TMK-based cell surface auxin signaling activates cell wall acidification in Arabidopsis

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TMK-based cell surface auxin signaling activates cell wall acidification in Arabidopsis

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Abstract

The phytohormone auxin controls a myriad of processes in plants, at least in part through its regulation of cell expansion. The "acid growth hypothesis" has been proposed to explain auxin-stimulated cell expansion for five decades, but the mechanism underlying auxin-induced cell wall acidification is poorly characterized. Auxin induces the phosphorylation and activation of the plasma membrane (PM) H⁺-ATPase that pumps protons into the apoplast, yet how auxin activates its phosphorylation remains elusive. Here, we show that the transmembrane kinase (TMK) auxin signaling proteins interact with PM H⁺-ATPases and activate their phosphorylation to promote cell wall acidification and hypocotyl cell elongation in Arabidopsis. Auxin induced TMK's interaction with H⁺-ATPase on the plasma membrane within 1-2 minutes as well as TMK-dependent phosphorylation of the penultimate Thr residue. Genetic, biochemical, and molecular evidence demonstrates that TMKs directly phosphorylate PM H⁺-ATPase and are required for auxin-induced PM H⁺-ATPase activation, apoplastic acidification, and cell expansion. Thus, our findings reveal a crucial connection between auxin and PM H⁺-ATPase activation in regulating apoplastic pH changes and cell expansion via TMK-based cell surface auxin signaling.

One Sentence Summary: TMK-based cell surface auxin signaling directly activates the phosphorylation of the plasma membrane H⁺-ATPase, leading to H⁺ pump activation, cell wall acidification, and cell elongation in Arabidopsis hypocotyls.

Main

Embedded in a rigid cell wall, the plant cell must modify its wall to gain the adjustable elasticity to regulate cell expansion in space and time. Auxin induces rapid cell expansion by acidifying the cell wall space (apoplast), leading to the activation of cell wall-localized proteins for wall loosening, a growth mechanism that has been
known as the acid-growth theory for half of a century. Auxin triggers the efflux of protons resulting in apoplastic acidification by activating the plasma membrane (PM) localized P-type H\(^+\)-ATPase. In Arabidopsis, PM H\(^+\)-ATPase is encoded by an AHA (Autoinhibited, H\(^+\)-ATPase) gene family with 11 members. Phosphorylation of the conserved penultimate Thr residue (Thr-948 in AHA1, Thr-947 in AHA2) has been proposed to release the autoinhibition of the ATPase pump activity by the cytoplasmic C-terminal region. Recently, Fendrych et al. demonstrated that auxin-induced apoplastic acidification and growth were mediated by TIR1/AFB-Aux/IAA nuclear auxin perception in hypocotyls. Auxin induces the TIR1/AFB-dependent expression of SAUR proteins that act as inhibitors of PP2C.D phosphatases, which dephosphorylate the penultimate Thr. While this mechanism can sustain H\(^+\)-ATPase activity by preventing penultimate Thr dephosphorylation, it cannot account for how the PM H\(^+\)-ATPase is initially phosphorylated to become activated.

The PM-localized TMK receptor-like kinases play a vital role in auxin signaling in regulating pavement cell morphogenesis, differential growth of the apical hook, and lateral root (LR) formation in Arabidopsis. Auxin rapidly promotes TMK-dependent activation of PM-associated ROP GTPases within seconds, providing a mechanism for rapid auxin responses on the cell surface in addition to TIR1/AFB-based intracellular auxin signaling. To identify new components in TMK-mediated auxin signaling pathways, we performed immunoprecipitation coupled with mass spectrometry (IP-MS) to isolate potential interactors of TMK1 in Arabidopsis. Briefly, GFP-trap agarose beads were used to immunoprecipitate the TMK1-GFP protein complex from pTMK1::TMK1-GFP transgenic plants, which was further analyzed by MS. The proteins that were identified only from the pTMK1::TMK1-GFP transgenic plants but not from pTMK1::GFP control plants were considered as candidates for TMK1-associated proteins. Among them, we were especially interested in the PM H\(^+\)-ATPases (AHAs) (Fig1. a), as the previous study showed that auxin triggers the activation of the PM H\(^+\)-ATPase, which promotes hypocotyl cell elongation. We further confirmed that GFP-AHA1 immunoprecipitated TMK1 and...
TMK4 in the 35S::GFP-AHA1 transgenic plants as detected by immunoblot with α-
TMK1 and α-TMK4 antibodies, respectively (Fig. 1b, Extended Data Fig. 1a).
Furthermore, TMK1-GFP immunoprecipitated AHA(s) from pTMK1::TMK1-GFP
transgenic plants as detected by immunoblot analysis with an α-AHA2-cat antibody
upon an α-GFP-trap antibody immunoprecipitation (Extended Data Fig. 1b). In vitro
pull-down assay showed the kinase domain of TMK1 (TMK1KD), when fused to
maltose-binding protein (MBP), directly interacted with the AHA2 C-terminal domain
fused to glutathione S-transferase (GST) (GST-AHA2-C) (Extended Data Fig. 1c),
suggesting that the kinase domain of TMK1 directly binds the C-terminal region of
AHA2. We postulated that TMK1 interacts with AHAs in vivo since both proteins are
predominantly localized on the PM\textsuperscript{18,23,24}. We used fluorescence resonance energy
transfer (FRET) analysis to test this hypothesis. Due to the poor expression of TMK1
in \textit{Nicotiana benthamiana}, we expressed a kinase-dead form of TMK1
(TMK1K616E). As shown in Fig 1c, strong FRET signals were detected on the PM in
the cells co-expressing AHA1-YFP with TMK1Km-CFP but not with TMK1ext-CFP
(the extracellular domain of TMK fused with CFP), confirming that TMK1 directly
interacts with AHA1 in vivo (Fig. 1c). This result also suggested that the kinase
activity of TMK1 is not a prerequisite for the interaction with AHA in vivo. To
investigate the relevance of auxin in the TMK1-AHA interaction, we performed a
coimmunoprecipitation (Co-IP) assay using protoplasts co-expressing Myc epitope-
tagged TMK1 and HA epitope-tagged AHA1. As shown in Fig. 1d, the in vivo
association of TMK1 with AHA1 was enhanced within 1 minute upon NAA treatment
(Fig. 1d), suggesting that auxin rapidly promotes TMK1-AHA interactions.
**Fig. 1.** **a**, Summary of LC-MS/MS analysis of AHAs associated with TMK1-GFP. The number of unique AHA peptides identified in the immunoprecipitates from pTMK1::TMK1-GFP transgenic seedlings is shown. IP-MS did not identify any AHA peptides from control pTMK1::GFP seedlings. **b**, co-immunoprecipitation of TMK1 with AHA1 in transgenic plants. Membrane proteins from 4-week-old 35S::GFP and 35S::GFP-AHA1 plants were immunoprecipitated with α-GFP-Trap antibody and analyzed with Western blotting using an α-TMK1 antibody (Top). The expression of AHA1-GFP and GFP control in transgenic plants is shown in the bottom panel. The experiments were repeated three times with similar results. **c**, TMK1 interacted with AHA1 on the PM. FRET analysis in *Nicotiana benthamiana* leaf epidermal cells transiently co-expressing TMK1Km-CFP and AHA1-YFP showed TMK1Km-CFP directly interacted with AHA1-YFP on the PM. The transient expression was achieved by agrobacterium infiltration. The extracellular domain of TMK1 (TMK1ext-CFP) did not interact with AHA1-YFP in *N. benthamiana*. More than 10 cells were determined with similar results. **d**, TMK1’s association with AHA1 in Arabidopsis protoplasts was rapidly enhanced by auxin treatments within 1 minute. TMK1-Myc was co-expressed with AHA1-HA in Arabidopsis protoplasts. Co-IP was carried out with an α-Myc antibody (IP: α-Myc), and the proteins were analyzed by using Western blot with an α-HA antibody. Top shows that AHA1-HA coimmunoprecipitated with TMK1-Myc (IP: α-Myc; WB: α-HA, WB: α-Myc). The Middle and Bottom show the expression of AHA1-HA and TMK1-Myc proteins, respectively (WB: α-HA or α-Myc for input control). Protoplasts were treated with 1 μM NAA for 1, 2, and 5 minutes as indicated. The number under the immunoblot is the relative signal intensities as determined by ImageJ. Similar results were reproduced twice in experiments involving 2 min and 5 min NAA treatments.

The penultimate threonine (Thr) residue of the H^+-ATPase protein is conserved among the AHA family members, and the phosphorylation of this residue is a primary mechanism by which the H^+-ATPase is activated in response to multiple signals.
including phytohormones, sucrose, NaCl, blue light, and the fungal toxin fusicoccin\textsuperscript{11,12,22,25-27}. Thus, we tested whether auxin activates AHA phosphorylation through TMKs. We examined the phosphorylation status of the penultimate Thr of the H\textsuperscript{+}-ATPase in the aerial parts of Arabidopsis seedlings by phosphoproteomics. The phosphorylation levels of the penultimate Thr of AHA1, AHA2, and AHA11 were compromised in the \textit{tmk1-1 tmk4-1} mutant compared to wild type (Extended Data Fig. 2a), implying a general reduction of H\textsuperscript{+}-ATPase activity in the mutant. TMK1 and TMK4 are functionally redundant in the regulation of Arabidopsis seedling growth, as neither of \textit{tmk1} and \textit{tmk4} single knockout mutants exhibits a visible growth defect, while \textit{tmk1 tmk4} double mutants show severe growth retardation, especially in hypocotyl elongation\textsuperscript{23} (also see below).

To further investigate the role of TMK1 and TMK4 in auxin-induced PM H\textsuperscript{+}-ATPase activation, we analyzed the phosphorylation status of the penultimate Thr residue by immunoblot analysis using anti-pThr-947 antibody, which recognizes the unique phosphorylation of the penultimate Thr in all of the AHA isoforms\textsuperscript{22}. Fusicoccin (FC) promotes the binding of 14-3-3 to the phosphorylated C-terminal region of PM H\textsuperscript{+}-ATPase, resulting in the pump activation. As shown previously\textsuperscript{12}, FC increased the level of penultimate Thr phosphorylation in wild type Col-0 seedlings (Fig. 2a, b). Similarly, auxin treatment greatly increased its phosphorylation level (Fig. 2a, b). Compared with untreated wild type, the level of phosphorylated penultimate Thr was reduced in the \textit{tmk1-1 tmk4-1} mutant (Fig. 2.a, b). Importantly, auxin-induced phosphorylation of this residue was dramatically reduced in the \textit{tmk1-1 tmk4-1} mutant (Fig. 2a,b). Thus, TMK1 and TMK4 are required for the auxin-induced increase in penultimate Thr phosphorylation. We were unable to assess whether TMKs directly phosphorylate AHA at this penultimate Thr residue using AHA1-C terminal recombinant proteins purified from \textit{E. coli}, because we did not detect a TMK-induced increase in the phosphorylated T947 residue using the anti-pThr-947 antibody possibly due to its phosphorylation prior to the addition of TMKs. Thus, we immunoprecipitated AHA1-GFP from Arabidopsis protoplasts that transiently
expressed this fusion protein for _in vitro_ phosphorylation assay. Recombinant TMK1KD, but not kinase-dead (TMK1KDKm), greatly increased the phosphorylation of AHA1-GFP on the T948 (T947 of AHA2) residue _in vitro_ (Fig. 2c). We further determined whether TMK1 phosphorylated a synthetic peptide containing the C-terminal 16 amino acid residues from AHA1 (AHA1-C16) in an _in vitro_ phosphorylation assay. Mass spectrometry analysis showed that the penultimate Thr residue (T15 of the peptide, T948 of AHA1) of the AHA1-C16 peptide was highly phosphorylated by the recombinant TMK1KD, but not by TMK1KDKm (Fig. 2d, Extended Data Fig. 2b). The second to the last Thr residue (T9T15) of the AHA1-C16 peptide was weakly phosphorylated by TMK1KD. Neither TMK1KD nor TMK1KDKm phosphorylated the scrambled synthetic peptide (Fig. 2d). Thus, TMK1 specifically phosphorylates the penultimate Thr residue of AHA1. Together with auxin-induced rapid interaction of TMK1 with AHA1 and the requirement of TMK1 and TMK4 for auxin-induced AHA phosphorylation _in vivo_, these results strongly indicate a role for TMK1 in the direct phosphorylation of AHA1 activated by auxin.

We next investigated whether TMK1 and TMK4 are required for the activation of PM H⁺-ATPase by auxin. The activation of PM H⁺-ATPase couples with the ATP hydrolysis. As shown previously, auxin treatment for 30 minutes increased ATP hydrolysis in the aerial parts of wild type Arabidopsis seedlings (Fig. 2e). Neither tmk1-1 nor tmk4-1 mutations significantly affected auxin-induced changes in ATP hydrolysis. In contrast, auxin-induced enhancement of ATP hydrolysis was abolished in the tmk1-1 tmk4-1 mutant (Fig. 2e), indicating that TMK1 and TMK4 are essential for auxin-induced H⁺-ATPase activation. In agreement with the compromised H⁺-ATPase activity in the tmk1-1 tmk4-1 mutant, we found that the tmk1-1 tmk4-1 mutant was more tolerant to lithium than the wild type (Extended Data Fig. 2c, d), a toxic alkali cation, whose uptake is coupled with the activation of H⁺-ATPase and hyperpolarized PM. In particular, the aerial part of wild type seedlings became chlorotic but tmk1-1 tmk4-1 remained green after growth on lithium. This is in contrast to SAUR19-OX lines, which display increased H⁺-ATPase activity and thus
exhibit much higher sensitivity to lithium\textsuperscript{15}. These findings together demonstrate that TMK1 and TMK4 are required for auxin-induced PM H\textsuperscript{+}-ATPase activation.

Fig. 2. TMK1 and TMK4 are required for auxin-induced phosphorylation and activation of the PM H\textsuperscript{+}-ATPase. a, Auxin-induced AHA phosphorylation is compromised in the tmk1-1 tmk4-1 (tmk1/4) mutant. The endogenous auxin-depleted aerial sections of seedlings were incubated with 100 nM IAA for 10 minutes or 10 μm fusicoccin (FC) for 5 minutes, respectively. The amounts of H\textsuperscript{+}-ATPase and the phosphorylation status of the penultimate Thr in the C-terminus were determined by immunoblot analysis with anti-H\textsuperscript{+}-ATPase (H\textsuperscript{+}-ATPase) and anti-pThr-947 (pThr 947) antibodies, respectively. b, Quantification of the phosphorylation level of the H\textsuperscript{+}-ATPase. Values are means ± SD; n = 3 independent experiments. c, MBP-TMK1KD phosphorylated AHA1-GFP in vitro. AHA1-GFP was transiently expressed in Arabidopsis protoplasts and immunoprecipitated by GFP-trap. The GFP-trap beads immobilized AHA1-GFP proteins were incubated with TMK1 (TMK1KD, WT) or kinase-dead form (TMK1KD,Km) for 1 hr. The amounts of H\textsuperscript{+}-ATPase and phosphorylated penultimate Thr were determined by immunoblot analysis with anti-H\textsuperscript{+}-ATPase (H\textsuperscript{+}-ATPase) and anti-pThr-947 (pThr 947) antibodies, respectively. The AHA1-GFP proteins were determined by immunoblot analysis with an anti-GFP antibody. The input MBP-TMK1KD and MBP-TMK1KDm recombinant proteins were detected by coomassie brilliant blue staining (CBB). The number under the immunoblot is the relative signal intensities as determined by ImageJ. d, MBP-TMK1KD phosphorylated the synthetic AHA1-C16 peptide in vitro. MBP-TMK1KD or MBP-TMK1KDm (1 μg) was incubated with the AHA1-C terminal synthetic peptide (KLKGLDIDTAGHHITV) or a scrambled peptide (GDHVKITHLSDKGLIT) (10 μg) in 100 μl phosphorylation buffer, respectively. The peptides were then analyzed by mass spectrometry. The graph shows the abundance of phosphorylated peptides at the indicated residues analyzed mass spectrometry. Values are means ±SD; n =3. * P ≤ 0.01, results of One-way ANOVA-tests. Two biological replicates with three technical replicates/each produced similar results. e, Auxin induction of H\textsuperscript{+}-ATPase activity in the aerial
Aerial sections of 14-days old seedlings were treated with 10 μm IAA for 30 minutes and used for vanadate-sensitive ATP hydrolysis assay by determining the inorganic phosphate released from ATP as described previously. The values shown are relative ATP hydrolytic activity of indicated samples to that of control Col-0 without auxin treatment. Values are means ± SD; n =3. * P ≤ 0.05, results of paired Student’s t-tests.

To assess the consequence of the reduction of PM H\(^+\)-ATPase activity in tmk1-1 tmk4-1, we introduced membrane-impermeable 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) as a ratiometric fluorescent pH indicator for assessing changes in the apoplastic pH at the cellular resolution in Arabidopsis thaliana hypocotyls. Two different forms of HPTS (the protonated and deprotonated) were visualized in 2 independent channels with excitation wavelengths of 405 and 458 nm, respectively. The apoplastic pH correlates with the ratiometric values (signal intensity from the 458-nm channel divided by that from the 405-nm channel). As a positive control for the HPTS-based pH indicator, we monitored the apoplastic pH in hypocotyls of the ost2-2D mutant harboring the constitutively activated PM H\(^+\)-ATPase AHA1. As shown previously, the ost2-2D mutant exhibited lower 458/405 values compared with the wild type (Fig. 3a, b), confirming the apoplastic acidification in the ost2-2D mutant. In contrast, significantly higher 458/405 values were observed in tmk1-1 tmk4-1 hypocotyls, suggesting apoplastic alkalization in the mutant (Fig. 3a, b). Furthermore, the apoplastic pH of the tmk1-1 tmk4-1 mutant was restored to the wild type level when this mutant was complemented with wild type TMK1 (Extended Data Fig. 3a,b), indicating that TMK1 is essential for the regulation of the apoplastic pH.

Importantly, we found that hypocotyl cell length was correlated with the pH value of the mutant when compared to the wild type (Fig. 3c). In tmk1-1 tmk4-1, the mean length of hypocotyl cells was significantly shorter than in wild type (Fig. 3a, and Fig. 3c). In contrast, increased apoplastic acidification is linked to an increase in cell length and hypocotyl length in ost2-2D (Fig.3a, and Extended Data Fig. 3c and d).
Fig. 3. TMK1 and TMK4 are required for apoplastic acidification and cell elongation in Arabidopsis hypocotyl. a and b, Comparison of the apoplastic pH in wild type (Col-0), ost2-2D, and the tmk1-1 tmk4-1 (tmk1/4) mutant. Changes in pH were visualized with ratiometric values of fluorescent HPTS. Y-Axis: the mean 458/405 values of ost2-2D and the tmk1-1 tmk4-1 mutant relative to the WT. c, Epidermal cell lengths of hypocotyls from two days-old etiolated seedlings were measured using Image J. Hypocotyl epidermal cells in the 100-500 μM region after apical hook were measured. Values are means ±SD; (n>20 cells per line). * P ≤ 0.05. Scale bar=100 μM

Auxin promotes the acidification of the apoplast in hypocotyls via the activation of PM H⁺-ATPase, contributing to auxin-induced cell elongation. We found that the auxin-induced acidification in the apoplast was completely abolished in the tmk1-1 tmk4-1 mutant (Fig. 4a, b), suggesting an essential role of TMK1 and TMK4 in auxin-triggered PM H⁺-ATPase activation. Moreover, exogenous NAA promoted the elongation of auxin-depleted hypocotyl segments in wild type, but not in the tmk1-1 tmk4-1 mutant (Fig. 4c). The tmk1-1 tmk4-1 mutant complemented with TMK1 exhibited a normal response to auxin in promoting hypocotyl segment elongation (Fig. 4c). The severe defect in tmk1-1 tmk4-1 hypocotyl elongation was partially rescued when tmk1-1 tmk4-1 seedlings were grown on the medium with lower pH (pH 5.0 and pH 4.3) compared to standard medium (pH 5.7) (Extended Data Fig. 4a,b). Moreover, ost2-2D, which caused activation of the PM H⁺-ATPase, partially rescued
the hypocotyl elongation defect of *tmk1-1 tmk4-1* (Extended Data Fig. 4c,d). TMK1 and TMK4 likely activate other downstream pathways to regulate hypocotyl elongation in addition to the PM H⁺-ATPase activation, such as ROP GTPase signaling to the organization of the cytokinetic18,30. Such additional downstream pathways may explain the incomplete rescue of the hypocotyl elongation defect in *tmk1-1 tmk4-1* by *ost2-2D*. Taken together, our results indicate that TMK1 and TMK4 are required for apoplastic acidification via the auxin-triggered PM H⁺-ATPase activation, contributing to auxin regulation of hypocotyl cell elongation.

**Fig. 4.** TMK1 and TMK4 are required for auxin-induced apoplastic acidification and hypocotyl elongation. **a,** and **b,** The *tmk1-1 tmk4-1* (*tmk1/4*) mutant is insensitive to auxin-induced apoplastic pH changes. Effect of auxin on the apoplastic change as visualized by HPTS staining. 2-day etiolated seedlings were treated with 100 nM NAA for 15 minutes. Error bar represents SEM (n>10 hypocotyls per line). **c,** Auxin-induced hypocotyl elongation was compromised in the *tmk1-1 tmk4-1* (*tmk1/4*) mutant. The auxin-depleted hypocotyl sections were treated with/without 10 μM NAA for 30 minutes. The hypocotyl sections were measured by Image J at 0 and 30 minutes after treatments. The Y-axis represents the relative length when comparing the hypocotyl segments at 30 minutes to that at 0 minute. TMK1 restored the defect of auxin-induced hypocotyl elongation in the *tmk1-1 tmk4-1* mutant (*tmk1/4 TMK1*). Values are
In this work, we showed that TMK1 directly interacts with PM H\(^+\)-ATPases on the PM, and this interaction was induced rapidly (within 1-2 min) by auxin treatment (Fig. 1d), preceding an auxin-induced increase in cell elongation\(^{14}\). Therefore, the auxin-induced TMK-AHA association can be considered as the very early response for auxin signal transduction. Once interacting with AHAs upon auxin stimulation, TMK1 directly phosphorylates AHA1 on the penultimate Thr residue (Fig. 2c, d).

Furthermore, auxin induced the phosphorylation of AHA's penultimate Thr in a TMK1/TMK4-dependent manner (Fig. 2a, b, and Extended Data Fig. 2a). This auxin-induced phosphorylation of AHA's penultimate Thr occurred in root tissues within 2 minutes after auxin treatment (Li et al. accompanying manuscript), nearly as rapid as the auxin-induced interaction between TMK1 and AHA1 (Fig. 1d). Therefore, TMK regulates AHA activation by directly affecting the phosphorylation status of the penultimate Thr. Consequently, auxin induced apoplastic acidification in a TMK1/TMK4-dependent manner in hypocotyl cells (Fig. 3a, b, and Fig. 4a, b).

Moreover, reducing the apoplast pH either genetically by ost2-2D or growing seedlings in an acidic environment partially restored the hypocotyl elongation defect of tmk1-1 tmk4-1 (Extended Data Fig. 4a-d). These data shows that the cell surface auxin signaling component TMKs act as a protein kinase that responds to auxin to rapidly and directly initiate the phosphorylation of PM H\(^+\)-ATPases, triggering their activation and apoplastic acidification, and thereby promoting cell expansion. In contrast, the TIR1/AFB-dependent nuclear auxin signaling pathway activates the expression of SAUR proteins that inhibit the PP2C.D-mediated dephosphorylation and inactivation of PM H\(^+\)-ATPase\(^{15,31,32}\). The mechanism for the perception of auxin that activates the TMK-based cell surface signaling remains to be determined. Nonetheless, the current findings strongly support the hypothesis that the cell surface and intracellular auxin signaling pathways respectively initiate and sustain PM H\(^+\)-ATPase activation in cells where auxin promotes cell expansion, such as in hypocotyls, and collectively explain the acid growth theory. In roots, TMK-dependent
auxin signaling also promotes ATPase activation, but to counter the rapid alkalization (or membrane depolarization) activated by TIR1/AFBs\textsuperscript{33,34} (Li et al. accompanying manuscript). Importantly, these findings, together with the recent findings on the TMK-mediated noncanonical auxin signaling in regulating pavement cell morphogenesis\textsuperscript{18,35} (Perez et al. manuscript in preparation), differential growth of the apical hook\textsuperscript{16}, LRs formation\textsuperscript{17}, and root gravitropic response\textsuperscript{36} are emerging as a common theme that auxin regulates growth and developmental processes via the coordinate actions of intracellular and cell surface auxin signaling systems.

Materials and Methods

Plant materials and growth conditions. Columbia Col-0 was used as wild type in this study. ost2-2D seeds were obtained from Jeffrey Leung (Department of Institut Jean-Pierre Bourgin, INRA, Versailles, France). The tmkl-1 tmk4-1 mutant and pTMK1-TMK1-GFP transgenic lines (generated in the tmkl-1 tmk4-1 background) were described previously\textsuperscript{16,17}. The ost2-2D tmkl-1 tmk4-1 mutants were generated by genetic crosses and confirmed by genotyping. Arabidopsis plants were grown in soil (Sungro S16-281) in a growth room at 23 °C, 40% relative humidity, and 75 μE m\textsuperscript{-2} s\textsuperscript{-1} light with a 12-h photoperiod for approximate 4 weeks before protoplast isolations. To grow Arabidopsis seedlings, the seeds were surface sterilized with 50% bleach for 10 minutes (for tmkl-1 tmk4-1 seeds were sterilized with 75% (vol/vol) ethanol for 5 minutes), and washed 3 times with sterilized distilled H\textsubscript{2}O, and then placed on the plates with 1/2 MS medium containing 0.5% sucrose and 0.8% agar at pH 5.7 at dark with vertical growth. 2 to 3 days after germination (DAG) hypocotyls were used for cell characterization.

Plasmid construction and generation of transgenic plants. Full-length and truncated variants TMK1, AHA1, and AHA2 were amplified by PCR from Col-0 cDNA and cloned into a protoplast transient expression vector (HBT vectors obtained from Libo Shan & Ping He, Texas A&M) or plant expression vector pGWB641 and pGWB644. Stable transgenic lines were generated by using the standard
Agrobacterium tumefaciens-mediated transformation in the tmk1-1 tmk4-1 mutant or Col-0. AHA2-C terminal region was cloned into pDest-565, and expressed in E.coli (Rosetta, BL21).

**Determination of H⁺-ATPase phosphorylation levels.** The immunoblot was performed as described by Hayashi by using specific antibodies against the catalytic domain of AHA2 and phosphorylated Thr-947 in AHA2. These antibodies recognize not only AHA2 but also other H⁺-ATPase isoforms in Arabidopsis.

Briefly, ten pieces of auxin-depleted aerial sections were collected and grounded with a plastic pestle, followed by solubilization in 40 µL of SDS buffer (3% [w/v] SDS, 30 mM Tris-HCl [pH 8.0], 10 mM EDTA, 10 mM NaF, 30% [w/v] Sucrose, 0.012% [w/v] Coomassie Brilliant Blue, and 15% [v/v] 2-mercaptoethanol), and the homogenates were centrifuged at room temperature (10,000g for 5 min). 12 µL of the supernatant was loaded onto 10% (w/v) SDS PAGE gels to assess the H⁺-ATPase or the phosphorylated penultimate Thr levels by using the above-mentioned antibodies, respectively. A goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz) was used as a secondary antibody. The chemiluminescent signal was quantified using ImageJ software.

**HPTS staining and imaging.** HPTS staining and imaging were performed as described by Barbez with modification. Briefly, 2-day etiolated seedlings were transferred and incubated with 1 mM HPTS (from 100 mM water stock) with 0.01% Triton-X-100 under vacuum (10-15 pa) 5 minutes. The seedlings were then incubated with HPTS for 60 minutes in the liquid growth medium. The seedlings were subsequently mounted in the same growth medium on a microscopy slide and covered with a coverslip. For auxin treatment, seedlings were incubated in 1/2 MS growth medium supplemented with 1 mM HPTS and NAA in the stated concentration and subsequently mounted in the same growth medium on a microscopy slide and covered with a coverslip. Seedling imaging was performed using an inverted Zeiss 880 confocal microscope equipped with a highly sensitive GaAsP detector. Fluorescent signals for the protonated HPTS form (Excitation 405 nm, emission peak 514nm), as
well as the deprotonated HPTS form (excitation, 458 nm emission peak, 514 nm),
were detected with a 10× (water immersion) objective.

**Immunoprecipitation-mass spectrometry analyses.** One gram of *pTMK1-

gTMK1-GFP/tmk1-1 tmk4-1* seedlings grown on 1/2 MS medium was collected and
grounded in liquid nitrogen with a mortar and pestle. Total proteins were extracted by
extraction buffer (50 mM Tri-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton
X-100 with protease inhibitor and phosphatase inhibitor) on ice. The extracts were
centrifugated at 13,000g for 30 min, and the supernatants were incubated with GFP-
trap agarose beads (GFP-Trap®_A, gta-20, ChromoTek) at 4 ℃ for 2 hours to
immunoprecipitate TMK1-GFP proteins. The agarose beads were washed and
resuspended with 50 mM Tris-Cl buffer (pH7.8). One 10th of the beads were used for
immunoblot analysis with an anti-GFP antibody. The remaining agarose beads were
used for LC-MS/MS analysis. MS analysis was carried out by Orbitrap Fusion mass
spectrometry (Thermo Fisher Scientific, Waltham, MA).

**Phosphoproteomics Analyses.** Col-0 and the *tmkl-1 tmk4-1* seedlings were cultured
on ½ MS plate for 5 days, then the aerial parts of seedlings were transferred to ½ MS
liquid medium and incubated in KPSC buffer (10 mM potassium phosphate, pH 6.0,
2% sucrose, 50 μm chloramphenicol) in darkness overnight, and the buffer was
replaced every 1 hr for 12 hrs 40. Seedings were collected and flash-frozen in liquid
nitrogen. A total of 1 g of frozen shoots (fresh weight) was grounded with liquid
nitrogen pre-cold mortar and homogenized in 5 ml extraction buffer [50 mM Tris-HCl
buffer (pH 8), 0.1 M KCl, 30% sucrose, 5 mM EDTA, and 1 mM DTT in Milli-Q
water, 1x complete protease inhibitor mixture and the PhosSTOP phosphatase
inhibitor mixture] in a Dounce Homogenizer. At least 50 strokes were performed. The
homogenate was filtered through four layers of miracloth and centrifuged at 5000 × g
at 4-°C for 10 min. Half of the supernatant was used to resuspend the pellet, and the
mixture was centrifuged again at 5000g 4-°C for 10 min. The two fractions of the
supernatants were combined and mixed with 3, 1, and 4 volumes of methanol,
chloroform, and water, respectively. The mixtures were centrifuged at 5000 × g for 10
min, and the aqueous phase was removed. After the addition of 4 volumes of methanol, the proteins were pelleted via centrifugation at $4000 \times g$ for 10 min. Pellets were washed with 80% acetone and resuspended in 6 M guanidinium hydrochloride in 50 mM triethylammonium bicarbonate (TEAB) buffer (pH 8). The proteins were used for Tandem Mass Tag (TMT) labeling according to the Kit protocol (Thermo Scientific #90096) and quantitation by mass spectrometry (MS).

**Protoplast preparation and transient expression.** Protoplasts were prepared according to the protocol described by Yoo et al.\(^4\) Maxiprep DNA for transient expression was prepared using the Invitrogen PureLink Plasmid Maxiprep Kit. $2 \times 10^5$ protoplasts were transfected with indicated AHA1-HA or TMK1-myc and incubated at room temperature for 10 hours. The protoplasts were collected and stored at -80 °C for further usage.

**Coimmunoprecipitation (Co-IP) assay.** $2 \times 10^5$ protoplasts were transfected with indicated plasmids and incubated for 7-10 hours at room temperature. Protoplasts were then collected in 2 ml Eppendorf tubes and subjected to centrifuge with a swinging-basket centrifuge at 100 x $g$ for 1 minute. The supernatant was discarded, and the protoplasts were resuspended with 100 µl W5 solution (2 mM MES-KOH, pH 5.7, 5 mM KCl, 154 mM NaCl, and 125 mM CaCl\(_2\)). The protoplasts were treated with 1 µM NAA for the indicated time period, frozen in liquid N\(_2\) immediately and stored in -80 °C. The samples were lysed with 0.5 mL of extraction buffer (10 mM HEPES at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5% Triton X-100, and protease inhibitor mixture from Roche). After vortexing vigorously for 30 s, the samples were centrifuged at 12,470 $\times$ g for 10 min at 4 °C. The supernatant was incubated with an anti-GFP-Trap antibody for 2 h with gentle shaking. The beads were collected and washed three times with washing buffer (10 mM HEPES at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1% Triton X-100) and once with 50 mM Tris·HCl at pH 7.5. The immunoprecipitated proteins were analyzed by immunoblot with $\alpha$-GFP or $\alpha$-HA antibody. For seedling Co-IP, approximate 1 g of
10-day old seedlings were grounded in liquid N\textsubscript{2} and further grounded in 0.5 mL of ice-cold Co-IP buffer (10 mM HEPES at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1%Triton X-100, and protease inhibitor mixture from Roche). The homogenates were centrifuged at 12,470 \times g at 4 °C for 10 min. The resulting supernatants were used to perform the Co-IP assay with the same procedures as protoplast Co-IP assay with α-GFP-Trap antibodies.

**In vitro pull-down assay.**

MBP or GST fusion proteins were expressed in *E. coli* and affinity-purified using standard protocols. Briefly, 200 ml IPTG-induced cell culture pellet was lysed in 20 ml lysis buffer (containing 0.5% Triton-X-100) by sonication on ice. Centrifuge lysates were cleared by spinning at 10,000 x g for 30 minutes at 4 °C. The supernatant was then incubated with 100 μl amylose resins or glutathione-sepharose beads at 4 °C for 4 hrs with gentle rotation. The beads were then centrifuged and washed with lysis buffer for 3 times. Proteins were eluted with GST (10 mM reduced glutathione in 50 mM Tris. pH 8.0), or MBP (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM maltose, pH 7.4) buffer. The protein concentration was estimated by NanoDrop ND-1000 spectrophotometer and confirmed by the Bio-Rad Quick Start Bradford Dye Reagent. 10 μg of GST or GST fusion proteins (immobilized on glutathione-sepharose beads) were incubated with 10 μg prewashed MBP or MBP fusion proteins at 4 °C in 150 μl of incubation buffer (10 mM HEPES at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% Triton-X-100) for 1 hour. The beads were collected and washed three times with washing buffer (20 mM HEPES at pH 7.5, 300 mM NaCl, 1 mM EDTA, and 0.5% NP-40) and once with 50mM Tris.HCl (pH 7.5). Proteins in the beads were analyzed by immunoblot with an α-GST or α-MBP antibodies, respectively.

**Vanadate-sensitive ATPase activity measurement**

ATP hydrolysis by PM H\textsuperscript{+}-ATPase was measured in a vanadate-sensitive manner as previous described\textsuperscript{22}. Briefly, the aerial parts of 14-day-old seedlings (Col-0, *tmkl-1*,}
tmk4-1, and tmk1-1 tmk4-1) were incubated in KPSC buffer (10 mM potassium phosphate, pH 6.0, 2% sucrose, 50 μM chloramphenicol) in darkness for 10 hours. The buffer was replaced for every hour. The pretreated tissues were incubated in the presence of 10 μM IAA for 30 minutes in darkness. The tissues were homogenized with homogenization buffer (50 mM MOPS-KOH, pH 7.0, 100 mM KNO₃, 2 mM sodium molybdate, 0.1 mM NaF, 2 mM EGTA, 1 mM PMSF and 20 μM leupeptin) and the homogenates were centrifuged at 10,000 g for 10 minutes; the obtained supernatant was further ultra-centrifuged at 45,000 g for 60 minutes. The resultant pellet (microsomal fraction) was resuspended in the homogenization buffer. ATP hydrolytic activity of the microsomal fraction was measured in a vanadate-sensitive manner, and the inorganic phosphate released from ATP was measured.

In vitro phosphorylation

Protoplasts were isolated from plants expressing AHA1-GFP as described above. Agarose immobilized (GFP-Trap beads, Chromotek, #gta-100 ) AHA1-GFP proteins was incubated with 1 μg MBP-TMK1KD or MBP-TMK1KDKm recombinant proteins (expressed in E. coli and isolated by affinity purification) in 100 μl phosphorylation buffer (5 mM HEPES, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT and 50 μM ATP) at room temperature (24°C) for 1 hr. After incubation, the reaction was stopped by adding 4 x SDS loading buffer. Proteins in the beads were analyzed by immunoblot with an α-pT947, α-AHA1-cat, or α-GFP antibodies (Chromotek, #3h9), respectively.

For the phosphorylation assays of the synthetic AHA1-C16 peptides, 10 μg synthetic peptides were incubated with 1 μg MBP-TMK1KD or MBP-TMK1KDKm recombinant proteins in 100 μl phosphorylation buffer for 1 hr before the samples were analyzed by mass spectrometry to determine the sites and the levels of AHA1-C16 phosphorylation.
### Primers for construct cloning and genotyping

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Acknowledgements

We thank members of the Yang laboratory for stimulating discussion and critical comments on this work. We are grateful to Dr. Jeffrey Leung (Department of Institute Jean-Pierre Bourgin, INRA, Versailles, France) for ost2-2D seeds, Dr. Koh Iba (Kyushu University, Japan) for 35S::GFP-AHA1 seeds, and Dr. Libo Shan (University of Texas A&M) for protoplast transient expression vectors. K.T. (20K06685) and T.K. (20H05687 and 20H05910) were funded by MEXT/JSPS KAKENHI. W.M.G was funded by NIH (GM067203). H.Z. was funded by NIH (S10OD016400).

References


Extended Data

Extended Data Fig. 1. TMK interacts with AHAs in planta and in vitro.  

**a**, TMK4 associates with AHA1 in transgenic plants. The membrane proteins from 4-week-old 35S::GFP-only and 35S::GFP-AHA1 plants were immunoprecipitated with α-GFP-Trap antibody and analyzed with Western blots using an α-TMK4 antibody (Top). The expression of GFP-AHA1 and GFP control in transgenic plants is shown (Bottom).

**b**, TMK1 associates with AHA2 in transgenic plants. Membrane proteins from 4-wk-old 35S::GFP and pTMK1::TMK1-GFP/tmk1-1/4-1 transgenic plants were immunoprecipitated with α-GFP-Trap antibody and analyzed with Western blots using an α-AHA2 antibody (Top). The expression of TMK1-GFP and GFP control in transgenic plants is shown (Bottom).

**c**, TMK1's cytoplasmic kinase domain (KD) interacts with AHA2's C-terminal domain in vitro. *E. coli*-expressed maltose-binding protein (MBP)-TMK1KD or MBP proteins were incubated with glutathione bead-bound glutathione-S-transferase (GST)-AHA2-C or GST (Pull-down:GST), and the beads were collected and washed for Western blotting of immunoprecipitated proteins with α-MBP antibody (left). The input GST-AHA2-C, MBP-TMK1KD, MBP, and GST proteins were detected by Coomassie brilliant blue staining (CBB).
Extended Data Fig. 2. TMK1 and TMK4 impact the phosphorylation status of AHAs and the function of the PM H\textsuperscript{+}-ATPase pump.  

\textbf{a}, The phosphorylated status of AHAs was changed in the \textit{tmk1-1 tmk4-1} (\textit{tmk1/4}) mutant. The aerial part of 5-days auxin-depleted seedlings was used to prepare membrane proteins for TMT (Tandem mass tag) labelling and mass spectrometry quantification as described in Method. Mass spectrometry analysis showed that the abundance of the peptides containing phosphorylated penultimate threonine from AHA1, AHA2, and AHA11 was significantly decreased in \textit{tmk1-1 tmk4-1} (\textit{tmk1/4}) mutant relative to wild type.

High-resolution fragmentation spectra of peptides containing phosphorylated penultimate threonine are presented in the middle, phosphorylated peptides mass spectrometry information is presented at the bottom.  

\textbf{b}, High-resolution fragmentation spectra of peptides containing phosphorylated penultimate threonine of AHA1-C16 synthetical peptide (pT948 of AHA1) (see Fig. 2d).

\textbf{c}, Lithium tolerance in the \textit{tmk1-1 tmk4-1} (\textit{tmk1/4}) mutant. Wild type (Col-0) and \textit{tmk1-1 tmk4-1} (\textit{tmk1/4}) mutant seedlings were grown on 1/2 MS medium with or without 18 mM LiCl for 5 days. LiCl treatment caused severe seedling growth retardation and severe chlorosis of
the aerial parts in Col-0, whereas the tmk1-1 tmk4-1 (tmk1/4) mutant was tolerant to LiCl, especially in the aerial parts. d, The root length of the seedlings was measured by ImageJ. Error bar represents SEM (n>10 hypocotyls per line). The number above the columns indicates the percentage of root growth inhibition induced by LiCl.

Extended Data Fig. 3. TMK1 and TMK4 regulate apoplastic pH and hypocotyl elongation.
a, TMK1 (pTMK1::TMK1-GFP) complemented the apoplastic pH defect of the tmk1-1 tmk4-1 (tmk1/4) mutant. Comparison of the apoplastic pH in WT, the tmk1-1 tmk4-1 mutant, and the tmk1-1 tmk4-1/TMK1 (tmk1/4/TMK1) complemented line. Visualized by HPTS staining (a). Y-Axis: the mean 458/405 values of the tmk1-1 tmk4-1 mutant and the TMK1 complemented line relative to wild type (b). c and d, The tmk1-1 tmk4-1 mutant showed a defect in hypocotyl elongation (c). Hypocotyl lengths of 3 days-old etiolated seedlings were measured by Image J (d). Error bar represents SD (n>10 hypocotyls per line). Scale bar= 100 μM.
Extended Data. Fig. 4. Acidic environments and activation of the PM H\(^{+}\)-ATPase pump partially restored hypocotyl elongation defect in tmk1-1 tmk4-1.  

a and b, Low pH in the medium was able to partially restore the tmk1-1 tmk4-1 (tmk1/4) defect in hypocotyl elongation. 

Seedlings growth on 1/2 MS medium with indicated pH (a), and the length was measured by ImageJ (b). c and d, ost2-2D mutation partially restored the hypocotyl elongation defect of tmk1-1 tmk4-1 mutant. Seedlings were grown on 1/2 MS medium for 4 days, and the length was measured by ImageJ. Values are means ±SD; Error bar represents SD (n>20 hypocotyl per line), * P ≤ 0.05.
Figures

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Figure 1

TMK1 directly interacts with AHAs. a, Summary of LC-MS/MS analysis of AHAs associated with TMK1-GFP. The number of unique AHA peptides identified in the 99 immunoprecipitates from pTMK1::TMK1-GFP transgenic seedlings is shown. IP-MS did not identify any AHA peptides from control pTMK1::GFP seedlings. b, co-immunoprecipitation of TMK1 with AHA1 in transgenic plants. Membrane proteins from 4-week-old 35S::GFP and 35S::GFP-AHA1 plants were immunoprecipitated with α-GFP-Trap antibody and analyzed with Western blotting using an α-TMK1 antibody (Top). The expression of AHA1-GFP and GFP control in transgenic plants is shown in the bottom panel. The experiments were repeated three times with similar results. c, TMK1 interacted with AHA1 on the PM. FRET analysis in Nicotiana benthamiana leaf epidermal cells transiently co-expressing TMK1Km-CFP and AHA1-YFP showed TMK1Km-CFP directly interacted with AHA1-YFP on the PM. The transient expression was achieved by agrobacterium infiltration. The extracellular domain of TMK1 (TMK1ext-CFP) did not interact with AHA1-YFP in N. benthamiana. More than 10 cells were determined with similar results. d, TMK1’s association with AHA1 in Arabidopsis protoplasts was rapidly enhanced by auxin treatments within 1 minute. TMK1-Myc was co-expressed with AHA1-HA in Arabidopsis protoplasts. Co-IP was carried out with an α-Myc antibody (IP: α-Myc), and the proteins were analyzed by using Western blot with an α-HA antibody. Top shows that AHA1-HA coimmunoprecipitated with TMK1-Myc (IP: α-Myc; WB: α-HA, WB: α-Myc). The Middle and Bottom show the expression of AHA1-HA and TMK1-Myc proteins, respectively.
Protoplasts were treated with 1 μM NAA for 1, 2, and 5 minutes as indicated. The number under the immunoblot is the relative signal intensities as determined by ImageJ. Similar results were reproduced twice in experiments involving 2 min and 5 min NAA treatments.

**Figure 2**

TMK1 and TMK4 are required for auxin-induced phosphorylation and activation of the PM H⁺-ATPase. a, Auxin-induced AHA phosphorylation is compromised in the tmk1-1 tmk4-1(tmk1/4) mutant. The endogenous auxin-depleted aerial sections of seedlings were incubated with 100 nM IAA for 10 minutes or 10 μm fusicoccin (FC) for 5 minutes, respectively. The amounts of H⁺-ATPase and the phosphorylation status of the penultimate Thr in the C terminus were determined by immunoblot analysis with anti-H⁺-ATPase (H⁺-ATPase) and anti pThr-947 (pThr 947) antibodies, respectively. b, Quantification of the phosphorylation level of the H⁺-ATPase. Values are means ± SD; n = 3 independent experiments. The amounts of H⁺-ATPase and phosphorylated penultimate Thr were determined by immunoblot analysis with anti-H⁺-ATPase (H⁺-ATPase) and anti-pThr-947 (pThr 947) antibodies, respectively. The AHA1-GFP proteins were determined by immunoblot analysis with an anti-GFP antibody. The input MBP-TMK1KD and MBP-TMK1KDKm recombinant proteins were detected by coomassie brilliant blue staining (CBB). The number under the immunoblot is the relative signal intensities as determined by ImageJ. d, MBP-
TMK1KD phosphorylated the synthetic AHA1-C16 peptide in vitro. MBP-TMK1KD or MBP-TMK1KDKm (1 μg) was incubated with the AHA1-C terminal synthetic peptide (KLKGLIDTAGHHITV) or a scrambled peptide (GDAHVKITHLDKGLIT) (10 μg) in 100 μl phosphorylation buffer, respectively. The peptides were then analyzed by mass spectrometry. The graph shows the abundance of phosphorylated peptides at the indicated residues analyzed mass spectrometry. Values are means ±SD; n =3. * P ≤ 0.01, results of One-way ANOVA-tests. Two biological replicates with three technical replicates/each produced similar results.

Auxin induction of H+-ATPase activity in the aerial parts of wild type and the tmk1-1 tmk4-1 mutant. Aerial sections 208 of 14-days old seedlings were treated with 10 μm IAA for 30 minutes and used for vanadate-sensitive ATP hydrolysis assay by determining the inorganic phosphate released from ATP as described previously 25. The values shown are relative ATP hydrolytic activity of indicated samples to that of control Col-0 without auxin treatment. Values are means ± SD; n =3. * P ≤ 0.05, results of paired Student’s t-tests.
Figure 3

TMK1 and TMK4 are required for apoplastic acidification and cell elongation in Arabidopsis hypocotyl. a and b, Comparison of the apoplastic pH in wild type (Col-0), ost2-2D, and the tmk1-1 tmk4-1 (tmk1/4) mutant. Changes in pH were visualized with ratiometric values of fluorescent HPTS. Y-Axis: the mean 458/405 values of ost2-2D and the tmk1-1 tmk4-1 mutant relative to the WT. c, Epidermal cell lengths of hypocotyls from two days-old etiolated seedlings were measured using Image J. Hypocotyl epidermal
cells in the 100-500 μM region after apical hook were measured. Values are means ±SD; (n>20 cells per line). * P≤0.05. Scale bar=100 μM