Proteome-wide cellular thermal shift assay reveals unexpected cross-talk between brassinosteroid and auxin signaling

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Despite the growing interest in using chemical genetics in plant research, small molecule target identification remains a major challenge. The cellular thermal shift assay coupled with high-resolution mass spectrometry (CETSA MS) that monitors changes in the thermal stability of proteins caused by their interactions with small molecules, other proteins, or posttranslational modifications, allows the discovery of drug targets or the study of protein–metabolite and protein–protein interactions mainly in mammalian cells. To showcase the applicability of this method in plants, we applied CETSA MS to intact Arabidopsis thaliana cells and identified the thermal proteome of the plant-specific glycogen synthase kinase 3 (GSK3) inhibitor, bikinin. A comparison between the thermal and the phosphoproteomes of bikinin revealed the auxin efflux carrier PIN-FORMED1 (PIN1) as a substrate of the Arabidopsis GSK3s that negatively regulate the brassinosteroid signaling. We established that PIN1 phosphorylation by the GSK3s is essential for maintaining its intracellular polarity that is required for auxin-mediated regulation of vascular patterning in the leaf, thus revealing cross-talk between brassinosteroid and auxin signaling.

Significance

Chemical genetics, which investigates biological processes using small molecules, is gaining interest in plant research. However, a major challenge is to uncover the mode of action of the small molecules. Here, we applied the cellular thermal shift assay coupled with mass spectrometry (CETSA MS) to intact Arabidopsis cells and showed that bikinin, the plant-specific glycogen synthase kinase 3 (GSK3) inhibitor, changed the thermal stability of some of its direct targets and putative GSK3-interacting proteins. In combination with phosphoproteomics, we also revealed that GSK3s phosphorylated the auxin carrier PIN-FORMED1 and regulated its polarity that is required for the vascular patterning in the leaf.

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Competing interest statement: D.M.M. is the inventor of patents related to the CETSA method and is a cofounder, board member, and employee of Pelago Bioscience AB. This article is a PNAS Direct Submission. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

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and BES1/BZR2 transcription factors (14), BIN2/AtSK21 interacts and phosphorylates the AUXIN RESPONSE FACTOR2 (ARF2) (21). This phosphorylation results in a loss in the DNA binding and repressor activity of ARF2 and facilitates auxin responses (21). BIN2/AtSK21 also phosphorylates and activates ARF7 and ARF19 to promote lateral root development through an increase in auxin response (22). Moreover, BRs have been shown to control posttranscriptionally the endocytic sorting of PIN-FORMED 2 (PIN2) (23) and stimulate the nuclear abundance and signaling of auxin via repressing the accumulation of PIN-LIKES (PILS) proteins at the endoplasmic reticulum (24).

Here, by adapting the CETSA MS to Arabidopsis intact cells and combining it with phosphoproteomics, we discovered that the auxin efflux carrier PIN1 is a substrate of the AtSKs. We found that phosphorylation mediated by the AtSKs is required for PIN1 polarity and for leaf venation. In summary, we demonstrate that CETSA MS is a powerful method for the identification of small molecule targets as well as for the discovery of new protein–protein interactions in plant cells.

Results

CETSA Monitoring of Small Molecule–Protein Interactions in Intact Arabidopsis Cells. Previously, we had used the Western blot–based CETSA for small molecule target validation in cell lysates of Arabidopsis seedlings (6). To extend the use of CETSA to intact plant cells, we adapted the available protocol (10) to Arabidopsis cell suspension cultures (SI Appendix, Fig. S1). First, we tested whether the plant cell wall complicated the protein isolation by evaluating the efficiency of the freeze–thaw lysis method applied to mammalian cells (10). After washing and resuspension in protein extraction buffer, 100–μL aliquots of cells were freeze–thawed multiple times and the protein concentration of the supernatant was measured with the Bradford protein assay. The protein concentration in the lysate plateaued after seven freeze–thaw cycles and centrifugation. As expected, the Arabidopsis proteins unfolded and precipitated at high temperature (SI Appendix, Fig. S24). Moreover, considering that plants grow over a wide temperature range, we assessed whether in intact Arabidopsis cells, proteins follow a melting profile similar to that in lysates when heated (5, 26). To this end, we heated 100-μL aliquots of Arabidopsis cells in protein extraction buffer, heated to different temperatures (from 25°C to 80°C) for 2 min, and analyzed the lysates by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after seven freeze–thaw cycles and centrifugation. As expected, the Arabidopsis proteins unfolded and precipitated at high temperature (SI Appendix, Fig. S2 B and C).

Subsequently, as a proof of concept, we aimed to apply CETSA to cells treated with bikinin, an inhibitor of several AtSKs in Arabidopsis (12). Application of bikinin at concentrations of 30 to 50 μM to Arabidopsis seedlings induces BR responses that can be measured by changes in the phosphorylation status of the transcription factor BES1/BZR2 (14). To check whether bikinin is effective in Arabidopsis cell suspension cultures, we treated cells with 50 μM bikinin for 30 min. As expected, bikinin induced the dephosphorylation of BES1 in the cell cultures similarly to the most active BR, brassinolide (BL) (SI Appendix, Fig. S2D). Taken together, bikinin can induce BR responses in Arabidopsis cell suspension cultures.

Next, we investigated the effect of bikinin on the thermal stability of its direct targets, the ten AtSKs (12), by means of the adjusted CETSA protocol (SI Appendix, Fig. S1) and by using Western blots for detection. In brief, after 30 min of treatment with bikinin or dimethyl sulfoxide (DMSO), the Arabidopsis cells were washed, resuspended in protein extraction buffer containing either bikinin or DMSO, and aliquoted into PCR tubes. Then, the aliquots were heated to 12 distinct temperatures (30, 35, 40, 43, 46, 49, 52, 55, 58, 61, 65, and 70°C) for 2 min. Afterward, the heated cells were lysed through seven freeze–thaw cycles followed by Western blot–based protein detection. As specific antibodies for all AtSKs are not available, Arabidopsis cell suspension cultures overexpressing the hemagglutinin (HA)-tagged AtSKs were utilized to generate protein-melting curves and to assess the bikinin-induced Tm shifts. First, we examined whether bikinin induced Tm shifts for AtSK12 and AtSK13 at a 50-μM concentration, but, surprisingly, found that it stabilized AtSK13 with a Tm shift of 5.94°C (SI Appendix, Fig. S34), but not AtSK12 (SI Appendix, Fig. S38). By contrast, the thermal stability of the ATP synthase β (ATPβ), used as a control, was not affected by bikinin. We next performed an isothermal dose–response fingerprinting (ITDRCETSA) for AtSK12 at 45°C, a temperature selected between the Tm of AtSK12 and Tm of AtSK13 under control (DMSO) conditions and determined the bikinin half-maximum effective concentration (EC50) to be around 70 μM (SI Appendix, Fig. S3C). To ensure saturation and achieve sufficiently sized Tm shifts for all AtSKs, we used 250 μM bikinin (Fig. 1). Staining of the cells with the cell viability tracer, fluorescein diacetate (FDA), excluded the potential cytotoxic effect of bikinin when used at high concentrations (SI Appendix, Fig. S2E). Of the 10 putative bikinin targets (12), AtSK11, AtSK12, AtSK13, BIN2/AtSK21, AtSK22, and AtSK41 showed Tm shifts, whereas the thermal stability of AtSK23, AtSK31, AtSK32, and AtSK42 was not affected by the small molecule at 250 μM (Fig. 1) as well as the thermal denaturation of the DMSO control (Dataset SI Appendix). Collectively, these results showed that bikinin stabilized most of its targets, indicating that the CETSA protocol was applicable to intact Arabidopsis cells.

CETSA MS of Bikinin in Intact Arabidopsis Cells. Several recent studies in mammalian cells reported the use of proteome-wide CETSA MS for obtaining a comprehensive view on small molecule–protein interactions through determination of individual thermal temperature shifts (8–10, 27). Therefore, we extended the CETSA protocol as described above (SI Appendix, Fig. S1) to the Arabidopsis proteome (SI Appendix, Fig. S2D) by using 50 μM bikinin, the BR response-inducing concentration in cell cultures. Briefly, following the heating at 25, 30, 35, 40, 45, 50, 55, 60, 70, and 80°C and freeze–thaw cycles, samples were analyzed with a nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS), whereafter they were digested with trypsin and labeled with 10-plex tandem mass tag (TMT10).

In total, 6,000 proteins were identified, of which the melting profiles were defined for 4,225 proteins in samples treated with both bikinin and DMSO (Dataset SL4). Approximately 96% of the identified proteins had a melting temperature within the range of 35°C to 60°C (Fig. 2 A and Dataset SL4), of which only 61 proteins displayed a significant change in thermal stability (27 were stabilized and 34 were destabilized) in the presence of 50 μM bikinin (absolute value of Tm shift ≥ 2°C, analysis of variance [ANOVA]-based F test P < 0.01) (Fig. 2B and Dataset S1). However, because none of the AtSKs were identified by the CETSA MS, we examined the protein expression of AtSKs in Arabidopsis cell suspension cultures. Although qRT-PCR revealed expression of all AtSKs in the cell cultures (SI Appendix, Fig. S54), only AtSK11, AtSK21, AtSK31, and AtSK41, albeit at a low intensity, were detected with shotgun proteomics (SI Appendix, Fig. S5B and Dataset S2). Given that all AtSKs were efficiently extracted using the CETSA protocol when overexpressed (Fig. 1), probably the low number of peptides identifying these proteins in cell cultures obstructed the creation of their melting curves (Dataset S2).

As changes in thermal stability had also been observed for downstream effectors of the direct small molecule target, possibly as a result of altered posttranslational modifications or interactions with other proteins (4, 9), we examined whether any of the 61
proteins \((P < 0.01)\) (Dataset S2B) functioned together with the AtSKs and found that only the MITOGEN-ACTIVATED PROTEIN KINASE3 (MPK3), which acts downstream of the known BIN2/AtSK21 interactor YODA (YDA) (28), exhibited a Tm shift (−2.96°C, \(P < 0.01\)). Furthermore, we checked whether any of the 61 proteins with significant Tm shifts were putative AtSK interactors (SI Appendix, Fig. S6) according to the STRING database (29). Based on this analysis, the HOPW1-1-INTERACTING2 (WIN2, PLANT BIOLOGY Lu et al. Proteome-wide cellular thermal shift assay reveals novel cross-talk between brassinosteroid and auxin signaling proteins (\(P < 0.01\)) (Dataset S2B) functioned together with the AtSKs and found that only the MITOGEN-ACTIVATED PROTEIN KINASE3 (MPK3), which acts downstream of the known BIN2/AtSK21 interactor YODA (YDA) (28), exhibited a Tm shift (−2.96°C, \(P < 0.01\)). Furthermore, we checked whether any of the 61 proteins with significant Tm shifts were putative AtSK interactors (SI Appendix, Fig. S6) according to the STRING database (29). Based on this analysis, the HOPW1-1-INTERACTING2 (WIN2,
AT4G31750) \( (T_m \text{ shift} = 7.80 \, ^\circ\text{C}, \, P < 0.01) \) (Fig. 2C) was selected as a possible AtSK-interacting protein. Interaction between HA-tagged WIN2 and each of the 10 GFP-tagged AtSKs was observed by coimmunoprecipitation experiments carried out using tobacco (Nicotiana tabacum) cells transiently overexpressing the proteins (Fig. 2D). In summary, in cell suspension cultures bikinin affected the thermal stability of the Arabidopsis proteome and induced Tm shifts in several proteins that might be putative AtSK-interacting proteins or AtSK downstream effectors.

**The Phosphoproteome of Bikinin.** As, besides MPK3, the proteins with altered thermal stability identified by the CETSA MS (Dataset S1) were neither direct bikinin targets nor known downstream AtSK effectors, we carried out a phosphoproteomics analysis on bikinin-treated Arabidopsis cell suspension cultures. We hypothesized that the putative AtSK-interacting proteins or AtSK downstream effectors might modify their phosphorylation state upon inhibition of their kinase activity. Arabidopsis cell suspension cultures were treated with 50 \( \mu\text{M} \) bikinin or DMSO for 30 min, under the same CETSA MS conditions. The phosphoproteomics analysis revealed that, in total, 9,351 phosphopeptides were mapped to 1,751 proteins (Dataset S3A). Bikinin treatment significantly down-regulated \( (P < 0.05) \) the phosphorylation intensities of 972 phosphosites that belong to 665 proteins (Fig. 3A and SI Appendix, Fig. S7 and Dataset S3 B–E), of which six proteins were known AtSK-interacting proteins, including BZR1 (30), ARF2 (21), YDA (28), GLUCOSE-6-PHOSPHATE DEHYDROGENASE6 (G6PD6) (31), TETRATRICOPETIDE-REPEAT THIOREDOXIN-LIKE3 (TTL3) (32), and OCTOPUS (33). In addition, the phosphorylation intensities of 101 phosphosites belonging to 84 proteins were significantly up-regulated \( (P < 0.05) \) (Fig. 3A and SI Appendix, Fig. S7 and Dataset S3 B–E). Of note, both down-regulated and up-regulated phosphosites were identified in 35 proteins (Fig. 3A and Dataset S3 B–E). The gene ontology (GO) enrichment analysis (34) revealed that most of the enriched terms for the identified phosphorylation-regulated proteins were related to mRNA splicing, metabolic process, and transport (SI Appendix, Fig. S8A and Dataset S3G). However, of the bikinin-regulated phosphoproteins, only six (phosphorylation down-regulated) proteins showed significant Tm shifts in the CETSA MS (Dataset S3E), including the auxin efflux carrier PIN1 \( (T_m \text{ shift} = 3.83 \, ^\circ\text{C}) \) (Fig. 3A and B). Taken together, only a few proteins identified in the bikinin phosphoproteome displayed bikinin-induced changes in their thermal stability.

**AtSKs Phosphorylate PIN1 and Regulate Its Polarity.** We hypothesized that PIN1 is a direct substrate of the AtSKs, because the PIN1 protein had been identified in the CETSA MS (Fig. 3B
and D and Dataset S1) and the phosphorylation intensities of five residues in the hydrophilic loop (HL) (Ser 271, Ser 282, Thr 286, Ser 290, and Ser 337) (SI Appendix, Fig. S9A) were reduced in the presence of 50 μM bikinin (Fig. 3C and Dataset S3C). To verify this observation, we carried out a protein kinase assay by incubating the polyhistidine (HIS)-tagged HL of PIN1 with HIS-small ubiquitin-like modifier (HIS-SUMO)-tagged AtSK proteins in an in vitro phosphorylation reaction. The results showed that eight AtSK proteins phosphorylated the His-PIN1-HL (SI Appendix, Fig. S9B). Remarkably, four of the five PIN1 residues, of which the phosphorylation was altered by bikinin, had previously been identified as targets of the serine/threonine protein kinases PINOID/AGCVIII kinases (PID/WAGs) (35), D6 Protein Kinases (D6PKs) (36), and MPKs (MPK3, MPK4, and MPK6) (37, 38). Moreover, these residues were conserved (SI Appendix, Fig. S9A) and essential for the polar localization of the PIN proteins with long HLs (39).

Given the prominent role of phosphorylation in regulating polarity, we next assessed whether treatments with bikinin and BL affected the polar localization of PIN1 and PIN2 in the root meristem by means of immunolocalization. Wild-type Arabidopsis plants were treated in liquid medium with 50 μM bikinin, 10 nM BL, and 0.1% (vol/vol) DMSO for 12 h. As expected, PIN1 displayed a more apolar localization in the presence of bikinin and BL (Fig. 4A and B) than with DMSO, whereas the PIN2 polarity remained unaffected (SI Appendix, Fig. S10). To provide genetic evidence for the AtSK function in PIN1 polarity regulation, we analyzed the PIN1 localization in the root tips of the atskquad (atsk13RNAi bin2 bil1 bil2, designated atskquad) and sextuple (atsk11RNAi atsk12RNAi atsk13RNAi bin2-3 bil1 bil2, designated atsksext) mutants (40). PIN1 had a more apolar localization in the root tips of the atskquad mutant than the wild-type (Fig. 4C and D). Because of the seedling lethality of the atsksext mutant (40), the plant phenotypes were analyzed in the T1 generation. The atsksext mutant also displayed an aberrant PIN1 polar localization (Fig. 4C and D). Given that PIN1-mediated polar auxin transport regulates the foliar vascular patterning (41), we examined whether AtSKs were involved in the regulation of leaf venation. Five-day-old wild-type plants were treated in liquid medium with 50 μM bikinin, 10 nM BL, and 0.1% (vol/vol) DMSO for 2 d. As expected, plants treated with bikinin and BL had an abnormal leaf vascular patterning, when compared to the mock control (SI Appendix, Fig. S11). Consistent with BR and bikinin treatments, the atskquad and atsksext mutants exhibited an aberrant vascular patterning, with missing loops and disconnected upper loops (Fig. 4E and F). Subsequently, we examined the PIN1 localization in the leaf veins of atskquad and atsksext mutants. As anticipated, atskquad and atsksext mutants displayed impaired PIN1 localizations, including missing PIN1 in...
Fig. 4. PIN1 polarity modulation by the AtSKs. (A) Immunolocalization of PIN1 in root tips after 12 h of 50 μM BIK, 10 nM BL, or 0.1% (vol/vol) DMSO treatments. Zoom-ins of areas marked with dashed boxes are shown below each main image. (B) Quantitative evaluation of A showing mean of PIN1 lateral-to-basal signal intensity ratio in endodermal cells. n > 180 cells corresponds to a minimum of 15 roots per treatment from three independent experiments. (C) Immunolocalization of PIN1 in root tips of Wassilewskija (Ws), AtSK quadruple (atskquad), and sextuple (atsksext) mutants. Zoom-ins of areas marked with dashed boxes are shown below each main image. (Scale bars, 20 μm.) (D) Quantitative evaluation of C showing mean of PIN1 lateral-to-basal signal intensity ratio in endodermal cells. n > 95 cells corresponds to a minimum of 15 roots per treatment from two independent experiments. (E) Representative images of venation patterning defects in cotyledons of Ws, atskquad, and atsksext mutants. (Scale bars, 1 mm.) (F) Quantification of venation defects in atskquad and atsksext mutants (n > 60 of each genotype from three independent experiments). (G) Immunolocalization of PIN1 in the leaf veins of Ws, atskquad, and atsksext mutants. Red arrows indicate the defects in PIN1 localization, including missing PIN1, and more PIN1 localizations parallel to the vein axis. (H) Quantitative evaluation of G showing defects in PIN1 localization; n > 45 leaves from five independent experiments. (I) Subcellular localization of PIN1-GFP and PIN1-GFP5A in root tip cells. Arrowheads indicate the lateral membrane signal in the endodermal cells. (J) Quantitative evaluation of I showing mean of PIN1-GFP and PIN1-GFP5A lateral-to-basal signal intensity ratio in endodermal cells. n > 60 cells corresponds to a minimum of 15 roots per genotype from three independent experiments. (K) Representative images of venation patterning defects in cotyledons of PIN1pro::PIN1-GFP and two transgenic lines of PIN1pro::PIN15A-GFP. (Scale bars, 1 mm.) (L) Quantification of venation defects in PIN1pro::PIN1-GFP and two PIN1pro::PIN15A-GFP transgenic lines (n > 60 of each genotype from three independent experiments). (B, D, and J) Scatter dot plots showing all the individual points with means and SEs. One-way ANOVA with Tukey’s post hoc test compared to DMSO or PIN2-GFP, respectively. ***P < 0.0001.
some cells and more PIN1 localizations parallel to the vein axis, when compared to the wild-type control (Fig. 4 G and H).

To test whether the identified five phosphorylation sites were relevant for the AtSK-mediated regulation of PIN1, we generated phospho-inactive His-PIN1-HEL5 by substituting the Thr and Ser residues with Ala. The phosphorylation of His-PIN1-HEL5 by BIN2/AtSK in vitro was reduced but not abolished (SI Appendix, Fig. S12A), suggesting that more sites were phospho

To demonstrate the capability of CESTA MS to identify proteins that might bind the AtSKs directly or function in downstream pathways, we carried out a phosphoproteomics analysis in the presence of bikinin. Phosphorylation sites in 665 proteins were down-regulated upon bikinin treatment, comprising six known substrates of the AtSKs, hence confirming the quality of our data. Noteworthy, the phosphorylation intensities of 84 proteins were up-regulated upon bikinin treatment, indicating that these proteins might be indirect AtSK targets. The GO enrichment analysis for the bikinin-regulated phosphoproteins revealed that AtSKs might be involved in the regulation of RNA splicing and protein intracellular transport, like their mammalian homologs (49, 50). Moreover, of all proteins with differential phosphorylation, 76 proteins were also identified by proximity labeling with BIN2/AtSK21 as a bait (51) (Dataset S3F). Thus, we speculate that some of these proteins might be substrates of the AtSKs. By comparing the CESTA MS and phosphoproteome datasets, we found that the thermal stability of most of these proteins was not affected, even though their phosphorylation intensities were altered by the bikinin treatment. A possible reason might be that either the phosphorylation cannot affect the protein melting behavior or that the bikinin-induced changes in the phosphorylation intensity are not sufficient to induce an important Tm shift for most of these proteins. However, for a few proteins, bikinin had an impact on both the phosphorylation and melting behaviors, indicating that the phosphorylation status might affect the thermal stability of some proteins. Although some studies in human cells reported that phosphorylation affected the protein thermal stability (52), others demonstrated that for most of the proteins, the melting behavior of phosphorylated and nonphosphorylated forms was concordant (53, 54).

Discussion

Given the challenges in small molecule target identification in plants (44), experimental strategies, especially label-free methods, are required to facilitate chemical genetics studies. CETSA is such a method that has been proven useful for the detection of direct drug targets and downstream effects of drug-induced perturbations in several cellular systems and tissues (9, 27, 45, 46). In plants, CETSA has been successfully applied for small molecule target validation in cell lysates of Arabidopsis (6) and, recently, for the thermal profiles of more than 2,000 proteins in Arabidopsis lyses have been reported (5, 26). Here, we explored the potential of CETSA to identify the protein targets of the AtSK kinase inhibitor bikinin (12) in intact Arabidopsis cells. In Western blot–based CETSA, bikinin stabilized six of the 10 AtSKs. As expected, bikinin had no effect on either AtSK31 or AtSK42, of which the kinase activity was not inhibited in vitro (12), but surprisingly, on AtSK23 and AtSK32 as well, of which the in vitro inhibition of the kinase activity had previously been reported (12). One reason might be that the binding affinity of bikinin to AtSK23 and AtSK32 is lower than that to other AtSKs, thus requiring higher bikinin concentrations to induce the noticeable Tm shifts. Moreover, in a previous study, ~30% of the target proteins of the promiscuous kinase inhibitor staurosporine did not show Tm shifts (9), suggesting that some protein kinases are not responsive in CETSA.

By combining CETSA with MS, we identified the thermal profiles of 4,225 proteins in the presence of bikinin in Arabidopsis cell suspension cultures, of which 61 (1.44%) showed significant Tm shifts (absolute value ≥ 2°C). ANOVA-based F test P < 0.01. Although the CETSA MS assay did not detect any AtSKs, probably due to their low protein abundance in the cell cultures, MPK3, the substrate of the known BIN2/AtSK21 interactor YDA (28), was detected. In addition, the thus far unknown interaction between WIN2 (At4G31750) and AtSKs was validated in vivo. WIN2 is implicated in the modulation of the defense responses induced by the Pseudomonas syringae effector protein HopW1-1 (47). AtSK11 has been shown to regulate the pattern-triggered immunity and the susceptibility to P. syringae, probably through G6PD6 phosphorylation (48). Our data suggest that AtSK11, as well as its homologs, might regulate the immune responses to P. syringae via direct interaction with WIN2.
studies have shown that BRs affect PIN proteins through different mechanisms (23, 58), including transcriptional activation of PIN4 and PIN7 (58). Additionally, BRs and bikinin stabilized PIN2, but not PIN1, to interfere with the auxin distribution in gravistimulated roots (23), whereas BRs partially altered PIN2 polarity via the actin cytoskeleton (59). However, the actin cytoskeleton and stress response were not required for the polar localization of PIN2 (60). Interestingly, BRs did not affect the PIN2 polarity in our study, although four of the five identified phosphorylation sites are conserved in the HL of PIN2. Together, these data indicate that the mechanisms controlling the polarity and the stability of PINs by BRs differ.

PIN1 has been shown to be expressed in early leaf veins and margins and PIN1-mediated polar auxin transport is important for the leaf vein pattern in Arabidopsis (40). Moreover, MPK6 could regulate the leaf vein pattern probably by regulating the PIN1 phosphorylation and polarity, although the main MPK6-targeted phosphorylation site Ser157, also targeted by AtSKs, was not involved (37). In addition, the auxin-regulated receptor CAMEL controls the cotyledon venation through modulation of the PIN1 phosphorylation and polarization (55). Our data showed that chemical activity inhibition or genetic knockout of the AtSKs resulted in impaired PIN polarity and defective cotyledon venation patterns, suggesting that BRs control leaf venation via AtSK-mediated phosphorylation of PIN1.

In summary, we adapted the CETSA MS method for intact Arabidopsis cells and demonstrated its ability to identify direct targets and downstream components of the small molecule target proteins. Such information is useful to understand the mode of action of the small molecules and the function of their target proteins. Moreover, we identified PIN1 as a substrate of AtSKs, uncovering an unknown BR mechanism and auxin cross-talk.

Materials and Methods

Plant Materials and Growth Condition. A. thaliana (L.) Heynh. (accession Columbia-0 [Col-0]) was used, except for the atsk13R2N1 bin2-3 b1il2 b1il2 quadruple and atsk11R2N1 atsk12R2N1 atsk13 b1il2 b1il2 sextuple mutants that were generated in Wassilewskija (Ws) background as previously described (40). The transgenic Arabidopsis plant expressing PIN1:pro:PIN1-GFP had been described previously (45). The growth conditions are described in detail in SI Appendix, Materials and Methods.

The experimental procedures for CETSA, CETSA MS, phosphoproteomics, confocal microscopy, in vitro kinase assay, whole-mount in situ immunolocalization of PIN1, immunoprecipitation, qRT-PCR, FDA measurement, and statistical analysis are described in detail in SI Appendix, Materials and Methods. The data of CETSA MS and phosphoproteomics analysis are provided in Datasets S5–S53. The primers used in this study are listed in SI Appendix, Table S1.

Data Availability. The CETSA MS data have been unloaded to MassIVE (ID: MSV000088548). The phosphoproteomics data have been uploaded to ProteomeXchange (ID: PXD029936). The shotgun proteomics data have been uploaded to ProteomeXchange (ID: PXD030309). All other study data are included in the article and/or supporting information.

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