The membrane-localized protein kinase MAP4K4/TOT3 regulates thermomorphogenesis

Plants respond to mild warm temperature conditions by increased elongation growth of organs to enhance cooling capacity, in a process called thermomorphogenesis. To this date, the regulation of thermomorphogenesis has been exclusively shown to intersect with light signalling pathways. To identify regulators of thermomorphogenesis that are conserved in flowering plants, we map changes in protein phosphorylation in both dicots and monocots exposed to warm temperature. We identify MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE4 (MAP4K4)/TARGET OF TEMPERATURE3 (TOT3) as a regulator of thermomorphogenesis that impinges on brassinosteroid signalling in Arabidopsis thaliana. In addition, we show that TOT3 plays a role in thermal response in wheat, a monocot crop. Altogether, the conserved thermal regulation by TOT3 expands our knowledge of thermomorphogenesis beyond the well-studied pathways and can contribute to ensuring food security under a changing climate.
Almost every organism is exposed to variation in temperature, on a daily and on a seasonal basis. This is especially true for plants that, as sessile organisms, need to continuously alter their growth, development and physiology in response to temperature variation\textsuperscript{1–3}. To sense and respond to temperature changes, several molecular sensors and downstream signalling and response networks have evolved\textsuperscript{4}. Despite that our knowledge of temperature perception and response in plants has increased in recent years, research mainly focussed on transcriptional regulation. Hence, we still know relatively little about the cellular signalling cascades that control architectural adaptations to high ambient temperatures (referred to as thermomorphogenesis). Thermomorphogenesis is characterized by traits such as upward leaf movement (thermonasty) and petiole and hypocotyl elongation in dicots, such as Arabidopsis thaliana, cabbages and tomatoes\textsuperscript{5–7,12–14}. The resulting open rosette architecture improves the cooling capacity in unfavourable warm-temperature conditions within the physiological range\textsuperscript{7,12–14}. In monocots, mild warm-temperatures typically affect growth rate, including leaf elongation and the length of leaf internodes\textsuperscript{8–11}.

In A. thaliana, the basic helix-loophelix transcription factors PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PIF7 are central and required regulators of warm-temperature-mediated elongation growth\textsuperscript{12–14}. Two upstream negative regulators of PIF4, namely the red light receptor phytochrome B (phyB) and the circadian clock protein EARLY FLOWERING 3 (ELF3), are thermosensors that perceive temperature information directly\textsuperscript{15,16}. High temperature promotes the dark reversion of active nuclear-localized phyB into the cytosolic inactive form and at the same time induces reversible liquid–liquid phase separation of ELF3. In parallel, an RNA thermoswitch controls the translation of PIF7 independently from thermosensory pathways regulating PIF4\textsuperscript{14}. Nevertheless, both PIF4 and PIF7 dimerize and are functionally dependent on each other to regulate the transcription of a set of common genes, including auxin biosynthesis genes, such as YUCCA8 (YUC8), and other auxin-responsive genes that are required for thermomorphogenesis\textsuperscript{13,14}. In addition to phytochromes, blue light and ultraviolet-B light perceived by cryptochrome 1 (CRY1) and UV RESISTANCE LOCUS 8 (UVR8), respectively, inhibit PIF4 activity to negatively regulate thermomorphogenesis\textsuperscript{17,18}. Altogether, PIF4 is an important signalling hub that integrates thermomorphogenesis with different light signalling components.

Considering the diverse effects of high temperature on multiple molecular components, it is likely that PIF4- and PIF7-dependent pathways are not the only thermomorphogenic pathways in Arabidopsis. Many cellular signalling events are mediated by protein phosphorylation, including plant responses to biotic and abiotic stresses\textsuperscript{19}. For example, during response to freezing, the stability of the transcription factor INDUCER OF CBF EXPRESSION 1 (ICE1) is regulated by phosphorylation by multiple protein kinases, including a MITOGEN-ACTIVATED PROTEIN (MAP) kinase cascade. In contrast to freezing response\textsuperscript{20,21}, little is known about phosphorylation events that regulate thermomorphogenesis. In this study, we explore the cellular high-temperature-responsive phosphorylation landscape using a phosphoproteomics approach. Hereby, we identified TARGET OF TEMPERATURE 3 (TOT3), a MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE KINASE (MAP4K), which controls warm-temperature-responsive growth in plants in conjunction with two closely related MAP4Ks. Importantly, we demonstrate that there is a signalling pathway regulating thermomorphogenesis independently from PIF4 and light-signalling pathways, and this pathway requires TOT3 activity. We also show that TOT3 impinges on brassinosteroid-mediated growth control under warm temperature. Finally, we provide evidence that the function of TOT3 as a regulator of high-temperature-mediated growth responses is conserved in monocots.

**Results**

**Phosphoproteomics reveals conserved thermoresponsive phosphorylation events.** Reversible and dynamic phosphorylation of proteins is essential for many cellular signalling cascades. We therefore analysed the phosphoproteome of A. thaliana Col-0 seedlings transferred from control (21 °C) to warm temperature (27 °C) (Fig. 1a). As expected, this relatively high ambient temperature induced the open rosette architecture, typical for thermomorphogenesis (Fig. 1b). To capture early and dynamic changes in protein phosphorylation status, we sampled before (0 min) and at 12, 24 and 60 min after exposure to 27 °C (Fig. 1a). This time frame aligned with the up-regulation of HSP70 expression, a transcriptional marker associated with temperature perception status\textsuperscript{22} (Supplementary Fig. 1). The phosphoproteome data subjected to a multiple-sample comparison between the time points revealed 212 differentially regulated phosphosites, which mapped to 180 functionally diverse proteins (Supplementary Fig. 2a and Supplementary Data 1). We manually included 14 additional phosphosites, which mapped to 12 proteins, to our selection, as these were not detected for at least one time point and thus not part of the multiple-sample comparison (Supplementary Data 1).

Next, to identify proteins involved in thermomorphogenesis and temperature perception and signalling that are regulated in both monocot and dicot plants, we compared the A. thaliana dataset with similar phosphoproteome datasets from wheat leaves and spikelets\textsuperscript{23}, and from soybean leaves exposed to increased temperature for 60 min (Supplementary Fig. 2b and Supplementary Data 2–4). This comparison revealed 42 putative orthologues that were also differentially phosphorylated in soybean or wheat and we named these TARGETS OF TEMPERATURE (TOTs) (Fig. 1c and Supplementary Data 1).

**TOT3 encodes a plasma membrane-localized MAP4K4.** Although temperature perception in many organisms, such as (cyanobacteria), occurs in the plasma membrane\textsuperscript{24,25}, warm-temperature-sensing mechanisms residing in the plasma membrane are largely unknown in plants. We therefore prioritized TOTs that were predicted to be membrane-associated, and subsequently focused on TOT3 as the only candidate that contains a protein kinase domain (Fig. 1c, Supplementary Fig. 4a and Supplementary Data 1). TOT3 encodes MAP4K4, a conserved member of a clade of protein kinases referred to as the MAP4K family\textsuperscript{26} (Supplementary Fig. 3). The MAP4K family is evolutionarily conserved and related to yeast Ste20\textsuperscript{27}, and MAP4Ks can activate MAPK cascades or phosphorylate diverse substrates\textsuperscript{28}. In Arabidopsis, BLUE LIGHT SIGNALING1 (BLUS1)/MAP4K10 functions downstream of phototropins to control stomatal opening\textsuperscript{28}. In addition, both TOT3/MAP4K4 and SIK1/MAP4K3 were shown to play a role in ROS production during plant immunity response\textsuperscript{29,30}. The expression of TOT3 remained stable during a warm-temperature time course (Supplementary Fig. 4b), indicating that regulation of the transcription of TOT3 cannot explain its effect on thermomorphogenesis. Next, we confirmed that at least a part of GFP:TOT3 is indeed plasma membrane-localized in the hypocotyl, in cotyledons and in the primary root meristem (Fig. 1d, e and Supplementary Figs. 5 and 6).

**TOT3 is important for thermoresponsive growth in plants.** Next, we genetically explored a role for TOT3 in thermoresponsive growth in plants. Two independent A. thaliana mutant lines carrying alleles lacking full-length TOT3 transcript (tot3-1 and tot3-2) displayed a significantly shorter hypocotyl at 28 °C when compared to Col-0; but showed no significant differences at 21 °C (Fig. 2a, b and Supplementary Figs. 7 and 8). In
contrast, we did not detect any obvious differences with respect to flowering time, another hallmark of warm-temperature responsiveness, at 21 or 28 °C (Supplementary Fig. 9). A pTOT3::GFP:TOT3 construct complemented the tot3-2 hypocotyl phenotype at 28 °C (Fig. 2c, d), confirming that TOT3 activity is required for warm-temperature-mediated elongation and that the GFP:TOT3 fusion is functional.

Since we also identified differential phosphorylation of a TOT3 orthologue in wheat, we investigated whether regulation of thermomorphogenesis by TOT3 is functionally conserved. We identified Cadenza wheat TILLING lines with a premature stop codon in the coding sequence (CDS) of the three wheat TOT3 homeologues (Fig. 3a). Little is known about thermomorphogenesis in monocots, but wild-type Cadenza wheat seedlings displayed high-temperature-triggered growth promotion of the leaf sheath when grown at 24 °C compared to 14 °C (Supplementary Fig. 11a). Interestingly, Cadenza wheat TILLING lines with mutations in the wheat TOT3 gene TraesCS7D02G232400 showed a significantly shorter second leaf sheath at 24 °C compared to 14 °C (Fig. 3b, c), whereas those with mutations in the homeologous TraesCS7A02G232300 and TraesCS7B02G130700 genes did not show any significant difference (Supplementary Fig. 11b, c). In contrast, a temperature increase up to 34 °C repressed wheat seedling growth (Supplementary Fig. 12). However, tot3 mutant wheat seedlings were more stunted than wild-type plants at 34 °C (Supplementary Fig. 12). Taken together, our data support that TOT3 is a conserved regulator of thermoresponsive growth in plants.

Thermomorphogenesis in Arabidopsis requires TOT3 kinase activity. Since some MAP4Ks can act as molecular adaptors rather than as bona fide kinases, we evaluated if the TOT3 kinase domain and its kinase activity are required for its function. TOT3 protein variants that lacked the kinase domain (3xHA:TOT3309–674) or in which aspartate 157 in the conserved DFG motif of the kinase domain was replaced by asparagine (GFP:TOT3D157N), which abolishes kinase activity, could not rescue the tot3-2 short hypocotyl phenotype at 28 °C (Supplementary Figs. 13 and 14a, b). In addition, MBP-TOT3WT-6xHIS
recombinantly expressed in Escherichia coli showed autophosphorylation in vitro while MBP-TOT3D157N-6xHIS did not, indicating that TOT3 is an active kinase and that the D157N mutation abolishes its activity (Supplementary Fig. 14c). This supports that the TOT3 kinase domain and activity are indeed required for TOT3-mediated growth at warm temperature.

Fig. 2 TOT3 is required for thermomorphogenesis in Arabidopsis. a, b Hypocotyl length of 7-day-old Col-0 wild-type and loss-of-function tot3-1, tot3-2 under short-day conditions at 21 and 28 °C (33 ≤ n ≤ 44). c, d Hypocotyl length of 7-day-old tot3-2 plants complemented with pTOT3::GFP:TOT3 (three independently transformed lines: #5.4, #6.8 and #7.4) under short-day conditions at 21 and 28 °C. Representative pictures (a, c) and hypocotyl length quantification (b, d). Scale bar, 5 mm. Box plots show median with Tukey-based whiskers and outliers. The number of individually measured seedlings (n) is indicated above the X-axis. Fold-change is indicated for each genotype. Letters indicate significant differences based on two-way ANOVA and Tukey’s test (p < 0.01). The p-value for the interaction (genotype × temperature) is shown at the top.

TOT3 regulates thermomorphogenesis independently of phyB and PIF4. Next, we evaluated the genetic interaction between TOT3 and known temperature-signalling components, and hypocotyl growth regulators phyB and PIF4. While tot3-2 and phyb-9 displayed a short and long hypocotyl compared to Col-0 at 28 °C, respectively, the tot3-2 phyb-9 double mutant has an intermediate phenotype (Fig. 4a, b), suggesting that TOT3 and phyB signalling have additive effects on thermomorphogenesis. On the contrary, the pif4-101 tot3-2 double mutant showed significantly less increase in hypocotyl length than either single mutant at 28 °C, pointing to a possible additive effect of both factors (Fig. 4c, d). Next, the expression of a core set of temperature-responsive genes, including PIF4 and its two downstream target genes YUC8 and ATHB2, which are involved in thermomorphogenesis and act downstream of phyB, was assessed. Although some of these genes displayed a slightly lower expression level in tot3-2, their temperature-triggered up-regulation was not significantly affected (Fig. 4e). The observation that transcript levels of YUC8 (a rate limiting enzyme in warm-temperature-induced auxin biosynthesis) and of IAA29 (an auxin response gene involved in hypocotyl growth) were not or hardly affected in the tot3-2 mutant background (Fig. 4e and Supplementary Fig. 15a) suggests that auxin responses are largely not affected by TOT3. This was confirmed by pharmacological application of picloram, a synthetic auxin that promotes hypocotyl growth in Col-0. Picloram triggered hypocotyl elongation in tot3-2 to a comparable extent as in Col-0 at control temperature (21 °C) (Supplementary Fig. 15b), further indicating that auxin signalling is not perturbed in the tot3-2 mutant.

Both phyB and PIF4 are light signalling components that are, in addition to thermomorphogenesis, also involved in other responses, such as shade avoidance. The observation that tot3 hypocotyl length at 21 °C is similar to that of Col-0 (Fig. 2a, b) may indicate that TOT3 is not required for hypocotyl growth in the absence of a warm-temperature cue. Indeed, hypocotyl length of tot3 at 21 °C in the presence of a low red-to-far-red (R/Fr) light ratio, a typical canopy shade signal that leads to elongated hypocotyls, is similar to wild-type (Supplementary Fig. 15c). Next, we examined thermo-responsive hypocotyl growth in darkness at 21 and 28 °C. During etiolation, the activity of PIFs should be maximized due to the lack of active phytochromes in the absence of light. Strikingly, in this setup, we observed a similar increase in hypocotyl length of both pif4-101 and Col-0 at 28 °C, compared to 21 °C in darkness (Fig. 4f, g). In contrast, hypocotyl...
length of *tot3-2* seedlings remained the same at both 21 and 28 °C in darkness (Fig. 4f, g and Supplementary Fig. 15d). Furthermore, *pif4-101 tot3-2* seedlings exhibited a similar phenotype as the *tot3-2* mutant. This observation agrees with the fact that the transcript levels of *PIF4* and its targets were not increased at 28 °C compared to 21 °C in both Col-0 and *tot3-2* plants in darkness (Fig. 4h). This contrasts with what has been routinely observed in other light regimes36 (Fig. 4e). In conclusion, our results indicate that TOT3 defines a temperature-signalling pathway controlling hypocotyl thermomorphogenesis independent from the light signalling components phyB and PIF4.

**TOT3 interacts with MAP4K6/TOI4 and MAP4K5/TOI5 to regulate thermomorphogenesis.** Next, we explored (direct) downstream targets and interacting proteins of TOT3, to position TOT3 in a cellular signalling cascade triggering warm-temperature-induced growth. We therefore performed a protein–protein interaction study using tandem affinity purification (TAP) on GShino::TAP TOT3 expressed in an *A. thaliana* cell culture. The interactome revealed 23 TOT3-INTERACTING proteins (TOIs), including MAP4K5, MAP4K6, MAP4K7, MAP4K8 and MAP4K9, which are all MAPK4Ks closely related to TOT3 (Fig. 5a, Supplementary Fig. 3 and Supplementary Data 4). A subsequent co-immunoprecipitation experiment using pTOT3::GFP:TOT3-expressing *tot3-2* seedlings confirmed the interactions between TOT3 and TOI4/MAP4K6 or TOI5/MAP4K5 and showed that these interactions were temperature-independent (Fig. 5a and Supplementary Data 5). The TOT3–TOI4/5 interaction was further confirmed by yeast two-hybrid (Y2H) analyses (Fig. 5b). Although stress-related gene ontology terms were enriched among potential interactors identified in the TAP and GFP pull-down experiments (Supplementary Data 5), the above-described protein–protein interaction datasets did not identify an

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**Fig. 3 TOT3 is required for thermomorphogenesis in wheat.** a Position of stop codons (*) in wheat TILLING lines Cadenza1111 (Q233*), Cadenza0743 (Q191*), Cadenza1716 (W122*), Cadenza0256 (Q191*) and Cadenza0235 (W203*) in protein models for three wheat TOT3 homeologues. b, c Length of the visible leaf sheath (in those cases where the second leaf has not emerged, this leaf sheath is underneath the one of the first leaf) in homozygous F3 wild-type (WT) and *tot3* two-week old Cadenza wheat seedlings (all lines were backcrossed once with WT Cadenza and WT plants were selected from the backcrossing with each Cadenza TILLING line) grown at 14 and 24 °C in long-day conditions (16 h light/ 8 h dark). Representative pictures (b) and leaf sheath length quantification (c). Scale bar, 2 cm. Blue overlay in (b) marks the leaf sheath. Box plots show median with Tukey-based whiskers and outliers. The number of individually measured seedlings (n) is indicated above the X-axis. The respective quantification letters indicate significant differences based on two-way ANOVA and Tukey’s test (p < 0.01); n.s: not significant (c). The p-value for the interaction (genotype × temperature) is shown at the top.
obvious signature associating it with known (temperature) signalling pathways. Nevertheless, a role for TOI4 and TOI5 in TOT3-mediated warm-temperature signalling is indirectly supported by phosphoproteome data, which revealed that TOI4 and TOI5 phosphopeptides were less abundant in \textit{tot3-2} at 28 °C compared to Col-0 (Fig.5a, c and Supplementary Data 6). Furthermore, warm-temperature-induced changes in phosphorylation status of TOI4 and TOI5 orthologues were also observed in soybean and wheat23 (Supplementary Fig. 17). Although TOI4 and TOI5 expression levels are not affected in \textit{tot3} (Supplementary Fig. 16), we cannot rule out that TOI4 and TOI5 protein levels might be affected in \textit{tot3-2} and/or that the stability of TOI4 and TOI5 in a complex might be higher than that of the individual proteins.

Next, to further explore TOT3-mediated phosphorylation of TOI4/5, we transiently co-expressed the kinase-dead RFP-TOI4D188N and RFP-TOI5D174N with wild-type TOT3 or the kinase-dead TOT3D157N in tobacco leaves and analysed the phosphorylation status of the immunoprecipitated RFP-TOI4D188N and RFP-TOI5D174N. An increased phosphorylation at S499, S516 and S654 on TOI5D174N was observed when co-expressed with wild-type TOT3, compared to the kinase-dead TOT3D157N (Supplementary Fig. 18 and Supplementary Data 7). However, no differences in phosphorylation were observed for the kinase-dead TOI4D188N (Supplementary Fig. 18 and Supplementary Data 6). In addition, in vitro phosphorylation analysis of recombinant kinase-dead TOI4 D188N or TOI5 D174N proteins showed no difference between incubation with wild-type TOT3 or with kinase-dead TOT3D157N (Supplementary Data 8). This suggests that TOT3, at least in vitro, does not directly phosphorylate TOI4 and TOI5, and that phosphorylation of TOI4 and TOI5—although both interact with TOT3—is likely...
controlled in planta by an unknown regulator. Future analyses will have to reveal the role of the direct interaction between TOT3 and TOI4/5 in thermoresponsive growth.

To test for an anticipated role for TOI4 and TOI5 in temperature signalling, we generated loss-of-function toi4 and toi5 mutants (Supplementary Fig. 19), as well as various double-mutant combinations with toi3. Both toi4-1 and toi5-2, as well as the toi4-1 toi5-2 double mutant, displayed similar warm-temperature-induced hypocotyl elongation at 28 °C under short-day conditions as Col-0 (Fig. 5d, e). Furthermore, tot3-2 toi5-2 plants exhibited only a slight difference in thermomorphogenic hypocotyl length compared to tot3-2 plants, whereas the response is largely abolished in tot3-2 toi4-1 plants (Fig. 5d, e). These observations seem to emphasize a dominant genetic role of TOT3 in the thermomorphogenic pathway, probably in conjunction with TOI4, whereas TOI5 may play a minor role. However, when grown in darkness at 28 °C, toi4-1 toi5-2 had a slightly shorter hypocotyl than the wild-type (Supplementary Fig. 20). Since tot3-2 has an even shorter hypocotyl when grown in darkness at 28 °C (Supplementary Fig. 20), there is possibly genetic redundancy with other MAP4Ks or, alternatively, other TOIs are involved. Nevertheless, our data position TOT3 as a central regulator of temperature-mediated growth.

**TOT3 impinges on brassinosteroid signalling.** We also observed that at 28 °C several brassinosteroid signalling components were less phosphorylated in toi3-2 compared to Col-0 (Supplementary Fig. 21). Given the prominent role of brassinosteroid signalling in controlling thermomorphogenesis, we explored a possible link between TOT3 and this phytohormone. Despite the lower phosphorylation levels of several upstream brassinosteroid signalling components, such as BSU1, BSK7 and BSK8, tot3-2 did not affect the brassinolide-induced accumulation of non-phosphorylated BES1, a hallmark for brassinosteroid signalling activation, at 28 °C compared to wild-type (Fig. 6a and Supplementary Fig. 22). Treatment with brassinolide increases hypocotyl length at 21 °C similarly in Col-0 and tot3-2 (Fig. 6b). In our hands, however, the same brassinolide treatment resulted in a shorter hypocotyl at 28 °C (Fig. 6b), in contrast to previously reported results. This can be due to differences in the experimental setup and/or the different source or concentration of brassinolide (see “Methods”), but both growth promoting and repressing activities are not uncommon for a hormone. Nevertheless, a strong reduction in hypocotyl length in the presence of brassinolide at 28 °C was not observed in toi3-2 plants (Fig. 6b), suggesting that TOT3 might impact brassinosteroid signalling.

We next focused on BZR1, one of the major transcription factors regulated by brassinosteroid signalling that is required for thermomorphogenesis. The *bzip1-1D* gain-of-function mutant...
**Fig. 6** TOT3 impinges brassinosteroid signalling pathway.  

**a** Representative western blot (of 3 biological replicates) of BES1 phosphorylation status in 4-day-old Col-0 and tot3-2 seedlings grown on mock (DMSO) or 100 nM brassinolide (BL) at 28 °C under short-day conditions. BES1 was detected with anti-BES1 antibody. BES1-P, phosphorylated BES1; BES1, dephosphorylated BES1. Detection of TUBULIN (TUB) was used as a loading control.

**b** Hypocotyl length of 5-day old tot3-2 and Col-0 wild-type plants grown at 21 and 28 °C in short-day conditions on medium containing DMSO or 50 nM brassinolide.  

**c** Hypocotyl length of 4-day old tot3-2, bzr1-1D, tot3-2 bzr1-1D and Col-0 wild-type seedlings grown at 21 and 28 °C in darkness on MS/2 medium containing DMSO (c) or 1 µM brassinazole (d).  

**d** Hypocotyl length of 4-day old tot3-2, bzr1-1D, tot3-2 bzr1-1D and Col-0 wild-type seedlings grown at 21 and 28 °C in darkness on MS/2 medium containing DMSO (c) or 1 µM brassinazole (d).  

**e** Relative expression of BZR1 target genes PRE6, PAR1 and PAR2 in Col-0 and tot3-2 plants grown at control (21 °C) and warm temperature (28 °C) under short-day conditions.

**f** Relative expression of BZR1 target genes PRE6, PAR1 and PAR2 in Col-0 wild-type and tot3-2 plants grown at control (21 °C) and warm temperature (28 °C) under dark conditions. Box plots show median with Tukey-based whiskers and outliers. The number of individually measured seedlings (n) is indicated above the X-axis (b, c, d). Bar diagram shows mean of 3 biological replicates (individual dots) with standard error of the mean (e, f). Letters indicate significant differences based on two-way ANOVA and Tukey's test (p < 0.01); n.s: not significant. The p-value for the interaction (genotype × temperature or genotype × treatment) is shown at the top.
largely suppresses brassinosteroid-deficient and brassinosteroid-insensitive phenotypes and shows a BR hyperresponsive phenotype, for example, during etiolation in darkness44,45. However, the bzr1-1D mutation was not able to rescue the short tot3-2 hypocotyl in the tot3-2 bzr1-1D double mutant at 28°C in darkness (Fig. 6c). Treatment with brassinazole, an inhibitor of brassinosteroid biosynthesis, largely suppressed hypocotyl growth of both Col-0 and tot3-2 seedlings at both 21 and 28°C in darkness (Fig. 6d). However, while the bzr1-1D mutant largely rescued this phenotype, the effect of bzr1-1D is significantly dampened in the tot3-2 bzr1-1D mutant (Fig. 6d). Taken together, these results suggest that TOT3 affects BZR1-mediated brassinosteroid responses, possibly through regulating active BZR1.

To further explore this possible explanation, we assessed the expression of genes that are directly regulated by BZR1 in the tot3-2 mutant: PACLOBUTRAZOL RESISTANCE1 (PRE1), PRE5, PRE6, PHY RAPIDLY REGULATED 1 (PAR1) and PAR2.56-58. In nearly all cases, the 28°C-mediated change in transcript levels is different in tot3-2 compared to Col-0 under short-day conditions or in darkness (Fig. 6e, f and Supplementary Fig. 23). Considering the cell expansion-promoting effect of PREs and the cell expansion-repressing effect of PAR124,49, the different expression profiles of these genes at 28°C cannot fully explain the reduced hypocotyl length in tot3-2. However, the reprogramming of their expression in tot3-2 mutants again points to altered activity of their upstream regulators, including BZR1, and suggests that the impact on the gain-of-function bzr1-1D is representative for the endogenous role of TOT3 on BZR1.

Finally, the tot3-2 toi4-2 toi5-1 triple mutant exhibited a striking dwarf phenotype that resembles the phenotype of briel-116, a null mutant for the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE1 (BRI1)50 (Supplementary Fig. 24), suggesting that both brassinosteroid signalling and TOT3 pathways may affect the same growth-controlling component(s).

In conclusion, these results indicate that TOT3 controls brassinosteroid-mediated hypocotyl growth under warm temperature in darkness, through gating BZR1 activity.

Discussion

Plants incorporate temperature information into their life cycles in several ways. Therefore, climate warming strongly impacts plant growth and yield.2,51 Here, we present the membrane-associated protein kinase TOT3/MAP4K4 as a new key player in plant responses to warm temperature. In Arabidopsis, thermoresponsive hypocotyl growth intersects with light-signalling pathways and is mainly controlled by the transcription factors PIF4 and PIF7 and their (co)-regulators.4 Considering temperature is a non-ligand environmental stimulus that affects every cellular component, it is unlikely that there is only a single regulatory pathway that controls thermomorphogenesis. Indeed, pif4 mutants grown in a normal dark/light regime often show a partial thermoresponsive hypocotyl elongation56,52 (Fig. 4c, d). Additionally, about 21% of the warm-temperature-regulated transcriptome is not deregulated in phyABCDE quintuple null mutant plants.15 Here, we propose a thermomorphogenesis pathway governed by TOT3 that operates independently of phyB and PIF4 (Supplementary Fig. 25). This is supported by our observation that in the dark, where activity of PIFs reaches the maximum due to the lack of active phytochromes, both Col-0 and pif4-101 hypocotyl still elongated at 28°C, whereas this response is abolished in both tot3-2 and tot3-2 pif4-101 mutants. Moreover, the intermediate hypocotyl length of the tot3-2 phyb-9 double-mutant compared to the respective single-mutant plants further strengthens our model.

While the MAP kinase signalling cascades in plants have been extensively studied in the context of development and stress responses, not much is known about the function of plant MAP4Ks and whether these proteins function independently from the canonical MAP3K-MAP2K-MAPK cascades.52 Here, we show that MAP4K4/TOT3 interacts with related MAP4Ks (MAP4K6/TOI4 and MAP4K5/TOI5) and that these related kinases also play a role in thermomorphogenesis in conjunction with TOT3 to varying extents. In plants, interaction of closely related kinases has been observed for several receptor-like kinases, which act together in ligand perception and downstream phosphorylation.53 Another well-known example is OPEN STOMATA 1 (OST1)/SUCROSE NON-FERMENTING RELATED KINASE 2.6 (SnRK2.6), which forms a complex with SnRK2.2, SnRK2.3 and SnRK2.8, potentially amplifying SnRK2 signalling upon salt stress or osmotic stress.54 Of note, while these kinases show significant functional redundancy, they also exhibit differences in their function and regulation.55 Similarly, homo- and heterodimer formation is also observed for mammalian MAP4Ks, while they also have distinct functions and interactors.56 The thermomorphogenic phenotype of toi4 and toi5 double-mutants, and especially the phenotype of the tot3 toi4 toi5 triple mutant, suggest the existence of functional redundancy between TOT3, TOI4 and TOI5 with respect to particular phenotypes. However, the toi4 toi5 double-mutant phenotype does not exhibit an obvious phenotype at high temperature, indicating that TOT3 plays a dominant (genetic) role in the response. Alternatively, the role of TOI5 might largely overlap with TOT3, as both interact and TOI5 phosphorylation depends on TOT3, while TOI4 appears to play a largely redundant or alternative role. Given their interaction, future work should address if these MAP4Ks function in the same complex in planta and to what extent (de)phosphorylation events contribute to relaying the temperature signal to downstream signalling components. For example, in our Arabidopsis phosphoproteome dataset, the serine 334 (S333) of TOT3 shows significant increased phosphorylation at 60 min. This residue is highly conserved among MAP4Ks and also among TOT3 homologues in other land plants (Supplementary Fig. 26), indicating a possibly conserved functional importance for this phosphosite. Similarly, the phosphorylation of S348 in BLUS1 is triggered by blue light and is important for its activity.29 In addition, we observed indirect, but TOT3-dependent phosphorylation of TOI4 and TOI5 that needs to be explored in the future.

TOT3 is potentially required for conveying the stimulatory effect of brassinosteroids on warm-temperature-triggered hypocotyl elongation in darkness (Fig. 6), whereas a link with auxin is less prominent (Fig. 4 and Supplementary Fig. 15). Our data suggest that brassinosteroid signalling might be severely impaired in the tot3-2 toi4-1 toi5-2 mutant. However, it remains to be investigated how TOT3 controls BZR1 activity and how the output of the TOT3 complex with TOI4 and TOI5 affects growth, and particularly its interactions with light signalling networks need to be considered (Supplementary Fig. 26), especially since TOT3 seemingly acts independent of shade avoidance and parallel to PIF4-mediated thermosignalling (Fig. 4).

Finally, while elongation of aerial organs in dicots is a mechanism to cope with high temperature deviating from the optimal temperature, in wheat the role of high-temperature-mediated growth responses is not well studied. Nevertheless, TOT3 is also involved in regulating growth responses in wheat at temperatures above the optimal growth temperature (Fig. 3 and Supplementary Fig. 12). While we indeed only found differential phosphorylation of TOT3 orthologues in wheat spikelets, we also found TOI4 and TOI5 orthologues in the soybean and wheat leaf data. This suggests that components of the TOT3 signalling cascade are conserved and may also be involved in high temperature responses in these species. However, given that mass
spectrometry does not give a full proteome-wide overview in every independent analysis, the lack of identifying TOT3 phosphorylation does not necessarily indicate that it does not occur in wheat leaves. Thus, the conserved TOT3 complex has great potential for knowledge-based breeding of warm-temperature-resilient crops that can contribute to upkeep future food security in a warming climate.

Methods

Plant materials and growth conditions. All *A. thaliana* plants used in this study were grown in the greenhouse under short-day condition and background wild-type. The following *A. thaliana* lines were used: to6-1 (SALK_064174), toc-3 (SALK_086087), phy-b (ref. 37), pfts-101 (ref. 38), liz1-1D (ref. 44). For soybean phosphoproteome profiling, we used Glycine max [L] Merr. cv. "Benning HP" (ref. 38). Arabidopsis seeds were sown on Murashige and Skoog (MS) growth medium containing 1% sucrose. MS salts (0.1 g/mL), 0.5 g of MES, 10 g of sucrose and 8 g of plant tissue culture agar; pH 5.7). For treatment with picloram (Sigma-Aldrich, CAS: 1918-02-1) and brassinolide (0.5 g of MES, 10 g of sucrose and 8 g of plant tissue culture agar; pH 5.7).

Wheat TILLING lines. The wheat TOT3 ortholog ID in the D genome (TraesCS7D02G232400) was searched on the EnsemblPlants website (plants.db). Seedlings were then either left at 21 °C or put at 28 °C where they remained for an extended period. For cotyledon images of GFP:TOT3 localization, after 24 days after transplanting to induce reproductive development. When plants were 30 °C day/20 °C night, with a 9 h photoperiod and 2 h darkness. Daytime photosynthetic photon flux density in the growth chambers was approximately 700 μmol m−2 s−1. The 2 h darkness interruption was removed 24 days after transplanting to induce reproductive development. When plants were in full flower (~38 days after transplanting), the temperature was increased to 35 °C for 1 h, before the whole leaflets from the uppermost fully expanded leaf were excised and flash-frozen for phosphoproteomics. Four plants were sampled as one treatment temperature. For cotyledon images of GFP:TOT3 localization, after germination in the continuous light growth chamber at 21 °C, plates were wrapped in aluminium foil to prevent formation of the autofluorescence chlorophyll. Seedlings were then either left at 21 °C or put at 28 °C where they remained for an additional 3 days prior to imaging. Western blot. For the TOT3 western blot, finely ground plant materials were homogenized in ice-cold extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 and a Roche Complete protease inhibitor; 1 tablet per 10 mL). The tubes were centrifuged twice for 20 min at 20,817 g at 4 °C. The protein concentration and supernatant were determined using a BCA assay. The proteins were immunoreacted with anti-HA (Sigma-Aldrich, USA) (1:2000) and mouse IgG HRP linked whole antibody (GE Healthcare, USA) (1:10,000). For the BES1 western blot, 4-day-old seedlings were grown at 28 °C in short-day conditions on solid medium with mock (DMSO) or brassinolide (100 nM). Finely ground plant materials were homogenized in ice-cold homogenization buffer (1% SDS, 25 mM TRIS pH 7.5, 150 mM NaCl, 10 mM DTT and a Roche Complete protease inhibitor 1 tablet per 10 mL). For immunodetection, antibody-bES1 antibody (1:5000) was used as primary antibody, anti-Rabbit (at 1:10,000) was used as secondary antibody. The proteins were detected by ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., USA). For the BES1 dephosphorylation assay, the ratio of the dephosphorylated BES1 to the total BES1 protein level was quantified based on the signal intensity. The loading was adjusted to an equal level based on the amount of α-tubulin. Signal intensities were determined using Image Lab (Bio-Rad).

Generation of constructs and transgenic lines. PCR was performed using Q5 High-Fidelity polymerase according to the manufacturer’s instructions (New England Biolabs). The CS9 of TOT3 and a fragment of 199 base pairs upstream of TOT3 CDS amplified by PCR. To allow reverse-transcribed RNA and genomic DNA extracted from Col-0 seedlings, respectively. The CDS of TO14 and TO5 was synthesized using the BioXPC200TM system (SGI-DNA) with DNA sequence being modified so that the coding information was retained. For Golden Gate cloning, the TOT3 promoter and TOT3 CDS were ligated into pGAGp0000 (ref. 38). To generate the GFP:TOT3 and pT03:GFP-T03 constructs, respectively. The respective constructs were assembled into the destination vector pG-pGAP (Supplementary Table 2). Plant vectors were transformed in Agrobacterium tumefaciens C58Cl1 using the freeze-thaw method (ref. 38). Plant transformation was performed using the floral dip method (ref. 40). All transgenic plants were screened at a BBR resistance screen. For site-directed mutagenesis, the pGCG0000 (TOT3 CDS) was used as a template for PCR using specific primers containing the mutation site. Then, the DNA template plasmid was digested with DpnI for 1 h before being transformed into DH5α. E. coli cells. For the Y2H assay and TAP analysis, the CDS of interest was cloned into the pDONR221 entry vector pDONR221 and then recombined into the host vector pGAL424gate or pGAL424gate and pGB7tagate. All the primers are listed in Supplementary Table 1.

Confocal microscopy. Whole seedlings were immersed in an aequorin solution of 2 or 10 µM N (3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexa-trienyl)pyridinium dibromide (FM64-44). Seedlings were stained for 5 min and then quickly washed with Milli-Q water to remove excessive FM4-44. Seedlings were imaged in a fluorescence microscope (Olympus, Japan). The fluorescent photon flux density in the growth chambers was approximately 700 μmol m−2 s−1. The 2 h darkness interruption was removed 24 days after transplanting to induce reproductive development. When plants were in full flower (~38 days after transplanting), the temperature was increased to 35 °C for 1 h, before the whole leaflets from the uppermost fully expanded leaf were excised and flash-frozen for phosphoproteomics. Four plants were sampled as one treatment temperature. For cotyledon images of GFP:TOT3 localization, after germination in the continuous light growth chamber at 21 °C, plates were wrapped in aluminium foil to prevent formation of the autofluorescence chlorophyll. Seedlings were then either left at 21 °C or put at 28 °C where they remained for an additional 3 days prior to imaging.

Low red-far/red (R/Fr) treatment. Seedlings were cultured on full-strength MS medium without sucrose containing 0.8% agar. Plants were cultured in white light conditions for 2 days at 22 °C (control) under short-day photoperiod (8 h light/16 h darkness) (Snijders, Microclima 1000, approximately 120 μmol m−2 s−1 photosynthetic active radiation (PAR)). Plates were subsequently subjected to 6 days to a low red-Fr regime at 22 °C (700 nm) of 0.98 μmol m−2 s−1). Plants were then transferred to room temperature for germination. Plants that germinated uniformly were selected and grown in plastic pots containing soil at 24 and 34 °C under 16 h light/8 h dark (100 μmol m−2 s−1 photosynthetically active radiation, supplied by cool-white, fluorescent tungsten tubes, Osram). In order to generate loss-of-function toi4 and toi5 mutants, we applied the FAST CRISPR-Cas9 system combined with Golden Gate cloning (ref. 42). Two different guide RNAs (gRNAs) were designed for each gene (Supplementary Table 1). The oligos were synthesized using the BsuDIG (Supplementary Table 1). The oligos were synthesized using the BsuDIG and ligated into the BbsI-digested entry vector containing the compatible DNA-overhang using T4 DNA ligase (New England Biolabs) (Supplementary Tables 1 and 2). The entry modules were assembled into the destination vector pFASTRK24GW which contains the red fluorescent protein (GFP) (ref. 40). Six 9-day-old seedlings were subsequently scanned using a flatbed scanner and hypocotyl lengths were measured using ImageJ image-analysis software (https://imagej.nih.gov/ij/).
The prey and bait CDSs were recombined into pGAL4-2xP6 or pGB7q-9ate, respectively. The Saccharomyces cerevisiae strain Y2H7A (a strain co-transformed with the bait and prey plasmid using polyethylene glycol/PEG/lithium acetate method) and the target gene were inserted in separate plasmids. Yeast two-hybrid assays were performed by mating the strains to the target gene. Selections which contained homologous –1 or +1 indel mutations at TOI4 and TOI5 loci were selected as TOI4 and TOI5 loss-of-function mutants, since this type of mutation causes a frame shift in the resulting mRNA and subsequently leads to mistranslated proteins. Further, TOI4 TOI5 loss-of-function mutants were checked for TOI4 and TOI5 expression levels by qPCR using the primers listed in Supplementary Table 1. Genomic DNA isolated from the bait and prey plasmid using the polyethylene glycol/PEG/lithium acetate method was amplified by PCR and sequenced. The primers were selected in non-redundant (SD) media devoid of <0.01 was carried out to test for differences between the genotypes. For the statistical test, the number of randomizations was set at the default value of 250, the technical replicates were preserved during the randomizations. In addition, we retained candidates that were not detected in all biological and technical replicates of at least one time point (and thus could not be subjected to statistical analysis), but for which a phosphosite was detected in one of the other time points. For the soybean phosphoproteome analysis unabridged data of Col-0 seedlings transferred to 27 °C, phosphorylation and dephosphorylation were at least two out of three unique phosphosites for at least one time point were retained. Log2 phosphosite intensities were centered by subtracting the median of the entire set of protein ratios per sample. A multiple-sample test (one-way ANOVA) with a p-value cut-off of <0.01 was carried out to test for differences between the time points. For the statistical test, the number of randomizations was set at the default value of 250, the technical replicates were preserved during the randomizations. In addition, we retained candidates that were not detected in all biological and technical replicates of at least one time point (and thus could not be subjected to statistical analysis), but for which a phosphosite was detected in one of the other time points. For the soybean phosphoproteome analysis unabridged data of Col-0 seedlings transferred to 27 °C, phosphorylation and dephosphorylation were at least two out of three unique phosphosites for at least one time point were retained. Log2 phosphosite intensities were centered by subtracting the median of the entire set of protein ratios per sample. 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A multiple-sample test (one-way ANOVA) with a p-value cut-off of <0.01 was carried out to test for differences between the genotypes.
described. Beads were washed three times with 500 μL TAP extraction buffer and one time with 500 μL TAP extraction buffer without detergent. For on-bead protease digestion, the beads were washed with 500 μL 50 mM NH4HCO3 (pH 8.0). The wash buffer was removed and 50 μL 50 mM NH4HCO3 was added together with 1 mg Trypsin/Lys-C and incubated at 37 °C for 4 h. Next, the digest was separated from the beads and overnight incubated with 0.5 mg Trypsin/Lys-C at 37 °C. Finally, the digest was centrifuged at 20,800g in an Eppendorf centrifuge for 5 min, and the supernatant was transferred to a new Eppendorf tube and the peptides were vacuum dried and stored at −20 °C until LC-MS/MS analysis. The peptides were analysed on a Q Exactive (ThermoFisher Scientific). After MS-based identification of co-purified proteins, specific proteins were detected by comparison with the non-specific proteins26, built from 213 pull-downs with 42 different bait proteins. True interactors that might have been missed because of their presence in the list of non-specific proteins were retained through a semi-quantitative analysis. In this approach, average normalized spectral abundance factors (NSAF) of the identified Arabidopsis proteins in the TOT3 samples were compared against the corresponding average NSAF deduced from the control pull-down dataset. For stringent filtering of specific proteins, only the proteins identified with at least two peptides were retained that were highly (at least 10-fold) and significantly [−log10(p-value(t-test)) ≥ 10] enriched compared to the control dataset. The mass spectrometry proteomics data for these experiments have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD015483.

Co-immunoprecipitation. Col-0, pTOT3::GFP:TOT3 seedlings and 35S::GFP seedlings were grown in similar conditions as used for the time-dependent phosphoproteome profiling. Ten-day-old light-grown seedlings were collected at 0 min at 21 °C and at 60 min after being transferred to 28 °C. For each condition and genotype, three biological replicates were obtained. Next, 1 g of finely ground plant material was homogenized with 2.2 mL extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) and sonicated. Debris was removed from the samples by centrifugation, and 2 mL of sample was diluted 5-fold with extraction buffer without NP40, then mixed with 50 μL pre-equilibrated GFP-Trap™ MA beads (ChromoTek) and rotated for 2 h at 4 °C to maximize the protein binding. Subsequently, the solution was removed, the beads were washed three times with wash buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl), and then once with 1 mL 50 mM TEAB (pH 8.0) (Thermo Fisher). On-bead digestion was performed on the bound proteins with 0.5 μg trypsin (Promega) for 2 h at 37 °C. The supernatant was retained. The beads were washed twice with 25 μL 50 mM TEAB and the wash solution was pooled with the previous supernatant. Disulfide bonds were reduced by adding TCEP and iodoacetamide (Thermo Fisher) to a final concentration of 10 mM and 15 mM, respectively, and incubated at 30 °C. The remaining iodoacetamide was quenched by adding DTT to a final concentration of 4 mM. Then 0.5 μg trypsin was added and the sample was incubated overnight at 37 °C to complete the digestion. The digestion was stopped by adjusting the sample to 1% TFA and samples were desalted using C18 Bond Elut tips (Agilent Technologies). MS analysis of the peptides was performed identically to TAP analysis. After MS-based identification of co-immunoprecipitated proteins, proteins were considered as potential interactors of TOT3 if they were present in pTOT3::GFP:TOT3 and not in the non-specific samples or if the fold change of the LFQ intensity was 10-fold higher in the pTOT3::GFP:TOT3 samples, compared against a list of non-specific proteins as described above. MS/MS spectra were searched against the combined protein database of GFP/RFP, GFP-TOT3/ TOT3Δ1157N and RFP-TOT4Δ1885/TOT3Δ174N, and using the Nicotiana benthamiana proteome downloaded from SolGenomics database containing 57,140 protein entries by the MaxQuant software (version 1.6.0.43) using UseGalaxy server. Data processing was similar to the analysis of Arabidopsis phosphoproteome. All the phosphosites with at least 3 valid values at least in one treatment group were retained as reproducibly quantified phosphosites for statistical analysis. The two-sample test with p < 0.05 was carried out to test the differences among the treatments.

Statistics and reproducibility. For box plots, the lower Tukey-based whisker shows the smallest value that is greater than the lower quartile minus 1.5 × interquartile range, the upper Tukey-based whisker shows the greatest value that is smaller than the upper quartile plus 1.5 × interquartile range and data points outside this range (outliers) are plotted as individual dots.

Venn diagrams and heatmaps. Comparing lists was performed using Venny v2.1 (http://bioinfogp.cnb.csic.es/tools/venny/). Heatmaps were generated using Perseus v1.5.5.3 (ref. 49) or MeV v4.9.0 (ref. 41).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The raw mass spectrometry proteomics datasets are publicly available through ProteomeXchange with identifiers PXD015468 and PXD015483. All other data are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

L.D.V and K.G. initiated and managed the project and designed experiments. L.D.V., E.S., D.d.J., X.X, M.v.Z., T.V., T.Z., A.L., L.P., Y.W, N.D.W. and B.v.d.C. performed experiments. I.D.S., K.G., M.v.Z., C.U., G.D.J., E.R. and D.V.D. contributed ideas, interpreted results and critically revised the manuscript. All authors discussed the results and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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