1 **ABP1/ABL3-TMK1 cell-surface auxin signaling directly targets PIN2-mediated auxin**

2 **fluxes for root gravitropism**

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4 Lesia Rodriguez[1*], Lukáš Fiedler[1*], Minxia Zou[1], Caterina Giannini[1], Aline Monzer[1], Dmitrii

5 Vladimirtsev[1], Marek Randuch[1], Yongfan Yu[1], Zuzana Gelová[1], Inge Verstraeten[1], Jakub Hajný[1,2],

6 Meng Chen[3], Shutang Tan[1,3], Lukas Hoermayer[1], Lanxin Li[1], Maria Mar Marques-Bueno[4,5], Zainab

7 Quddoos[1], Gergely Molnár[1], Tongda Xu[6], Ivan Kulich[1], Yvon Jaillais[4] and Jiří Friml[1#]

8

9 [1]Institute of Science and Technology Austria (ISTA), Am Campus 1, 3400, Klosterneuburg, Austria

10 [2]Laboratory of Growth Regulators, The Czech Academy of Sciences, Institute of Experimental Botany

11 & Palacký University, Šlechtitelů 27, CZ-78371 Olomouc, Czech Republic

12 [3]MOE Key Laboratory for Cellular Dynamics, Center for Advanced Interdisciplinary Science and

13 Biomedicine of IHM, Hefei National Laboratory for Physical Sciences at the Microscale, Division of

14 Life Sciences and Medicine, University of Science and Technology of China, Hefei 230027, China.

15 [4]Laboratoire Reproduction et Développement des Plantes Université de Lyon, ENS de Lyon, UCB Lyon

16 1, CNRS, INRA, F-69342 Lyon, France

17 [5]Present Address: Center for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB,

18 Barcelona 01893, Spain

19 [6]Plant Synthetic Biology Center, Haixia Institute of Science and Technology, and College of Life

20 Sciences, Fujian Agriculture and Forestry University, Fuzhou, Fujian, P.R.China

21 [*] Equal contributions

22 [#] Corresponding author: jiri.friml@ista.ac.at

23 Lead Contact: Further information and requests for resources and reagents should be directed to and will
24 be fulfilled by the lead contact, Jiří Friml (jiri.friml@ista.ac.at).

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SUMMARY

Phytohormone auxin and its directional transport mediate much of the remarkably plastic development of higher plants. Positive feedback between auxin signaling and transport is a key prerequisite for (i) self-organizing processes including vascular tissue formation and (ii) directional growth responses such as gravitropism. Here we identify a mechanism, by which auxin signaling directly targets PIN auxin transporters. Via the cell-surface ABP1-TMK1 receptor module, auxin rapidly induces phosphorylation and thus stabilization of PIN2. Following gravistimulation, initial auxin asymmetry activates autophosphorylation of the TMK1 kinase. This induces TMK1 interaction with and phosphorylation of PIN2, stabilizing PIN2 at the lower root side, thus reinforcing asymmetric auxin flow for root bending. Upstream of TMK1 in this regulation, ABP1 acts redundantly with the root-expressed ABP1-LIKE auxin receptor ABL3. Such positive feedback between cell-surface auxin signaling and PIN-mediated polar auxin transport is fundamental for robust root gravitropism and presumably also for other self-organizing developmental phenomena.

INTRODUCTION

Plant developmental mechanisms differ fundamentally from animals. With cells encapsulated in rigid cell walls without the possibility of migration, plants mainly rely on oriented cell divisions and expansions and have the capacity to self-organize complex tissues. Being rooted in the soil, plants are also highly adapted to cope with changing environments. Much of the adaptability and self-organization is mediated by the phytohormone auxin with examples including the formation of an embryonic axis, regular arrangement of leaves and flowers, establishment of leaf vein patterns, or flexible vasculature regeneration around a wound[1]. Auxin also acts as a key endogenous signal positioning sessile plants in their environment during directional growth responses such as gravitropism and phototropism[2]. Both self-organizing development and translation of environmental signals into directional growth rely on mechanistically elusive feedback between auxin signaling and polar auxin transport[3,4].

Directional cell-to-cell auxin transport is a plant-specific mechanism[5] dependent upon plasma membrane-localized transporters[6,7]. Chief among these are AUX1/LAX importers[8] and PIN auxin exporters. The latter inhabit polarized plasma membrane domains to determine vectorial auxin fluxes through tissues[9,10]. Inside cells, auxin triggers a well-studied transcriptional pathway through predominantly nuclear TIR1/AFB receptors. This leads to developmental reprogramming[11,12] and contributes to growth regulation[13]. Historically, several auxin responses showed such rapidity that transcriptional cascades did not suffice for their explanation. While several of these were later found to also depend on TIR1/AFB receptors, other responses require extracellular (apoplastic) auxin perception[14]. This has been formalized recently as comprising AUXIN-BINDING PROTEIN1 (ABP1),

65 ABP1-LIKEs (ABLs), and TRANSMEMBRANE KINASEs (TMKs) [ABP1/ABL-TMK] co-receptor
66 complexes at the cell-surface[15–17].

67 Sensitive phospho-proteomic pipelines recently revealed that auxin triggers a global
68 phosphorylation response via ABP1 and TMK1[17,18]. Notably, the lack of auxin-induced phosphorylation
69 in *abp1* and *tmk1* mutants correlates with strong defects in auxin canalization[17], a mysterious process
70 underlying self-organized plant development including regeneration of vasculature and formation of
71 polarized auxin-transporting channels from a local auxin source. Canalization also requires TIR1/AFB
72 receptors[19], suggesting that both intracellular and apoplastic signaling contribute to auxin feedback
73 regulation of PIN-dependent auxin transport. This is consistent with computational predictions
74 exploring potential mechanism of PIN polarization by auxin feedback[20], however, no such mechanism
75 linking auxin signaling and transport has been discovered.

76 Feedback between auxin and its transport has also been proposed for gravitropic root bending.
77 Contrary to canalization, this would not involve adjustments of PIN polarity but rather the stabilization
78 of the root-specific PIN2 transporter[21]. Among the latest novelties in the quest of plants to grow upright
79 is evolution of fast root gravitropism, which was enabled by functional innovations in the PIN2 protein[22].
80 Fast gravitropism occurs through directional auxin transport from the site of gravity perception towards
81 the elongation zone where growth response takes place. After gravity sensing in the columella at the
82 root tip[23], auxin flux becomes redirected to the lower root side[24,25]. This initial asymmetry is then
83 propagated by AUX1- and PIN2-mediated transport[26,27] from the root tip to the elongation zone and
84 translated into root bending through local inhibition of cell elongation[28].

85 During the gravitropic response, PIN2 distribution itself becomes asymmetric with increased and
86 decreased PIN2 stability at the lower and upper root sides, respectively[21,29]. Such lateral PIN2 gradient
87 not only propagates but also reinforces the initial root tip auxin asymmetry, contributing to the
88 robustness of root bending as well as to the fine-tuning of gravitropism by other hormonal cues[30]. How
89 auxin regulates its own transport via PIN2 in the context of root gravitropism remains an outstanding
90 question.

91 In search of a possible mechanism for cell-surface auxin signaling effect on auxin transport, we
92 mined the auxin-inducible, ABP1-TMK1-mediated phospho-proteome and identified enrichment of
93 PINs with PIN2 as the main target. We find that an auxin-induced interaction of TMK1 with PIN2 and
94 its phosphorylation are directly responsible for the PIN2 gradient that reinforces gravitropic root
95 bending. This pathway perceives auxin through the newly identified root-expressed ABL3 receptor
96 acting redundantly with ABP1. Our findings identify a direct mechanism for feedback regulation
97 between auxin signaling and auxin transport.

98

99 **RESULTS**

100 **ABP1-TMK1 cell-surface auxin signaling induces phosphorylation of PIN auxin transporters**

101 To identify components of feedback regulation of auxin transport downstream of ABP1-TMK1 auxin

102 signaling, we took advantage of a rapid phospho-proteomic dataset (100 nM IAA, 2 min) recorded in

103 roots of *Arabidopsis thaliana* (Arabidopsis) wild-type (WT) or the respective mutants[17]. We queried

104 proteins concurrently hypo-phosphorylated in both *abp1-TD1* and *tmk1-1* mutants for molecular

105 function using a gene ontology (GO) analysis. When partitioned by significance, the most dominant

106 terms were rather general and included "binding" or "protein binding". On the other hand, partitioning

107 significant terms by effect size (fold enrichment) always recovered "auxin efflux transmembrane

108 transporter activity" as the most strongly enriched GO term (Figure S1A and S1B). Inspection of the

109 corresponding enriched phospho-proteins showed the presence of PINs and ABCB/ABCG transporters.

110 We further focused on PINs as dominant auxin transporters with many established developmental roles.

111 There were in total nine PIN phospho-peptides (phospho-sites) significantly downregulated in both

112 *abp1-TD1* and *tmk1-1* (Figure 1A and Figure S1C). To verify the genetic specificity of these results, we

113 examined in parallel a recent matched auxin phosphorylation dataset[18] from the mutant of the

114 intracellular AFB1 auxin receptor[31]. Except for PIN1^{S337}, none of the ABP1-TMK1-dependent PIN

115 phospho-sites were deregulated in *afb1-3* (Figure 1A). This suggests that auxin activates PIN

116 phosphorylation specifically through the cell-surface ABP1-TMK1 module independently of

117 intracellular TIR1/AFB signaling. Two of the nine PIN phospho-sites mapped to PIN1, five to PIN2,

118 and two to PIN3 (Figure 1A). Notably, all these targeted hydrophilic PIN loops, the expected location

119 for post-translational modifications regulating PIN function[32]. The two PIN1 sites, PIN1^{S271} and

120 PIN1^{S337}, were previously ascribed to shoot functions as targets of the D6PK protein kinase[33] and the

121 MKK7-MPK6 module[34], respectively. Another previously studied phospho-site was PIN2^{S439}, which

122 participates in root adaptation to varying nitrogen sources[35,36].

123 Given that cell-surface auxin signaling mutants show perturbed phospho-proteomes even under

124 mock conditions[17,18], we next assessed auxin inducibility of these phospho-sites in WT roots. Notably,

125 four out of five PIN2 phospho-sites strongly responded to 100 nM IAA within 30 seconds of treatment

126 (Figure 1B). The PIN3^{S389} phospho-site showed a similar behavior. Conversely, while the auxin profile

127 of PIN1^{S337} also significantly deviated from mock conditions, the site only underwent a delayed negative

128 fluctuation (Figure S1D). This correlates with PIN1^{S337} not being specifically targeted by ABP1-TMK1

129 (Figure 1A). We also confirmed average to low evolutionary conservation of PIN1^{S337} (Figure S1E),

130 altogether suggesting minor biological relevance of this particular site for the ABP1-TMK1 auxin

131 phospho-response. When extending evolutionary conservation analysis to the remaining PIN phospho-

132 sites, we observed moderate conservation of PIN1^{S271} and poor conservation of the auxin-inducible

133 PIN3^{S389} (Figure S1E and S1F). On the other hand, four out of five PIN2 phospho-sites showed high

134 ConSurf scores and perfect conservation in PIN2 orthologs from Arabidopsis to Gymnosperms (Figure
135 1C and 1D).

136   Given that rapid auxin phospho-response represents an ancient auxin pathway[18], we next asked
137 whether PIN phosphorylation is conserved across the green lineage. Unlike in Arabidopsis, we found
138 no significantly regulated PIN phospho-sites in the auxin phospho-proteomes (100 nM IAA, 2 min) of
139 two bryophytes (*Physcomitrium*, *Marchantia*) and two streptophyte algae (*Penium*, *Klebsormidium*).
140 Conversely, the fern *Ceratopteris* showed auxin-regulated PIN phosphorylation under the same
141 conditions[37]. This suggests a co-option of an ancestral auxin response for PIN phosphorylation after the
142 divergence of Bryophyta from the green lineage, probably in the common ancestor of vascular plants.

143   These analyses identified PIN auxin transporters, particularly PIN2, as prominent targets of
144 ultrafast ABP1-TMK1-mediated auxin phospho-response, representing a recent evolutionary
145 innovation.

146

147 ABP1-TMK1-dependent PIN2 phospho-sites are crucial for PIN2 stability and root gravitropism

148 In further investigations, we focused on PIN2, as it was most extensively targeted, its phosphorylation
149 strongly responded to auxin, and it showed remarkable conservation at the majority of its phospho-sites.

150   To test the physiological relevance of ABP1-TMK1-dependent PIN2 phosphorylation, we mutated
151 the five candidate phospho-sites (Figure 2A and 2B) to either aspartate or alanine and introduced the
152 resultant phospho-variants in the agravitropic *eir1-4* mutant[21] under the native PIN2 promoter. This
153 yielded *PIN2::PIN2^WT^-GFP;eir1-4* (*PIN2^WT^-GFP*), *PIN2::PIN2^5-MIMIC^-GFP;eir1-4* (*PIN2^5-MIMIC^-GFP*)
154 and *PIN2::PIN2^5-DEAD^-GFP;eir1-4* (*PIN2^5-DEAD^-GFP*). Given the rapidity of the auxin effect on PIN2
155 phosphorylation (Figure 1B), we specifically focused on early stages of gravitropic root bending. While
156 *PIN2^WT^-GFP* complemented *eir1-4* close to WT levels, the phospho-mimic *PIN2^5-MIMIC^-GFP* provided
157 only partial rescue (Figure 2C); an effect highly reproducible among independent lines. The phospho-
158 dead *PIN2^5-DEAD^-GFP* showed a weaker effect, which was pronounced during the first two hours of
159 bending and then slowly dissipated (Figure 2C). Interestingly, the *PIN2^5-MIMIC^-GFP* phenotype extended
160 beyond early gravitropic stages and was apparent even 12 hours after gravistimulation (Figure S2A),
161 suggesting that chronic ABP1-TMK1-like phosphorylation of PIN2 strongly perturbs root gravitropism.
162 These results collectively demonstrate the importance of ABP1-TMK1-dependent phospho-sites for the
163 physiological function of PIN2 in root gravitropism.

164   Phosphorylation of PIN2 by AGC3 kinases at $PIN2^{S237}$, $PIN2^{S258}$, and $PIN2^{S310}$ was previously
165 established as regulating polar PIN2 localization[38]. However, the five ABP1-TMK1-dependent PIN2
166 phospho-sites were non-overlapping (Figure 2A). Indeed, we observed stereotypical polarity of apical
167 PIN2 in epidermal cells and basal PIN2 in young cortical cells in our *PIN2^5-MIMIC^-GFP* and *PIN2^5-DEAD^-*

168 *GFP* phospho-lines (Figure S2B). While the PIN2 phospho-lines showed no obvious polarity defects,

169 we did observe reproducible differences in their GFP signal intensity. These were stronger than

170 insertion-dependent variation when selecting T1 transformants and were also apparent in independent,

171 single-insert, GFP-positive phospho-lines (Figure 2D). The *PIN2^5-MIMIC^-GFP* variants showed

172 occasional weak stabilization compared to *PIN2^WT^-GFP* (Figure 2D and 2E). On the other hand,

173 *PIN2^5-DEAD^-GFP* lines showed a highly consistent strong destabilization compared to *PIN2^WT^-GFP* roots

174 (Figure 2D and 2E). Isolation of lines with comparable transgene mRNA levels unequivocally ruled out

175 insertion-dependent expression artifacts and established the loss-of-phosphorylation stability defect as

176 post-transcriptional (Figure S2C and S2D).

177 Altogether, our results show the relevance of ABP1-TMK1-dependent PIN2 phospho-sites for

178 steady-state PIN2 stability and root gravitropism, suggesting a role of cell-surface auxin signaling in

179 these processes.

180

Root-expressed ABL3 auxin receptor acts redundantly with ABP1 in root gravitropism

182 Next, we investigated the genetic basis of PIN2 phosphorylation by cell-surface auxin signaling. It

183 recently became recognized that apoplastic auxin perception shows multi-level redundancy[39,40]. This

184 includes a presumably abundant pool of poorly understood ABP1/ABL auxin receptors communicating

185 with four possible TMKs, together activating global phosphorylation reprogramming of the cellular

186 proteome[15,17,18]. Although we identified PINs as major phospho-targets of this signaling pathway (Figure

187 1A), the precise composition and redundancy of the upstream auxin signaling complexes remain elusive.

188 TMKs form a redundant family with single mutants having rather subtle phenotypes and higher-

189 order mutants showing strong defects in growth and development[41]. To study TMK expression in roots

190 we used global transcriptomic data and generated *TMK1::GUS*, *TMK2::GUS*, *TMK3::GUS,* and

191 *TMK4::GUS* lines reporting the corresponding promoter activities. The dominant family member highly

192 expressed in roots was *TMK1*, followed by *TMK3* and *TMK4* with lower expression levels (Figure S3A-

193 D). To examine the role of TMK1 in root gravitropism, we performed sensitive phenotyping of the *tmk1-

194 1* mutant (Figure 3A, Figure S3F, and Figure S4D). This revealed an early root-bending defect that was

195 complemented by a *TMK1::gTMK1-GFP* construct (Figure S3F). These data support TMK1 as the

196 dominant TMK upstream of PIN2 phosphorylation.

197 Unlike *tmk1-1*, the well-established *abp1* mutant lines (*abp1-C1*, *abp1-TD1*) do not show any

198 appreciable defects in gravitropism[42]. Nevertheless, complementation of *abp1-TD1* by native expression

199 of an auxin-binding-deficient ABP1 variant exerts a dominant negative effect on root gravitropism,

200 indicating the existence of unknown redundant ABLs interacting with TMK1 in the root[15]. A recent

201 report[15] described the redundant action of ABP1 with two auxin receptors, ABL1 and ABL2. The

202   *abp1;abl1;abl2* triple mutant shows normal gravitropism, however, consistent with the predominantly

203   shoot-specific expression of *ABL1* and *ABL2* (Figure S4A).

204   Sensitive gravitropic phenotyping led us to identify a T-DNA insertion knock-out of an

205   ABL1/ABL2 paralog, which we named ABP1-LIKE 3 (ABL3). The *abl3-1* mutation phenocopied the

206   early root gravitropism defects of *tmk1-1* but only in a double mutant constellation with either *abp1-C1*

207   (Figure 3A and 3B) or *abp1-TD1* (Figure S4D and S4E). The double mutant phenotype was reproduced

208   with an independent T-DNA insertion line, *abl3-2* (Figure S4F). We confirmed *ABL3* (AT4G14630)

209   expression in the root by mining public RNAseq data and using an *ABL3::GUS* line (Figure S4A-C).

210   The ABL3 protein encompasses 222 residues and does not harbor a KDEL endoplasmic reticulum

211   retention sequence. Superimposition of Arabidopsis ABL3 AlphaFold2 structure with the 1-NAA-bound

212   maize ABP1 crystal structure revealed a potential auxin-binding cleft in AtABL3 (Figure 3C). This also

213   highlighted that ABL3 conforms to the ancient cupin fold of ABP1[43]. Importantly, ABL3 showed perfect

214   conservation of three metal-coordinating residues known to be indispensable for auxin binding in ABP1,

215   ABL1, and ABL2 (Figure 3C). The sequence surrounding these residues resembled ABL1 and ABL2

216   more than ABP1 (Figure S4G), as expected from members of the same GLP family[16].

217   Next, we tested whether ABL3 binds auxin using a cellular thermal shift assay (CETSA) followed

218   by western blotting. The natural auxin IAA conferred protection from thermal denaturation on ABL3-

219   HA in protein extracts from Arabidopsis root protoplasts transformed with *35S::ABL3-HA* (Figure 3D).

220   Likewise, IAA protected ABL3-6xHIS-3xFLAG (or ABL3-HF) in protein extracts from Arabidopsis

221   seedlings stably transformed with *35S::ABL3-HF* (Figure S4H). These results qualify ABL3 as an auxin-

222   binding protein.

223   To transmit signals from auxin-bound ABL3, TMK1 would be expected as an ABL3 interaction

224   partner. Indeed, in tobacco leaves, TMK1-HA co-immunoprecipitated with ABL3-mCherry but not with

225   anti-mCherry beads alone (Figure 3E). Reciprocally, we further confirmed this interaction in

226   Arabidopsis root protoplasts where ABL3-HA co-immunoprecipitated with TMK1-mCherry but not

227   with anti-mCherry beads alone (Fig. S4I).

228   Thus, we identified ABL3 as a root-expressed auxin-binding protein interacting with TMK1 and

229   acting redundantly with ABP1 in root gravitropism. These observations are consistent with the notion

230   that the cell-surface ABP1/ABL3-TMK1 module represents a root-specific pathway targeting PIN2

231   phosphorylation for early stages of gravitropic root bending.

232

Exogenous and endogenous auxin activates TMK1 and downstream ROP signaling in roots

234   Despite recent progress, the cellular and molecular readouts of cell-surface TMK1-dependent auxin

235   signaling remain poorly established. Previous data showed that the cytoplasmic part of TMK1 harbors

236   an ABP1-dependent phospho-site[17]. Furthermore, the TMK1 kinase domain shows a capacity to auto-

237   phosphorylate[44], and research on other leucine-rich repeat receptor-like kinases (LRR-RLKs) suggests

238   that phosphorylation of their cytoplasmic domains leads to LRR-RLK activation[45,46].

239   Therefore, we examined TMK1 phosphorylation in response to auxin. We immunoprecipitated

240   TMK1-FLAG from auxin-treated (IAA, 10 nM, 1 hour) *TMK1::TMK1-FLAG;tmk1-1* roots. After

241   confirming successful IP with an anti-FLAG antibody, we stripped and re-probed the membranes with

242   a Phos-tag Biotin Probe that coordinates tetrahedral phosphate moieties. We observed significant

243   induction of TMK1 phosphorylation by auxin, presumably corresponding to increased TMK1 activity

244   (Figure 4A and S5A).

245   As a downstream response, we investigated the root-specific activation of small GTPases from the

246   ROP family implicated downstream of ABP1/ABL-TMKs. Previous ROP activation assays relied

247   extensively on ROP overexpression, used the synthetic auxin 1-NAA, and were usually performed with

248   leaf tissue[15,47,48]. We specifically asked if the natural auxin IAA activates ROPs in roots under non-

249   overexpressing conditions. Immunoblotting microsomal protein extracts from auxin-treated (IAA, 5 nM,

250   0–120 minutes) roots with an anti-ROP6 antibody revealed auxin-induced enrichment of ROP6 in WT

251   but not *tmk1-1* (Figure 4B and S5B). Given that membrane association is a prerequisite for ROP

252   activation[49], enrichment in the microsomal fraction likely reports TMK1-dependent ROP6 activation by

253   auxin. Interestingly, we also observed that auxin stabilized TMK1 itself (Figure 4A-B and S5B) but did

254   not induce *TMK1* mRNA over time (IAA, 10 or 100 nM, 0–120 minutes: Figure S5C). Such TMK1

255   stabilization at the membrane might be related to the recently reported auxin-mediated TMK1 nano-

256   clustering effect[50].

257   We next used an orthogonal method to study ROP activation by auxin. As usual for small GTPases,

258   only GTP-bound (active) but not GDP-bound (inactive) ROP proteins engage in protein-protein

259   interactions with their effectors. We purified the Cdc42/Rac-interactive binding motif (CRIB) domain

260   of the RIC1 ROP effector from bacteria and used it to pull down active ROPs from native root protein

261   extracts. Immunoblotting with an anti-ROP2 antibody revealed a strong auxin-induced (IAA, 100 nM,

262   10 minutes) ROP2 activation in WT but much weaker activation in *tmk1-1*, *abp1-C1*, or *abp1-TD1* roots

263   (Figure 4C and S5D). This confirms that auxin activates root ROP2 through the ABP1-TMK1 module.

264   To test whether the above observations remain valid also when auxin levels are changed

265   endogenously, we repeated TMK1-FLAG immunoprecipitation followed by a Phos-tag Biotin Probe

266   blotting on gravistimulated roots. This revealed a significant increase in TMK1 phosphorylation (Figure

267   4D and S5E), indicating that gravistimulation activates TMK1. Accordingly, gravistimulation also

268   induced a TMK1-dependent enrichment of ROP6 in the microsomal fraction (Figure 4E and S5F).

269   Overall, these data show that both exogenous and endogenous manipulations of auxin levels

270   promote TMK1 phosphorylation and activation of downstream ROP GTPase signaling.

271

Asymmetric activation of TMK1 and downstream ROP signaling in root gravitropism

Having established auxin-induced TMK1 activation in the root (see Figure 4 and S5) and the importance of ABP1-TMK1-dependent PIN2 phosphorylation for its stability and in early gravitropic root bending (see Figure 1, 2, S1, and S2), we assessed the role of these regulations in root gravitropism.

Our experiments with native ROP activation suggested auxin-responsive ROP signaling as a suitable proxy for TMK1 activity in the root tissue (Figure 4 and S5). However, blotting-based assays do not provide sufficient spatiotemporal resolution. For this reason, we constructed an *in situ* ROP sensor by inserting (i) ROP2 and (ii) the CRIB domain of the ROP effector RIC1 on opposite ends of a circularly permuted GFP (cpGFP). The cpGFP fluorescence should decrease when activated GTP-bound ROP2 interacts with the nearby CRIB domain (Figure S6D). Expression of CRIB-cpGFP-ROP2 and ROP2-mCherry from the same cassette yielded a ratiometric ROP sensor, which we called the CpGFP ROP Activity Probe (CRAP). As expected, the 561/488 nm CRAP ratio sensitively reported auxin (IAA, 10 nM) pulses in *CRAP;WT* roots in a microfluidic root chip setup (Figure S6E).

Strikingly, within 5 minutes of gravistimulation, CRAP-expressing roots developed asymmetric signal distribution with significantly more ROP activation at the lower side of the root (Figure S6F and Figure 5A). A GDP-locked CRAP (CRIB-cpGFP-ROP2^{T20N}) failed to show this asymmetry, confirming that CRAP indeed reports ROP activation rather than e.g. local fluctuations of the cpGFP root microenvironment (Figure S6F). This identifies a novel asymmetric rapid response to gravity-induced auxin flux redirection in roots, as confirmed by the lack of CRAP asymmetry after inhibition of auxin transport by NPA (Figure S6I).

Given that auxin-induced ROP activation is TMK1-dependent (Figure 4 and S5), the gravitropic CRAP gradient likely mirrors asymmetric TMK1 activation by auxin flow from the root rip. Indeed, the *tmk1-1* mutation abolished asymmetric ROP activation in *CRAP;tmk1-1* roots (Figure 5B). These data collectively establish rapid asymmetric activation of the TMK1 kinase and downstream ROP signaling by redirection of auxin fluxes during gravitropic root bending.

297

Asymmetric TMK1 activation mediates PIN2 asymmetry in root gravitropism

The rapid TMK1 activation along the lower root side corresponds well with the timeframe in which auxin induces PIN2 phosphorylation through TMK1 (Figure 1B), inferring that asymmetric TMK1 activation likely results in asymmetric PIN2 phosphorylation. Given that TMK1-regulated phospho-sites mediate PIN2 stability (see Figure 2), we decided to follow the fate of PIN2-GFP in gravistimulated

303   roots. We observed asymmetric PIN2-GFP stabilization at the lower root side, as shown before[21,29,51],

304   and this was completely abolished in the *tmk1-1* mutant (Figure 5C and 5D).

305   We further assessed the role of TMK1 and its kinase activity by cloning a kinase-dead TMK1

306   construct carrying a mutation in the ATP-binding site and generating *UBQ10::TMK1^K616R^-mCherry*

307   (TMK1^DN^) in a WT background. Notably, TMK1^DN^ expression perturbed the gravity-induced PIN2-

308   GFP asymmetry (Figure S6G). Accordingly, it also conveyed an early defect in gravitropic root bending

309   (Figure S6A and S6B), phenocopying the *tmk1-1* mutant (Figure 3A, S3F and S4D). The TMK1^DN^-

310   expressing plants showed unperturbed levels of the endogenous TMK1 protein (Figure S6C), ruling out

311   transgene-induced silencing of the endogenous *TMK1* gene. This shows that TMK1^DN^ causes a dominant

312   negative phenotype, underscoring the importance of TMK1 kinase activity for both, gravity-induced

313   PIN2 asymmetry and rapid bending response.

314   The requirement of TMK1 for the PIN2-GFP gradient suggests that TMK1 stabilizes PIN2 to

315   enhance PIN2-mediated auxin flux from the root tip along the lower root side. To test this, we monitored

316   the *DR5rev::GFP* auxin response reporter, which revealed a significantly decreased gravity-induced

317   auxin asymmetry in *tmk1-1* compared to WT (Figure 5E and 5F, Figure S6H). Accordingly, inhibition

318   of PIN-mediated auxin transport by NPA interfered not only with CRAP-reported asymmetric TMK1

319   activation (Figure S6I) but also the PIN2-GFP asymmetry (Figure S6J), confirming that polar auxin

320   transport itself contributes to asymmetric TMK1 activation and subsequent PIN2 stabilization for further

321   asymmetric auxin flow reinforcement. Finally, we followed PIN2-GFP gradient formation in the *abp1-*

322   *TD1;abl3-1* background, which revealed a perturbed profile that was especially deficient in the

323   maintenance phase (Figure S6K). The residual PIN2-GFP asymmetry contrasts with the complete

324   abolishment in *tmk1-1* and likely indicates the existence of further unexplored ABL proteins upstream

325   of TMK1.

326   Altogether, these data identify a positive feedback loop, in which, following gravistimulation, PIN2

327   redistributes auxin from the root tip to the lower root side, activating the TMK1 kinase, which promotes

328   PIN2 phosphorylation and stabilization, channeling even more auxin along the lower root side and

329   reinforcing the original gravity-induced auxin flow asymmetry.

330

331   Auxin induces TMK1 interaction with PIN2 and phosphorylation of its hydrophilic loop

332   Our hitherto results demonstrate a strong functional relevance of TMK1-dependent PIN2

333   phosphorylation during root gravitropism. Co-localization of TMK1-GFP and PIN2-mCherry expressed

334   from native promoters suggested a possibility for their direct interaction (Figure S7A). To test this, we

335   immunoprecipitated TMK1-FLAG from *TMK1::TMK1-FLAG;tmk1-1* roots and used an anti-PIN2

336   antibody for detection of native PIN2. PIN2 did not co-immunoprecipitate with TMK1-FLAG in

337   untreated samples. On the other hand, in auxin-treated roots (IAA, 5 or 20 nM, 15 or 30 minutes), PIN2
338   co-immunoprecipitated with TMK1-FLAG in a time-dependent and auxin concentration-dependent
339   manner (Figure 6A and S7B). This suggests that auxin promotes the formation of a TMK1-PIN2
340   complex at the plasma membrane.

341   To verify the biochemical evidence for TMK1-PIN2 interaction, we performed fluorescence
342   lifetime imaging on FRET-pair-tagged proteins (FRET-FLIM), a technique that quantitatively reports
343   protein interactions. We introduced *35S::TMK1-GFP* and *35S::PIN2-mCherry* in Arabidopsis root
344   protoplasts and measured the fluorescence lifetime of the GFP signal. PIN2-mCherry strongly reduced
345   the lifetime of TMK1-GFP, demonstrating an interaction between TMK1 and PIN2 (Figure 6B).
346   Notably, a truncated TMK1$^{\Delta KD}$-GFP variant without a kinase domain caused a significant ~35 % drop
347   in the interaction strength compared to TMK1-GFP. The interaction of an unrelated receptor-like kinase
348   FLS2-GFP with PIN2-mCherry was ~60 % weaker than that of TMK1-GFP (Figure 6B and S7C). These
349   results establish both the contribution of the TMK1 kinase domain and the specificity of the TMK1-
350   PIN2 interaction.

351   An auxin-induced TMK1-PIN2 interaction provides a plausible mechanism for the TMK1-
352   dependent PIN2 phosphorylation observed in phospho-proteomic data (Figure 1 and S1). To test this,
353   we performed an *in vitro* phosphorylation assay with ^{32}P-ATP as a phosphate donor. We incubated a
354   purified N-terminally HIS-tagged PIN2 hydrophilic loop (HIS-PIN2-HL) with TMK1-3HA
355   immunoprecipitated from a root protein extract. The results showed that intact TMK1-3HA but not the
356   kinase-dead version TMK1^{K616R}-3HA was able to phosphorylate HIS-PIN2-HL (Figure 6C). We did not
357   observe any auxin effect in this kinase assay (Figure S7D), presumably due to saturation of TMK1-3HA
358   activity by endogenous auxin during immunoprecipitation from TMK1-3HA roots.

359   Taken together, these data demonstrate that the receptor-like kinase TMK1 interacts, in an auxin-
360   dependent manner, with the PIN2 auxin efflux carrier and phosphorylates its hydrophilic loop to
361   stabilize it.

362

363   **DISCUSSION**

364   **Co-option of ancient auxin phospho-response for auxin feedback on its transport in vascular**
365   **plants**

366   Previous work indicated that while the rapid ABP1-TMK1-mediated auxin phospho-response is relevant
367   for some rapid cellular auxin effects, specifically cytoplasmic streaming and apoplast
368   acidification[13,17,18,52], *abp1* and *tmk* mutants also show severe phenotypes in the long-term establishment
369   of auxin- and auxin transport-positive channels after wounding and from externally applied auxin
370   source, leading to vasculature formation[17]. The underlying mechanism of this so-called auxin

371   canalization is largely unclear but at its center lies feedback regulation between auxin signaling and

372   directional auxin transport[3,4].

373   Here, mining of a root ABP1-TMK1 phospho-proteome revealed PIN auxin transporters as major

374   phospho-targets of the ABP1-TMK1 cell-surface auxin perception. It follows that PIN phosphorylation

375   by ABP1-TMK1 likely modulates directional auxin transport to delineate auxin channels for subsequent

376   vascular differentiation and eventually other processes involving feedback regulation of auxin transport.

377   Consistently, a 15-year-old model predicted extracellular auxin perception as a key signaling input

378   parameter for auxin canalization[20]. Although the auxin phospho-response evolved in unicellular algae[18],

379   we find that it began targeting PINs only after the divergence of Bryophyta from the green lineage, likely

380   in the common ancestor of vascular plants. The ABP1-TMK1-mediated PIN phosphorylation thus

381   represents a recent evolutionary novelty that arose through the co-option of ancient rapid auxin response,

382   presumably to enable the formation and regeneration of vasculature.

383

ABP1-TMK1-mediated phosphorylation encodes PIN2 stability

385   Focusing on the dominant phospho-target PIN2, we report five ABP1-TMK1-dependent phospho-sites,

386   the majority of which are induced by auxin and remarkably conserved. Strikingly, neither of these

387   overlaps with previously published polarity-regulating PIN2 phospho-sites[38]. Indeed, both PIN2 stability

388   and the physiological function of PIN2 in root gravitropism require ABP1-TMK1-dependent phospho-

389   sites, implying the existence of two PIN2 phospho-codes; one for stereotypical maintenance of PIN2

390   polarity via AGC3 kinases[38], and the other for dynamic adjustments of PIN2 stability in response to

391   auxin.

392

ABL3: Root-expressed auxin receptor acting redundantly with ABP1 in root gravitropism

394   Overall lack of *tmk1*-like phenotypes in *abp1* mutants, despite the strong similarity of phospho-

395   proteomic signatures between *abp1* and *tmk1*[17], contributed to the historical controversy surrounding

396   ABP1. Indeed, while we confirmed an early gravitropic phenotype in the *tmk1* mutant, *abp1* mutant

397   alleles show normal root bending, as reported before[42]. Hidden genetic redundancy with distant but

398   structurally conserved ABL proteins has been invoked to explain this discrepancy, however, the recently

399   identified ABL1 and ABL2 show minimal expression in the root, and the *abp1;abl1;abl2* triple mutant

400   shows normal gravitropism[15].

401   We identified the first root-expressed ABL protein, ABL3, through genetic redundancy with ABP1,

402   and as an auxin binder and TMK1 interactor. Notably, ABL3 is paralogous to both ABL1 and ABL2

403   because they all belong to the 32-member Arabidopsis GLP family, which is distantly related to ABP1

404   by the ancient cupin fold[43]. ABP1 and ABL3 likely form part of an auxin-sensing complex docking on

405   TMK1 in the root, providing a plausible model for auxin perception upstream of PIN2 phosphorylation.

406   While the field so far only scratched the surface of the real diversity of potential cell surface auxin

407   receptors, this paints a picture in which specialized expression patterns of ABL auxin receptors confer

408   specific functions on the rather ubiquitously expressed TMKs. There are likely more root-expressed

409   ABLs awaiting discovery because *tmk1* and *abp1;abl3* mutants show a weaker phenotype than both the

410   dominant-negative *ABP1::ABP1-5;abp1-TD1* line[15] and higher-order *tmk* mutants[53,54].

411

412   Model for TMK1-based auxin feedback on PIN2-mediated transport in root gravitropism

413   The PIN2 transporter evolved as a specific component of efficient root gravitropism in seed plants[22] and

414   it is well documented that its abundance during gravistimulation becomes asymmetric with more PIN2

415   found at the lower root side[21,29]. This reinforces an initial auxin gradient and contributes to the robustness

416   of root bending[30]. Nonetheless, the molecular mechanism behind this regulation has remained unknown

417   since its discovery almost 20 years ago. Here, the wealth of our data together argues for a model

418   encompassing auxin feedback on its transport.

419   A change in the gravity vector is sensed in the root columella, which establishes an initial auxin

420   flow redirection to the lower side of the root[23]. This initial auxin asymmetry activates the TMK1 kinase

421   specifically at the lower root side. Activated TMK1 then interacts with PIN2 in the epidermis and

422   phosphorylates its hydrophilic loop at several conserved stability-regulating phospho-sites, leading to

423   PIN2 stabilization in these cells. The resulting PIN2 abundance gradient further enhances auxin transport

424   along the lower root side to the elongation zone, where it activates intracellular TIR1/AFB auxin

425   signaling for growth inhibition and downward root bending. This demonstrates the existence of a

426   positive feedback loop representing the first direct molecular mechanism for auxin feedback on its

427   transport.

428   The TMK-based auxin feedback regulation likely represents a more general mechanism acting in

429   various developmental contexts with different PIN transporters, thereby mediating specialized aspects

430   of adaptive and self-organized plant growth and development. This is supported by the identification of

431   TMK1-mediated PIN1 phosphorylation in the context of PIN polarity and auxin canalization[55]. The

432   TMK-PIN mechanism evolved recently in vascular plants through the co-option of an ancestral auxin

433   phospho-response from unicellular algae[18] and it likely diversified to guide the auxin-mediated

434   development of morphologically complex plants.

435

436   Limitations of the study

437 Our work establishes a phosphorylation-based feedforward loop between ABP1/ABL3-TMK1 cell-
438 surface auxin signaling and PIN2-mediated polar auxin transport in root gravitropism. Importantly, the
439 newly identified root-expressed ABL3 auxin receptor extends the concept of ABP1/ABL signaling to
440 roots, mirroring hypocotyl- and pavement cell-centered functions of the dominantly shoot-expressed
441 ABL1 and ABL2 receptors[15]. Nevertheless, several lines of evidence indicate the existence of further
442 unknown ABL proteins in the root. First, the gravitropic PIN2-GFP asymmetry is fully absent in *tmk1*
443 but partially persists in *abp1;abl3* mutants. Second, *tmk1* and *abp1;abl3* mutants show a weaker root
444 growth and bending phenotype than both the dominant-negative *ABP1::ABP1-5;abp1-TD1* line[15] and
445 higher-order *tmk* mutants[53,54]. Future studies should undertake a systematic effort to define which of the
446 32 Arabidopsis GLP proteins act as ABL auxin receptors. Furthermore, it remains a persistent mystery
447 in the auxin transport field, how PIN phosphorylations on specific residues downstream of different
448 signaling pathways[32] selectively affect PIN activity, polarity, or stability.

449

450

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696

697 **ACKNOWLEDGEMENTS**

709

AUTHOR CONTRIBUTIONS

711 Conceptualization: J.F., L.R.; Methodology: L.R., L.F. I.K.; Investigation: L.R., L.F., M.Z., C.G., D.V.,
712 A.M., M.R., Y.Y., Z.G., I.V., J.H., M.C., S.T., L.H., L.L., M.M.M.-B., Z.Q., G.M.; Resources: L.R.,
713 L.F., M.M.M.-B., Y.J., T.X.; Writing: J.F., L.F., L.R.; Review & Editing: all authors; Visualization:
714 L.R., L.F.; Supervision: J.F., L.F., L.R.; Funding Acquisition: J.F.

715

DECLARATION OF INTERESTS

717 The authors declare no competing interests.

718

719

FIGURE LEGENDS

Figure 1. Rapid auxin phospho-response targets PINs in Arabidopsis

(A) Overview of PIN phospho-sites downregulated (FDR < 0.05) in *abp1-TD1* and *tmk1-1* auxin-treated (100 nM IAA, 2 min) roots. 4 biological replicates, Mean + SD, permutation-based t-tests with FDR-controlled p-values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

(B) Significant PIN2 phospho-site auxin profiles (FDR ≤ 0.01, 100 nM IAA).

(C) Localization of ABP1-TMK1-dependent phospho-sites on a ConSurf conservation-colored AlphaFold2 structure of PIN2.

728   (D) Multiple sequence alignment of eight PIN2 orthologs with a highlight of Arabidopsis ABP1-TMK1-

729   dependent PIN2 phospho-sites.

730

731   **Figure S1. Identification of PINs as ABP1-TMK1 phospho-targets by gene ontology analysis**

732   (A) Treemap of GO terms enriched for the ABP1-TMK1 phospho-proteome under mock conditions.

733   Box sizes scale with -log10(p-value) (top) or fold enrichment (bottom).

734   (B) Treemap of GO terms enriched for the ABP1-TMK1 phospho-proteome under auxin treatment (100

735   nM IAA, 2 min) conditions. Box sizes scale with -log10(p-value) (top) or fold enrichment (bottom).

736   (C) Overview of phospho-peptides pertaining to PIN phospho-sites from Fig. 1A.

737   (D) Significant PIN1 and PIN3 phospho-site auxin profiles (FDR $\leq$ 0.01, 100 nM IAA).

738   (E) Localization of ABP1-TMK1-dependent phospho-sites on a ConSurf conservation-colored

739   AlphaFold2 structure of PIN1.

740   (F) Localization of ABP1-TMK1-dependent phospho-sites on a ConSurf conservation-colored

741   AlphaFold2 structure of PIN3.

742

743   **Figure 2. ABP1-TMK1-dependent PIN2 phospho-sites for gravitropism and PIN2 stability**

744   (A) Schematic of ABP1-TMK1-dependent phospho-sites mapped on the PIN2 hydrophilic loop.

745   (B) Schematic summarizing properties of the studied PIN2 phospho-sites.

746   (C) Root gravitropism of PIN2-GFP phospho-lines on medium with sucrose (1 %). Mean + or – SD.

747   (D) Representative maximum intensity projection images of PIN2-GFP phospho-lines.

748   (E) Quantification of GFP signal from (D). Kruskal-Wallis analysis followed by Holm-corrected

749   Wilcoxon rank sum tests relative to WT. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

750

751   **Figure S2. Supplementary data on PIN2-GFP phospho-lines**

752   (A) Normalized root angles after 12-hour gravistimulation of PIN2-GFP phospho-lines on medium with

753   sucrose (1 %).

754   (B) Representative median root sections showing PIN2-GFP phospho-line polarity. Scale bar, 20 μm.

755 (C) Quantification of GFP signal in two independent PIN2$^{5\text{-DEAD}}$-GFP phospho-lines compared to a

756 PIN2^{WT}-GFP phospho-line.

757 (D) qPCR quantification of *PIN2-GFP* mRNA in phospho-lines corresponding to (C). No *PIN2-GFP*

758 was amplified in a wild-type Columbia-0 sample.

759

760 **Figure 3. ABP1/ABL3-TMK1 signaling during early root gravitropism**

761 (A) Mutant root gravitropism profiles on medium without sucrose. Mean + or – SD.

762 (B) Representative images of mutant roots gravistimulated for 1 hour. The full set of images is shown

763 in Fig. S4E. Scale bar, 100 μm.

764 (C) Superimposition of Arabidopsis ABL3 AlphaFold2 structure with 1-NAA-bound maize ABP1

765 crystal structure highlighting a potential auxin-binding cavity of Arabidopsis ABL3.

766 (D) CETSA assay on *35S::ABL3-HA* Arabidopsis root protoplasts in the presence of 100 μM IAA.

767 (E) Co-immunoprecipitation from tobacco leaves of TMK1-HA with ABL3-mCherry but not with anti-

768 mCherry beads alone.

769

770 **Figure S3. Expression of *TMK* genes and analysis of the *tmk1-1* mutant**

771 (A) Relative transcription levels of *TMK1, TMK2, TMK3,* and *TMK4* in Arabidopsis roots from 803

772 samples assayed with the Affymetrix Arabidopsis ATH1 Genome Array. Data was obtained using the

773 Genevestigator databases (https://genevestigator.com). Mean + SD.

774 (B) Root tip expression pattern of *TMK1, TMK2, TMK3,* and *TMK4* promoters fused to the GUS

775 transcriptional reporter. Scale bar, 100 μm.

776 (C) Relative expression levels of *TMK1, TMK2, TMK3* and *TMK4* in different root cell types obtained

777 from high-resolution spatiotemporal microarray analysis of 5-6-day-old roots reported in[56] (ePlant

778 http://bar.utoronto.ca/eplant).

779 (D) Representative intensity-colored image of a 4-day-old *TMK1::TMK1-GFP;WT* root.

780 (E) *TMK1* qPCR in *tmk1-1*.

781 (F) Root gravitropism profiles on medium without sucrose. Mean + or – SD.

782

783 **Figure S4. Characterization of ABL3 properties**

784   (A) Comprehensive expression profiles of *ABL3* and *ABP1/ABL/TMK* genes obtained using[57].

785   (B) Snapshot of *ABL3* expression pattern obtained using ePlant/TAIR.

786   (C) *ABL3::GUS* staining (2h). Scale bar, 100 μm.

787   (D) Mutant root gravitropism profiles on medium without sucrose. Mean + or – SD.

788   (E) Representative images of mutant roots gravistimulated for 1 hour. Scale bar, 100 μm.

789   (F) Mutant root gravitropism profiles on medium without sucrose. Mean + or – SD.

790   (G) Multiple sequence alignment of ABP1/ABL protein sequences surrounding the auxin pocket with

791   metal-coordinating residues (orange). Purple coloring highlights conservation.

792   (H) CETSA assay on *35S::ABL3-6xHIS-3xFLAG* seedlings in the presence of 100 μM IAA.

793   (I) Co-immunoprecipitation from Arabidopsis root protoplasts of ABL3-HA with TMK1-mCherry but

794   not with anti-mCherry beads alone.

795

796   **Figure 4. Auxin-induced activation of TMK1 and downstream ROP signaling in roots**

797   (A) Auxin effect on TMK1 phosphorylation in *TMK1::TMK1-FLAG;tmk1-1* roots assayed through

798   TMK1-FLAG immunoprecipitation and Phos-tag Biotin Probe analysis. Refer to Fig. S5A for

799   quantification of three experimental replicates.

800   (B) Auxin effect on ROP6 and TMK1 levels in the WT or *tmk1-1* root microsomal protein fractions.

801   Refer to Fig. S5B for quantification of three experimental replicates.

802   (C) Auxin effect on ROP2 activation assayed by native ROP pulldown from roots of the indicated

803   genotypes. Empty well was edited out from the upper blot for visualization purposes. Refer to Fig. S5D

804   for unedited blot image.

805   (D) Gravistimulation effect on TMK1 phosphorylation in *TMK1::TMK1-FLAG;tmk1-1* roots assayed

806   through TMK1-FLAG immunoprecipitation and Phos-tag Biotin Probe analysis. Refer to Fig. S5E for

807   quantification of three experimental replicates.

808   (E) Gravistimulation effect on ROP6 and TMK1 levels in the WT or *tmk1-1* root microsomal protein

809   fractions. Refer to Fig. S5F for quantification of three experimental replicates.

810

811   **Figure S5. Supplementary data for ROP activation through TMK1**

812   (A) Quantification of auxin effect on TMK1 phosphorylation in *TMK1::TMK1-FLAG;tmk1-1* roots

813   assayed through TMK1-FLAG immunoprecipitation and Phos-tag Biotin Probe analysis.

814   Phosphorylation levels detected with a Phos-tag Biotin Probe were normalized to signal obtained from

815   anti-FLAG detection, and subsequently to the mock value. Mean ± SD of 3 biological replicates.

816   Unpaired two-tailed t-test, **$p < 0.01$.

817   (B) Quantification of auxin effect on ROP6 and TMK1 levels in the WT or *tmk1-1* root microsomal

818   protein fractions. Band intensities were normalized to the loading control, and subsequently to the first

819   timepoint T0. Mean ± SD of 3 biological replicates. Two-way ANOVA with Dunnett's multiple

820   comparisons, ****$p < 0.0001$.

821   (C) RT-qPCR of *TMK1* expression after auxin treatment. Normalization was done to *PP2A* as a

822   reference gene. The expression level at the start of the experiment (T0) was set to a relative value of 1.

823   (D) Auxin effect on ROP2 activation assayed by native ROP pulldown from roots of the indicated

824   genotypes. Full unedited blot relating to Fig. 4C.

825   (E) Quantification of gravistimulation effect on TMK1 phosphorylation in *TMK1::TMK1-FLAG;tmk1-*

826   *1* roots assayed through TMK1-FLAG immunoprecipitation and Phos-tag Biotin Probe analysis.

827   Phosphorylation levels detected with a Phos-tag Biotin Probe were normalized to signal obtained from

828   anti-FLAG detection, and subsequently to the first timepoint T0. Mean ± SD of 3 biological replicates.

829   One-way ANOVA with Dunnett's multiple comparisons to timepoint 0 (****$p < 0.0001$).

830   (F) Quantification of gravistimulation effect on ROP6 and TMK1 levels in the WT or *tmk1-1* root

831   microsomal protein fractions. Band intensities were normalized to the loading control, and

832   subsequently to the first timepoint T0. Mean ± SD of 3 biological replicates. Two-way ANOVA with

833   Dunnett's multiple comparisons, ****$p < 0.0001$.

834

835   **Figure 5. Asymmetric TMK1 activation for PIN2 asymmetry in root gravitropism**

836   (A) Image of a root with strongly asymmetric ROP activity (reported by the CRAP sensor) in response

837   to 15-minute gravistimulation.

838   (B) Rapid gravistimulation-induced establishment of asymmetric ROP activity in WT or *tmk1-1* roots.

839   (C) Representative images of *PIN2::PIN2-GFP* in 4-day-old WT and *tmk1-1* seedlings after 4 hours of

840   gravistimulation. g, gravity vector. Scale bar, 20 μm.

841   (D) Quantification of gravistimulation-induced PIN2-GFP asymmetry. Normalization to the first

842   timepoint T0 for the upper or lower root side independently. Mean ± SD. Two-way ANOVA with

843   Dunnett's multiple comparisons, **** $p < 0.0001$.

844 (E) Representative confocal images of asymmetric auxin response (visualized by *DR5rev::GFP*) at the

845 lower side of the root after a 3-hour gravistimulation in WT and *tmk1-1* 4-day-old roots. Refer to Fig.

846 S6H for corresponding images taken before gravistimulation.

847 (F) Quantification of *DR5rev::GFP* asymmetry as a ratio of fluorescence intensity at the lower side to

848 the upper side of gravistimulated roots at the indicated time points, normalized to the initial fluorescence

849 value. Mean ± SD, n = 10. Two-way ANOVA with Dunnett's multiple comparisons, $*p < 0.05$, $**p <$

850 0.01. g, gravity vector.

851

852 **Figure S6. Requirement of TMK1 kinase activity for early root gravitropism**

853 (A) Representative images of primary roots expressing the *UBQ10::TMK1^{K616R}-mCherry* construct.

854 (B) Root gravitropism profiles on medium without sucrose. Mean + or – SD.

855 (C) Western blotting shows that native TMK1 protein expression is not silenced in *UBQ10::TMK1^{K616R}-*

856 *mCherry* roots.

857 (D) Schematic showing the function of the CRAP sensor.

858 (E) CRAP sensor reports IAA pulses (10 nM IAA, highlighted in magenta) in a microfluidic root chip

859 device.

860 (F) Dominant-negative mutation in the CRAP sensor abolishes rapid gravistimulation-induced

861 establishment of CRAP asymmetry in WT roots. Mean + or – SD.

862 (G) Dominant negative effect of *UBQ10::TMK1^{K616R}-mCherry* on gravistimulation-induced PIN2-GFP

863 asymmetry. Mean + or – SD.

864 (H) Representative confocal images of *DR5rev::GFP;WT* and *DR5rev::GFP;tmk1-1* roots before

865 gravistimulation.

866 (I) 10 μM NPA treatment abolishes rapid gravistimulation-induced establishment of CRAP asymmetry

867 in WT roots. Mean + or – SD.

868 (J) 10 μM NPA treatment abolishes gravistimulation-induced PIN2-GFP asymmetry. Mean + or – SD.

869 (K) PIN2-GFP asymmetry defect in *abp1-TD1;abl3-1* double mutant. Mean + or – SD.

870

871 **Figure 6. Auxin-mediated TMK1 interaction with and phosphorylation of PIN2**

872 (A) Auxin promotes the interaction between TMK1 and PIN2 in a co-immunoprecipitation (Co-IP)

873 assay. Microsomal protein fraction from 20 nM IAA- or DMSO-treated roots expressing *TMK1::TMK1-*

874   *FLAG/tmk1-1* was immunoprecipitated with an anti-FLAG antibody, and endogenous PIN2 was

875   detected by blotting with an anti-PIN2 antibody. See Figure S3A for Co-IP with 5 nM IAA where input

876   PIN2 abundance in the protein microsomal fraction is increased.

877   (B) FRET-FLIM analysis on transiently expressed *35S::TMK1-GFP, 35S::TMK1^{ΔKD}-GFP, 35S::FLS2-*

878   *GFP* and *35S::PIN2-mCherry* in root protoplasts. Fluorescence lifetime values are displayed as a heat

879   map (for additional images see Figure S3B). One-way ANOVA with Holm-corrected post hoc tests, *p

880   < 0.05, ****$p < 0.0001$.

881   (C) *In vitro* kinase assay showing that TMK1-3xHA directly phosphorylates the 6His-PIN2 hydrophilic

882   loop (HL). Top panel, ^{32}P autoradiography; middle panel, CBB staining; bottom panel, immunoblot with

883   an anti-HA antibody.

884

885   **Figure S7. Supplementary data on TMK1-PIN2 interaction**

886   (A) Co-localization of TMK1-GFP and PIN2-mCherry.

887   (B) Co-IP assay showing the interaction of TMK1 with PIN2 upon low auxin treatment (5 nM IAA).

888   Microsomal protein fraction from WT was used as a control for unspecific binding of endogenous PIN2

889   (shown in Figure 3A, same experiment).

890   (C) FRET-FLIM analysis on transiently expressed *35S::TMK1^{ΔKD}-GFP, 35S::TMK1^{ΔKD}-*

891   *GFP/35S::PIN2-mCherry, 35S::FLS2-GFP* and *35S::FLS2-GFP/35S::PIN2-mCherry* in root

892   protoplasts (the same experiment is shown in Figure 3B). GFP fluorescence lifetime was calculated as

893   described in the Methods section. The heat map depicts fluorescence lifetime values (see Figure 3B for

894   lifetime analysis).

895   (D) *In vitro* kinase assay showing phosphorylation of the 6His-PIN2 HL substrate by TMK1-3xHA in

896   the presence or absence of auxin. Top panel, ^{32}P-autoradiography; middle panel, CBB staining; bottom

897   panel, immunoblot with an anti-HA antibody.

898

899   **METHODS**

900   **Molecular cloning, plant material, and growth conditions**

901   All mutant alleles were in the *Columbia-0* (*Col-0*) background except *abp1-TD1*, which was in

902   *Columbia-4* (*Col-4*). The T-DNA insertion lines *eir1-4* (SALK_091142) and *tmk1-1* (SALK_016360)

903   were previously reported[21,58], as was *abp1-C1* and *abp1-TD1*[59]. The T-DNA insertion line

904   SAIL_441_G04, which harbors four insertions, was obtained from NASC and crossed with Col-0.

905   Insertion-specific primers (Supplementary Table 3) were then used to out-segregate the

906 SAILSEQ_441_G04.2 insertion in *ABL3*/AT4G14630 from SAILSEQ_441_G04.0 (AT4G09510),

907 SAILSEQ_441_G04.1 (AT4G02930) and SAILSEQ_441_G04.3 (AT5G09590), yielding *abl3-1*. To

908 obtain *abp1-C1;abl13-1* and *abp1-TD1;abl3-1*, we crossed the respective *abp1* mutants with *abl3-1*.

909 The T-DNA insertion line SALK_203089C was obtained from NASC, verified (Supplementary Table

910 3), and used as *abl3-2*. The *abp1-TD1;abl3-2* double mutant was obtained by genetic crosses.

911 *PIN2::PIN2-GFP*, *DR5rev::GFP*, *TMK1::TMK1-FLAG;tmk1-1*, *TMK1::GUS*, *TMK2::GUS*,

912 *TMK3::GUS*, and *TMK4::GUS* were described before[17,58,60,61]. We generated *PIN2::PIN2-GFP;tmk1-1*

913 and *DR5rev::GFP;tmk1-1* by genetic crosses of the respective markers with *tmk1-1*. We generated

914 *PIN2::PIN2-GFP;abp1-TD1;abl3-1* by a genetic cross with *abp1-TD1;abl3-1*. Shuta Asai kindly

915 provided the *ABL3::GUS* and *35S::ABL3-6xHIS-3xFLAG* lines[62]. *DEX::TMK1^{K616R}-HA* line

916 (unpublished) was kindly provided by William Gray. To generate *TMK1::TMK1-GFP* and

917 *35S::TMK1^{ΔKD}-GFP*, the *TMK1* full length or *TMK1^{ΔKD}* (amino acid 1-587) genomic DNA without a

918 stop codon were amplified from *Col-0* DNA through PCR with *TMK1-FL-B1-F* and *TMK1-FL-B2-*

919 *R/TMK1-ΔKD-B2-R* primers (Supplemental Table 3), respectively. The resulting *TMK1* sequences were

920 inserted into pDONR221 by a BP reaction. Next, for the *TMK1::TMK1-GFP* construct, *pDONR P4-*

921 *P1R pTMK1*, *pDONR221 gTMK1,* and *pDONR P2R-P3 GFP*; for *35S::TMK1^{ΔKD}-GFP*, *pDONR P4-*

922 *P1R p35S*, *pDONR221 gTMK1^{ΔKD}* and *pDONR P2R-P3 GFP* were recombined into pB7m34GW vector

923 by a MultiSite Gateway LR reaction. To generate *UBQ10::TMK1^{K616R}-2xmCherry*,

924 *TMK1^{K616R}noSTOP/pDONR221* was obtained by site-directed mutagenesis, amplifying *pDONR221*

925 *TMK1* with the *TMK1KD-K616F* and *TMK1KD-K616R* primer pair. *pDONR221-TMK1^{K616R}* was then

926 recombined by LR reaction with *pDONR P4-P1R pUBQ10*[63], *pDONR P2R-P3 2xmCHERRY-4xMyc*[64]

927 and pH7m34GW[65] to obtain *UBQ10::TMK1^{K616R}-2xmCherry* in pH7m34GW.

928 To clone PIN2 phospho-lines, Gibson Assembly (NEBuilder® HiFi DNA Assembly Master Mix,

929 E2621L) was used to insert EGFP into the PIN2 coding sequence between Thr405 and Arg406

930 (according to[35]) and to assemble this fragment with an attL sites-containing pDONR221 backbone

931 (Supplemental Table 3), yielding *pDONR221 PIN2^{WT}-EGFP*. For *5-MIMIC* and *5-DEAD* constructs, we

932 used gene synthesis (Integrated DNA Technologies, IDT) to obtain 1533-bp blocks containing EGFP

933 and its flanking PIN2 CDS sequences with Ser179, Ser183, Thr233, Ser393, Ser439 triplets mutated to

934 either GAC (Asp, *5-MIMIC*) or GCC (Ala, *5-DEAD*) (Supplementary table 3). These were Gibson-

935 Assembled with a fragment amplified from the *pDONR221 PIN2^{WT}-EGFP* plasmid (Supplementary

936 table 3), yielding *pDONR221 PIN2^{5-MIMIC}-EGFP* and *pDONR221 PIN2^{5-DEAD}-EGFP*. Finally, the

937 pDONR221 plasmids were recombined with pDONR-P4-P1R-pPIN2[22] into pB7m24GW.3 by a

938 multisite LR reaction (Gateway), and *eir1-4* (*pin2* null)[21] was used for dipping.

939 The CRAP sensor was cloned using a combination of Gibson Assembly and the GreenGate

940 approach. First, each GreenGate block was generated by fusing two PCR fragments – vector backbone

941 and the respective CDS. ROP2 promoter fragment was subcloned into pGGA backbone fragment

942 digested by BsaI. To obtain a non-ratiometric CRAP sensor, the following fragments were fused:

943 pGGA-ROP2p. + pGGB-CRIB + pGGC-cpGFP + pGGD-ROP2 + pGGE009(UBI10term.) + pGGF-

944 HYG. The resulting destination vector was sequenced and used as a template for cloning the entire

945 CRAP CDS into a pGGD vector. This vector was then used in the GreenGate reaction to add the

946 mCHERRY ratiometric control: pGGA-ROP2p + pGGB-mCHERRY-ROP2_UBI10term. +

947 pGGC015(mCHERRY)-pGGD-CRAP-pGGE-HSP18.2term + pGGF005(HYG). ROP2-UBI10

948 fragment was amplified from the non-ratiometric CRAP sensor. Point mutagenesis to generate the

949 dominant negative T20N mutation into ROP2 was performed as a single fragment Gibson Assembly

950 with point-mutated compatible cohesive ends. The common building blocks were obtained from[66]

951 (pGGC015, pGGE-009, pGGF005). The pGGZ001 block with exchanged bacterial selection cassette

952 (kanamycin) and pGGE-HSP18term was kindly provided by Dr. Andrea Bleckmann. The CRAP sensor

953 was dipped into Col-0 to obtain *CRAP;WT* and this line was subsequently crossed with *tmk1-1* to obtain

954 *CRAP;tmk1-1*.

955 To obtain seedlings co-expressing TMK1-GFP and PIN2-mCherry, we crossed *TMK1::TMK1-*

956 *GFP;tmk1-1*[67] with *PIN2::PIN2-mCherry;eir-1-4* (kind gift by Christian Luschnig) and imaged the F1

957 generation.

958 All constructs were transformed into the *Agrobacterium tumefaciens* strain pGV3101 by

959 electroporation and further into plants by the floral dip method. The T2 generation was screened for

960 single insertions and homozygous T3 lines were used for experiments.

961 Arabidopsis seeds were surface-sterilized with 70% (v/v) ethanol for 20 min, followed by

962 commercial bleach (2.5% [v/v] sodium hypochlorite) containing 0.05% (v/v) Triton X-100 for 10 min,

963 and finally washed four times with sterile water. Seed stratification was conducted in the dark at 4°C for

964 2 days. Unless indicated otherwise, seedlings were grown at 22°C on ½ MS plates with 1% agar and 1%

965 sucrose, or in soil with 16h light/8h dark cycles, photoperiod at 80 to 100 mE m^{-2} sec^{-1}.

966

967 **Bioinformatics**

968 Phospho-proteomic analyses used data from[17,18,37]. Time-course profiles of auxin-induced

969 phosphorylation were obtained with the AuxPhos tool (https:// weijerslab.shinyapps.io/AuxPhos)[18].

970 Evolutionary rates for PIN1, PIN2, and PIN3 amino acids were calculated as ConSurf[68,69] conservation

971 scores and projected on the respective AlphaFold structures for visualization. Multiple sequence

972 alignment of PIN2 orthologs (Uniprot IDs: Q9LU77, F6GXI9, E5KGD3, A0A251QTL1,

973 A0A1D6P5D8, Q651V6, W1PK04, B5TXD0) or ABP1/ABLs (Uniprot IDs: P33487, P13689, P94040,

974 P94072, Q9LEA7) was done with the MUSCLE tool available from EMBL-EBI[70] and visualized in

975 Jalview[71].

For gene ontology enrichment, the "mock ABP1-TMK1 phospho-proteome" comprised Ensembl protein IDs of proteins concurrently hypo-phosphorylated in both *abp1-TD1* and *tmk1-1* mutants under mock conditions or—in the case of the "auxin-treated ABP1-TMK1 phospho-proteome"—auxin (2 min, 100 nM IAA) conditions[17]. These were submitted to the PANTHER extension of the TAIR database[72,73] and queried for molecular function with all *Arabidopsis thaliana* proteins as a reference list. Annotation version and release date: [GO Ontology database DOI: 10.5281/zenodo.12173881 Released 2024-06-17]. Enrichment calculation was using Fisher's Exact Test with a Bonferroni correction ($p<0.05$). The resultant terms were processed in REVIGO[74] (parameters: medium list size of 0.7, clustering variables: p-value or fold enrichment, removal of obsolete GO terms, whole Uniprot reference set, SimRel semantic similarity) and visualized as treemaps with arbitrary coloring.

Plant pictograms were obtained from the Bioicons project under the MIT license. Attributions: Arabidopsis_thaliana icon by DBCLS https://togotv.dbcls.jp/en/pics.html is licensed under CC-BY 4.0 Unported https://creativecommons.org/licenses/by/4.0/. Zea_mays_cartoon icon by Daniel Carvalho https://figshare.com/authors/Plant_Illustrations/3773596 is licensed under CC-BY 4.0 Unported https://creativecommons.org/licenses/by/4.0/. DicotSeedling icon https://github.com/ginavong by Gina-Vong is licensed under CC0 https://creativecommons.org/publicdomain/zero/1.0/. The conifer branch pictogram was obtained from the free-for-use Pixabay repository.

The crystal structure of maize ABP1 (PDB ID: 1LRH) was superimposed with the AlphaFold2 structure of ABL3 (Uniprot ID: Q9LEA7) with the "super" command in Pymol. Tissue-specific expression profiles for *ABL1*, *ABL2*, *ABP1*, *ABL3*, *TMK1*, *TMK2*, *TMK3* and *TMK4* were compiled using a database of ~20,000 public Arabidopsis RNAseq experiments[57].

RT-qPCR

Total RNA was prepared from max. 100 mg of roots of 4-day-old seedlings according to the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from 1µg of total mRNA using the QuantiNova Reverse Transcription Kit (Qiagen). Mutant expression analyses used 3-4 biological and 3 technical replicates pipetted into a 384-well plate using an automated JANUS Workstation (PerkinElmer). According to the manufacturer's instructions, 5 µL reaction volume contained 2.5 µL Luna® Universal qPCR mastermix (NEB). RT-qPCR analyses were performed using the Real-time PCR Roche Lightcycler 480 and control PP2AA3 (At1G13320) primers were used as in[75]. For each of the evaluated genes, three different primer pairs were tested (Supplemental Table 3); except the *PIN2-GFP* phospho-line transgene, which was quantified using a single pair of primers without testing multiple sets.

Fluorescence lifetime imaging

FRET-FLIM experiments were performed in protoplasts isolated from Arabidopsis root cell suspension as described previously[76]. 10 µg of plasmid DNA (*TMK1-GFP, TMK1$^{\Delta KD}$-GFP, PIN2-mCherry, FLS2-*

1012   *GFP*) were used for protoplast transfection, followed by incubation in a sterile 24-well microtiter plate

1013   overnight in the dark at room temperature. FRET-FLIM experiments were performed using a TriM

1014   Scope II inverted 2-photon microscope equipped with a FLIM X16 TCSPC Detector for time-correlated

1015   single photon counting (LaVision BioTec). Fluorescence lifetime image stacks (150 slices, with 0,082

1016   ns time interval) were acquired. Image analyses were done in Fiji by performing a threshold mask from

1017   the sum projection of each stack and by averaging all the pixels at each time point of the stack. To yield

1018   an exponential decay with offset, the intensity at time point 0 was normalized.

1019

1020   **GUS staining**

1021   4-day-old seedlings were stained in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% X-GlcA

1022   sodium salt, 1 mM $K_3[Fe(CN)_6]$, 1 mM $K_4[Fe(CN)_6]$ and 0.05% Triton X-100 for 20 min (*TMK1::GUS*),

1023   2 h (*TMK4::GUS*, *TMK2::GUS*, *ABL3::GUS*) or 6 h (*TMK3::GUS*) at 37°C. Further, samples were

1024   incubated overnight in 80% (v/v) ethanol at room temperature. Tissue clearing was conducted as

1025   previously described[77]. DIC microscopy for analysis of the GUS staining assay was performed using an

1026   Olympus BX53 microscope equipped with 10x and 20x air objectives and a DP26 CCD camera.

1027

1028   **Root gravitropic assays**

1029   For sensitive phenotyping of the gravitropic response, unless indicated otherwise, seeds were

1030   germinated on sucrose-free ½ MS plates with 1% agar[26,78,79]. 5-day-old seedlings were transferred to

1031   fresh plates and incubated in a vertical position for 40 minutes for recovery. After rotation by 90°, the

1032   roots were imaged every 30 min on a vertical flatbed scanner (Epson Perfection V370 Photo). Image

1033   time series were stabilized using the StackReg Fiji plugin. Root curvature was analyzed with the Manual

1034   Tracking Fiji plugin and angles were calculated from root tip positions over time in Microsoft Excel.

1035

1036   **Imaging of transgenic lines**

1037   Confocal microscopy was performed on a vertical Zeiss LSM800 microscope[80] equipped with a 20X

1038   Plan Apochromat air objective (NA = 0.8). GFP- and mCherry-tagged proteins [PIN2-GFP, TMK1-

1039   GFP, CRAP (mCherry/GFP), DR5rev::GFP] were excited at 488 and 561 nm, respectively, with

1040   emission collected in the following ranges: 490-576 nm or 560-700 nm, respectively.

1041   PIN2-GFP phospho-lines were imaged by taking 12 Z-sections spanning the whole volume of each

1042   root. These were processed through "maximum intensity" projection in Fiji and total GFP signal was

1043   quantified across epidermal and cortical regions.

1044   For imaging of *PIN2::PIN2-GFP* and *DR5rev::GFP* asymmetric distribution, 5-day-old seedlings

1045   were placed in a 1-well chambered coverglass (VWR, Kammerdeckglaser, Lab-Tek, Nunc, catalog

1046   number 734-2056) with a block of solid ½ MS medium[80], optionally supplemented with mock or 10 μM

1047   NPA according to the experiment. For recovery, the chamber was incubated vertically in darkness for 2

1048   h before imaging. Ten Z-sections spaced 1 μm for *PIN2::PIN2-GFP* and 4.5 μm for *DR5rev::GFP* were

1049   collected in the median root section before and after 90° rotation at the indicated time points. The "sum

1050   slices" intensity projection in Fiji was then applied. Marking epidermal and cortical regions together,

1051   the mean grey value was quantified as per[29].

1052   The CRAP sensor was validated by auxin treatments in a microfluidic vRootchip setup described

1053   previously[13,28]. For CRAP sensor imaging during gravistimulation, 5-day-old seedlings were placed

1054   vertically in a 1-well chambered coverglass with a block of solid 1/2 MS agar. The chamber was fitted

1055   inside a rotational stage. Seedlings were gravistimulated by turning the stage by 90°, achieving

1056   horizontal root position, and subsequently flipping the stage by 180°. Each root was imaged every 8.94

1057   s for 15 min three times (with 180° flips in between) as technical replicates. GFP and mCherry signals

1058   were recorded simultaneously as a single track. Mean grey values at the lower and upper root sides were

1059   quantified, averaging the three technical replicates for each single root. Next, the 561/488 nm ratio

1060   indicative of ROP activity was calculated for the upper and lower root sides. Finally, to compare ROP

1061   activity between the two sides, the lower root side ratio was divided by the upper root side ratio and

1062   plotted over time. For visual representation, a root with a strong CRAP asymmetry was used. The last

1063   10 time points of the 15-min imaging time course were averaged, and the pixel ratios reflecting CRAP

1064   activation were calculated as [mCherry - mCherry background] / [GFP - GFP background]. For plotting,

1065   the alpha value was derived from intensity and the Gaussian difference of the mCherry reference channel

1066   to suppress artifacts from numeric instability in low-intensity regions.

1067

1068 **Microsomal protein extraction**

1069   4-day-old-seedling roots were harvested, ground to powder in liquid nitrogen, and vortexed vigorously

1070   in extraction buffer 1 [50 mM Tris–HCl pH 7.5, 150 mM NaCl, Complete EDTA-free Protease Inhibitor

1071   cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche)] in 1/10 (w/v) ratio. The resulting

1072   homogenate was centrifuged at 20000 g for 30 min at 4°C. The pellet was resuspended in extraction

1073   buffer 2 [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1% TritonX100, Complete

1074   EDTA-free Protease Inhibitor cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche)], and

1075   centrifuged at 12000 g for 20 min at 4°C. Bradford assay was used to quantify protein in the supernatant.

1076   Samples were separated by SDS-PAGE (4-15% Mini-PROTEAN®TGX™ Precast Protein Gels (Bio-

1077   RAD)), transferred to a PVDF (polyvinylidene difluoride) membrane, and immuno-blotted using the

1078   following primary antibodies: affinity-purified TMK1 (1:1000)[58], AHA2 (1:1000)[81], PIN2 (1:1000)[21]

1079   antibodies and anti-ROP6 (1:1000, Abiocode). The secondary antibody was anti-Rabbit-HRP (1:5000,

1080   GE Healthcare). Detection was done using the SuperSignal West Femto Maximum Sensitivity Substrate

1081   detection kit (ThermoFisher Scientific) and the Amersham 600RGB imager (GE Healthcare).

1082

1083 **CETSA**

1084   For CETSA from protoplasts, 10 µg of plasmid DNA (*35S::gGLP9-HA*) was used for protoplast

1085   transfection, followed by incubation in a sterile 24-well microtiter plate overnight in the dark at room

1086   temperature. The incubation buffer was exchanged for protein extraction buffer (50 mM Tris-HCl, pH

1087   7.6, 150 mM NaCl, 10 % glycerol, 5 mM DTT, 1 mM PMSF, 0.5 % NP-40, complete Roche protease

1088   inhibitors) and protoplasts were lysed by 10 vigorous ice-vortexing(5s)-ice cycles, 30 min rotation at

1089   4°C, and again 10 vigorous ice-vortexing(5s)-ice cycles. The lysate was centrifuged twice at maximum

1090   speed for 10 minutes (4°C) in a table-top centrifuge. The final supernatant represented the protein

1091   extract, which was first sampled for Western blotting and then split into two halves supplemented with

1092   either 100 µM IAA or mock treatment. IAA- or mock-treated extracts were incubated for 1 h on ice with

1093   occasional mixing. Next, the extracts were aliquoted for a 3 min incubation at temperatures between 42

1094   and 47°C in a PCR machine (Bio-Rad) and returned to ice immediately. Finally, the samples were spun

1095   down in a tabletop centrifuge (12,000 rpm, 6 min, 4°C) and the supernatants were carefully transferred

1096   to new tubes for Western blotting (Anti-HA-HRP as described above).

1097   CETSA from 5-day-old *35S::ABL3-6xHIS-3xFLAG* Arabidopsis seedlings used the same buffer

1098   and protocol with the only difference being the protein extraction procedure and the use of a different

1099   antibody for Western blotting (anti-FLAG®M2-Peroxidase (HRP), Sigma). Protein was extracted by

1100   adding ice-cold buffer to liquid-nitrogen-ground tissue, followed by centrifugation and supernatant

1101   collection as described for protoplast proteins.

1102

1103   **Western blot analysis of phosphorylated proteins**

1104   To analyze the phosphorylation status of TMK1 *in planta*, after corresponding treatment, 4-day-old

1105   *tmk1-1* roots expressing *TMK1::TMK1-FLAG* were ground to powder in liquid nitrogen and

1106   homogenized in ice-cold sucrose buffer [20 mM Tris–HCl pH 8, 0.33 M sucrose, Complete EDTA-free

1107   Protease Inhibitor cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche)], followed by a

1108   centrifugation step at 5000 g for 10 min at 4°C. To obtain the membrane protein fraction, the supernatant

1109   was centrifuged at 20000 g for 30 min at 4°C and the resulting pellet was solubilized with lysis buffer

1110   [20 mM Tris–HCl pH 8, 150 mM NaCl, 1% TritonX100, Complete EDTA-free Protease Inhibitor

1111   cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche)] and centrifuged at 20000 g for 10

1112   min at 4°C. The corresponding supernatant was used for immunoprecipitation assay with anti-FLAG

1113   microbeads according to the manufacturer´s instructions (µMACS Epitope Tag Protein Isolation Kit

1114   (MACS Miltenyi Biotec)). Samples were separated by SDS-PAGE (4-15% Mini-PROTEAN®TGX™

1115   Precast Protein Gels (Bio-RAD)) and transferred to a PVDF membrane, which was blotted with anti-

1116   FLAG®M2-Peroxidase (HRP) (Sigma) to detect total immunoprecipitated protein. Next, the membrane

1117   was stripped in 7 mL of mild stripping buffer (Abcam, 1 L: 15 g glycine, 1 g SDS, 10 mL Tween-20,

1118   pH 2.2) for 5 min, and the incubation was repeated with fresh buffer. The membrane was washed (2x

1119   10 min in PBS, 2x 5 min in TBST) and re-blocked. The Phos-tag BTL-111 probe was then used

1120 according to the manufacturer's instructions (www.wako-chem.co.jp) to detect phosphorylated protein

1121 levels.

1122

1123 **Co-immunoprecipitation assays**

1124 4-day-old *tmk1-1* seedlings expressing TMK1::TMK1-FLAG were treated with 5, 20 nM IAA or DMSO

1125 control for 30 minutes. Roots were harvested, ground to powder in liquid nitrogen, and subjected to

1126 protein microsomal fraction extraction. Solubilized proteins from microsomal fraction were

1127 immunoprecipitated using super-paramagnetic μMACS beads coupled to monoclonal anti-FLAG

1128 antibody according to the manufacturer's instructions (Miltenyi Biotec). WT Col-0 extract was used as

1129 a control of the unspecific binding of endogenous PIN2. Proteins immunoprecipitated with anti-FLAG

1130 antibody were separated by SDS-PAGE (4-15% Mini-PROTEAN®TGX™ Precast Protein Gels (Bio-

1131 RAD)), transferred to a PVDF membrane and analyzed by immunoblot with anti-FLAG®M2-

1132 Peroxidase (HRP) (Sigma) antibody to detect TMK1-FLAG and with anti-PIN2 antibody to detect co-

1133 immunoprecipitated endogenous PIN2.

1134 Tobacco leaves were infiltrated or co-infiltrated with overnight LB suspensions of *Agrobacterium*

1135 *tumefaciens* GV3101 carrying the desired expression plasmids (*35S::ABL3-HA*, *35S::ABL3-mCherry*,

1136 *35S::TMK1-HA*, *35S::TMK1-mCherry*). Including an overnight dark incubation, the infiltrated plants

1137 were grown for 36 hours, and leaves were subsequently harvested in liquid nitrogen. Frozen leaves were

1138 ground on ice in ice-cold extraction buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 % glycerol, 5

1139 mM DTT, 1 mM PMSF, 0.5 % NP-40, complete Roche protease inhibitors). The extracts were

1140 centrifuged in a tabletop centrifuge at top speed for 15 min and the supernatant was harvested, followed

1141 by a repetition of the same. The protein extracts were incubated with ChromoTek RFP-Trap Magnetic

1142 Agarose (rtma-20, Proteintech) for 1 hour at 4 °C (rotating), washed 5 times with the extraction buffer,

1143 and analyzed by SDS-PAGE. Blotting was with an Abcam anti-mCherry antibody (ab167453) and anti-

1144 HA-HRP (described above).

1145

1146 **Active ROP assay on non-overexpressing plants**

1147 5-day-old seedling roots were harvested and incubated in ½ MS liquid medium with 100 nM IAA or

1148 mock treatment for 10 minutes and frozen in liquid nitrogen. Total protein was extracted in extraction

1149 buffer (25 mM HEPES pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1 mM PMSF, 5 mM Na3VO4, 5 mM

1150 NaF, 1 mM TCEP, cOmplete Protease Inhibitor Cocktail, Roche) in 2 steps. For every 100 mg of ground

1151 tissue, 400 μl of extraction buffer was added, then incubated with another 400 μl of extraction buffer

1152 containing 5% Triton X-100 for one hour. Active ROP proteins were pulled down using His-MBP-CRIB

1153 (BL21 *E. coli* total cell extract) conjugated to HisPurTM Ni-NTA Magnetic Beads (Thermo Fisher) by

1154 incubation of 200 μl total protein extract with 25 μl (initial volume) of beads for 2 hours at 4°C. The

1155 beads were washed with Washing buffer (25 mM HEPES pH 7.4, 300 mM KCl, 10 mM MgCl2, 12.5

1156   mM imidazole) 3 times and boiled at 95°C with 30 µl SDS loading dye (BioRad). Protein samples were

1157   separated by 12% SDS-PAGE and analyzed by immune-blotting with an anti-ROP2 antibody (1:10,000,

1158   Abiocode). Input samples were blotted for total ROP2 content.

1159

1160   **Recombinant protein expression and purification from *E. coli.***

1161   The 6xHis-PIN2 HL recombinant protein was expressed using the 6xHis-PIN2 HL vector[82] in *E. coli*

1162   BL21 (DE3) strain upon induction by 0.5 mM IPTG (Isopropyl β-D-1-Thiogalactopyranoside) at 16°C

1163   for 12 h. Cells were harvested by centrifugation at 4000 g for 15 min and washed with water. They were

1164   then lysed by sonication in lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM

1165   DTT, and 1% Triton X-100). The lysed solution was centrifuged at 12000 g for 15 min. The supernatant

1166   was then purified using Ni-NTA His affinity agarose (Thermo Scientific) according to the

1167   manufacturer's instructions. 6xHis-PIN2 HL protein was eluted from the beads in elution buffer (50 mM

1168   Tris-HCl pH7.4, 150 mM NaCl, and 250 mM imidazole). Eluted protein samples were checked by SDS-

1169   PAGE and Coomassie brilliant blue staining (Bio-Safe™ Coomassie Stain, Bio-Rad). The protein

1170   concentration was determined with the Bradford method (Quick Start™ Bradford Reagent, Bio-Rad).

1171

1172   **TMK1-HA immunoprecipitation**

1173   To immunoprecipitate HA-tagged TMK1 protein, roots of 7-day-old *pUBQ10::TMK1-3xHA* and

1174   *DEX::TMK1^{K616R}-HA* (previously induced for 48 h with 30 µM dexamethasone) seedlings were frozen

1175   and ground in liquid nitrogen, and subsequently homogenized in protein extraction buffer [PEB: 50 mM

1176   Tris-HCl pH7.5, 200 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 1 mM $MnCl_2$, Complete (Roche)

1177   protease cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche)), followed by a 20 min

1178   centrifugation step at 14000 g, 4°C. In a fresh Eppendorf tube, 50 µL anti-HA agarose beads were added

1179   (Anti-HA Affinity Matrix, SIGMA; pre-washed with 200 µL PEB) to the supernatant. After 4 h of

1180   rotating at 4°C, the samples were spun down again at 2000 g, 4°C, and the supernatant was discarded.

1181   The agarose beads were washed twice with 200 µL PEB, and finally re-suspended into 50 µL PEB for

1182   further work.

1183

1184   *In vitro* **kinase assay**

1185   The *in vitro* kinase assay with [γ-^{32}P]-ATP was conducted as reported[82] with minor modifications.

1186   Immunoprecipitated TMK1-HA and TMK1^{K616R}-HA (5 µL) from 7-day-old Arabidopsis seedlings,

1187   together with the recombinant 6xHis-PIN2 HL (10 µL) from *E. coli*, were added to the kinase reaction

1188   buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM NaCl, 2.5 mM cold ATP (adenosine 5'-

1189   triphosphate), and 1 mM DTT) in the presence of 5 µCi [γ-^{32}P]-ATP (NEG502A001MC; Perkin-Elmer).

1190   Reactions were incubated at 25°C for 90 min and terminated by adding 10 µL 5×SDS loading buffer.

1191   20 µL reaction samples were then separated with 10% SDS-PAGE gel, developed with a phosphor-plate

1192   overnight. Eventually, the phosphor plate was imaged with a Fujifilm FLA 3000 plus DAGE system.

1193

1194   **Statistical analysis**

1195   Data processing and visualization was done in R version 4.0.4.

1196

1197   **Accession Numbers**

1198   Gene sequence data from this article can be found in the Arabidopsis Genome Initiative databases under

1199   the following accession numbers: AT4G02980 (*ABP1*), AT1G66150 (*TMK1*), AT1G24650 (*TMK2*),

1200   AT2G01820 (*TMK3*), AT3G23750 (*TMK4)*, AT5G57090 (*PIN2*), AT1G20090 (*ROP2*), AT4G35020

1201   for (*ROP6*), AT4G30190 (*AHA2*), AT5G46330 (*FLS2*), AT1G72610 (*ABL1*), AT5G20630 (*ABL2*),

1202   AT4G14630 (*ABL3*).

Figure 1

A PIN2

S179
S183
T233
S439
S393

B

	S179	S183	T233	S393	S439
ABP1-TMK1 dependency					
Induction by IAA					
Conservation					

C

- PIN2::PIN2^5-MIMIC-GFP;eir1-4 (1) — WT
- PIN2::PIN2^5-DEAD-GFP;eir1-4 (1) — eir1-4
- PIN2::PIN2^WT-GFP;eir1-4 (1)

Normalized root angle (°)

Time since gravistimulation (h)

D

PIN2::PIN2^WT-GFP; eir1-4 (1)   PIN2::PIN2^5-MIMIC-GFP; eir1-4 (1)   PIN2::PIN2^5-DEAD-GFP; eir1-4 (1)

100 μm   100 μm   100 μm

E

Mean grey value (AU)

PIN2::PIN2^()-GFP;eir1-4

WT (1) 5-MIMIC (1) 5-MIMIC (2) 5-DEAD (1) 5-DEAD (2)

Figure 2

A

Root angle (°) vs Time since gravistimulation (h)

- WT
- *abl3-1*
- *abp1-C1*
- *abp1-C1;abl3-1*
- *tmk1-1*

B

WT | *abl3-1*
abp1-C1 | *abp1-C1;abl3-1*
tmk1-1

C

■ *Arabidopsis thaliana* ABL3 AlphaFold2 structure
■ *Zea mays* 1-NAA-bound ABP1 crystal structure

1-NAA
E63
Zn
H59
H57
H106

D

ABL3-HA

42°C | 43°C | 44°C | 45°C | 46°C | 47°C
IAA: 100 μM − + | − + | − + | − + | − + | − +

HA
34
(kDa)
START

E

ABL3-mCherry + − +
TMK1-HA − + + (kDa)

INPUT
mCherry — 55
HA — 130

IP
mCherry — 55
HA — 130

Figure 3

Figure 4

A TOP / BOTTOM / WT

B ROP activation (Lower/Upper) vs Time since gravistimulation (min) — WT, *tmk1-1*

C 4 h — g ↓ — WT / *tmk1-1*

D PIN2::PIN2-GFP / WT — Upper, Lower; PIN2::PIN2-GFP / *tmk1-1* — Upper, Lower; PIN2-GFP signal (T/T0) vs Time since gravistimulation (h)

E 3 h — g ↓ — WT / *tmk1-1*

F DR5rev::GFP ratio (Lower/Upper) vs Time since gravistimulation (h) — WT, *tmk1-1*

Figure 5

A

TMK1::TMK1-FLAG/

WT | *tmk1-1*

DMSO | DMSO | 30 | Time (min), 20 nM IAA

INPUT:
α-PIN2 — -70 kDa ◄ PIN2

α-AHA2 — -100 kDa

IP: α-Flag — -130 kDa ◄ TMK1

α-PIN2 — -70 kDa ◄ PIN2

B

GFP | mCherry | Merge | Lifetime

TMK1-GFP

TMK1-GFP
PIN2-mCherry

5ns / 0ns

Lifetime

TMK1 / TMK1 PIN2 | ΔKD / ΔKD PIN2 | FLS2 / FLS2 PIN2

**** ** **** * **

C

In vitro kinase assay

WT | K616R | WT | K616R | — TMK1-3xHA
− | − | + | + | + — 6xHis-PIN2 HL

^{32}P — -130 kDa
-55 kDa
◄ ^{32}P-PIN2 HL
-35 kDa

CBB — -55 kDa
◄ PIN2 HL
-35 kDa

α-HA — -130 kDa
◄ TMK1

Figure 6