A conserved signaling axis integrates conflicting environmental drought and heat signals to control stomatal aperture in plants

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Abstract

Plants continuously respond to changing environmental conditions to prevent damage and maintain optimal performance. To regulate gas exchange with the environment and to control abiotic stress relief, plants have pores in their leaf epidermis, called stomata 1. Multiple environmental signals affect the opening and closing of these stomata 2. Heat promotes stomatal opening (to cool down) and drought induces stomatal closing (to prevent water loss). Coinciding stress conditions, however, may evoke conflicting stomatal responses, but the cellular mechanisms to resolve these conflicts are unknown. Here, we demonstrate that the high temperature-associated kinase TARGET OF TEMPERATURE 3 (TOT3) directly controls the activity of plasma membrane H+-ATPases to induce stomatal opening. This TOT3 activity is directly antagonized by OPEN STOMATA 1 (OST1), to prevent water loss during drought stress. This signaling axis harmonizes conflicting heat and drought signals to regulate stomatal aperture. In the context of global climate change, understanding how conflicting stress signals converge on stomatal regulation allows the development of climate change-ready crops.

Main Text

Plants are exposed to environmental stresses, such as high temperature and drought, and respond to changing conditions to prevent damage and maintain optimal performance [3–5]. Stomata – pores in the epidermis of plant organs – contribute to abiotic stress relief and avoidance, by regulating the rate of gas and water vapor exchange with the environment [6]. Under drought conditions, leaf temperature rises when stomata close in a phytohormone abscisic acid (ABA)-dependent manner [7–9] *(Extended Data Fig.1a-c)*. Supra-optimal air temperatures on the contrary, promote stomatal opening in both dicot and monocot species, such as the model dicot plant *Arabidopsis thaliana* and the monocot crop species *Triticum aestivum* (wheat) (Fig. 1a-d and Extended Data Fig.1d). This high-temperature-triggered response increases stomatal conductance and can stimulate leaf cooling through evaporation under well-hydrated conditions [10–14] *(*Fig. 1e-h *and* Extended Data Fig. *1d-i)*. However, how the high temperature signal is perceived and transmitted to regulate stomatal aperture is poorly understood [11,12]. In contrast, under drought – which results in dehydration and eventually wilting – closing stomata limits water loss [1,15]. Since both drought and heat stresses often coincide [16,17], this prompts a regulatory conflict as the individual signals trigger opposite stomatal responses. To resolve this conflict, likely a shared, so far unknown signaling module exists providing a tight regulation of stomatal aperture by integrating coinciding heat and drought stress signals [6,18]. Opening and closing of stomata are rapid responses that likely require switch-like signaling mechanisms that can swiftly alternate when environmental conditions change, making kinase-mediated phosphorylation relays ideal for this.

Stomatal opening is partially mediated through the activation of plasma membrane H^+-ATPases, which requires phosphorylation of their penultimate threonine [19–22]. Our previous phosphoproteome analysis revealed warm temperature-mediated phosphorylation of ARABIDOPSIS H^+-ATPase 1 (AHA1) and AHA2 at their penultimate residues T948 and T947, respectively, in wild-type seedlings [23] (Extended

Data Fig. 2a-b). This suggests increased activity of H^+-ATPases at high temperature, which is confirmed by increased proton (H^+) release from seedlings at high temperature (Extended Data Fig. 3). Our previous phosphoproteome analyses furthermore revealed that AHA1 phosphorylation is downregulated in a loss-of-function line for the kinase TARGET OF TEMPERATURE 3 (TOT3)/MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE KINASE 4 (MAP4K4), a conserved regulator of moderate high temperature-mediated plant growth [23] (Extended Data Fig. 2c-d). *TOT3* is expressed in stomata and its protein localization and promoter activity were not affected by high temperature (Fig. 1i and Extended Data Fig. 4) [23]. Notably, loss of TOT3 function does not appear to modify stomatal development (fig. S5). These observations together suggest a role for TOT3 in controlling AHA1-mediated stomatal aperture. Indeed, we observed that not only Arabidopsis, but also wheat *tot3* mutants had their stomata significantly more closed at high temperature compared to their respective wild-type control lines (Fig. 1a-d and Extended Data Fig. *1d,* 6 and 7), indicating a conserved role for TOT3 in regulating dicot and monocot stomatal aperture. This role in stomatal aperture aligned with a difference in stomatal conductance of both Arabidopsis and wheat *tot3* mutants at high temperature, compared to wild-type control lines (Fig. 1e-h and Extended Data Fig. 8-9). Furthermore, the stomatal aperture of the Arabidopsis *tot3-2* mutant at high temperature correlated with a higher leaf temperature, compared to the *tot3-2* mutant complemented with a functional GFP:TOT3 (*tot3-2 pTOT3::GFP:TOT3*) (Extended Data Fig. 10).

To explore AHA1 as a potential TOT3 kinase substrate, we first confirmed that TOT3 and AHA1 proteins interact, through co-immunoprecipitation (co-IP), bimolecular fluorescence complementation (BiFC) and yeast-two hybrid (Y2H) (Fig. 2a and Extended Data Fig. 11). Transiently co-expressing *TOT3* or *TOT3*^{D157N} (a kinase-dead version) together with *AHA1* in tobacco revealed elevated AHA1 phosphorylation at T948 in the presence of TOT3 compared to TOT3^{D157N} (Fig. 2b and Extended Data Table 1), indicating that kinase active TOT3 positively regulates AHA1 phosphorylation status. An immunohistochemical assay showed reduced phosphorylation of the penultimate AHA threonine in *tot3-2* mutant guard cells exposed to high temperature compared to *tot3-2 pTOT3::GFP:TOT3* (Fig. 2c-d and Extended Data Fig. 12), supporting that AHA1 phosphorylation occurs downstream of TOT3. Subsequently, an *in vitro* kinase assay showed direct phosphorylation of the C-terminal part of AHA1 (from E905-V949, referred to as AHA1$_{905\text{-}949}$) by TOT3, which is abolished when using the non-phosphorylatable AHA1$_{905\text{-}949}^{T948A}$ variant (Fig. 2e). These results place AHA1 directly downstream of TOT3 as a substrate, to control its activity. Accordingly, the high temperature-mediated increase in proton release was abolished in the *tot3-2* mutant compared to *tot3-2 pTOT3::GFP:TOT3* (Extended Data Fig. 13). Finally, we uncoupled AHA1 activity from external cues or (TOT3-mediated) phosphorylation of the penultimate T948 both genetically through testing *open stomata2-2D* (*ost2-2D*), an *aha1* mutant with constitutively activated H^+-ATPase activity [21], and pharmacologically through using fusicoccin, an activator of plasma membrane H^+-ATPases [20–22,24]. Introducing *ost2-2D* in *tot3-2* and treating *tot3-2* with fusicoccin both rescued the stomatal aperture phenotype of *tot3-2* at high temperature (Fig. 2f-g and Extended Data Fig. 14). Taken together, we show that TOT3 regulates stomatal aperture through controlling AHA1 activity by direct phosphorylation of T948.

AHA activity is down-regulated by a SnRK2-dependent ABA pathway to induce stomatal closure [25–27]. One of these, OPEN STOMATA 1 (OST1)/SnRK2.6, is specifically expressed in guard cells [27,28] (Extended Data Fig. 15) and is involved in ABA-mediated stomatal closure under drought stress. Hence, the *ost1* mutant exhibits constitutively open stomata and consequently cooler leaves [27–29] (Extended Data Fig. 16), which contrasts to the *tot3* phenotype (Fig. 1a-b and Extended Data Fig. 6). The partial reversion of the *ost1-3* constitutively open stomata phenotype in *tot3-2 ost1-3* double mutants (Extended Data Fig. 17), is consistent with TOT3 and OST1 acting in the same pathway. We next showed that TOT3 and OST1 co-localize in stomata and interact (Fig. 3a and Extended Data Fig. 15 and 18). This interaction was not dependent on, nor affected by, ABA or high temperature [30] (Extended Data Fig. 18). Our genetic and biochemical data confirm previous results where TOT3 was listed as an OST1-INTERACTING PROTEIN [30], and indicate that TOT3 and OST1 could function together during high temperature and/or drought signaling at the level of stomatal aperture control.

Subsequently, an *in vitro* kinase assay using TOT3 from heat-treated Arabidopsis plants revealed heat-mediated autophosphorylation, pointing to increased TOT3 kinase activity (Extended Data Fig. 19). However, in the presence of ABA or drought, signals that are known to activate OST1 [27,31,32], heat-activated TOT3 autophosphorylation was reduced (Extended Data Fig. 19). These data imply that TOT3 functions downstream of ABA and drought. We therefore tested whether TOT3 could be phosphorylated by ABA-activated OST1. Indeed, mass spectrometry analyses revealed several TOT3 phosphosites that were up-regulated *in planta* by ABA and/or OST1 compared to control conditions (Fig. 3b-c and Extended Data Fig. 20, Extended Data Table 2). Since some ABA-responsive TOT3 phosphosites, such as S324 or S481, were not directly regulated by OST1 (Extended Data Fig. 21), we focused on those TOT3 phosphosites that were regulated by ABA-activated OST1: S382, S448 and S451 (Fig. 3b-c). An *in vitro* kinase assay using an *E. coli*-expressed *TOT3* fragment encompassing those phosphosites (GST:TOT3$_{365\text{-}465}$) and His:MBP:OST1 confirmed that OST1 directly phosphorylates TOT3 (Fig. 3d). In contrast, a TOT3 phosphomutant variant where these sites, together with two additional adjacent serine residues (S381 and S383; generating GST:TOT3$_{365\text{-}465}^{S381A/S382A/S383A/S448A/S451A}$, here referred to as GST:TOT3$_{365\text{-}465}$-5A) were mutated, exhibited largely reduced OST1-mediated phosphorylation (Fig. 3d), indicating that direct OST1 phosphorylation target site(s) is/are among these residues.

To assess if OST1-mediated phosphorylation of TOT3 affected TOT3 function, we tested the phosphomutant GFP:TOT3-5A and phosphomimetic (GFP:TOT3$^{S382E/S448E/S451E}$, here referred to as GFP:TOT3-3E) proteins for their ability to rescue the *tot3-2* stomatal aperture phenotype at high temperature. Two independent *tot3-2 pTOT3:GFP:TOT3-3E* lines could not rescue the *tot3-2* stomatal aperture phenotype, while two independent *tot3-2 pTOT3:GFP:TOT3-5A* lines did rescue the *tot3-2* stomatal aperture phenotype (Fig. 3e-f and Extended Data Fig. 22). Moreover, expression of *GFP:TOT3-3E* did not alter the partial rescue of the *ost1-3* stomata aperture phenotype by the *tot3-2* allele (Extended Data Fig. 23). Taken together, this suggests that OST1-mediated phosphorylation of TOT3 leads to a less functional TOT3 protein. To assess if TOT3-3E was unable to rescue the *tot3-2* mutant due to altered kinase activity, we performed *in vitro* kinase assays. This revealed reduced kinase activity for TOT3-3E,

considering autophosphorylation and AHA1 phosphorylation, while TOT3-5A showed increased kinase activity, compared to the wildtype TOT3 protein (Fig. 3g). Consistently, transient expression of *TOT3-5A* or *TOT3-3E* in *N. benthamiana* leaves resulted in increased (*TOT3-5A*) or decreased (*TOT3-3E*) phosphorylation of AHAs (Extended Data Fig. S24). This aligned with an increased proton release, independent of high temperature, in *tot3-2 pTOT3:GFP:TOT3-5A* (Extended Data Fig. 24). These results support that TOT3-5A is hyper-active and TOT3-3E is inactive, and thus that OST1-mediated phosphorylation of TOT3 impairs TOT3 kinase activity.

We next evaluated if OST1-mediated regulation of TOT3 activity is functionally relevant under drought and/or heat stress in Arabidopsis seedlings. We first observed reduced phosphorylation of TOT3-S448 at high temperature and increased phosphorylation upon both ABA treatment (Fig. 4a and Extended Data Fig. 25, Extended Data Table 3) and drought stress (Fig. 4b and Extended Data Table 4). Furthermore, drought-regulated TOT3-S448 phosphorylation depends on OST1 (Fig. 4c). These results indicate that under drought stress and under high ABA levels, where stomatal closure occurs, phosphorylation of TOT3 on OST1-target residues occurs, to inactivate TOT3. Indeed, *tot3-2 pTOT3:GFP:TOT3-5A* lines were less sensitive to ABA with respect to a reduction of stomatal aperture (Fig. 4d). These results support that specific OST1-mediated phosphorylation controls TOT3 activity under drought stress conditions. Furthermore, the *tot3-2* mutant and *tot3-2* expressing *GFP:TOT3-3E* were more tolerant to progressive drought stress than *tot3-2* expressing *GFP:TOT3* or *GFP:TOT3-5A* (Fig. 4e-f), suggesting that TOT3 activity contributes to drought responsiveness and resilience.

Our results support that opposite regulation of stomatal opening under high temperature and during drought stress correlates with TOT3 activity. This suggests that these conflicting stress conditions converge on TOT3 activity to achieve an integrated response. The opposing effect of both stress conditions on TOT3 activity and stomatal aperture highlights an apparent signaling conflict during combined high temperature and drought stress. To reveal the hierarchy of TOT3 signaling, we exposed a line with increased OST1-independent TOT3 kinase activity (*pTOT3::GFP:TOT3-5A* in *tot3-2*) to the combination of drought and increased temperature. This revealed that preventing TOT3 inactivation leads to cooler leaves under these combined stresses, compared to the *tot3-2 pTOT3::GFP:TOT3* control line and compared to exposure to the individual stresses (Fig. 4g and Extended Data Fig. 26). This implies that drought-induced, OST1-mediated inactivation of TOT3 is essential to close stomata under drought conditions if, at the same time, a high-temperature signal pushes to keep these open.

In conclusion, we propose a model where high temperature activates TOT3 to promote leaf cooling via direct activation of AHA1-regulated stomatal opening (Fig. 4h). This high temperature response is negatively regulated by OST1-mediated phosphorylation of TOT3 under drought stress conditions (Fig. 4h). This gas-and-brake mechanism allows fast and flexible harmonization of conflicting signals arising from heat and drought cues to optimize stomatal aperture to effectively deal with combined stresses. These insights are increasing our understanding of how different environmental signals are interpreted to fine-tune plant responses, which provides opportunities for crop resilience improvement.

Methods

Plant materials and growth conditions

All *A. thaliana* mutants and transgenic lines used in this study are in the Col-0 genetic background, unless otherwise indicated. TOT3 (AT5G14720), OST1 (AT4G33950), AHA1 (AT2G18960) and AHA2 (AT4G30190) are the main factors studied in this paper. The following *A. thaliana* lines were used: *tot3-1 (SALK_065417)* [23], *tot3-2 (SALK_086087)* [23], *ost1-3 (SALK_008068)* [29,33] and *ost1 (SALK_067550C)* [33]. Lines were ordered from the Eurasian Arabidopsis Stock Centre (NASC). The *ost2-2D* mutant and *tot3-2* complementation line *tot3-2 pTOT3::GFP:TOT3 #5 and #7 (TOT3 tot3-2 #5 and TOT3 tot3-2 #7)* have been described previously [21,23]. The TOT3 phosphomutant *tot3-2 pTOT3::GFP:TOT3 S381A/S382A/S383A/S448A/S451A (TOT3-5A tot3-2)* and phosphomimetic *tot3-2 pTOT3::GFP:TOT3 S382E/S448E/S451E* (*TOT3-3E tot3-2)* are generated in this study (see below and Extended Data Table 6). Arabidopsis seeds were sterilized with 70% ethanol and sown on half Murashige and Skoog (MS) growth medium containing 1% sucrose (per liter: 2.15 g of MS salts, 0.1 g of myo-inositol, 0.5 g of MES, 10 g of sucrose and 8 g of plant tissue culture agar; pH 5.7). Seeds were stratified at 4°C for 2 days in the dark and then moved to 21°C under continuous light for germination for 48 h. After that, seedlings were grown at 21°C under 70% ± 10% relative humidity under long-day conditions (16 h light/ 8 h darkness) (unless indicated otherwise). All *Triticum aestivum* plants in this study are in the Cadenza accession genetic background. Wheat TOT3 homologue (TraesCS7D01G232400) mutants Cadenza 1716 (*tot3* W122*) and Cadenza0256 (*tot3* Q191*) have been described previously [23]. The seeds were put on wet paper enclosed by plastic wrap for two days at 4 °C. After stratification, the seeds were transferred to 21°C in darkness for 2 days to allow germination. Seeds that germinated uniformly were selected and grown in plastic pots containing Jiffy 7c pellets (Jiffy Products International AS, Norway) under 21°C with 70% ± 10% RH condition and 16h light/8h dark, 90 µmol m^{-2} s^{-1} photosynthetically active radiation cool-white (fluorescent tungsten tubes, Osram). Detailed information on the growth conditions for high temperature experiments, drought experiments, and stomatal conductance analyses can be found in the ***Supplementary Information***.

Plasmid constructs and generation transgenic lines

PCRs were performed by Q5® High-Fidelity polymerase according to the manufacturer's instructions (New England Biolabs, USA) with primer pairs (Extended Data Table 5). The CDS of TOT3-3E and TOT3-5A were synthesized (Twist Bioscience, USA). For the various plasmids, the construct information and cloning system are provided (Extended Data Table 6). The GoldenGate cloning system, Gateway cloning system, Gibson assembly and restriction cloning were used to generate the plasmids according to the associated protocols (Extended Data Table 6). Plasmids were checked by PCR and sequenced with suitable primers (Extended Data Table 5). Plant vectors were transformed in *Agrobacterium tumefaciens* C58C1 and plant transformation was performed using the floral dip method as previously described [34].

Leaf temperature under abscisic acid (ABA) treatment

The experiments were performed with soil-grown Col-0 plants (10 leaf stage) at 21°C sprayed with 10 µM ABA (10 mM ABA stock dissolved in EtOH and diluted in 0.025% (v/v) Silwet or Mock (0.025% (v/v) Silwet with identical volume of EtOH). Thermal images were taken every 15 min during the photoperiod continuously using FLIR ResearchIR Max software (version 4, FLIR Inc., USA).

Thermal imaging and leaf temperature quantification

Thermal images of plants on plates were captured using a PI 200 infrared thermography camera, (Germany) from the top of the plants from a fixed distance. All genotypes in each plate were recorded together. Leaf temperature quantification was processed in optris PIX Connect (version 2.18.2239.0, Germany). The middle region of each leaf of one plant was measured to get the average leaf temperature of each plant. Thermal images of plants on soil were recorded by a FLIR A655sc High Resolution LWIR thermal imaging (IR) camera (Teledyne FLIR LLC, USA) using FLIR ResearchIR Max software (version 4, USA). Acquired thermal images were analyzed using ImageJ software. To measure the leaf temperature of each plant a region of interest was drawn on one mature leaf per plant and measurements were taken every 4^{th} hour, at ZT = 0, 4, and 8 h (8h photoperiod) and ZT = 12, 16, 20 (16 h darkness period), throughout the experimental period. Every 6^{th} image (i.e., equaling every 24 h) the measured region of interest was manually adjusted for each leaf per plant to correct for leaf growth and movement, assuring that the same region of each leaf was measured throughout the experimental period.

Stomatal aperture assay with fusicoccin treatment

Stomatal aperture assays were performed as previously described [11]. Three-week-old plants grown on petri plates under long-day conditions at 21°C were used. Mature leaves were incubated overnight in a buffer containing 10 mM MES-KOH and 50 mM KCl, pH 6.15 at 21°C in the dark to induce stomatal closure. Subsequently, leaves were transferred to the same buffer containing 10 µM fusicoccin dissolved in DMSO or 0.1% DMSO (mock) as the negative control. The leaves were incubated at 21°C or 34°C for 2 h before imaging with Zeiss 710 inverted confocal microscope. The images were used for stomatal aperture measurement.

Stomatal aperture assay with abscisic acid (ABA) treatment

The experiments were performed using plants grown on half-strength MS medium petri plates covered with sterilized nylon mesh (PROSEP, Belgium). Seeds of different genotypes were sown of divided parts of plates in replicates to avoid position effects. After 2 days stratification at 4°C, the plates were kept in long-day conditions (16h light/8h dark, 90 µmol m^{-2} s^{-1} photosynthetically active radiation and 70% RH) for 3 weeks at 21 °C. After 3 weeks, the mesh with growing plants was transferred to half MS plates with 10 µM ABA or DMSO (mock) and treated for another 2 hours at the above condition. And the 3rd leaf was used for leaf imprinting.

Leaf imprint and stomatal quantification

To detect stomatal aperture from intact plants, leaf imprint assays were performed with modifications as described [35–37]. After 2h exposure to 21°C or 34°C, the 3rd true leaf of 3-week-old Arabidopsis or wheat plants was used for taking the imprint. Moderate pressure was used to mix the catalyst and polysiloxane (3M™ ESPE Express light body, USA). The mixture was applied to the abaxial surface of the leaf using toothpicks with minimal pressure. After the mixture was hardened, the leaf imprint was separated from the leaf. Transparent nail varnish was covered on the hardened imprints and dried to make a positive imprint. Scanning electron microscope (SEM) TM1000 (Hitachi, Japan) was used to scan the leaf center part of the positive imprints (Extended Data Fig. 5a). The obtained images were measured and quantified using ImageJ software (version 1.53f). The aperture of normal-looking, well-imaged stomata was calculated as a ratio by using the length divided by the width of the aperture.

Quantitative analysis of stomatal density and index

The stomatal density and index measurement and analysis were performed as described [38]. Detailed information can be found in the ***Supplementary Information***.

Histochemical β-Glucuronidase (GUS) Staining

Two-week-old plants grown under continuous light were used for GUS staining. Plants were fixated in cold 90% (v/v) acetone until the leaves turned white. Leaves were rinsed with NT buffer (100 mM Tris/50 mM NaCl). After that, the samples were incubated in NT buffer containing 2 mM K3[Fe(CN)6] and 2 mM X-Gluc for staining at 37°C for 2h. The plants were rinsed with NT buffer to remove the staining agents and mounted on glass slides in 80% (v/v) lactic acid. The images were taken by using an Olympus BX53 (Olympus, Japan).

Determination of plant proton release under high temperature

The assay was performed as described [39,40]. Briefly, seeds were germinated and grown on vertical half MS petri plates for 7 days. Seedlings were transferred to a flask (5 seedlings/flask) and washed with assay solution (one-quarter strength MS and 2 mM MES buffer, pH 6.8) for 5 min. In each flask, 6 mL of half MS liquid medium was added and the seedlings were allowed to grow for 2 weeks. Acidification assays were then performed in fresh assay solution under indicated temperatures while shaking in the growth chamber (TC 445S, Tintometer GmbH) set at identical environmental conditions (70% RH and 90 µmol m^{-2} s^{-1} PAR) for 8 hours. H^+ release was calculated by taking the difference between the initial and final pH and normalized to the total fresh weight of the plants.

Immunohistochemical detection of AHA and the phosphorylation of the AHA penultimate residue in guard cells

Immunohistochemical detection was performed according to a previously published method by using an immune-robot; InsituPro Vsi II (Intavis) [25,41]. A detailed description can be found in the ***Supplementary Information***. The fluorescence signal of guard cells was captured by a Leica SP8 confocal microscope (Leica, Germany) and gating technology was applied for autofluorescence removal. The fluorescence intensities of stomata were quantified using ImageJ (version 1.53f), for which each stomate was circled and the average intensity of the area was calculated as previously described [25,42,43] .

Tobacco infiltration

Agrobacterium tumefaciens strain C58 was transformed with the indicated plasmids (Extended Data Table 6). Five- to six-week-old *Nicotiana benthamiana* leaves were infiltrated as previously described [23].

Bimolecular fluorescence complementation and quantification.

The BiFC method was modified based on the previously described protocol [44]. A detailed description can be found in the ***Supplementary Information***. The obtained images were measured using ImageJ (version 1.53f) as described [45]. The separate images with the YFP channel or RFP channel were exported to ImageJ and the average signal of each channel on the whole image was calculated. The ratio of YFP/RFP was calculated.

Protein extraction, co-immunoprecipitation, and Western blot

Plant materials were collected and ground in liquid nitrogen with a mortar and pestle until becoming fine powder. Total proteins were extracted as previously described [23]. A detailed description can be found in the ***Supplementary Information***.

Recombinant protein purification

The plasmids (Extended Data Table 6) were transformed into *E. coli* strain BL21 (DE3). The protein purification method was modified based on the previously described [23]. Detailed information can be found in the ***Supplementary Information***.

Co-immunoprecipitation of recombinant proteins

One µg recombinant GST or GST-OST1 proteins were mixed with 50 µL Glutathione Sepharose 4B beads in 1 mL PBS buffer at 4°C for 2 h with rotation. After that, the supernatant was removed by centrifugation. One µg MBP-TOT3-His recombinant proteins was added onto GST- or GST-OST1-bound beads and incubated at 4°C for 2 h with rotation. The supernatant was separated from the beads by centrifugation and removed. Beads were washed 5 times with 1 mL PBS each. Proteins were separated by SDS-PAGE and S-gel and Western Blot was performed as described above using anti-His at 1:2000 dilution (Qiagen, Germany) as the primary antibody and anti-mouse antibody at 1:10000 dilution (Cytiva, USA) as the secondary antibody.

Radioactive kinase assay for recombinant proteins

Three µg of purified substrate protein and one µg purified kinase were incubated at 30°C for 1 h in kinase assay buffer (10 mM Tris-HCl pH 7.5, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 0.5 mM DTT, 5 µM ATP and 5 µCi [γ-32P]ATP) in a total volume of 30 µL. The kinase reaction was stopped by adding Laemmli sample buffer and Sample Reducing Agent and incubation for 10 min under 70°C. Proteins were separated by SDS–PAGE. Phosphorylation was visualized by autoradiography. Coomassie staining was performed as the loading control.

Radioactive kinase assay for in planta immunoprecipitated proteins

For western blots or kinase assays using the proteins extracted from plants, 2-week-old *tot3-2 pTOT3::GFP:TOT3* plants that were grown at 21°C under continuous light were used. Plants were firstly moved to a half-strength MS petri plate with 50 µM ABA or DMSO at 21°C for 2 h and then the plates were transferred to 21 or 34°C for another 2 h. Leaves were collected and ground into powder for protein

extraction and co-immunoprecipitation with GFP or RFP magnetic beads as described above. After immunoprecipitation and washing, the beads were resuspended in kinase assay buffer as described above and incubated at 30°C for 1 h. The kinase reaction was stopped by adding Laemmli sample buffer and Sample Reducing Agent and incubation for 10 min at 70°C. Proteins were separated by SDS–PAGE. Phosphorylation signals were detected by autoradiography. Silver staining or Coomassie staining was performed as the loading control. The phosphorylation intensity of the target protein in autoradiographs was measured using ImageJ (version 1.53f). The protein amount was measured to normalize phosphorylation intensity. The mock condition was set as 1 as the basal level to calculate the relative phosphorylation level under treatments.

Radioactive kinase assay of TOT3 under combined heat and drought treatment

The radioactive kinase assay was performed as described previously with adaptations [46]. A detailed description on plant growth conditions and radioactive kinase assay can be found in the ***Supplementary Information***.

Yeast two-hybrid assay

To test for interaction between TOT3 and OST1, the yeast strain AH109 was co-transformed with *pBRIDGE-OST1* and *pGADT7-TOT3* and the corresponding controls with empty vector. Co-transformed yeast colonies were selected in a liquid synthetic defined (SD) medium lacking Leu and Trp, and two independent colonies were grown for interaction assays. A Y2H growth assay was performed to determine the interaction on medium lacking Leu, Trp and His. Dilutions (10^{-1}, 10^{-2}, 10^{-3}) of saturated cultures were spotted onto the plates and photographs were taken after 5 days at 30ºC. For the interaction of TOT3 and AHA1, the assay was performed as described before [47]. NubG-TOT3 with AHA1-Cub-PLV and the corresponding controls were transformed into Yeast strain THY.AP 4 [*MATa ura3 leu2 lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2*]. Co-transformed yeast colonies were selected in a liquid synthetic defined (SD) medium lacking Leu and Trp, and followed by selection on SD medium lacking Leu, Trp, His and Ade. Dilutions (10^{-1}, 10^{-2}, 10^{-3}) of saturated cultures were spotted onto the plates and photographs were taken after 3 days at 30ºC.

RT-qPCR

Two-week-old Arabidopsis seedlings were grown at 21°C under continuous light and transferred to 34°C or kept at 21°C for 2 h. The shoot of the seedlings was harvested for detection of gene expression. At least three biological replicates were performed for each experiment. RNA Tissue Miniprep (Promega, USA) was used for RNA extraction and purification under the manufacturer's instruction. cDNA synthesis

was performed from 1 µg RNA with qScript cDNA Synthesis Kit (Quantabio, USA). qRT-PCR was performed with primers provided in (Extended Data Table 5) on a LightCycler 480 (Roche Diagnostics) with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's instructions.

Mass spectrometry (MS) analysis of proteins expressed in tobacco leaves

Two separate experiments were performed. In the first experiment, plasmids containing *35S::GFP:TOT3* and *35S::RFP:OST1* (Extended Data Table 6) were co-infiltrated in tobacco leaves. After 3 days, the leaves were infiltrated with 50 µM ABA or DMSO for 2 h and then collected. In the second experiment, plasmids containing *35S::GFP:TOT3* or its mutated variants *TOT3*^{D157N} [23], *TOT3-3E* and *TOT-5A* (Extended Data Table 6) were co-infiltrated with *35S::RFP:AHA1*. The samples were harvested after 3 days. For all experiments, four biological replicates were performed. Immunoprecipitation of GFP-TOT3 (for the first experiment) or RFP-AHA1 (for the second experiment) was performed, and LC-MS/MS analyses were carried out as previously described to detect the phosphorylation of the immunoprecipitated proteins [23]. Peptide searches were performed using Maxquant software (version 1.6.10.43) on the UseGalaxy.be server. A database containing all *Nicotiana benthamiana* protein sequences from SolGenomics and the protein sequences of GFP, RFP, GFP-TOT3 and RFP-OST1 (for the first experiment) or GFP, RFP, GFP-TOT3 and RFP-AHA1 (for the second experiment) were used as the search database. All phosphosites with at least 3 valid values in one treatment group were retained as reproducibly quantified phosphosites for statistical analysis. Multiple sample test or Students' t-test with $p < 0.05$ was carried out to test the differences among the treatments.

Immunoprecipitation-Mass Spectrometry

Two-week-old *tot3-2 pTOT3::GFP:TOT3 #5* seedlings grown on square petri plates with half-strength MS under long-day conditions (16 h light/8 h dark, 90 µmol m^{-2} s^{-1} photosynthetically active radiation and 70% relative humidity (RH)) were used. Whole seedlings were moved to flasks with liquid half-strength MS, containing DMSO (mock) or 50 mM ABA (ABA) at 21°C, or DMSO at 34°C (heat) under identical light and humidity conditions for 1 h (see Extended Data Fig. 1d for the conditions). The shoot of the seedings was collected. Protein extraction and GFP-immunoprecipitation were performed as described above (***Mass spectrometry (MS) analysis of proteins expressed in tobacco leaves***). Peptide searches were performed using Maxquant software (version 1.6.17.0) on the UseGalaxy.be server. A database (Araport11_genes.201606.pep) containing *Arabidopsis thaliana* protein sequences and the protein sequences of GFP-TOT3 was used as the search database. All phosphosites with at least 3 valid values in one treatment group were retained as reproducibly quantified phosphosites for statistical analysis. Multiple sample test with $p < 0.05$ was carried out to test the differences among the different treatments.

Phosphopeptide enrichment and LC-MS/MS analysis

Phosphoproteome analyses on plants under drought conditions were performed as described above (see ***Drought experiments*** for experimental set-ups). Protein extraction and phosphoproteomics were performed as previously described [23]. A detailed description can be found in the ***Supplementary Information***. MS/MS spectra were searched against the Araport11 database for *Arabidopsis thaliana* (Araport11_genes.201606.pep) by the MaxQuant software (version 1.6.17.0). The 'Phospho(STY).txt' output file generated by the MaxQuant search was loaded into Perseus software (version 1.6.7) for analysis. All data were selected first by a localization probability cut-off of > 0.75. Phosphosites that were quantified in at least three out of biological replicates for at least one genotype under one condition were retained. Log2 phosphosite intensities were centred by subtracting the median of the entire set of protein ratios per sample. Imputation was subsequently processed with default values for "Width = 0.3" and "Down shift = 1.8" by the mode of separately for each column [48]. Differential TOT3 phosphosites were selected in Perseus based on the two-sample test ($p < 0.05$) between (i) well-watered and drought in Col-0 wild-type and (ii) Col-0 and *ost1-3* under drought conditions.

Experimental control, quantification and statistical analyses

The majority of the experiments were randomized, and relevant analyses were performed mainly blinded with respect to sample identity during experiments and outcome assessment. The quantification of BiFC, stomatal assays and guard cell H^+-ATPase immunohistochemical detection were done in a blinded set-up involving several co-authors either labelling or measuring the samples/images. Most experiments were repeated two to three times and all biological replicates gave similar results. Statistical analyses were performed in GraphPad Prism (version 9.5.1).

References

1. Hetherington, A. M. & Woodward, F. I. The role of stomata in sensing and driving environmental change. *Nature* **424**, 901–908 (2003).

2. Murata, Y., Mori, I. C. & Munemasa, S. Diverse Stomatal Signaling and the Signal Integration Mechanism. *Annu. Rev. Plant Biol.* **66**, 369–392 (2015).

3. Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E. & Mittler, R. Abiotic and biotic stress combinations. *New Phytol.* **203**, 32–43 (2014).

4. Ciais, P. *et al.* Europe-wide reduction in primary productivity caused by the heat and drought in 2003. *Nature* **437**, 529–533 (2005).

5. Zhang, H., Zhu, J., Gong, Z. & Zhu, J.-K. Abiotic stress responses in plants. *Nat. Rev. Genet.* **23**, 104–119 (2022).

6. Pennisi, E. How plants learned to breathe. *Science (80-.).* **355**, 1110–1111 (2017).

7. Gupta, A., Rico-Medina, A. & Caño-Delgado, A. I. The physiology of plant responses to drought. *Science (80-.).* **368**, 266–269 (2020).

8. Hõrak, H., Fountain, L., Dunn, J. A., Landymore, J. & Gray, J. E. Leaf temperature responses to ABA and dead bacteria in wheat and Arabidopsis. *Plant Signal. Behav.* **16**, 1899471 (2021).

9. Munemasa, S. *et al.* Mechanisms of abscisic acid-mediated control of stomatal aperture. *Curr. Opin. Plant Biol.* **28**, 154–162 (2015).

10. Crawford, A. J., McLachlan, D. H., Hetherington, A. M. & Franklin, K. A. High temperature exposure increases plant cooling capacity. *Curr. Biol.* **22**, R396–R397 (2012).

11. Kostaki, K.-I. *et al.* Guard Cells Integrate Light and Temperature Signals to Control Stomatal Aperture. *Plant Physiol.* **182**, 1404–1419 (2020).

12. Gommers, C. Keep Cool and Open Up: Temperature-Induced Stomatal Opening. *Plant Physiol.* **182**, 1188–1189 (2020).

13. Urban, J., Ingwers, M., McGuire, M. A. & Teskey, R. O. Stomatal conductance increases with rising temperature. *Plant Signal. Behav.* **12**, e1356534 (2017).

14. Urban, J., Ingwers, M. W., McGuire, M. A. & Teskey, R. O. Increase in leaf temperature opens stomata and decouples net photosynthesis from stomatal conductance in Pinus taeda and Populus deltoides x nigra. *J. Exp. Bot.* **68**, 1757–1767 (2017).

15. Dubois, M. & Inzé, D. Plant growth under suboptimal water conditions: early responses and methods to study them. *J. Exp. Bot.* **71**, 1706–1722 (2020).

16. Mazdiyasni, O. & AghaKouchak, A. Substantial increase in concurrent droughts and heatwaves in the United States. *Proc. Natl. Acad. Sci.* **112**, 11484–11489 (2015).

17. Coughlan de Perez, E., Ganapathi, H., Masukwedza, G. I. T., Griffin, T. & Kelder, T. Potential for surprising heat and drought events in wheat-producing regions of USA and China. *npj Clim. Atmos. Sci.* **6**, 56 (2023).

18. Mittler, R. & Blumwald, E. Genetic Engineering for Modern Agriculture: Challenges and Perspectives. *Annu. Rev. Plant Biol.* **61**, 443–462 (2010).

19. Falhof, J., Pedersen, J. T., Fuglsang, A. T. & Palmgren, M. Plasma Membrane H + -ATPase Regulation in the Center of Plant Physiology. *Mol. Plant* **9**, 323–337 (2016).

20. Svennelid, F. *et al.* Phosphorylation of Thr-948 at the C Terminus of the Plasma Membrane H + -ATPase Creates a Binding Site for the Regulatory 14-3-3 Protein. *Plant Cell* **11**, 2379–2391 (1999).

21. Merlot, S. *et al.* Constitutive activation of a plasma membrane H+-ATPase prevents abscisic acid-mediated stomatal closure. *EMBO J.* **26**, 3216–3226 (2007).

22. Kinoshita, T. Blue light activates the plasma membrane H+-ATPase by phosphorylation of the C-terminus in stomatal guard cells. *EMBO J.* **18**, 5548–5558 (1999).

23. Vu, L. D. *et al.* The membrane-localized protein kinase MAP4K4/TOT3 regulates thermomorphogenesis. *Nat. Commun.* **12**, 2842 (2021).

24. Lin, W. *et al.* TMK-based cell-surface auxin signalling activates cell-wall acidification. *Nature* **599**, 278–282 (2021).

25. Hayashi, M., Inoue, S., Takahashi, K. & Kinoshita, T. Immunohistochemical Detection of Blue Light-Induced Phosphorylation of the Plasma Membrane H+-ATPase in Stomatal Guard Cells. *Plant Cell Physiol.* **52**, 1238–1248 (2011).

26. Miao, R., Russinova, E. & Rodriguez, P. L. Tripartite hormonal regulation of plasma membrane H+-ATPase activity. *Trends Plant Sci.* **27**, 588–600 (2022).

27. Mustilli, A.-C., Merlot, S., Vavasseur, A., Fenzi, F. & Giraudat, J. Arabidopsis OST1 Protein Kinase Mediates the Regulation of Stomatal Aperture by Abscisic Acid and Acts Upstream of Reactive Oxygen Species Production. *Plant Cell* **14**, 3089–3099 (2002).

28. Belin, C. *et al.* Identification of Features Regulating OST1 Kinase Activity and OST1 Function in Guard Cells. *Plant Physiol.* **141**, 1316–1327 (2006).

29. Takahashi, Y. *et al.* MAP3Kinase-dependent SnRK2-kinase activation is required for abscisic acid signal transduction and rapid osmotic stress response. *Nat. Commun.* **11**, 12 (2020).

30. Waadt, R. *et al.* Identification of Open Stomata1-Interacting Proteins Reveals Interactions with Sucrose Non-fermenting1-Related Protein Kinases2 and with Type 2A Protein Phosphatases That Function in Abscisic Acid Responses. *Plant Physiol.* **169**, 760–779 (2015).

31. Soma, F., Takahashi, F., Suzuki, T., Shinozaki, K. & Yamaguchi-Shinozaki, K. Plant Raf-like kinases regulate the mRNA population upstream of ABA-unresponsive SnRK2 kinases under drought stress. *Nat. Commun.* **11**, 1373 (2020).

32. Sun, Z. *et al.* RAF22, ABI1 and OST1 form a dynamic interactive network that optimizes plant growth and responses to drought stress in Arabidopsis. *Mol. Plant* **15**, 1192–1210 (2022).

33. Devireddy, A. R., Arbogast, J. & Mittler, R. Coordinated and rapid whole-plant systemic stomatal responses. *New Phytol.* **225**, 21–25 (2020).

34. Clough, S. J. & Bent, A. F. Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana. *Plant J.* **16**, 735–743 (1998).

35. Jinn, T.-L. Using silicon polymer impression technique and scanning electron microscopy to measure stomatal apertures. *BIO-PROTOCOL* **7**, (2017).

36. Huang, Y.-C. *et al.* PECTIN METHYLESTERASE34 Contributes to Heat Tolerance through Its Role in Promoting Stomatal Movement. *Plant Physiol.* **174**, 748–763 (2017).

37. Scarpeci, T., Zanor, M. & Valle, E. Estimation of Stomatal Aperture in Arabidopsis thaliana Using Silicone Rubber Imprints. *BIO-PROTOCOL* **7**, (2017).

38. Vanhaeren, H. *et al.* UBP12 and UBP13 negatively regulate the activity of the ubiquitin-dependent peptidases DA1, DAR1 and DAR2. *Elife* **9**, (2020).

39. Pizzio, G., Regmi, K. & Gaxiola, R. Rhizosphere Acidification Assay. *BIO-PROTOCOL* **5**, (2015).

40. Pizzio, G. A. *et al.* Arabidopsis Type I Proton-Pumping Pyrophosphatase Expresses Strongly in Phloem, Where It Is Required for Pyrophosphate Metabolism and Photosynthate Partitioning. *Plant Physiol.* **167**, 1541–1553 (2015).

41. Abas, L. *et al.* Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nat. Cell Biol.* **8**, 249–256 (2006).

42. Wang, Y. *et al.* Overexpression of plasma membrane H + -ATPase in guard cells promotes light-induced stomatal opening and enhances plant growth. *Proc. Natl. Acad. Sci.* **111**, 533–538 (2014).

43. Hayashi, Y., Takahashi, K., Inoue, S. I. & Kinoshita, T. Abscisic acid suppresses hypocotyl elongation by dephosphorylating plasma membrane H+-ATPase in arabidopsis thaliana. *Plant Cell Physiol.* **55**, 845–853 (2014).

44. Grefen, C. & Blatt, M. R. A 2in1 cloning system enables ratiometric bimolecular fluorescence complementation (rBiFC). *Biotechniques* **53**, 311–314 (2012).

45. Mehlhorn, D. G., Wallmeroth, N., Berendzen, K. W. & Grefen, C. 2in1 vectors improve in planta BiFC and FRET analyses. *Methods Mol. Biol.* **1691**, 139–158 (2018).

46. Lu, Q. *et al.* Proteome-wide cellular thermal shift assay reveals unexpected cross-talk between brassinosteroid and auxin signaling. *Proc. Natl. Acad. Sci. U. S. A.* **119**, (2022).

47. Obrdlik, P. *et al.* K + channel interactions detected by a genetic system optimized for systematic studies of membrane protein interactions. *Proc. Natl. Acad. Sci.* **101**, 12242–12247 (2004).

48. Tyanova, S. *et al.* The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods* **13**, 731–740 (2016).

Declarations

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Supplementary Information

Extended Data Figures 1 to 26

Extended Methods

Reference

Extended Data Tables 1 to 6

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