



Characterization of auxin transporter PIN6 plasma membrane targeting reveals a function for PIN6 in plant bolting

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1 **Characterization of auxin transporter PIN6 plasma membrane targeting reveals**
 2 **a function for PIN6 in plant bolting**

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57

58 **Summary**

- 59 • Auxin gradients are sustained by series of influx and efflux carriers whose
60 subcellular localization is sensitive to both exogenous and endogenous factors.
61 Recently the localization of the *Arabidopsis thaliana* auxin efflux carrier PIN-
62 FORMED (PIN) 6 was reported to be tissue specific and regulated through
63 unknown mechanisms.
- 64 • Here, we used genetic, molecular and pharmacological approaches to
65 characterize the molecular mechanism(s) controlling the subcellular localization of
66 PIN6.
- 67 • PIN6 localizes to endomembrane domains in tissues with low *PIN6* expression
68 levels such as roots, but localizes at the plasma membrane (PM) in tissues with
69 increased *PIN6* expression such as the inflorescence stem and nectary glands.
70 We provide evidence that this dual localization is controlled by PIN6
71 phosphorylation and demonstrate that PIN6 is phosphorylated by mitogen-
72 activated protein kinases (MAPKs) MPK4 and MPK6. The analysis of transgenic
73 plants expressing PIN6 at PM or in endomembrane domains reveals that PIN6
74 subcellular localization is critical for *Arabidopsis* inflorescence stem elongation
75 post-flowering (bolting). In line with a role for PIN6 in plant bolting, inflorescence
76 stems elongate faster in *pin6* mutant plants than in wild-type plants.
- 77 • We propose that PIN6 subcellular localization is under the control of
78 developmental signals acting on tissue specific determinants controlling PIN6-
79 expression levels and PIN6 phosphorylation.

80

81 **Key words:** *Arabidopsis thaliana*, auxin, bolting, inflorescence, stem

82

83

84 **Introduction**

85 Plant developmental plasticity involves the activity of series of plant hormones which
86 modulate stem cell fate activity during plant development (Wolters & Jurgens, 2009;
87 Rodriguez *et al.*, 2010). The plant hormone auxin plays a crucial role in this process
88 as it coordinates the patterning of the plant body plan, including the establishment of
89 apical–basal (Friml *et al.*, 2003), radial (Bjorklund *et al.*, 2007; Suer *et al.*, 2011;
90 Ameres & Zamore, 2013), and proximal–distal axes (Sabatini *et al.*, 1999; Cai *et al.*,
91 2014) and the determination of cell fate by positional information (Ditengou *et al.*,
92 2008; Finet & Jaillais, 2012). Auxin is polarly transported by auxin influx and efflux
93 carriers [(AUXIN RESISTANT 1/ Like AUX1 (AUX/LAX)) family (Ugaratechea-Chirino
94 *et al.*, 2010), ABCB/multi-drug resistance/P-glycoprotein (ABCB/MDR/PGP)(Paponov
95 *et al.*, 2005; Geisler & Murphy, 2006), and PIN-FORMED (PIN) proteins (Paponov *et al.*,
96 2005)]. These proteins have been suggested to coordinate the patterning of the
97 plant body plan (Sabatini *et al.*, 1999; Friml *et al.*, 2003; Bjorklund *et al.*, 2007;
98 Ditengou *et al.*, 2008; Cai *et al.*, 2014).

99 PAT relies on the proper subcellular localization of PIN proteins. PIN1, PIN2, PIN3,
100 PIN4 and PIN7 are targeted to the plasma membrane (PM) and they cycle between
101 the PM and endosomal compartments (Geldner *et al.*, 2001). PIN8 localizes to the
102 endoplasmic reticulum (ER) membranes (Mravec *et al.*, 2009; Dal Bosco *et al.*, 2012;
103 Ding *et al.*, 2012; Simon *et al.*, 2016), while PIN5 and PIN6 localize to both the ER
104 and PM (Ganguly *et al.*, 2014; Simon *et al.*, 2016). PIN5 was proposed to mediate
105 auxin flow from the ER lumen to the cytosol (Mravec *et al.*, 2009), while PIN8 and
106 PIN6 were proposed to export auxin in the opposite direction (Ganguly *et al.*, 2010;
107 Dal Bosco *et al.*, 2012; Ding *et al.*, 2012). Together these studies suggest that PM-
108 targeting of PIN-proteins probably depends on some tissue and/or cell specific
109 determinants. Although it is unclear which mechanisms regulate PIN5 and PIN6 dual
110 localization, it can be envisaged that PIN5 and PIN6 may be post-translationally
111 modified prior their ultimate subcellular targeting, suggesting that these proteins are
112 no longer recognized by the sorting machinery responsible for their retention in
113 endomembrane domains. Phosphorylation is the most common post-translational
114 modification involved in signal transduction. Three protein kinase families have been
115 shown to phosphorylate PIN proteins: (i) D6 PROTEIN KINASE (D6PK) regulates
116 auxin transport by phosphorylation of PIN1, PIN2, PIN3, PIN4 and PIN7 (Shen *et al.*,
117 2015); (ii) PINOID (PID) kinase and SERINE/THREONINE PROTEIN

118 PHOSPHATASE 2A (PP2A) antagonistically affect phosphorylation of the PIN
119 hydrophilic loop, which is important for polar targeting of PM-located PIN proteins
120 (Michniewicz *et al.*, 2007); and (iii) the recently characterized mitogen-activated
121 protein kinase (MAPK) pathway, which consists of the MKK7-MPK6 complex that
122 phosphorylates PIN1 serine 337 (S337) and impacts the polar localization of PIN1,
123 thereby modifying shoot branching (Jia *et al.*, 2016).

124 In the present study, we aimed at characterizing the molecular mechanism(s)
125 controlling PIN6 subcellular localization. Our study reveals that both *PIN6* gene
126 expression level and PIN6 phosphorylation modulate PIN6 subcellular localization in
127 Arabidopsis. Functional analysis of two phosphorylation sites, T392 and T393, which
128 were reported to be phosphorylated *in vivo* in Arabidopsis suspension cells treated
129 with bacterial elicitor flagellin (Benschop *et al.*, 2007), reveals that these sites play a
130 key role in PIN6-ER exit and regulate root and root hairs growth, as well as
131 inflorescence stem development. We demonstrate that PIN6 is phosphorylated by
132 both MPK4 and MPK6 *in vitro*, although T393 is not phosphorylated by these
133 kinases. Finally, the analysis of transgenic plants expressing PIN6 predominantly at
134 PM or in ER reveals a critical role for PIN6 subcellular localization on inflorescence
135 stem elongation post flowering. Hence, over-expressing a PIN6 mutant protein
136 (T392V-T393V) that is retained in the ER-PIN6 does not affect inflorescence stem
137 growth, while over-expressing native PIN6 or its PM-localized phosphomimetic
138 mutant (T392E-T393E) drastically repressed plant growth and delayed bolting. In line
139 with a role for PIN6 in plant bolting, the inflorescence stems elongated faster in *pin6*
140 mutant plants than in wild-type plants. We conclude that PIN6 may act as a gate
141 keeper ensuring that Arabidopsis plants efficiently develop the inflorescence stem at
142 the appropriate, possibly environmentally determined time.

143

144 **Materials and Methods**

145 **Materials and growth conditions**

146 *Arabidopsis thaliana* (L.) Heynh. Columbia (Col-0) and *Landsberg erecta* (Ler)
147 ecotypes were used. All T-DNA insertion lines as well as transgenic lines are
148 described in Methods S1. Seeds were surface sterilized and sown on solid
149 Arabidopsis medium (AM) (2.3 g/L MS salts, 1% sucrose, 1.6% agar-agar, 5 mM 2-
150 (N-morpholino)ethanesulfonic acid (MES) sodium salt (Sigma, Steinheim, Germany),

151 pH 6.0, adjusted with HCl). After vernalization for 2 days at 4°C, seeds were
152 germinated under a long-day period (16 h light, 8 h darkness) at 22°C. The same
153 growth conditions were applied in a phytochamber when plants were grown in soil.

154

155 **Pharmacological treatments**

156 For vesicular trafficking experiments, BFA (Invitrogen B7450) was used as previously
157 described (Geldner *et al.*, 2001) with slight modifications: plants were incubated in 25
158 μ M BFA (60 min). β -Estradiol was dissolved in 100% ethanol and added to AM
159 without exceeding an ethanol concentration of 0.1%.

160 **Free IAA level determination**

161 Approximately 15 mg (fresh weight) of 5 mm root sections was homogenized and
162 extracted for 16 h in methanol (Methods S2)

163

164 **Generation of the PIN6 antibody**

165 *AtPIN6* cDNA (nucleotides 177-396) corresponding to the antigen peptide was
166 inserted into the pET-28a(+) expression vector (Novagen). After expression in the
167 *Escherichia coli* Rosetta strain (Novagen), the His6-tagged recombinant protein was
168 affinity purified according to the Qiagen manual (Qiagen) and confirmed by SDS-
169 polyacrylamide gel electrophoresis (PAGE). The antigen peptide included in the
170 PAGE slice was used to immunize a rabbit (Eurogentec). The polyclonal antiserum
171 was affinity purified against the recombinant AtPIN6 peptide as previously described
172 (Hasumura *et al.*, 2005).

173

174 **Detection of PIN6 by western blot**

175 Proteins were extracted from 10, 3-week-old flower buds using extraction buffer
176 containing 50 mM Tris-HCl, 10 mM EDTA, 2 mM EGTA, 0.1% SDS, 1 mM DTT, 10
177 μ M protease inhibitor cocktail, 0.01 mM MG132 and 0.1 mM PMSF. After
178 centrifugation at 10,000 rpm at 4°C for 15 min, the supernatant containing total
179 protein was collected, and the protein concentration was measured using the Thermo
180 Scientific Pierce Micro BCA Assay according to the manufacturer's instructions. After
181 protein denaturation at 42°C in 5x Laemmli buffer (1:4), 7.4 mg/ml protein samples
182 were separated on a 10% SDS-PAGE gel and then transferred to a nitrocellulose
183 membrane. Blots were probed with a rabbit anti-PIN6 polyclonal antibody (1:1200),
184 and PIN6 signal was detected with an HRP-conjugated anti-rabbit antibody (1:5000)

185 (Agrisera). Plasma membrane H⁺-ATPase was used as a control for equal loading.
186 Signal detection was performed with a Fujifilm ImageQuantTM LAS 4000 CCD
187 camera using Super Signal West Pico Chemiluminescent substrate.

188

189 **Immunolocalization**

190 Plants were fixed with 4% paraformaldehyde in PBS (pH 7.3) and used for whole-
191 mount *in situ* immunolocalization as previously described (Friml *et al.*, 2004).

192

193 **Whole mount *in situ* hybridization**

194 *In situ* detection of *PIN6* mRNA in Arabidopsis seedling root tips was performed as
195 previously described (Riegler *et al.*, 2008; Begheldo *et al.*, 2013).

196

197 **Quantitative RNA analysis**

198 *PIN6* expression was assessed using semi-quantitative RT-PCR (*PIN6* mRNA in 6-
199 day-old PIN6ox plants) or qRT-PCR for *PIN6* expression throughout the plant
200 lifespan (Methods S4).

201

202 **Microscopy and image post processing**

203 Histological detection of β -glucuronidase (GUS) activity was performed as previously
204 described (Scarpella *et al.*, 2004). Fluorescent proteins were analyzed as described
205 in Methods S4. All images were assembled using Microsoft PowerPoint 2013.

206

207 **Kinase assay**

208 To test whether *PIN6* is phosphorylated by MPK4 or MPK6, the hydrophilic loop (HL:
209 residues 156-430) of *PIN6* cDNA was amplified and cloned into pGEM-T Easy vector
210 (Promega), and the sequence was verified. A non-phosphorylatable mutant HL
211 version (T226A, T242A, S286A, T304A, T320A, S326A, S337A, and T393A;
212 positions according to the full-length *PIN6* protein) was synthesized by GenScript.
213 The variant where T393 of the putative MAPK phosphosite is wild type was
214 generated by inserting the sequence corresponding to A156-D352 from the mutant
215 clone (including T226A, T242A, S286A, T304A, T320A, S326A and S337A) into the
216 WT construct. For *in vitro* transcription/translation, the HL sequence variants were
217 subcloned into the pEU3-NII-GLICNot vector with ligation-independent cloning
218 (Bardoczny *et al.*, 2008). *In vitro* mRNA synthesis was carried out using a

219 TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific) according to the
220 manufacturer's instructions. Cell-free translation was completed using a
221 WEPRO7240H Expression Kit (Cell Free Sciences, Japan). To activate His-tagged
222 MPK4 and MPK6 when included in the phosphorylation assay solution, mRNA
223 encoding constitutively active myc:MKK1 and myc:MKK4, respectively, were also
224 added to the translation mixture as described (Nagy & Meszaros, 2014).

225 *In vitro*-translated His6-AtMPK4 and His6-AtMPK6 proteins were purified by affinity
226 chromatography using TALON Magnetic Beads (Clontech), while *in vitro*-translated
227 wild-type and mutant GST-PIN6loop variants were purified by affinity chromatography
228 using Glutathione Magnetic Beads (Thermo Scientific)(Nagy & Meszaros, 2014). For
229 kinase assays, 300 and 100 ng of *in vitro*-translated, affinity-purified substrate and
230 kinase were used, respectively. As an activity control, 10 µg myelin basic protein
231 (MBP) was used as a generic MAPK substrate (not shown). The assay was carried
232 out in 20 mM HEPES, pH 7.5, 100 µM ATP, 1 mM DTT, 15 mM MgCl₂, 5 mM EGTA
233 and 5 µCi [γ -³²P]ATP with bead-bound GST-PIN6loop variants as substrates for 30
234 min at room temperature and then stopped by the addition of Laemmli SDS buffer.
235 Samples were fractionated by SDS-PAGE. The gel was fixed, stained with
236 Coomassie blue, dried and analyzed by autoradiography.

237

238 **Results**

239 ***PIN6* expression level is variable and increases highly during plant bolting**

240 *PIN6* was reported to be located at the plasma membrane and in the ER in different
241 cells and organs (Simon *et al.*, 2016), suggesting this dual localization may depend
242 on some tissue and/or cell specific determinants. To gain insight on regulation of
243 *PIN6*-localization, we first performed a quantitative RT-PCR analysis to ascertain
244 *PIN6* expression throughout the Arabidopsis lifespan. As previously shown by
245 qualitative *pPIN6:GUS* analysis (Nisar *et al.*, 2014), *PIN6* is expressed during both
246 vegetative and reproductive plant growth phases (Fig. 1a) with highest *PIN6* mRNA
247 levels in developing inflorescence stems (Fig. 1a). This observation was confirmed by
248 analysis of *pPIN6::GUS* plants where *PIN6* expression was present in elongating
249 inflorescence stems (Fig. Fig. S1b). These results are in agreement with publicly
250 available data sets (Ismagul *et al.*, 2014; Ivanova *et al.*, 2014) and indicate that *PIN6*
251 expression is under the control of developmental signals during bolting. Cross-
252 sections from the inflorescence stem of *pPIN6:GUS* plants showed *PIN6* expression

253 in xylem parenchyma cells (Xpc), the fascicular cambium (Fc) and the interfascicular
254 fiber tissues (IF) (Fig. S1f). Altogether, these observations show that *PIN6* is
255 ubiquitously expressed during Arabidopsis life span. The fact that *PIN6* expression is
256 increasing during plant bolting and stays relatively high in the stem, suggests a role
257 of *PIN6* in processes controlling both longitudinal and radial differentiation,
258 particularly during bolting.

259

260 ***PIN6* is localized at the PM in the shoot apical meristem, hypocotyl and** 261 **inflorescence stem**

262 To visualize *PIN6* subcellular localization, we generated a polyclonal anti-*PIN6*
263 antibody. In agreement with a recent report (Simon *et al.*, 2016), *PIN6* displayed dual
264 localization in endomembrane domains and at the PM. We used the anti-*PIN6*
265 antibody to detect *PIN6* at the root tip, the plant organ with the lowest *PIN6*
266 expression levels. *PIN6* was visible in the endomembrane compartments of the
267 epidermis and cortex cell files (Fig. 1B), the tissues in which *PIN6* mRNA were low
268 (Fig. 1a, Fig. S1E). However, in other tissues such as inflorescence stem vascular
269 cells (Nisar *et al.*, 2014), vegetative leaves and flower primordia, which displayed
270 higher *PIN6* mRNA levels, *PIN6* was detected at the PM co-localized with *PIN1* (Fig.
271 1a,c,d,f,g). To test the quality of the anti-*PIN6* antibody, we performed both western
272 blot analysis using the *pin6-5* mutant and immunolocalization using both the *pin6-5*
273 and *pin6-6* mutants (Fig. S2b). *PIN6* was recognized by the anti-*PIN6* antibody in WT
274 plants but as expected not in the mutants. This suggests that *pin6-5* and *pin6-6*,
275 which were previously described as knock-down mutants at the mRNA level, are
276 indeed null mutants at the protein level. This confirms that this antibody can be
277 considered specific for *PIN6*. However, additional higher and lower molecular weight
278 proteins were detected in both WT and *pin6-5* knock-out plants, which probably
279 resulted in the background signals which were observed in the immunolocalization
280 images (Fig. S2d-f, non-specific nuclear signals are indicated with an asterisk in WT
281 and *pin6* knock-outs).

282 These data show that tissues with low *PIN6* expression display *PIN6* in
283 endomembrane domains, while *PIN6* is at the PM in tissues with higher *PIN6*
284 expression. This suggests that the dual localization of *PIN6* may be dependent on
285 *PIN6* expression level. To substantiate this correlation, we extended our analysis to
286 other plant tissues reported to have strong *PIN6* expression, such as nectary glands

287 (Ludwig-Muller, 2014; Turi *et al.*, 2014). Observation of plants expressing the
288 *pPIN6:GFP-PIN6* construct revealed that the GFP signal co-localized almost perfectly
289 at the PM with the endosome tracker/PM-marker FM4-64 (Pearson's correlation
290 coefficient in co-localized volume (PCCCV)=0.97, with a value of 1 representing a
291 perfect correlation) (Fig. 2a-e). In contrast, very limited co-localization with the ER
292 marker Rhodamine B (Zhang *et al.*, 2014) (PCCCV=0.26) was observed in both the
293 median and lateral nectary glands (Fig. 2f-h). Taken together, these data support our
294 hypothesis that the PM localization of PIN6 most likely depends on the *PIN6*
295 expression level. Analysis of the PIN6-subcellular localization in nectary glands also
296 revealed basally (toward the root) localized GFP-PIN6, presumably exporting auxin
297 out of nectary glands, thus supporting the idea that nectary glands could be potential
298 sources of auxin (Aloni *et al.*, 2006) (Fig. 2i,j).

299

300 **PIN6 is targeted to the PM upon *PIN6* overexpression**

301 To confirm the relationship between *PIN6* expression level and PIN6 subcellular
302 localization, we generated transgenic lines overexpressing the *PIN6* genomic
303 sequence with (*GFP-PIN6ox*) or without (*PIN6ox*) a *GFP* tag and driven by the
304 constitutively active *CaMV35S* promoter. The non-tagged construct was used to
305 confirm that GFP does not affect PIN6 sub-cellular localization. We visualized PIN6
306 subcellular localization in *GFP-PIN6ox* and *PIN6ox* plants and analyzed its impact on
307 root and shoot growth. *PIN6* overexpression increased the *PIN6* mRNA level and
308 thus the PIN6 protein level (Fig.3a and Fig. S2b); both GFP-tagged and non-tagged
309 PIN6 were detected at the PM, where they co-localized with FM4-64 (PCCCV=0.7)
310 (Fig. 3e-h). In line with recent studies (Ganguly *et al.*, 2010; Cazzonelli *et al.*, 2013;
311 Simon *et al.*, 2016), the roots of both *GFP-PIN6ox* and *PIN6ox* plants were hairless
312 and displayed a strong waving phenotype, suggesting that GFP insertion did not
313 affect PIN6 functionality (Fig. 3b-d). More thorough inspection of the PIN6 sub-
314 cellular localization showed that PM-located PIN6 (PM-PIN6) exhibited polar
315 localization in root cells similar to our observations in nectary glands (see above). In
316 the cortex and stele cells, PIN6 co-localized basally with PIN1 (Fig. 3i and Fig. S3a).
317 The PIN6 PM-localization was most striking in the epidermis For clarity, the epidermis
318 cell file of the root meristematic zone was divided into two tiers (see Fig. 3i).
319 Epidermal cell tier 1 represents cells located in the upper part of the meristematic
320 zone, while tier 2 represents the lower part. The oldest cell of the most recent lateral

321 root cap cells (LRC) (solid white forward arrow in Fig. 3i) marks the limit between the
322 two tiers. In tier 1, PIN6 localized apically (toward the shoot) similarly to PIN2, with a
323 well-defined polarity in cells destined to elongate (Fig. 3i and Fig. S3b). In contrast, in
324 tier 2, PIN6 localized laterally and basally, pointing progressively towards the
325 quiescent centre (QC) and columella cells and presumably channeling auxin towards
326 this region (Fig. 3i and Fig. S3a). Indeed, PM-localized PIN6 perturbed auxin
327 distribution in *PIN6ox* roots as confirmed by both quantification of auxin levels and
328 the expression of the auxin-sensitive *DR5::GUS* reporter fusion protein at the *PIN6ox*
329 root tip (Fig. S3e-g) (Cazzonelli *et al.*, 2013).

330

331 **PID regulates PIN6 polarity despite altered phosphosite occurrence**

332 The unique localization dynamics of PIN6 suggests that it is under the control of a
333 unique regulatory mechanism. Phosphorylation has been already shown to be a key
334 determinant of PIN localization, the best characterized mechanism is the regulation of
335 PIN polarity by members of the AGCVIII protein kinase subfamily, PID and D6PK and
336 their close paralogues. The main D6PK site of PIN1 (S271) is conserved in all long-
337 HL PINs, including PIN6 (S291) (Zourelidou *et al.*, 2014). The three PID
338 phosphorylation sites are similarly well conserved in long-HL PINs with the exception
339 of PIN6, where the site corresponding to S252 of PIN1 is missing. Although PID is
340 known to regulate polarity, not localization to the PM, we first tested whether this
341 differential PID site composition can be associated with a differential regulation of
342 PIN6 by PID. In *PIDox* plants, PIN1 polarity was shifted from the basal to the apical
343 side in the stele (Friml *et al.*, 2004), whereas the cytoplasmic localization of PIN6 in
344 epidermal cells remained unchanged, implying that PID does not bring about PM
345 translocation of PIN6 (Fig. S5a-c) (Friml *et al.*, 2004; Rasmussen *et al.*, 2015).
346 Furthermore, we crossed PIN6 overexpression (*PIN6ox*) and *PINOID*-overexpression
347 (*PIDox*) plant materials. Similarly to *PIN6ox* plants, PIN6 was localized to PM in
348 *PIDoxPIN6ox* plants, but the PM-PIN6 basal localization in the stele shifted, similarly
349 to PIN1 (Fig. S5g-i). Moreover, the basal localization of PM-PIN6 in tier 2 epidermal
350 cells also shifted, whereas the apical localization of PIN6 in tier 1 epidermal cells did
351 not (in 100% of plants tested, $n > 15$; compare Fig. 3i and Fig. S5d-f with Fig. S5g-i).
352 These results imply that similarly to other PINs, PID plays a role in polarity regulation
353 of PIN6, however its ER to PM translocation is regulated by other means.

354 **Threonine-phosphorylation sites 392 (T392) and 393 (T393) are involved in PIN6**
 355 **localization at the PM**

356 A phosphoproteomic assay in Arabidopsis yielded PIN6 phosphopeptides from
 357 suspension-cell-derived PM vesicles (Benschop *et al.*, 2007). In that study, PM-
 358 localised PIN6 protein was shown to be phosphorylated at both or either of the two
 359 adjacent threonine residues at positions T392 and T393 in the hydrophilic loop
 360 (Benschop *et al.*, 2007). These phosphorylation sites are partially conserved among
 361 PIN6-like proteins in other species (Fig. S6b). Remarkably, these residues are not
 362 conserved in other members of the PIN family (Fig. S6b), raising the possibility of a
 363 PIN6-specific regulation through their phosphorylation. In order to test this hypothesis
 364 both T392 and T393 were converted by site-directed mutagenesis to valine, an amino
 365 acid that cannot be phosphorylated (PIN6^{T392EVT393V}) or to glutamic acid, which
 366 mimics constitutive phosphorylation (PIN6^{T392E/T393E}). Driven by a β -estradiol-
 367 inducible promoter, the intact *PIN6* gene (*PIN6ox-i*) and the mutated versions were
 368 separately introduced into WT plants. When grown in the presence of β -estradiol, the
 369 roots of *PIN6ox-i* and PIN6^{T392E/T393E} plants were dramatically affected in comparison
 370 to the roots of WT and PIN6^{T392V/T393V} plants (Fig. 4a-d, top panel). *PIN6ox-i* and
 371 PIN6^{T392E/T393E} plants developed hairless agravitropic roots (Fig. 4a-d, middle panel).
 372 Consistent with this, confocal microscope images revealed that, as for *PIN6ox* plants
 373 (Fig. 3i), PIN6 localized basally at the PM in *PIN6ox-i* root tip epidermal cells (Fig. 4b,
 374 lower panel). In PIN6^{T392E/T393E} plants, the GFP signal also localized to the PM but
 375 was non-polar (Fig. 4c, lower panel; Video S1). Altogether, these data indicate that
 376 the presence of PIN6 at the PM of epidermal cells, and not necessarily its polarity, is
 377 sufficient to perturb both the root gravity response and root hair development. In
 378 contrast, PIN6^{T392V/T393V} plants were indistinguishable from WT plants, and the GFP
 379 signal was mainly visible in the ER, where it co-localized with the ER marker
 380 rhodamine B hexyl (PCCCV=0.65) (Fig. 4d and Fig. S7a; Video S2). It is known that
 381 PM-localized PINs are sensitive to the fungal toxin brefeldin A (BFA), which blocks
 382 PIN protein recycling, while ER-PINs are BFA resistant (Mravec *et al.*, 2009).
 383 Accordingly, upon BFA treatment, PIN6^{T392E/T393E} co-localized with FM4-64 in BFA
 384 compartments, while PIN6^{T392V/T393V} localization was not affected, thus confirming
 385 their respective subcellular localizations (Fig. S7b-c). These data demonstrate that
 386 T392 and T393 phosphorylation sites are crucial the translocation of PIN6 to the PM.
 387

388 **PIN6 hydrophilic loop is phosphorylated by MPK4 and MPK6 but T393 is not a**
389 **MAP kinase phosphorylation site**

390 In order to predict putative kinase(s), which may mediate T392/T393 phosphorylation,
391 we screened the PIN6 protein sequence by using the Eukaryotic Linear Motif (ELM)
392 database (Dinkel *et al.*, 2016). This search resulted in a total of 59 predicted
393 phosphorylation sites targeted by eight types of kinases. In the region of interest
394 T393 was identified as a MAPK phosphorylation site, while no putative kinase was
395 associated with T292. T393 is one of eight potential proline-directed MAPK
396 phosphorylation sites in PIN6 HL (T226, T242, S286, T304, T320, S326, S337, and
397 T393) suggesting that PIN6 may be phosphorylated by MAP-kinase(s). In plants,
398 MAPK pathways are central regulators of various stress responses and
399 developmental processes (Rodriguez *et al.*, 2010; Xu & Zhang, 2015). In particular,
400 MPK6 of Arabidopsis, the best studied plant MAP kinase, has been reported to
401 specifically regulate developmental processes such as lateral root development and
402 plant height (Jia *et al.*, 2016), processes also shown to be altered in *pin6* mutants
403 (Cazzonelli *et al.*, 2013; Simon *et al.*, 2016). MPK4, another well-characterized plant
404 MAPK is involved in defense regulation, with *mpk4* mutants displaying severe
405 dwarfism and altered cell division and microtubule dynamics. Based on this
406 information we first tested whether PIN6 is phosphorylated by MPK4 and MPK6 using
407 *in vitro* kinase assays (Fig. 5).

408 The incorporation of radiolabeled phosphate in the hydrophilic loop (HL) of PIN6 in
409 the presence of activated MPK4 or MPK6 indicates that this protein is phosphorylated
410 by both kinases (Fig. 5). As a negative control, all eight MAPK phosphorylation
411 residues (S or T) were replaced with the non-phosphorylatable amino acid alanine
412 (T226A, T242A, S286A, T304A, T320A, S326A, S337A, and T393A in PIN6-mut8),
413 which results in the loss of MAPK-mediated phosphorylation (Fig. 5). Faint, residual
414 phosphorylation of the mutant proteins by MPK6 indicates weak, unspecific
415 phosphorylation on non-cognate residues, probably related to the strong activity of
416 MPK6. These results confirm MAPK-mediated phosphorylation of PIN6. In order to
417 test whether T393 is one of the residues actually phosphorylated by these MAPKs we
418 tested specific phosphorylation of T393 by using a septuple mutant, where T393 is
419 wild type (i.e. T226A, T242A, S286A, T304A, T320A, S326A, S337A; PIN6-mut7).
420 Neither MPK4 nor MPK6 phosphorylated PIN6 on T393 (Fig. 5), suggesting that this
421 residue is not a genuine MAP kinase site. These data reveal complex regulation of

422 PIN6 by at least two MAPK pathways, but also indicate that the modification of
 423 T392/T393 regulating PIN6 PM translocation is probably brought about by yet
 424 another type of protein kinase.

425

426 **PM-localized PIN6 represses plant growth**

427 To investigate the importance of PIN6 subcellular localization on plant development,
 428 we analyzed the phenotype of PIN6^{T392E/T393E} and PIN6^{T392V/T393V} plants grown in soil.
 429 Spraying plants with 100 μ M β -estradiol affected weakly the growth of Col-0 and
 430 PIN6^{T392E/T393E} plants, but strongly retarded the growth of PIN6^{T392E/T393E} and PIN6ox-i
 431 plants (Fig. S8a, b). Plants sprayed with water showed a similar elongation profile for
 432 the inflorescence stems of Col-0 and PIN6^{T392V/T393V}, while PIN6^{T392E/T393E} plants
 433 developed significantly smaller inflorescences. This result confirms the reported
 434 leakiness of the estradiol-inducible promoter (Kubo *et al.*, 2013), as indicated by the
 435 GFP signal observed in the leaves of non-induced PIN6^{T392V/T393V} and PIN6^{T392E/T393E}
 436 plants (Fig. S8c). β -estradiol also affected weakly the growth of Col-0 and
 437 PIN6^{T392V/T393V} plants, but it strongly delayed the growth of PIN6^{T392E/T393E}
 438 inflorescence stems and completely suppressed the bolting of PIN6ox-i plants (Fig.
 439 S8d). Taken together, these data demonstrate the importance of T392 and T393 for
 440 the regulation of plant inflorescence stem elongation and indicate that the
 441 phosphorylation-dependent PM targeting of PIN6 negatively controls inflorescence
 442 stem development, while preventing this phosphorylation results in the ER retention
 443 of PIN6. These data also suggested a role for PIN6-dependent auxin distribution
 444 during the elongation of the inflorescence stem.

445

446 **Inflorescence stem elongation is accelerated in *pin6* loss-of-function mutants**

447 In *Arabidopsis thaliana*, the floral transition (formation of an inflorescence meristem)
 448 marks the transition from the vegetative to the reproductive phase followed by
 449 elongation of the first internode, known as the bolting transition (Pritchard *et al.*,
 450 2012). This process is influenced by hormones but underlying mechanisms still
 451 remain poorly understood. To explore how PIN6-dependent PAT modulates
 452 inflorescence stem development, we analyzed the *pin6-5* (GK-430B01) and *pin6-6*
 453 (GK-711C09) T-DNA insertion lines (Supporting Information Fig. S2A; (Cazzonelli *et al.*,
 454 2013). We also analyzed two additional novel knock-out lines isolated from the
 455 *Arabidopsis* Genetrap collection in the *Landsberg erecta* (Ler) background

456 (Sundaresan *et al.*, 1995) (referred to here as *pin6-7* (GT7129) and *pin6-8*
457 (GT6906)), in which T-DNA is inserted in the 4th and 5th introns, respectively
458 (Supporting Information Fig. S2C). All *pin6* mutants developed inflorescence stems 3-
459 5 times longer than wild-type (WT) inflorescence stems while having the same
460 number of leaves. This phenotype suggests faster inflorescence stem elongation
461 rather than early flowering (Fig. 6a-d).

462

463 **PIN6 overexpression delays inflorescence stem elongation**

464 In contrast to *pin6* mutants and in line with previously published phenotypes
465 (Cazzonelli *et al.*, 2013), plants overexpressing *PIN6* developed significantly shorter
466 inflorescence stems (Fig. 6d). Compared to WT plants, these plants developed
467 siliques relatively close to the base of the inflorescence stem (indicated with an arrow
468 in Fig. S9a), suggesting that these plants were mature and capable of seed
469 production but that inflorescence stem elongation was arrested (Fig. 6a-d). To
470 confirm these results, we quantified the flowering time and the growth rates of WT,
471 *pin6-5* and *pin6-5* complemented with GFP-PIN6 driven by the *PIN6* native promoter
472 (*pPIN6:PIN6:GFP*). In our conditions, all plants (WT, *pPIN6:PIN6-GFP*, *pin6-5*, and
473 *PIN6ox*) flowered around the 28th day after sowing (DAS). However, although the
474 apical flower was visible in *PIN6ox* and *GFP-PIN6ox* plants, inflorescence stem
475 elongation required four additional days (Fig. 6e). WT and *pin6-5 pPIN6:PIN6-GFP*
476 plants displayed accelerated growth starting on the 34th day (open arrow in Fig. 6e),
477 but this acceleration occurred earlier in *pin6-5* plants (closed arrow in Fig. 6e). Thus,
478 six days after bolting, the growth rate of *pin6-5* mutant inflorescences was
479 approximately 2.42 cm/day versus 1.65 and 1.43 cm/day for WT and *pin6-5*
480 *pPIN6:PIN6-GFP* plants, respectively (Fig. S9b), demonstrating the rapid growth of
481 *pin6-5* plants and mutant complementation by the *pPIN6:PIN6-GFP* construct.
482 Conversely, *GFP-PIN6ox* and *PIN6ox* inflorescence stem elongation was slower
483 (only 0.25 and 0.13 cm/day, respectively (Fig. S9b)). The data also indicated that the
484 accelerated growth of *pin6* mutants is transient. Later, the WT and *pin6-6* mutant (a
485 trend visible in all mutants; not shown) growth rates became equal, while the *PIN6ox*
486 growth rate remained approximately 50% lower (Fig. S9c,d). Taken together, these
487 data demonstrate that *PIN6* activity regulates inflorescence stem elongation and
488 strongly suggest a role for auxin transport during plant bolting.

489

490

491

492 Auxin response is reduced in *pin6* mutants

493 Auxin transport capacities and auxin levels are crucial for shoot branching and
494 vascular tissue development (Muller & Leyser, 2011; Peer *et al.*, 2011; Bennett *et al.*,
495 2014). For example, a cambial auxin peak resulting from basipetally derived shoot-
496 apex auxin is essential for both primary and secondary growth (Bjorklund *et al.*, 2007;
497 Suer *et al.*, 2011; Ameres & Zamore, 2013). To determine how PIN6 activity
498 modulates auxin distribution and levels in bolting inflorescences, we used a
499 *pDR5:GFP* reporter (Ottenschlager *et al.*, 2003) to visualize auxin distribution and
500 activity in inflorescence stems six days after bolting. In transverse sections 50 mm
501 above the uppermost rosette leaf of the inflorescence stem of WT plants, the
502 DR5:GFP signal was mainly visible in phloem and xylem parenchyma cells (Fig.
503 7a,f,i). The DR5 signal was significantly lower in the *pin6-5* mutant (T-test, $P<0.05$,
504 $n=10$); in contrast, DR5 expression was significantly greater in *PIN6ox* plants (T-test,
505 $P<0.001$, $n=10$) (Fig. 7b-d). In addition, radial development was reduced in *PIN6ox*
506 plants but increased in *pin6-5* plants (Fig. 7b-e). Consequently, *pin6-5 DR5:GFP*
507 plants displayed a greater transversal stem surface area than *DR5:GFP* and
508 *PIN6oxDR5:GFP* plants (T-test, $P<0.001$, $n=10$). In *PIN6ox DR5:GFP* plants, the
509 number of xylem elements was significantly reduced and accompanied by ectopic
510 development of xylem parenchyma cells (Fig. 7f-l; xylem parenchyma cells are
511 indicated with a white arrow in Fig. 7i and k). Altogether, these data establish *PIN6* as
512 a key regulator of both the primary and secondary growth of Arabidopsis
513 inflorescence stems and show that impaired PIN6 activity strongly affects the auxin
514 response necessary for cambium proliferation and xylem differentiation (Hildebrandt
515 & Nellen, 1992; Nina Theis & Manuel Lerda, 2003; Suer *et al.*, 2011).

516

517 Discussion

518 Previous studies revealed the importance of the auxin transporter PIN6 in several
519 developmental processes, such as nectary development (Bender *et al.*, 2013), leaf
520 vein patterning (Sawchuk *et al.*, 2013), and root and lateral root development
521 (Cazonelli *et al.*, 2013; Simon *et al.*, 2016). Recently, Simon and colleagues
522 described the PM and ER subcellular localization of PIN6 and suggested its role in
523 controlling auxin transport and homeostasis in auxin-mediated development (Simon

524 *et al.*, 2016). In the present study, using genetic, molecular and pharmacological
 525 approaches, we characterized the mechanisms controlling PIN6 dual localization and
 526 uncovered a role for PIN6 during Arabidopsis inflorescence stem development.

527

528 **PIN6 subcellular localization is regulated by *PIN6* gene expression levels**

529 It was reported that auxin can induce the *PIN6* promoter in a tissue-specific manner
 530 (Cazzonelli *et al.*, 2013). Hence when combined to the finding that high *PIN6*
 531 expression results in PM-targeting of PIN6, it is not surprising to find PIN6 expressed
 532 at the PM in tissues reported to contain high levels of auxin such as the tip of young
 533 leaves, young flowers, vascular tissue of the stem and nectary glands (Muller *et al.*,
 534 2002; Benkova *et al.*, 2003; Aloni *et al.*, 2006; Cheng *et al.*, 2006). We did not
 535 observe PIN6 at the PM of root cells, despite the fact that auxin is synthesized and
 536 accumulates at the root tip. This suggests that at the root tip, besides auxin levels
 537 other cell determinants probably modulate PIN6 abundance and therefore its
 538 localization. In contradiction with our data, GFP-tagged PIN6 driven by the *PIN6*
 539 native promoter was recently reported to localize at the PM in the Arabidopsis root tip
 540 (Simon *et al.*, 2016), although whether this construct could rescue the described
 541 mutant phenotypes is not presented in that paper.

542

543 **T392/T393 phosphorylation sites modulate PIN6 subcellular localization**

544 PINOID (PID) kinase and SERINE/THREONINE PROTEIN PHOSPHATASE 2A
 545 (PP2A), were reported to be involved in the reversible phosphorylation of the PIN
 546 hydrophilic loop (Michniewicz *et al.*, 2007), although it is still unclear how PID
 547 regulates PIN trafficking (New ref), Nevertheless. our data indicate that despite loss
 548 of a PID site PIN6 retains a similar sensitivity to PID-dependent phosphorylation as
 549 PIN1, i.e. PIN6 basal localization is flipped to apical localization. However, we have
 550 no evidence that PID activity is responsible for PIN6 exit from the ER, since PIN6 is
 551 not targeted to PM in PIDox plants, since PIN6 is not targeted to the PM in 35S::PID
 552 plants; instead this observation indicates a role for other kinases.

553 Data mining retrieved *in vivo* phosphorylation at a tandem threonine pattern
 554 (T392/T393) unique to PIN6. Accordingly, functional analyses confirmed that the
 555 T392 and T393 residues are crucial for PIN6 ER exit and subsequent PM
 556 localization. In line with this, genetic modifications preventing phosphorylation of
 557 these residues resulted in PIN6 retention in the ER. A similar mechanism was

558 described for the PM-targeting of Arabidopsis PHOSPHATE TRANSPORTER1
559 (PHT1) (Bayle *et al.*, 2011), the nitrate transporter and auxin facilitator NRT1.1
560 (Krouk *et al.*, 2010; Habets & Offringa, 2014) and the aquaporin PIP2;1 (Weiste &
561 Droge-Laser, 2014). Phosphorylation of these proteins was shown to modulate their
562 targeting to the PM.

563

564 **PIN6 is phosphorylated by MPK4 and MPK6**

565 Phosphorylation of PIN6 at T392/T393 represents a novel regulatory mechanism,
566 thus we set out to identify the corresponding kinase(s). As there are 942 kinases
567 encoded in the Arabidopsis genome (Zulawski *et al.*, 2014), *in silico* pattern
568 screening appeared to be the feasible approach to predict the kinase(s)
569 phosphorylating T392 and/or T393. Accordingly, T393 is one of eight putative MAP
570 kinase phosphorylation sites in PIN6 HL. Here we provide evidence that PIN6 is
571 phosphorylated by both MPK4 and MPK6, thus revealing a novel regulatory
572 mechanism, although T393 is not phosphorylated by MPK4 or MPK6. Preference of
573 MAP kinases towards specific residues within a set of potential phosphosites has
574 been preceded in case of other substrates, e.g (Furlan *et al.*, 2017). Thus the
575 identity of the kinase(s) phosphorylating T392 and/or T393 remains elusive in light of
576 current kinase analysis tools. MAP kinases are involved in several adaptive and
577 developmental processes controlled by environmental stress (Pitzschke *et al.*, 2009;
578 Rodriguez *et al.*, 2010; Xu & Zhang, 2015). In particular, MKK7 is a repressor of PAT
579 (Dai *et al.*, 2006), and the MKK7-MPK6 cascade was recently shown to be involved
580 in PAT and to have a direct impact on auxin distribution in inflorescence stems (Jia *et al.*
581 *et al.*, 2016). MPK4 is known to modulate plant defense and development (Petersen *et al.*
582 *et al.*, 2000; Gawronski *et al.*, 2014). In light of MAPK-mediated phosphorylation of two
583 PINs and the involvement of two MAPK pathways [(Jia *et al.*, 2016); this study], a
584 complex regulatory network is emerging, which suggests an adaptive growth
585 mechanism allowing plants to rapidly respond to environmental or developmental
586 changes and fits well with the central role of MAPK pathways in adaptive regulation.
587 For this respect, *in vivo* analysis of PIN6 phosphorylation by MPK4 or MPK6 in
588 response to various stresses will be very informative.

589

590 **PIN6-dependent auxin transport regulates inflorescence stem elongation**

591 Our data show that *PIN6* expression is regulated by both developmental and tissue-
592 specific determinants throughout the entire plant lifespan. *PIN6* expression does not
593 regulate the transition to flowering, as flowering time in terms of leaf number at the
594 onset of bolting is unaltered in both *pin6* and *PIN6ox* genotypes. Therefore, PIN6-
595 dependent auxin transport is crucial for inflorescence stem elongation after floral
596 initiation. The significance of PIN6-mediated auxin transport in inflorescence stem
597 development is related to the degree of *PIN6* expression. Ectopic expression of PIN6
598 in *PIN6ox* plants causes the auxin accumulation (Cazzonelli *et al.*, 2013) responsible
599 for inhibiting inflorescence stem elongation, while lower auxin levels in the *pin6*
600 mutant promote both radial extension and faster inflorescence stem elongation. This
601 is in line with the well-accepted result that perturbing PAT alters the normal
602 development of Arabidopsis inflorescence stems (Okada *et al.*, 1991; Wilson *et al.*,
603 2013) and with the reported auxin concentration-dependent effect on stem
604 elongation, in which the application of high concentrations of auxin directly inhibits
605 the growth of shoots, while lowering auxin concentrations promotes growth (Thimann,
606 1939). It is possible that PIN6-dependent auxin gradients differentially regulate the
607 genes controlling cell expansion, thus inhibiting cell growth when auxin levels are
608 high, such as occurs in *PIN6ox* plants.

609 By delaying elongation of the inflorescence stem, PIN6-dependent auxin transport
610 allows the plant to optimally mature, hence optimizing seed yields. On the other
611 hand, lowering *PIN6* function appears to be a relevant tool for accelerating or
612 delaying inflorescence stem development. We propose that PIN6 acts as a gate
613 keeper, ensuring that Arabidopsis plants efficiently develop the inflorescence stem at
614 the appropriate, environmentally determined time and that inflorescence stem
615 development is timed in accordance with environmental conditions. The underlying
616 regulatory mechanisms probably involve upstream factors that sense environmental
617 changes and activate the kinases that phosphorylate PIN6, thereby stimulating its
618 exit from the ER. In this respect, it is remarkable that MAPK signaling is activated by
619 various environmental signals.

620 The fact that inflorescence stem elongation is repressed in plants overexpressing
621 *PIN6* [this study; (Cazzonelli *et al.*, 2013)] and in *PIN6*^{T392E/T393E} plants, where PIN6
622 localizes at the PM, suggests the existence of a correlation between PIN6
623 phosphorylation status, the PM-localization of PIN6 and the elongation of the
624 inflorescence stem during plant bolting. PM localization of PIN6 is crucial, as it may

625 contribute to the fine tuning of the tissue-specific auxin amounts necessary for the
626 optimal development of the Arabidopsis inflorescence stem. Furthermore, although
627 PIN6 is an auxin efflux carrier (Petrasek *et al.*, 2006; Simon *et al.*, 2016), its activity
628 once targeted to the PM is quite intriguing, particularly in relation to the other PM-
629 localized PIN proteins. The *pin1* mutant displays several developmental defects such
630 as naked, pin-shaped inflorescences (Galweiler *et al.*, 1998) and delayed bolting
631 (Okada *et al.*, 1991; Galweiler *et al.*, 1998), whereas *35S::PIN1* plants bolt similarly to
632 WT (Benkova *et al.*, 2003). In comparison, *pin6* mutants bolt faster, while PIN6ox
633 plants are severely delayed. Since PIN6 and PIN1 both localize to the PM in WT
634 stems, it is logical that their combined basipetal auxin transport activities are required
635 for inflorescence stem development. Interestingly, PAT was shown to be increased in
636 *35S::PIN1* plants but significantly reduced in plants overexpressing *PIN6* (Cazzonelli
637 *et al.*, 2013). Altogether, these observations suggest that PIN6 and PIN1 probably
638 have distinct activities during inflorescence stem development.

639 Taken together, our data suggest a mechanism in which
640 environmental/developmental cues act at both the transcriptional and
641 posttranscriptional levels by stimulating *PIN6* expression and inducing the
642 phosphorylation and subsequent translocation of PIN6 protein to the PM (Fig. S10).

643

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657

658 **Author contributions**

659 F.A.D., H.N., P.K., C.L., T. M., R.D. and K.P. conceived and designed the
 660 experiments. F.A.D., D.G., H.N., P.K., H.L., V.M., I.P., K.R., L.Q., X.L., C.B., S.N.,
 661 and T.V.N. performed the experiments. F.A.D., H.N., P.K., C.L., T. M., B.B., R.D. and
 662 K.P. analyzed the data. F.A.D. wrote the paper. All authors discussed the results and
 663 commented on the manuscript.

664

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878 **Supporting information**

- 880 **Fig. S1** PIN6 expression during plant development
- 881 **Fig. S2** Specificity of anti-PIN6 antibody
- 882 **Fig. S3** Localization of PIN6 and auxin distribution at the root tip
- 883 **Fig. S4** Evolutionarily conserved PINOID phosphorylation sites in AtPIN6 and PIN6-like
 884 proteins
- 885 **Fig. S5** A basal-to-apical shift in PM-resident PIN6 localization in plants overexpressing
 886 PINOID kinase
- 887 **Fig. S6** Phosphorylated residues in PIN proteins
- 888 **Fig. S7** ER-localized PIN6 is insensitive to BFA
- 889 **Fig. S8** The PIN6 phosphorylation sites T392 and T393 are involved in plant growth and plant
 890 inflorescence stem elongation
- 891 **Fig. S9** Inflorescence stem development

892 **Fig. S10** Developmental control of PIN6 subcellular localization

893 **Table S1** List of primers used

894 **Methods S1** Characterization of *pin6* mutants and generation of transgenic lines

895 **Methods S2** Free IAA level determination

896 **Methods S3** Immunolocalization

897 **Methods S4** Quantitative RNA analysis

898 **Methods S5** Microscopy and image post processing

899

900 **Notes S1** Methods references

901 **Video/Movie S1** Z-stack maximum projection of confocal microscope images showing

902 PM-located PIN6^{T392E/T393E}

903 **Video/Movie S2** Z-stack maximum projection of confocal microscope images showing

904 ER-located PIN6^{T392V/T393V}

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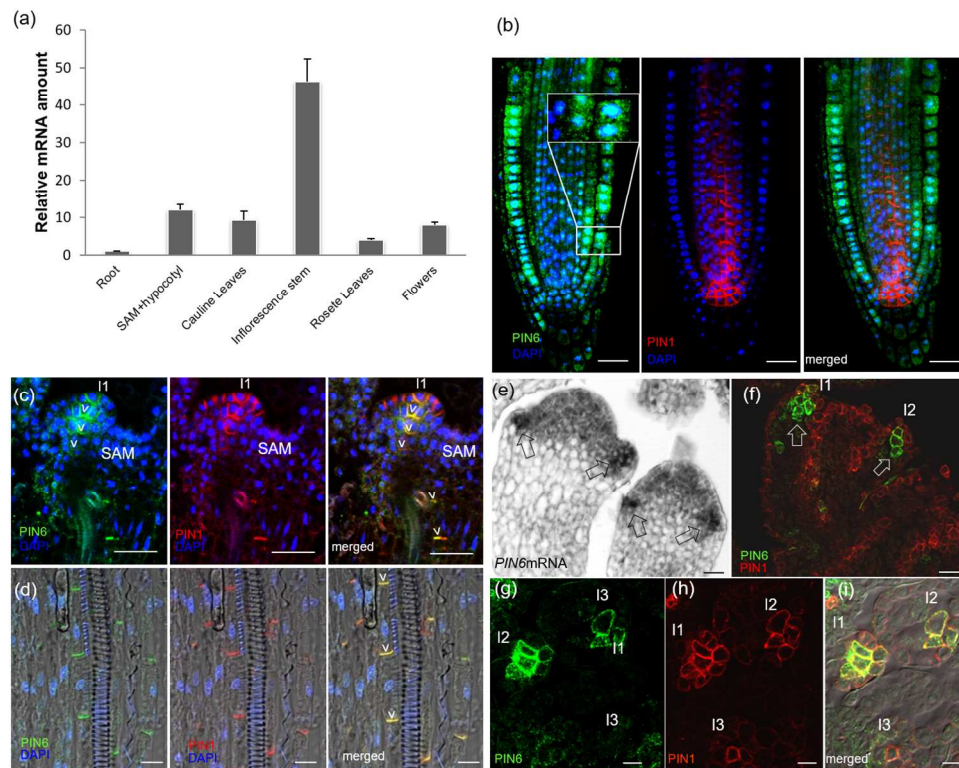


Figure 1. PIN6 expression and subcellular localization.

(a) PIN6 mRNA levels in different tissues were detected by qRT-PCR. (b-c) Immunolocalization of PIN6 and PIN1 in Arabidopsis WT seedlings. (b) Immunocytolocalization of PIN6 and PIN1 in a seedling root tip. PIN6 is mainly cytoplasmic in endomembrane domains. (c) PIN6 is localized mainly at the PM of developing vegetative leaf primordium and co-localizes with PIN1. (d) Co-localization of PIN6 and PIN1 at the PM in stem xylem parenchyma cells. (e) PIN6 expression in developing WT flowers. Arrows indicate PIN6 mRNA. (f-g), Immunolocalization of PIN6 and PIN1 in Arabidopsis inflorescences. (f) PIN6 and PIN1 subcellular localization in an inflorescence longitudinal section. Arrow indicates PIN6 localization at the PM in emerging incipient primordia (I1, I2). (g-i) Co-localization of PIN6 and PIN1 at the PM in WT Arabidopsis inflorescence transverse sections. (g), PM-localized PIN6 (green). (h) PM-localized PIN1 (red). (i) G and H merged. PIN6 and PIN1 co-localize at the PM in incipient leaf primordia (I1, I2, I3) tips. Scale bars, 20 μm in (b), (c), (e), (f), (g), (h), and (i); 10 μm in (d).

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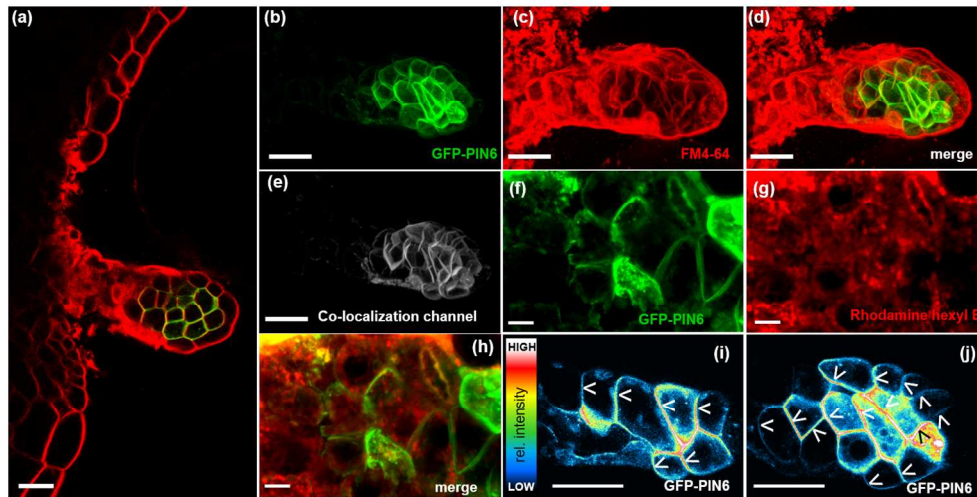


Figure 2. PIN6 is located at the PM in WT Arabidopsis nectary glands. (a) WT plants expressing a pPIN6::GFP-PIN6 fusion protein in nectary glands were labelled with endosome tracker/PM marker FM4-64. (b-e) Z-stack maximum projection of confocal microscope images showing GFP-PIN6 co-localizing with FM4-64. (b) GFP-PIN6 signal. (c), FM4-64 signal. (d), (b) and (c) merged. (e), Co-localization of GFP-PIN6 and FM4-64 in (D). (f-h) WT plants expressing a pPIN6::GFP-PIN6 fusion in nectary glands labelled with the ER marker rhodamine B hexyl ester. GFP-PIN6 is not localized to the ER. (f) GFP-PIN6 signal. (g) Rhodamine hexyl B signal. (h) (f) and (g) merged. (i-j) Semi-quantitative, color-coded heat map of PM-PIN6 displaying GFP-PIN6 polarity in confocal microscope virtual sections of a WT nectary. Dark and white pixels indicate low and high intensity, respectively; pixel values range from 0 to 4095. (i) Outer plane of the nectary gland. (j) Median plane of the nectary. Arrowheads indicate GFP-PIN6 polarity. Scale bar, 20 μ m.

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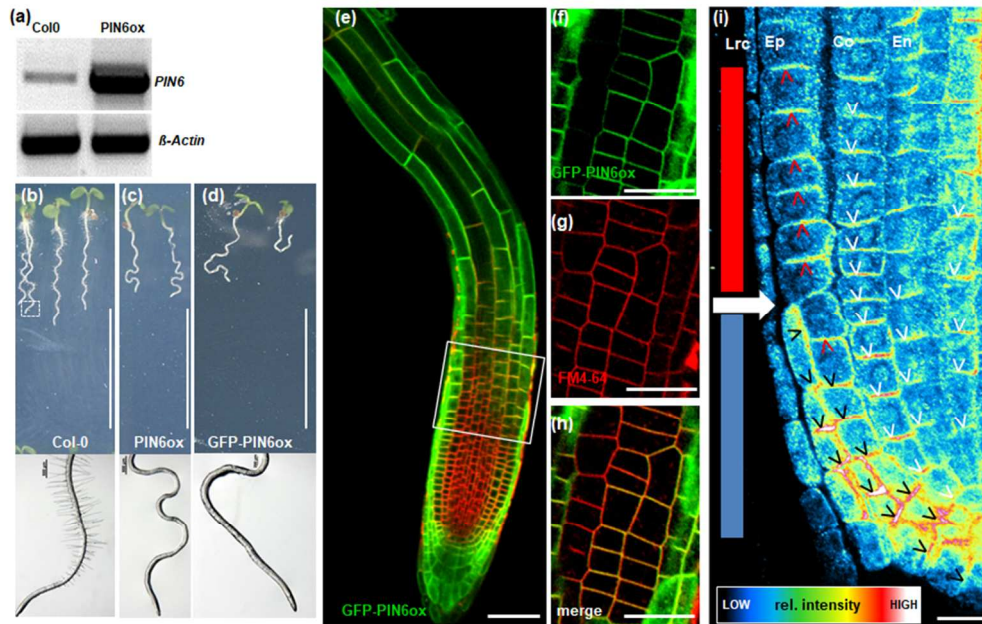


Figure 3. PM targeting of PIN6 in PIN6ox plants.

(a) PIN6 and β -Actin (as a reference gene) mRNA levels were detected by RT-PCR in WT (Col-0) and 35S::PIN6 (PIN6ox) Arabidopsis seedlings. (b-d) PIN6 overexpression perturbs root growth (upper panel) and abolishes root hair development (lower panel). (b) Col-0. (c) 35S::PIN6 (PIN6ox). (d) 35S::GFP-PIN6 (GFP-PIN6ox). (e) Overexpressed GFP-PIN6 (GFP-PIN6ox) co-localizes with the PM marker FM4-64. (f-h) Magnified view of epidermal cells showing co-localization of GFP-PIN6ox and FM4-64 at the PM. (i) Color-coded heat map showing high and low AtPIN6 signal intensities detected with the anti-PIN6 antibody. The solid white arrow marks the separation between meristematic cells of the epidermis in Tier 1 (red box) and Tier 2 (blue box). In Tier 1, PIN6 localizes apically (toward the shoot; red arrowheads), while in Tier 2, PIN6 localizes basally (toward the quiescent centre; black arrowheads). White arrowheads indicate PIN6 polarity in the cortex, endodermis and stele cells. Epidermis (Ep), cortex (Co), endodermis (En), lateral root cap cells (Lrc). Scale bar, 1 cm in a-c, 50 μ m in e, and 20 μ m in f-h and i.

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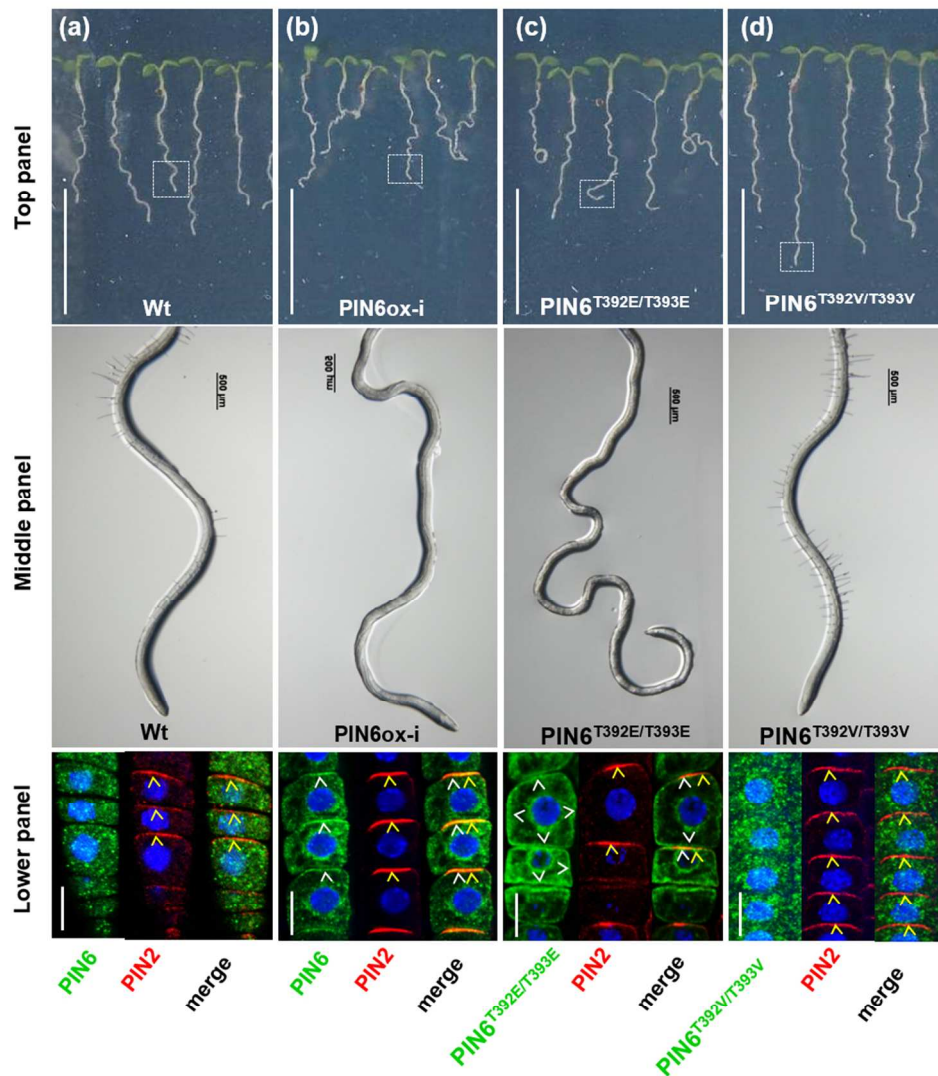


Figure 4. PIN6 ER exit is phosphorylation dependent. Four-day-old wild-type (WT) *Arabidopsis* and PIN6-inducible lines were induced with $0.1 \mu\text{M}$ β -estradiol for 3 days. (a) Neither the growth (top panel) nor root hair development (middle panel) of WT plants is affected. In the root tip epidermal cells (lower panel), immunolocalization shows that PIN6 localizes in endomembrane compartments, while PIN2 is detected at the PM. (b) After induction with β -estradiol, PIN6ox-i root growth is affected (top panel), root hair development is suppressed (middle panel) and PIN6 is targeted to the PM, where it colocalizes with PIN2 (lower panel). (c) Induced roots of EE plants are agravitropic (top panel) and hairless (middle panel), with GFP-PIN6 (detected with an anti-GFP antibody) being predominantly localized apolarly at the PM, where it partially co-localizes with PIN2 (lower panel). (d), Induced VV plants are not affected by β -estradiol induction (top and middle panels). GFP-PIN6 is predominantly localized in the ER. White and yellow arrowheads indicate PM-PIN6 and PM-PIN2, respectively, and nuclei are stained in blue with DAPI. Scale, 1 cm (top panel) and $10 \mu\text{m}$ (lower panel).

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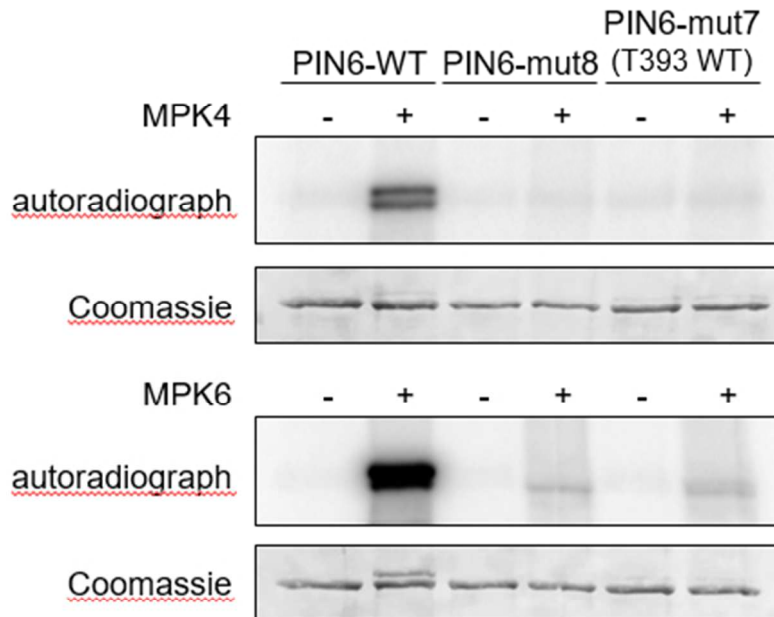


Figure 5. PIN6 is phosphorylated by MPK4 and MPK6. Kinase assay with in vitro-translated, affinity-purified WT GST:PIN6 (PIN6-WT), T226A, T242A, S286A, T304A, T320A, S326A, S337A, and T393A mutant GST:PIN6 (indicated as 'PIN6-mut8') and T226A, T242A, S286A, T304A, T320A, S326A and S337A mutant [indicated as 'PIN6-mut7 (T393-WT)'] GST:PIN6 variants. PIN6 variants were incubated in the absence (-) or presence (+) of in vitro-translated, affinity-purified, activated MPK4 (top panel) or MPK6 (bottom panel). Protein loading is visualized by Coomassie staining.

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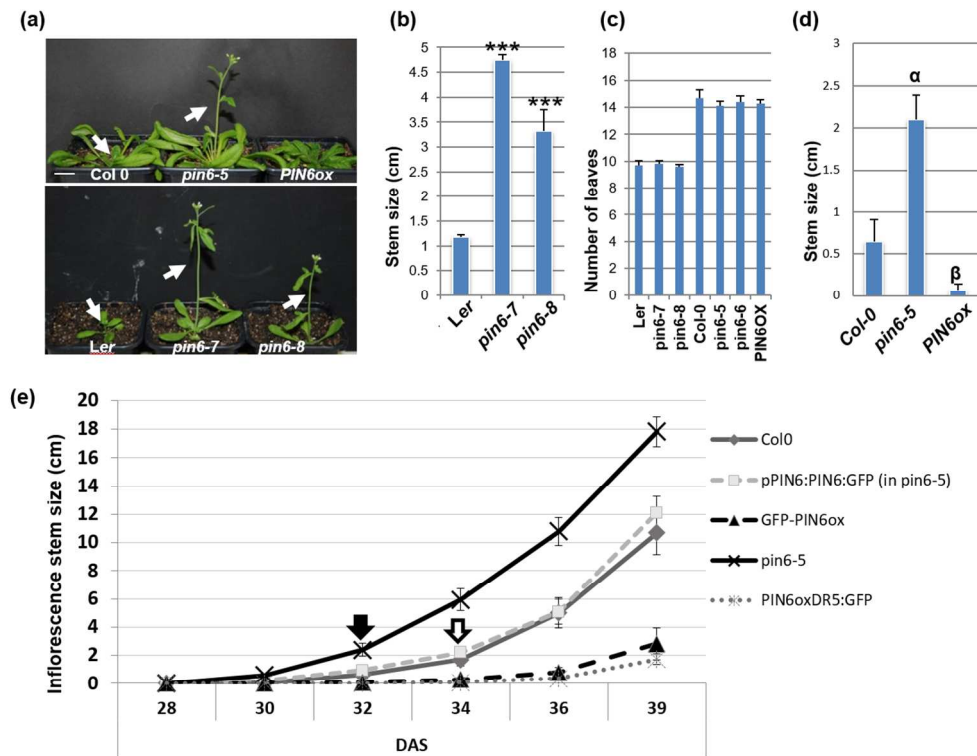


Figure 6. PIN6 regulates Arabidopsis inflorescence stem elongation. (a) WT Col-0, pin6-5, PIN6ox, WT Ler, pin6-7 and pin6-8 plants were grown in soil for 35 days (Col-0, pin6-5 and PIN6ox) or 23 days (Ler, pin6-7 and pin6-8). Scale bar, 1 cm. (b) pin6-6 and pin6-7 inflorescence stems are significantly longer than WT Ler inflorescence stems. Stars (*) indicate significant differences from WT control at $P < 0.01$ (T-test). The data are shown as means ($n = 40 \pm$ s.e.). (c) At flowering, pin6 mutants and WT plants display the same number of rosette leaves. (d) The inflorescence stem is significantly longer in pin6-5 plants than WT Col-0 plants, while PIN6ox plant inflorescence stems are significantly shorter. (e) Quantification of the inflorescence stem development of WT Col-0, pin6-5 mutant, pin6-5 complemented with pPIN6::PIN6-GFP, GFP-PIN6ox and PIN6oxDR5::GFP plants. DAS, days after sowing seeds. Symbols (α , β) in (d) indicate significant differences, as determined by ANOVA followed by Tukey's Honest significant difference (HSD) post hoc test ($P < 0.05$). The data are shown as means ($n = 20 \pm$ s.e.).

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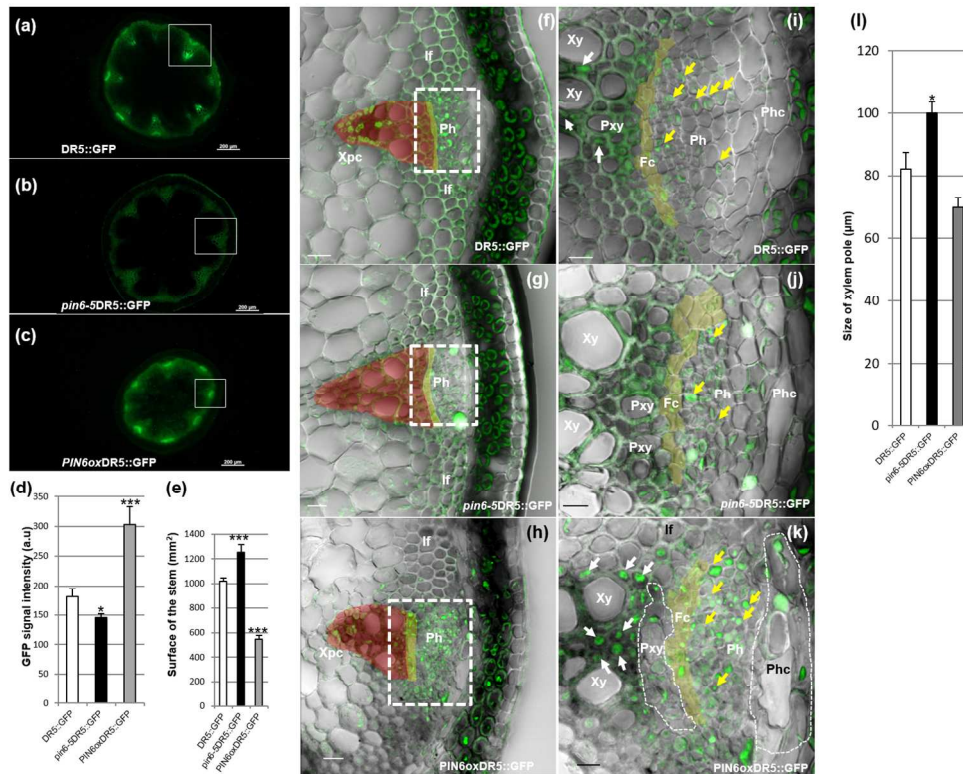


Figure 7. Auxin distribution and vascular tissue differentiation. Transverse sections taken 50 mm above the uppermost rosette leaf of the inflorescence stem of DR5rev::GFP, pin6-5DR5rev::GFP and PIN6oxDR5rev::GFP plants. (a), GFP signal in DR5rev::GFP vascular bundles. (b) GFP signal in pin6-5DR5rev::GFP vascular bundles. Note the reduction in DR5rev::GFP signal. (c) GFP signal in PIN6oxDR5rev::GFP vascular bundles. Note the increase in DR5rev::GFP signal. (d) Quantification of GFP signal from plants shown in (a), (b), and (c). (e) Stem surface area of plants shown in (a), (b), and (c). (f) Boxed area in (a). (g) Boxed area in (b). (h) Boxed area in (c). (i) Magnified view of boxed area in (f). Note the GFP signal in phloem cells (Ph, yellow arrows) and xylem parenchyma cells (Xpc, white arrows). (j) Magnified view of boxed area in (g). Note the reduced number of Xpc. (k) Magnified view of boxed area in (h). Note the DR5rev::GFP signal in nearly all phloem cells (yellow arrows), the increased number of Xpc displaying DR5rev::GFP signal (white arrows) and the presence of protoxylem cells (Pxy) and phloem cap cells (Phc) with an abnormal shape (dashed circles). Xylem tissues (Xy) and fascicular cambium cells (Fc) are overlaid in red and yellow, respectively. Interfascicular cambium (If). (l), Size of xylem pole. Stars (*) or (***) indicate significant differences at $P < 0.05$ or $P < 0.01$ (T-test), respectively. The data are shown as means ($n = 11-17 \pm$ s.e. for (d); $n = 8 \pm$ s.e. for (e); $n = 16-22$ for (m)). Scale bar, 20 μ m in (f-h), 10 μ m in (i-k).

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