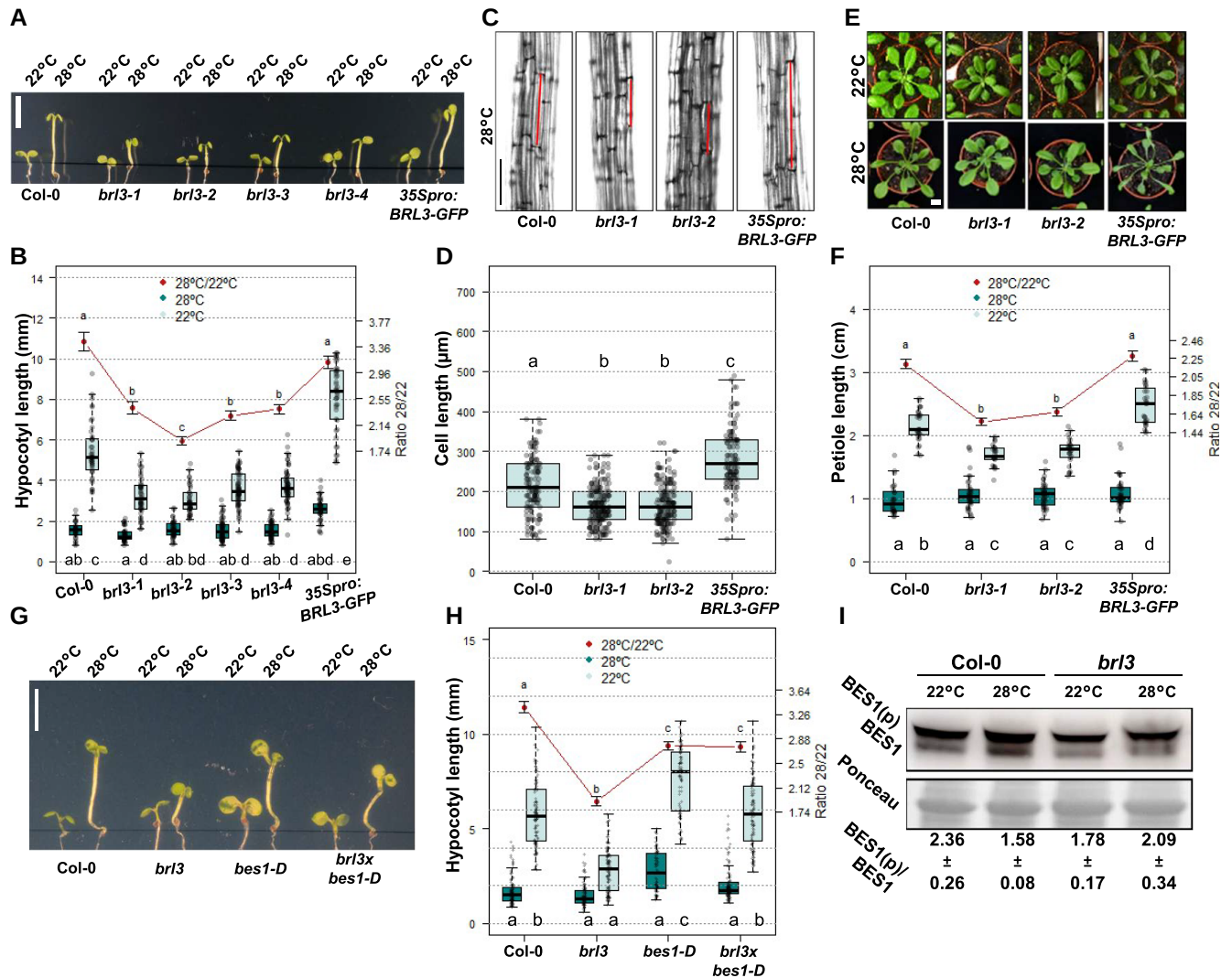


Figure 1. A functional BRL3 receptor cascade promotes *Arabidopsis* thermomorphogenesis. **A)** Seedling phenotypes and **B)** quantification of hypocotyl length for wild-type Col-0, *brl3-1*, *brl3-2*, *brl3-3* and *brl3-4* mutants, and 35S_{pro}:BRL3-GFP plants grown at 22 or 28 °C for 6 days. Scale bar: 5 mm. **C)** Hypocotyl cell phenotype and **D)** quantification of hypocotyl epidermal-cell length in wild-type (Col-0), the *brl3-1* and *brl3-2* mutants, and 35S_{pro}:BRL3-GFP seedlings grown at 28 °C for 6 days. Red lines in **(C)** depict cell length examples. Scale bar: 200 μm. **E)** Petiole-elongation phenotypes and **F)** quantification of petiole length for 15-day-old wild-type (Col-0), the *brl3-1* and *brl3-2* mutants, and 35S_{pro}:BRL3-GFP plants grown at long-day 22 °C condition and transferred to either 22 or 28 °C for 7 days before scoring at 22 days. Scale bar: 1 cm. **G)** Seedling phenotypes and **H)** quantification of hypocotyl length for wild-type (Col-0), *brl3*, *bes1-d*, and *brl3x bes1-d* seedlings. Scale bar: 5 mm. **I)** BES1 phosphorylation status in wild-type (Col-0) and *brl3-2* seedlings. Five-day-old seedlings grown at 22° were transferred to 22° or 28° for 24 h before harvest. BES1 phosphorylation status was analyzed by western blot with antiBES1 antibody. Numbers below the blot show the ratio between phosphorylated and de-phosphorylated BES1 levels across 3 independent western blots ± standard error of the mean (SEM). Ponceau staining of the membrane was used as a loading control. In **(B, D, F, H)**, boxplot boxes depict Q1, median, and Q3 of the data, while boxplot whiskers depict ±1.5 × interquartile range of each population. Points depict individual values. In **(B, H, F)** boxplots, letters depict statistical differences after a 2-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test. Error bars in ratio lines depict standard error (SE). In **(D)**, letters depict statistical differences after a 1-way ANOVA with Dunn's test. In **(I)**, numbers depict mean ± SEM. In all panels, P-value <0.05. Data assayed from 4 independent biological replicates (*n* > 50).

and HOMOLOG OF BEE2 INTERACTING WITH IBH 1 (HBI1) (Sun et al. 2010; Yu et al. 2011; Bai et al. 2012; Oh et al. 2012, 2014) were also found amongst BRL3-responsive genes at elevated temperatures (Fig. 2D). These results suggest that BRL3 is necessary for proper activation of high-temperature-response genes and that the BRL3 pathway is central for physiological and molecular adaptation to high-temperature stress.

BRL3 activity in phloem companion cells is sufficient for thermoresponsive growth

Given that BRL3 is expressed in the root stem-cell niche and in phloem vascular tissues throughout *Arabidopsis* plants

(Caño-Delgado et al. 2004; Fàbregas et al. 2013) (Supplementary Fig. S4, A to H), we sought to investigate the cell-specific contribution of BRL3 signals to the *brl3* mutant phenotype by expressing BRL3-GFP transgenes from various cell-type-specific promoters in the *brl3-2* mutant background (Fig. 3, A to L, Supplementary Fig. S5, A to F). BRL3-GFP expression in the stele (including xylem and phloem tissues from WOL_{pro}:BRL3-GFP; *brl3*) or phloem companion cell-enriched (from SUC2_{pro}:BRL3-GFP; *brl3*) rescued *brl3* thermomorphogenesis defects (Fig. 3, M and N, Supplementary Fig. S4, K and L). SUC2_{pro}:BRL3-GFP; *brl3* lines had higher BRL3 expression and BRL3-GFP fluorescence specifically in the phloem companion cells compared to that from its native promoter (Supplementary Fig. S4, M to O), which was accompanied by a

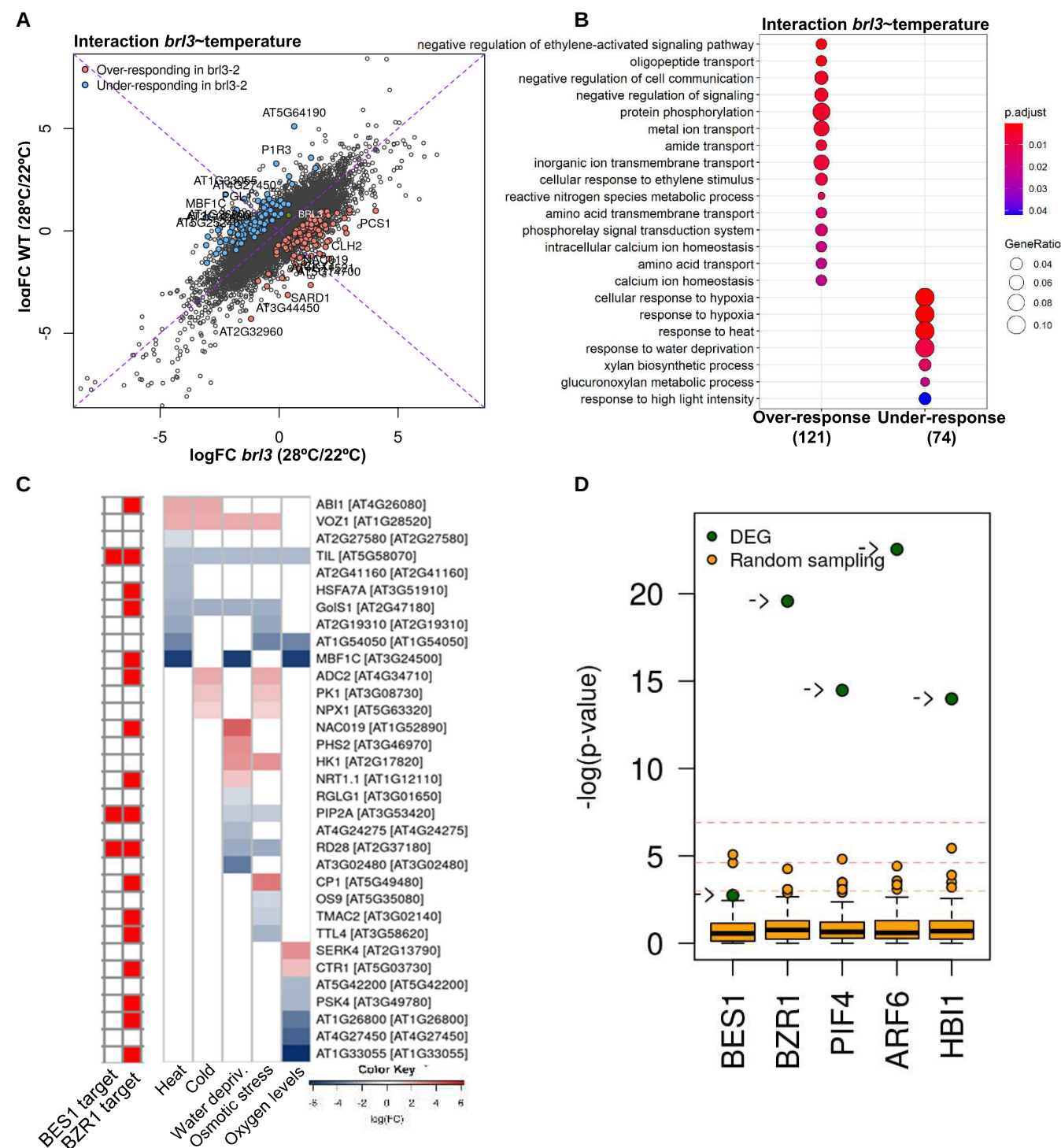


Figure 2. BRL3 is required for proper adaptation to high-temperature stress. **A)** Dot plot showing the comparison between transcript fold changes in Col-0 (28 vs 22 °C) and *brl3* (28 vs 22 °C). Red or blue dots represent genes identified as affected by the interaction genotype-temperature interaction in the linear model. **B)** Gene-ontology enrichment analysis based on genes affected by the interaction between *brl3* and temperature. Size of the nodes represents the number of annotated genes in a particular category and the color indicates its adjusted P-value upon enrichment test. **C)** Genes affected by the *brl3* and temperature interaction annotated as responding to abiotic stimulus [GO:0009628] and its children subcategories of responses to heat [GO:0009408], cold [GO:0009409], water deprivation [GO:0009414], osmotic stress [GO:0006970], and oxygen levels [GO:0070482]. Red on the heatmap indicates stronger high-temperature activation in *brl3* mutant than in wild-type (i.e. putatively repressed by BRL3) and blue the opposite (i.e. putatively activated by BRL3). Small red boxes next to the heatmap indicate the gene is among known direct targets of BES1 (left) or BZR1 (right). **D)** Enrichment in targets of core complex of cell elongation, i.e. BAP module (BES1/BZR1, PIF4, ARF6) and HBI1 in genes affected by the genotype-temperature interaction. In **(D)**, boxplot boxes depict Q1, median, and Q3 of the data, while boxplot whiskers depict $\pm 1.5 \times$ interquartile range of each population. Yellow points depict $-\log(P\text{-value})$ of a random sampling of genes (sample size = 248) being target of each TF. Green points depict $-\log(P\text{-value})$ of genes obtained in our experimental dataset (sample size = 248).

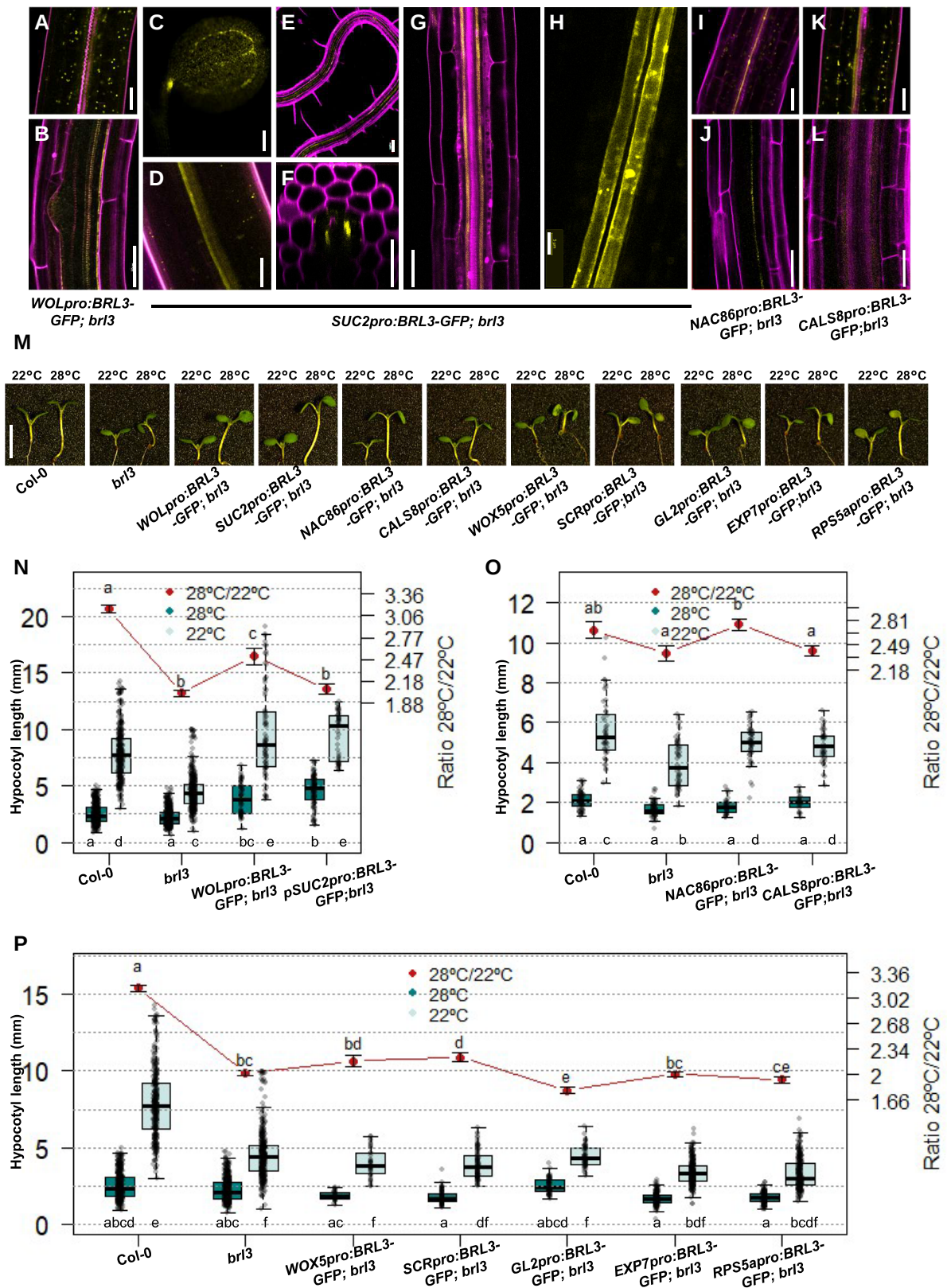


Figure 3. BRL3 functions in phloem companion cells to activate canonical brassinosteroid signaling. **A** to **L**) Representative confocal images of BRL3-GFP fluorescence expressed in tissue enriched manner from **(A)** hypocotyl and **(B)** root vasculature of *WOLpro:BRL3-GFP; brl3* line; **(C)** cotyledons and **(D)** hypocotyl phloem companion cells in *SUC2pro:BRL3-GFP; brl3* line; **(E** to **H)** roots in *SUC2pro:BRL3-GFP; brl3* line; **(I)** hypocotyl and **(J)** root of *NAC86pro:BRL3-GFP; brl3* line; **(K)** hypocotyl and **(L)** root of *CALS8pro:BRL3-GFP; brl3* line. **(M)** Representative images of high-temperature-induced hypocotyl elongation in *Arabidopsis* seedlings. Scale bar: 10 mm. **N** to **P**) quantification of **(M)**. Scale bars: **(A, D, E, I, and K)**: 100 μ m; **(B, F, G, J, and L)**: 50 μ m; **(C)**: 500 μ m; **(H)**: 5 μ m; and **(M)**: 5 mm. In boxplots, letters depict statistical differences after a 2-way ANOVA with Tukey's HSD post hoc test. In **(N** to **P**), boxplot boxes depict Q1, median, and Q3 of the data, while boxplot whiskers depict $\pm 1.5 \times$ interquartile range of each population. Points depict individual values. Letters depict statistical differences after a 2-way ANOVA with Tukey's HSD post hoc test. Error bars in ratio lines depict SE. In all panels, P -value < 0.05. Data assayed from 3 independent biological replicates ($n > 30$).

slight yet statistically significant increase in hypocotyl length even at 22 °C (Fig. 3, M and N). BRL3-GFP expression in proto-phloem sieve elements (from NAC86_{pro}:BRL3-GFP; *brl3*) and the phloem-pole pericycle (from CALS8_{pro}:BRL3-GFP; *brl3*) only partially rescued *brl3* thermomorphogenic defects (Fig. 3, M and O). This partial rescue was likely due to a reduced expression of BRL3, since CALLOSE SYNTHASE (CALS8) and NAC-domain containing protein 86 (NAC86) expression is confined to more reduced domains than SUC2 or BRL3. At 28 °C, hypocotyl cell elongation response in SUC2_{pro}:BRL3-GFP; *brl3* line was too restored to WT levels (Supplementary Fig. S5, A and B).

By contrast, BRL3-GFP expression in either the epidermis (atrichoblasts: GL2_{pro}:BRL3-GFP; *brl3* and trichoblasts: EXP7_{pro}:BRL3-GFP; *brl3*), endodermis (SCR_{pro}:BRL3-GFP; *brl3*), root stem-cell niche (WOX5_{pro}:BRL3-GFP; *brl3*) or the root apical meristem (RPS5A_{pro}:BRL3-GFP; *brl3*) all failed to rescue *brl3* thermomorphogenic defects (Fig. 3, M and P, Supplementary Fig. S5, C to H). It therefore seems plausible that the phloem companion cells can modulate cell elongation responses upon temperature cues through BRL3 signaling (Supplementary Fig. S2, A and B). Besides, phloem-enriched BRI1-GFP expression did not rescue the *brl3* hypocotyl-elongation defects using a SUC2_{pro}:BRI1-GFP; *brl3* cassette (Supplementary Fig. S5, I to N), adding to previous reports (Kang et al. 2017; Graeff et al. 2020) where tissue-specific BRI1-GFP expression could restore developmental phenotypes in the *bri1* mutant.

Histological analysis of BRL3_{pro}:GUS-GFP reporter expression in the Col-0 background revealed BRL3 promoter activity is spatially regulated upon elevated temperature, i.e. induced in hypocotyl vasculature (near the hypocotyl-root junction) and repressed in the root vasculature (Supplementary Fig. S4, I and J). This suggests that the BRL3 expression domain coincides with the organ and respective cellular region undergoing a growth response at elevated temperature. Collectively, these data demonstrate that BRL3 receptor signaling modulates adaptive growth to elevated temperature from the inner phloem companion cells.

Phloem companion cell-enriched BRL3 activity modulates gene expression related to energy flux during thermomorphogenesis

To elucidate BRL3-dependent transcriptional changes in the phloem companion cells that contribute to temperature adaptation, we compared transcriptomes between transgenic SUC2_{pro}:BRL3-GFP; *brl3* and *brl3* shoots grown at favorable (Supplementary Fig. S6A, Supplementary Data S3 and S4) and elevated temperatures (Supplementary Fig. S6B). At 22 °C, phloem companion cell-enriched BRL3-activated genes (those upregulated in SUC2_{pro}:BRL3-GFP; *brl3* as compared to *brl3*) were enriched in GO categories related to light responses and regeneration, while phloem companion cell-enriched BRL3-repressed genes (those down regulated in SUC2_{pro}:BRL3-GFP; *brl3* as compared to *brl3*) were enriched in categories related to response to biotic and abiotic stress (Supplementary Fig. S6A). At 28 °C, phloem companion cell-enriched BRL3-repressed genes pertain to categories related to defense and cellular response to abiotic stress (Supplementary Fig. S6B). Comparison of SUC2_{pro}:BRL3-GFP; *brl3* and *brl3* genotypes response to temperature was then analyzed (Fig. 4, A and B). Genes with a stronger response to temperature due to phloem companion cell-enriched BRL3 expression were enriched in photorespiration (over-response, Fig. 4B), which in turn impact redox homeostasis (Rhoads et al. 2006). Genes involved in auxin transport and negative regulators of ethylene and steroid

signaling (the latter probably due to feedback mechanisms) displayed less deregulation in SUC2_{pro}:BRL3-GFP; *brl3* lines than in the *brl3* mutant at elevated temperature (under-response, Fig. 4B), suggesting at least partial rescue by BRL3-GFP action in the phloem companion cells. These results suggest that phloem companion cell-enriched BRL3-GFP complementation potentially de-represses photorespiration machinery, resulting in improved energy usage at elevated temperatures, which could explain improved thermomorphogenic responses in the complementation line.

We intersected differentially expressed genes in *brl3* versus Col-0, and SUC2_{pro}:BRL3-GFP; *brl3* versus *brl3*, and found moderate overlap at 22 °C (16% of transcripts, Supplementary Fig. S6C) but major overlap at 28 °C (up to 60%) (Supplementary Fig. S6D), with 582 genes statistically significantly deregulated in both comparisons and in opposite directions (i.e. “core genes”, Supplementary Fig. S6, E and F, Supplementary Data S5 and S6). These comparisons support the increased relevance and consistency of BRL3 action under elevated temperature. As a control, enrichment of known BR-regulated genes (Goda et al. 2004; Nemhauser et al. 2004) was observed among the BRL3-responsive genes in both comparisons and core-gene sets (Fig. 4, C and D), suggesting BRL3-mediated regulation of BR responses. At 22 °C, BRL3-activated genes are enriched in GO categories related to cell communication and responses to stimulus while BRL3-repressed genes appear in categories regarding stress responses. At 28 °C, response to oxygen categories appear as enriched in BRL3-activated gene sets, while defense, immunity and response to wounding categories are enriched among BRL3-repressed gene sets. Collectively, these results demonstrate the physiological relevance of BRL3 signaling activity from inner phloem in balancing adaptive growth by modulating energy status and hormone responses.

As our transcriptome analysis revealed BRL3 to affect large number of genes belonging to abiotic stresses response categories, we tested responses of *brl3* and SUC2_{pro}:BRL3-GFP; *brl3* plants to a battery of stresses. Upon heat shock (42 °C, 150 min) *brl3* survival was greatly reduced compared to Col-0 (Fig. 5, A and B). The *brl3* mutant also had impaired resistance to osmotic stress compared to Col-0 in terms of hydrotropism (root growth reorientation toward water availability, Fig. 5, C and D) (Takahashi et al. 2002) and suffered lack of PCD signaling in the root apex and meristem regions (Fig. 5, E and F), probably due to faulty stress recognition mechanisms (Duan et al. 2010). All defects were restored by SUC2_{pro}:BRL3-GFP expression (Fig. 5, A to F) to Col-0 levels or in some cases even to 35S_{pro}:BRL3-GFP overexpressor levels. In summary, these findings confirm phloem companion cell-associated BRL3 signals play a pivotal role in promoting optimal plant growth and enhancing survival capabilities under these climate change conditions (Fig. 5G).

Discussion

Brassinosteroids are plant hormones that promote growth and confer resistance to various abiotic stresses, including temperature extremes, salinity and drought. Despite the stress-protective properties of BRs having been long recognized, deep knowledge of their molecular mechanisms underlying their function in abiotic stress responses has been lacking. Our results shed light on the distinctive roles of BR receptor BRL3 in growth acclimation and adaptation to adverse environmental conditions, especially upon elevated temperature. At the molecular level, BRL3 binds the brassinosteroid hormone ligand with similar high affinity as

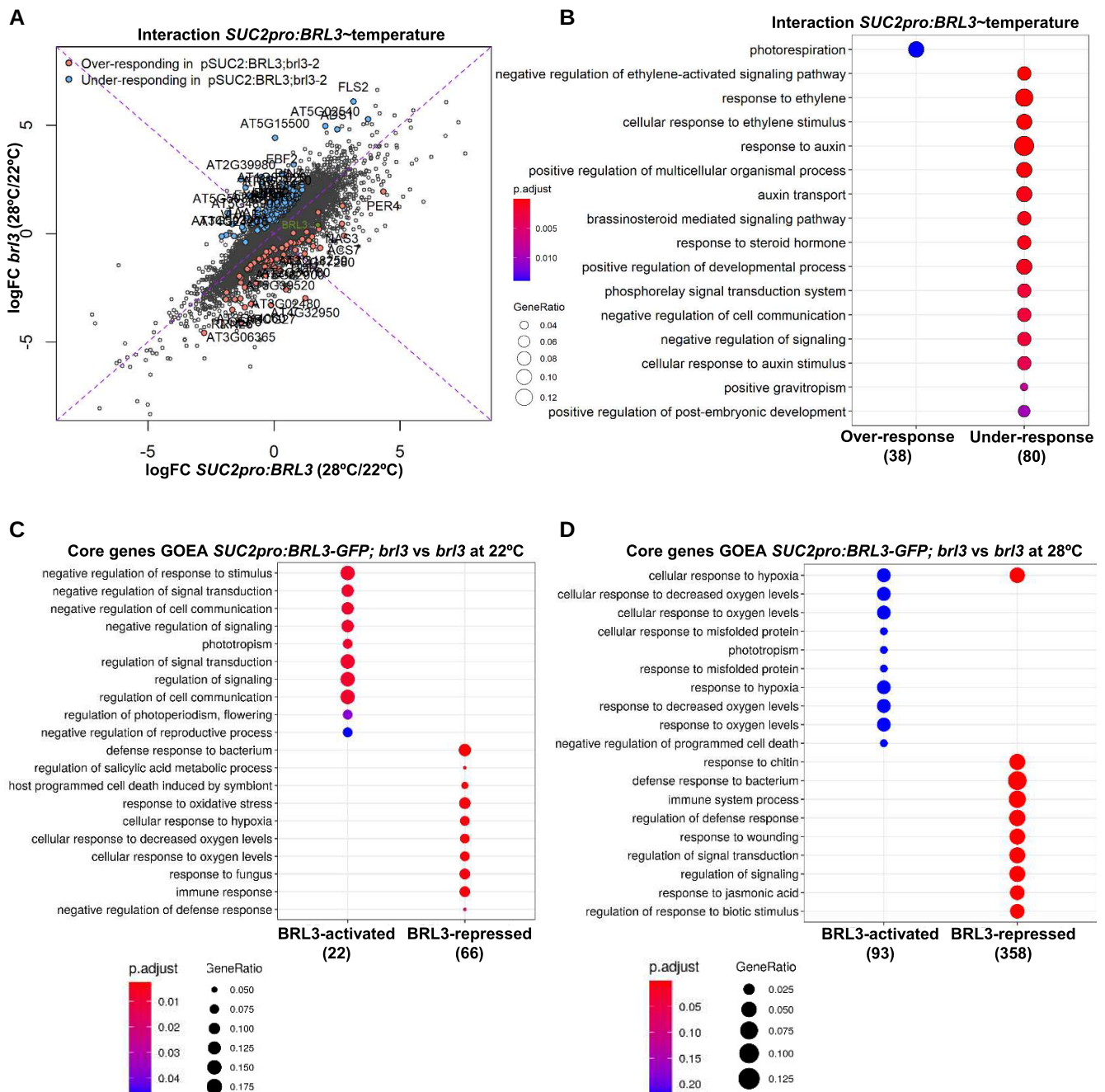


Figure 4. BRL3 signaling in the phloem companion cells modulates energy and photorespiration to control adaptive growth. **A)** Dot plot showing the comparison between transcript fold changes in *SUC2_{pro}:BRL3-GFP; brl3* (28 vs 22 °C) and *brl3* (28 vs 22 °C). Red or blue dots represent genes identified as affected by the genotype-temperature interaction in the linear model. **B)** Gene-ontology enrichment analysis of deregulated genes in the comparison affected by the interaction between *SUC2_{pro}:BRL3-GFP; brl3* and temperature, i.e. genes that are either over-responding or under-responding to temperature in the *SUC2_{pro}:BRL3-GFP; brl3* line with respect to the *brl3* mutant. The sizes of the nodes represent the number of annotated genes in a particular category and the color indicates its adjusted P-value upon enrichment test. **C** and **D)** Gene-ontology enrichment analysis of the core genes by separating the enriched categories among the BRL3-activated genes and BRL3-repressed genes in the comparison *SUC2_{pro}:BRL3-GFP; brl3* vs *brl3* at 22 and 28 °C. Core genes appear in the scatter plots present in [Supplementary Fig. S6, E and F](#).

the well-known BRI1 receptor (Caño-Delgado et al. 2004). Additionally, BRL3 physically interacts with known BRI1 interactors such as BAK1, BR-SIGNALING KINASE 1 AND 3 (BSK1 and BSK3) (Fàbregas et al. 2013), suggesting shared roles in the brassinosteroid-response pathway. The lack of any obvious phenotypic defect in *brl3* mutants under optimal growth conditions (Caño-Delgado et al. 2004) further supported a model of redundancy between BRL3 and BRI1, which has persisted for over 2

decades since the identification of BRLs. We found that *brl3* mutants show wild-type-like growth in favorable conditions (22 °C) but have obvious defects in hypocotyl- as well as petiole-elongation responses under elevated temperature (28 °C) (Fig. 1). By contrast, the *bri1* mutant had short hypocotyls irrespective of temperature in our experimental conditions. BRI1 degradation and subsequent inhibition of downstream signaling promotes primary-root elongation upon elevated temperatures

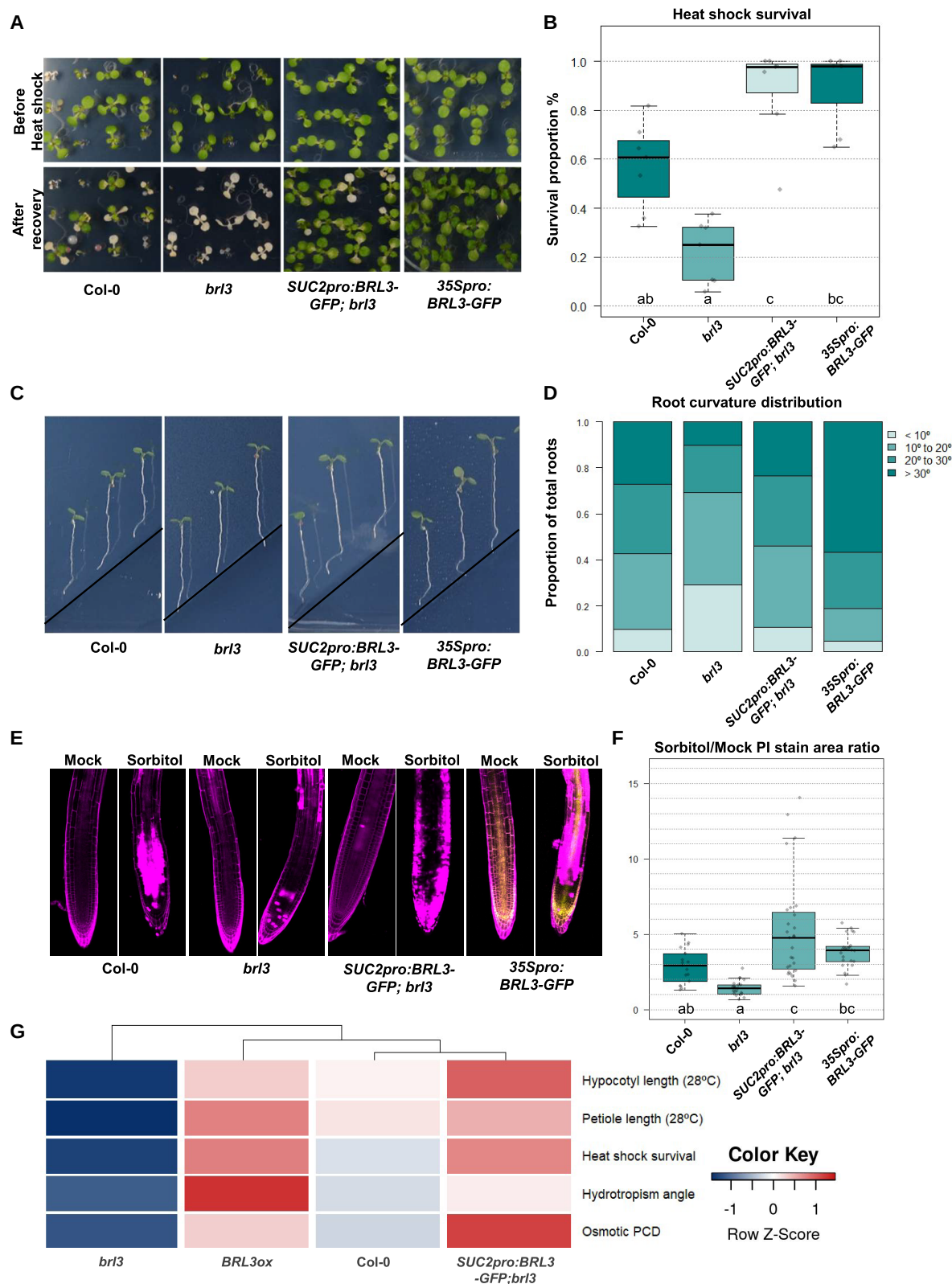


Figure 5. BRL3 signaling in the phloem companion cells promotes abiotic stress resistance and thermomorphogenesis. **A)** Seedling phenotypes and **B)** quantification of plant survival after heat-shock treatment (150 min at 42 °C) for wild-type (*Col-0*), *brl3*, *35Spro:BRL3-GFP*, and *SUC2pro:BRL3-GFP; brl3* seedlings. **C)** Representative pictures and **D)** measurements of root angle upon osmotic stress (270 mM sorbitol). Black bar in **(C)** depicts the angle in which the osmotic media is placed in the plate. **E)** Representative images and **F)** measurements of PCD in root cells upon osmotic stress (270 mM sorbitol), quantified as PI-stained root surface ratio. **G)** Stress trait matrix for physiological assays performed in this study on roots and shoots of *Col-0*, *brl3*, *35Spro:BRL3-GFP* and *SUC2pro:BRL3-GFP; brl3* lines. Color scale depicts values for scaled data. In all the conditions tested, phloem-enriched BRL3 expression was able to rescue stress-responsive defects of the *brl3* mutant. In **(B and F)**, boxplot boxes depict Q1, median, and Q3 of the data, while boxplot whiskers depict $\pm 1.5 \times$ interquartile range of each population. Points depict individual values. Letters depict statistical differences after a 1-way ANOVA followed by Dunn's post hoc test. In all panels, P -value < 0.05. Data assayed from >3 biological replicates.

(Martins et al. 2017). The *bri1-301* allele has been reported to be a temperature-sensitive kinase dead mutant, and shows defects in hypocotyl elongation in etiolated seedlings at elevated temperature (Zhang et al. 2018). Post-translational regulation such as SUMOylation has also emerged as a mechanism modulating BRI1 activity under stress conditions (Naranjo-Arcos et al. 2023), raising the possibility that similar regulation may influence BRL3's role during thermomorphogenic signaling. BRL3 was found to promote BES1 activity and regulate hypocotyl cell elongation during thermomorphogenic growth acclimation (Fig. 11). Transcriptome analysis further identified substantial overlap between BRL3-responsive genes and core cell-elongation-growth-related genes, particularly in terms of functional categories such as hormone homeostasis and abiotic stress responses (Fig. 2). This hints at coordinated signaling enabling plants to pre-adapt to rising temperatures. Unlike constitutive growth control exerted by BRI1 regardless of climate conditions (Xiang et al. 2021), our findings indicate that BRL3's role primarily contributes to adaptation to a range of adverse environments (Fàbregas et al. 2018).

The epidermis has been widely recognized as a key site for regulating plant organ growth (Savaldi-Goldstein et al. 2007; Procko et al. 2016; Kim et al. 2020), and recent work has highlighted the cortex as a layer where brassinosteroids promote root elongation (Nolan et al. 2023). Our findings expand this view by showing that BRL3, a vascular-enriched BR receptor expressed in a discrete set of cells including phloem companion cells (Salazar-Henao et al. 2016), plays a critical role not in general growth promotion but in the plant's ability to adapt to environmental stress, particularly elevated temperatures (Fig. 3).

Our transcriptomic analysis of phloem-enriched BRL3 activation shows a clear enrichment in genes related to energy metabolism and hormone regulation (Fig. 4). This indicates that BRL3 does not primarily drive general growth but instead reallocates internal

resources to support abiotic stress adaptation. Under high-temperature stress, BRL3 mobilizes energy and hormonal pathways that sustain cell elongation and enhance survival (Fig. 5). These findings position BRL3 as a spatially and functionally distinct BR receptor, specialized in coordinating abiotic stress responses rather than controlling baseline growth.

By acting from the phloem companion cells, BRL3 likely initiates signals that propagate outward to maintain growth across tissue layers under challenging conditions (Fig. 6). As broad manipulation of central growth regulators often leads to pleiotropic defects (Koorneef et al. 1985; Reid and Howell 1995; Hua and Meyerowitz 1998; Menand et al. 2002; Moore et al. 2003; Dharmasiri et al. 2005), the cell-type-specific function of BRL3 provides a promising entry point for precision strategies—whether via gene editing, transgenics, or molecular breeding—to engineer climate-resilient crops without compromising growth under optimal conditions.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis* (*Arabidopsis thaliana*) Col-0 seedlings were used as a control (N1092: NASC (Nottingham Arabidopsis Stock Centre number). Four T-DNA insertion-mutant alleles for BRL3 were used: *bri3-1* (Caño-Delgado et al. 2004) (20), *bri3-2* (SALK_006024C), *bri3-3* (SALK_079612) and *bri3-4* (SALK_111696) available from Arabidopsis Biological Resource Centre at Ohio State University, United States. The *bri3-2* (SALK_006024C) loss-of-function allele was used for all experiments described in this study unless specified. Seeds for *bri1-301*, *bak1-3*, *bri1*, *bri1 bri3*, *bri1 bri3 bak1*, *bri1 bri1 bri3 bak1*, BRI1 overexpression, BRL1 overexpression, BRL3 overexpression (35S_{pro}:BRL3-GFP) and *bes1-d* were obtained as previously described (Fàbregas et al.

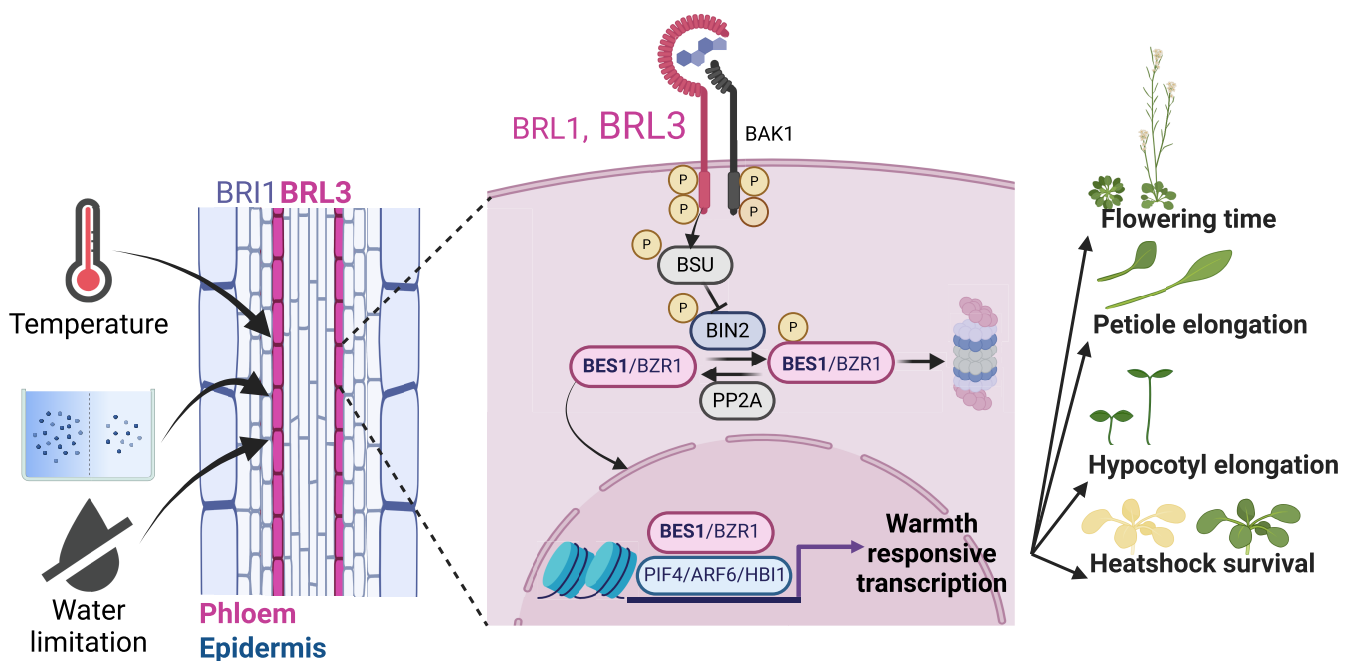


Figure 6. Vascular BR signaling via BRL3 regulates global transcriptome to control adaptive growth responses during adverse climate conditions such as increasing temperature and water limitation. Abiotic stress cues are sensed in the epidermis tissue. The signal is then relayed to the vascular tissue, where the brassinosteroid canonical pathway is activated in response, by heterodimerization of BAK1 with the BRL3 membrane receptor. The signaling pathway ends activating BES1/BZR1 transcription factors, which in turn modulate the expression of a plethora of genes (warmth responsive transcription) that enable thermomorphogenic adaptations in the plant body. Created in BioRender. <https://BioRender.com/9jbjny>.

2018). Double mutants *bri1-301 bri3* and *bri3 bes1-d* were created by genetic crossing. The complementation line was created by genetic crossing between a homozygous *BRL3_{pro}:BRL3-GFP* transgenic line (Fàbregas et al. 2013) and the *bri3-2* mutant. Primers used for genotyping are listed in Supplementary Table S1.

Arabidopsis thaliana seeds were surface-sterilized with 35% v/v NaClO for 5 min and washed 5 times for 5 min with sterile dH₂O. Sterilized seeds were stratified at 4 °C in darkness for 2 to 3 d. Seeds were placed on half-strength Murashige and Skoog medium with vitamins and without sucrose (0.5 MS-) solidified with 0.8% w/v agar. Plates were transferred to chambers with controlled growth conditions (16 h light/8 h dark; 22 °C temperature, 60% relative humidity and 50 to 70 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ of cool-white, fluorescent light).

Pharmacological treatments were performed in plates with hormones/drugs added to the media. Chemicals used were 1 μM BRZ₂₂₀ (T. Nakano, RIKEN, Japan) or 50 μM bikinin (200980, Sigma-Aldrich) for 6 d in control (long days, 22 °C), or 270 mM sorbitol (Duchefa, S0807) or high-temperature (long days, 28 °C) conditions. Hypocotyl length was measured using Image J software (v.1.48 v) (Schindelin et al. 2012) (<https://imagej.net/ij/>).

Phenotypic analysis of seedlings

For root growth analysis, seedlings were grown in vertical 0.5 MS-plates with 0.8% w/v agar in controlled growth conditions (Aralab 600; long days 16:8 h day/night cycle; 22 °C). MyROOT software (Betegón-Putze et al. 2019) was used to compare root growth. For thermomorphogenic hypocotyl-elongation analysis, seedlings were grown in horizontal 0.5 MS- media plates in controlled growth conditions (Aralab 600; long days 16:8 h day/night cycle 60% relative humidity and 70 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ of cool-white LEDs) at control (22 °C) or elevated (28 °C) temperature for 6 d. For the heat-shock assay, 7-d-old seedlings were subjected to a 150-min heat shock at 42 °C. Plants were moved back to control conditions for 5 d to check survival and recovery. To study the effect of elevated CO₂ conditions on seedling growth, seedlings were grown in horizontal 0.5 MS- plates in controlled growth conditions (Aralab 600; long days 16:8 h day/night cycle 60% relative humidity and 70 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ of cool-white LEDs) at ambient CO₂ (400 ppm) or elevated CO₂ (800 ppm) conditions for 6 d. For root hydrotropism, 5-d-old seedlings were placed on 0.5 MS-plates in which lower part of the media was replaced with 0.5 MS- media supplemented with 270 mM sorbitol to simulate a situation of reduced water availability and placed at a 45° angle to avoid a gravitropism effect. Root-curvature angles were measured and analyzed 24 h later using Image J. To assay cell death upon osmotic stress, 5-d-old seedlings grown on 0.5 MS- were shifted to either control or 270 mM sorbitol-containing media for 8 h. Roots were stained with propidium iodide (10 $\mu\text{g/mL}$, Sigma). Propidium iodide stains the cell wall and DNA in nuclei upon cell death. Images were acquired with a confocal microscope (FV1000, Olympus). Cell-death damage in primary roots was measured in a window of 500 μm from the quiescent center in the middle of root longitudinal sections (Image J). As an arbitrary setting to measure the stained area, a color threshold ranging from 160 to 255 in brightness was selected. Hypocotyl length was measured using ImageJ (v.1.48v) (Schindelin et al. 2012) (<https://imagej.net/ij/>).

Phenotypic analysis of adult plants

Two-week-old seedlings grown in 0.5 MS- plates were transferred individually to pots containing 30 $\pm$ 1 g of substrate (plus 1:8 v/v

vermiculite and 1:8 v/v perlite) in normal growth conditions (long days, 22 °C). For analysis of petiole-elongation responses to high temperatures, 2-week-old seedlings grown in 0.5 MS- plates were transferred individually to pots as above and left to acclimatize for 2 d under long days at 22 °C before moving to either control (long days, 22 °C) or high-temperature (long days, 28 °C) conditions. Petiole elongation in the longest rosette leaf was measured in 25-d-old plants using Image J (v.1.48v) (Schindelin et al. 2012) (<https://imagej.net/ij/>). For flowering-time analysis, plants were photographed, and the number of plants with >1 cm bolts were scored every day until all plants bolted and had flowers.

Plasmid construction and plant transformation

The *SUC2_{pro}:BRL3-GFP*, *WOL_{pro}:BRL3-GFP*, *WOX5_{pro}:BRL3-GFP*, *RPS5A_{pro}:BRL3-GFP*, *SCR_{pro}:BRL3-GFP*, *EXP7_{pro}:BRL3-GFP*, *GL2_{pro}:BRL3-GFP*, *NAC86_{pro}:BRL3-GFP*, *CALS8_{pro}:BRL3-GFP* and *SUC2_{pro}:BRI1-GFP* constructs were generated using a Gateway Multisite Cloning system (Invitrogen). Purified BRL3 and BRI1 PCR products were inserted by directional cloning into the Gateway pDONR221 donor vector (12536-017; Invitrogen) by BP reaction. The Gateway P4P1R vector containing cell-specific promoters *SUC2*, *WOL*, *WOX5*, *RPS5a*, *GL2*, *SCR*, *EXP7*, *CALS8* and *NAC86* were used as described in (Furuta et al. 2014; Marquès-Bueno et al. 2016; Ross-Elliott et al. 2017). All entry clones for cell/tissue-specific promoters are available at <http://www.ens-lyon.fr/DRP/SICE/Swelline.html>. A recombination LR reaction was performed using the 2 sequenced pENTRY vectors containing a specific promoter and the BRL3 coding sequence were subcloned into the modified pDEST gateway vector pR4pGWB604 (Nakamura et al. 2010) containing a GFP tag and NPT gene encoding resistance to phosphinothricin. All inserts were sequenced to verify sequence integrity. Constructs were introduced into Col-0 and *bri3* plants using *Agrobacterium tumefaciens* GV3101-mediated transformation by floral dipping (Clough and Bent 1998). Plants were genotyped by PCR isolated from genomic DNA (Aljanabi and Martinez 1997). GFP signals in different transgenic lines were observed and images were acquired with a confocal microscope (FV1000 Olympus and Zeiss LSM 980). Primers used for cloning, genotyping and sequencing are listed in Supplementary Table S1.

GUS staining

BRL3_{pro}:GFP-GUS reporter lines in the Col-0 background (Salazar-Henao et al. 2016) were used to analyze BRL3 promoter activity. The staining protocol was as follows: 6-d-old seedlings were immersed in ice-cold 90% (v/v) acetone and incubated for 20 min on ice. Acetone was rinsed twice with 1x PBS. Seedlings were then incubated with GUS staining solution [100 mM sodium phosphate buffer pH=7.2, 10 mM sodium EDTA, 0.1% v/v Triton X-100, 1 mg/mL 1.5-bromo-4-chloro-3-indolyl-beta-D-glucorinase (X-gluc; Duchefa, Netherlands), 10 mM potassium ferrocyanide and potassium ferricyanide] and incubated at 37 °C overnight in darkness. Seedlings were rinsed twice with 1x PBS and left overnight in 70% v/v ethanol. Samples were mounted on microscope slides and cover slips with chloral hydrate and observed at the following day under an epifluorescence vertical microscope (Leica DM6).

RT-qPCR

RNA was extracted from 6-day-old seedlings using the Maxwell Plant RNA Kit for Maxwell RSC instruments. Seedlings were collected, snap-frozen and ground to a powder using mortar and pestle. RNA was extracted following manufacturer instructions.

CDNA was prepared from 1 µg of RNA using iScript CDNA Synthesis Kit from Bio-Rad. qPCRs were performed loading 20 ng of CDNA per well and using a Bio-Rad CFX96 system. 18S gene (AT2G01010) was used as reference gene for normalizing relative expression using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). Primers are listed in Supplementary Table S1.

Western blot analysis

Five-day-old seedlings grown on 0.5 MS- plates were transferred to either 22 or 28 °C conditions for 24 h. Forty entire seedlings of 6-d-old seedlings were then collected and ground into powder in liquid nitrogen. Protein extraction buffer (50 mM Tris-Cl pH 6.8, SDS 10%, 5% v/v glycerol) supplemented with 5% v/v beta mercaptoethanol and 1× protease inhibitor cocktail (Roche) was added to each sample followed by centrifugation at 18,000×g at 4 °C for 10 min. The resulting supernatants were transferred to a new microfuge tube and 8 µL of 6× Laemmli buffer was added to the protein samples to be loaded (40 µL) and boiled at 95 °C for 3 min. SDS-PAGE was performed to resolve the protein extracts and equal loading was determined by Ponceau staining of membranes. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore) using the wet-transfer method (Bio-Rad) and immuno-detected with primary antibodies recognizing BES1 (Yu et al. 2011) and histone H3 (NEB). Membranes were then incubated with Anti-rabbit IgG HRP conjugated secondary antibody (Sigma) and the result was revealed with SuperSignal West PICO (Thermo-Fisher).

RNA-Seq

For RNA-seq analysis, shoot tissue was collected from 6-d-old seedlings (long days, 22 °C) and high-temperature (long days, 28 °C) conditions. Two biological replicates were collected per genotype per experimental condition. RNA was extracted with the Plant Easy Mini Kit (Qiagen) and quality checked using a Bioanalyser. Stranded cDNA libraries were prepared with TruSeq Stranded mRNA kit (Illumina). Paired-end sequencing, with 75-bp reads, was performed in with Illumina HiSeq500 sequencer at a minimum depth of 32 M. Reads were trimmed 9 bp at their 3' ends and quality filtered using Trimmomatic. Remaining reads were mapped against the TAIR10 genome with HISAT2. Mapped reads were quantified at the gene level with HtSeq using an Araport11 annotation file. Counts on chloroplast genes were eliminated prior to analysis due to an artifact. Raw counts were TMM-normalized and differential expression calculated with the edgeR R package. To identify genes with a different temperature response between genotypes, a linear model accounting for the temperature-genotype interaction was applied. Values for the interaction term were evaluated in such comparisons. For pairwise comparisons, results were filtered for adjusted P-value (FDR) <0.05 and FC >|1.5|. Raw reads and processed counts can be accessed at GEO under accession GSE190107. Comparisons and enrichment testing with brassinosteroid-regulated genes (Goda et al. 2004; Nemhauser et al. 2004) were done by a 2-sided Fisher's exact tests.

Statistical analyses

In quantitative measurements of plants, data distribution was tested for normality by Shapiro-Wilk test before statistical analysis. Data was compared by 1-way ANOVA or 2-way ANOVA coupled to specific post hoc tests, as required. The specific tests used are stated under each figure legend. A P-value threshold of <0.05 was used in all analyses. Barplot analysis depict mean ±

standard error of the mean of each population. Boxplot boxes depict Q1, median and Q3 of the data, while boxplot whiskers depict $\pm 1.5 \times \text{IQR}$ of each population. Points depict individual values. Letters were used to depict statistically significant different populations after analysis. For statistical analysis and plot deployment, R version 4.0.3 along with RStudio packages “HH”, “agricolae” and “multicompView” were used, as well as other utilities generated by the authors and available in GitHub.

Accession numbers

Sequence data from this article can be found in the GEO data libraries under accession numbers GSE190107. The accession numbers of relevant genes mentioned in this study are: BRL3 (AT3G13380), BRL1 (AT1G55610), BRI1 (AT4G39400), BAK1 (AT4G33430), BES1 (AT1G19350). And the accession numbers of genes whose promoters have been used to generate lines in this study are: SUC2 (AT1G22710), WOL (AT2G01830), CALS8 (AT3G14570), NAC86 (AT5G17260), WOX5 (AT3G11260), SCR (AT3G54220), GL2 (AT1G79840), EXP7 (AT1G12560), RPS5a (AT3G11940).

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Author contributions

Conceptualization: A.I.C.-D, A.G.. Methodology: A.G., A.R.-M., F.L.-E., M.-M.-B. Investigation: A.G., A.R.-M., F.L.-E., M.M.-B. Visualization: A.G., F.L.-E., M.M.-B., A.R.-M., J.B.F.-M. Funding acquisition: A.I.C.-D. Project administration: A.I.C.-D. Supervision: A.I.C.-D. Writing—original draft: A.I.C.-D, A.G., A.R.M. Writing—review & editing: A.I.C.-D, A.G., F.L.-E., M.M.-B., A.R.-M., J.B.F.-M.

Supplementary data

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

Supplementary Figure S1. Alleles of *brl3* and native complementation of thermomorphogenesis defects.

Supplementary Figure S2. Brassinosteroid receptor mutants and BR pathway control of thermomorphogenic response.

Supplementary Figure S3. Transcriptome analysis of *brl3* mutants by RNA-seq.

Supplementary Figure S4. BRL3 expression analysis and mutant phenotype complementation from transgenic expression in phloem companion cells.

Supplementary Figure S5. Local BRI1 expression from phloem companion cells cannot rescue *brl3* thermomorphogenic defects.

Supplementary Figure S6. BRL3 signals from phloem companion cells regulate plant adaptation to multiple climate stress.

Supplementary Table S1. List of primers used in this study.

Supplementary Data S1. List of deregulated genes Col-0 vs *brl3* at 22 °C.

Supplementary Data S2. List of genes with a different temperature response between *brl3* and Col-0 (genotype*treatment interaction-affected genes).

Supplementary Data S3. List of deregulated genes *SUC2_{pro}*: BRL3-GFP; *brl3* vs *brl3* at 22 °C.

Supplementary Data S4. List of genes with a different temperature response between *SUC2_{pro}*:BRL3-GFP; *brl3* and *brl3* (genotype*treatment interaction-affected genes).

Supplementary Data S5. List of differentially expressed genes overlapping between comparisons *brl3* vs. Col-0 and *SUC2^{pro}*: BRL3-GFP; *brl3* vs *brl3* at 22 and 28 °C in opposite directions (core genes).

Supplementary Data S6. Brassinosteroid-regulated genes that are BRL3-regulated from the phloem.

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Conflict of interest statement

The authors have filed a patent (EP 21 382 498.0.) on BRL3 function in the phloem in climate adaptation currently licensed to PLANET BIOTECH S.L. They declare no other competing interests.

Data availability

All data are available in the main text or the [Supplementary materials](#).

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