

1 **Regulation of intercellular TARGET OF MONOPTEROS 7 protein transport in**  
2 **the *Arabidopsis* root**

3  
4 Kuan-Ju Lu<sup>1</sup>, Bert De Rybel<sup>1,2,3</sup>, Hilda van Mourik<sup>1,4</sup>, and Dolf Weijers<sup>1\*</sup>

5  
6 **Affiliations:**

7 <sup>1</sup> Laboratory of Biochemistry, Wageningen University, Stippeneng 4, 6708 WE,  
8 Wageningen, The Netherlands.

9 <sup>2</sup> Ghent University, Department of Plant Biotechnology and Bioinformatics,  
10 Technologiemark 927, 9052 Ghent, Belgium

11 <sup>3</sup> VIB Center for Plant Systems Biology, Technologiemark 927, 9052 Ghent, Belgium

12 <sup>4</sup> Present address: Laboratory of Molecular Biology, Wageningen University,  
13 6708PB, Wageningen, The Netherlands.

14

15 **Running title:**

16 Control of TMO7 movement

17

18 \* Corresponding author: Dolf Weijers

19 E-mail: [dolf.weijers@wur.nl](mailto:dolf.weijers@wur.nl)

20

21 **Key words:** TMO7, Plasmodesmata, cell-cell communication, protein transport,  
22 embryogenesis, RAM

23

24 **Summary Statement:**

25 Unique protein motifs, subcellular localization and post-translational modification,  
26 rather than protein size regulate plasmodesmatal transport of TMO7 family proteins  
27 during *Arabidopsis* root development.

28

29

30

31

32

33

34 **Abstract**

35 Intercellular communication coordinates hypophysis establishment in the Arabidopsis  
36 embryo. Previously, TARGET OF MONOPTEROS 7 (TMO7) was reported to be  
37 transported to the hypophysis, the founder cell of the root cap, and RNA suppression  
38 experiment implicated its function in embryonic root development. However, it  
39 remained unclear what protein properties and mechanisms mediate TMO7 protein  
40 transport, and what role the movement plays in development. Here, we report that in  
41 the post-embryonic root, TMO7 and its close relatives are transported into the root  
42 cap through plasmodesmata in a sequence, but not size dependent manner. We also  
43 show that nuclear residence is critical for TMO7 transport, and postulate that  
44 modification, potentially phosphorylation, labels TMO7 for transport. Additionally,  
45 three novel CRISPR/Cas9-induced *tmo7* alleles confirmed a role in hypophysis  
46 division, but suggest complex redundancies with close relatives in root formation.  
47 Finally, we demonstrate that TMO7 transport is biologically meaningful, as local  
48 expression partially restores hypophysis division in a plasmodesmatal protein  
49 transport mutant. Our study identifies motifs and amino acids critical for TMO7  
50 protein transport and establishes the importance of TMO7 in hypophysis and root  
51 development.

52

## 53 **Introduction**

54

55 Intercellular communication is vital for development of multi-cellular organisms  
56 (Long et al., 2015), for example to coordinate distant events or locally organize  
57 tissues (Balkunde et al., 2017; Corbesier et al., 2007; Kurata et al., 2005; Nakajima et  
58 al., 2001; Pi et al., 2015; Sessions et al., 2000; Tamaki et al., 2007; Yadav et al.,  
59 2011). Plant cell walls provide robust physical support to build elaborate tissues and  
60 structures; however, cell walls also hamper cell migration, and it is therefore  
61 important for plant cells to receive positional cues from their surroundings for proper  
62 development (ten Hove et al., 2015). To overcome the physical barrier that cell walls  
63 pose to intercellular communication, plants evolved specialized channels connecting  
64 neighbouring cells, named plasmodesmata (PD). PD contain compressed ER tubes,  
65 allowing transport of molecules between cells (Otero et al., 2016).

66 Many plant tissue patterning events have been shown to involve cell-cell  
67 communication (Bernhardt et al., 2005; Helariutta et al., 2000; Kim et al., 2003;  
68 Kurata et al., 2005; Rodriguez et al., 2016; Schlereth et al., 2010). One of the earliest  
69 of such events in plant ontogeny occurs during early embryogenesis in the flowering  
70 plant *Arabidopsis thaliana* (ten Hove et al., 2015). Formation of the embryonic root  
71 requires the AUXIN RESPONSE FACTOR 5/MONOPTEROS (ARF5/MP)  
72 transcription factor, and *mp* mutants fail to form a root (Berleth and Jurgens, 1993;  
73 Hardtke and Berleth, 1998). The root forms from two cell populations: a set of  
74 embryonic cells and their extra-embryonic neighbour (the hypophysis) (Scheres et al.,  
75 1994). Both these cell populations develop abnormally in the *mp* mutant (Berleth and  
76 Jurgens, 1993; Hardtke and Berleth, 1998), but expression of MP only in the  
77 embryonic cells complements not only the embryonic but also the hypophysis defect,  
78 indicating a non-cell-autonomous function(Weijers et al., 2006). Imobility of MP  
79 protein suggests the existence of downstream mobile signals (Weijers et al., 2006). A  
80 direct MP downstream target, TARGET OF MONOPTEROS 7 (TMO7), is  
81 transcribed in embryonic cells but the protein is found in the neighbouring  
82 hypophysis, strongly suggesting protein transport. Fusing TMO7 to triple GFP  
83 (3xGFP) prevented protein accumulation in the hypophysis, suggesting that a size  
84 restriction to movement (Schlereth et al., 2010). Based on RNA suppression  
85 approaches and local expression, TMO7 appears to contribute either to establishing  
86 hypophysis identity or to controlling its cell division plane (Rademacher et al., 2012;

87 Schlereth et al., 2010). TMO7 is an atypical basic Helix-Loop-Helix (bHLH)  
88 transcription factor that lacks the basic region, and is significantly smaller than most  
89 mobile transcription factors studied to date. Key questions are how this protein is  
90 transported, what regulates transport, and what role this plays in development.

91 Several transcription factors that control plant development have been shown  
92 to move between cells. The *Zea mays* *KNOTTED 1* gene (*KNI*), and its homolog in  
93 *Arabidopsis thaliana*, *SHOOT MERISTEMLESS* (*STM*) encode homeobox-domain  
94 (HD) proteins which help maintain the shoot apical meristem (SAM) cells  
95 undifferentiated, and move directionally from inner to outer cell layers (Kim et al.,  
96 2003; Long et al., 1996; Lucas et al., 1995; Vollbrecht et al., 1991). The LEAFY  
97 (LFY) protein, a helix-turn-helix transcription factor that also participates in SAM  
98 development, appears to move by non-directional diffusion (Sessions et al., 2000; Wu  
99 et al., 2003). In addition, WUSCHEL (WUS), another key HD-containing  
100 transcription factor that regulates and maintains SAM activity, was also reported to  
101 move through PD (Daum et al., 2014; Yadav et al., 2011). Likewise, in root  
102 development there are several known mobile transcription factors, among which the  
103 SHORTROOT protein has been studied in most detail. *SHORTROOT* (*SHR*), a GRAS  
104 family transcription factor, is transcribed in stele tissues, but the SHR protein  
105 subsequently moves a layer outward to the endodermis and quiescent centre (QC)  
106 cells *SCARECROW* (*SCR*), another protein in GRAS family, is expressed (Helariutta  
107 et al., 2000; Nakajima et al., 2001). Together with SCR, SHR regulates the expression  
108 of C2H2 zinc-finger domain transcription factors, *JACKDAW* (*JKD*) and *MAGPIE*  
109 (*MGP*) in the endodermis, which forms a feed forward loop with SCR (Welch et al.,  
110 2007). SHR localizes in both cytoplasm and nucleus at stele; the interaction with  
111 SCR, JKD and MGP after movement, however, relocates the protein complex  
112 exclusively to the nucleus and prohibits the further movement of SHR (Gallagher et  
113 al., 2004; Nakajima et al., 2001; Welch et al., 2007). This transport regulation is  
114 critical for proper pattern formation of roots, as ectopic expression of SHR by the  
115 ubiquitous 35S promoter alters cell fate and creates multiplication of cell layers in  
116 root (Nakajima et al., 2001). Despite the importance of intercellular protein transport,  
117 the underlying mechanism is still largely elusive.

118 To reveal protein transport mechanisms, efforts have focused on identifying  
119 the critical transport elements in mobile transcription factors. However, the protein  
120 domains that mediate transport of different proteins do not seem to have common

121 features (Gallagher et al., 2014), and it is therefore unclear how mobile proteins are  
122 selected for transport. Currently, the accumulation and degradation of callose, a  $\beta$ -1,3-  
123 glucan, at the neck region of the PD aperture by Callose Synthase (CalS) and  $\beta$ -1,3-  
124 glucanase, respectively, is the most prominent mechanism regulating cell-cell  
125 communication (Burch-Smith and Zambryski, 2012; Gallagher et al., 2014). A  
126 dominant *CalS* mutant, *cals3-2d*, was identified to regulate the accumulation of  
127 callose in Arabidopsis. By constructing a inducible iCalSm system, transport of SHR  
128 and its regulatory microRNA (miR165/166) was demonstrated to be blocked by the  
129 accumulation of callose at PD (Vaten et al., 2011). Yet, the accumulation of callose  
130 regulates the dilation of PD by physically closure and would not be expected to  
131 contribute to selectivity. Thus, the molecular mechanism of selective protein transport  
132 is still not well understood.

133

134 Here, we report that the TMO7 protein moves through PD, and that its  
135 movement contributes to hypophysis division. We show that TMO7 protein mobility  
136 is shared with a small set of TMO7-like proteins. We further show that sequence, not  
137 protein size determines mobility, and identified protein motifs critical for subcellular  
138 localization and transport. Our study provides a framework for understanding  
139 selective transport of transcription factors in the Arabidopsis root.

140

## 141 **Results**

142

### 143 **TMO7 moves through plasmodesmata in the Arabidopsis root**

144 TMO7 is expressed in the early Arabidopsis embryo, is transported from the pro-  
145 embryo to the hypophysis, and RNA suppression interferes with embryonic root  
146 formation (Schlereth et al., 2010). However, expression levels are extremely low and  
147 immunofluorescence is required to visualise the TMO7-GFP fusion protein in the  
148 embryo. *TMO7* was originally identified as a MP/BDL-dependent gene in a  
149 transcriptome study on seedlings (Schlereth et al., 2010), and we therefore addressed  
150 whether the post-embryonic root would represent a more accessible model for  
151 studying protein movement. We first observed the expression pattern of  
152 *pTMO7::n3GFP* (nucleus localization signal - 3 times GREEN FLUORESCENT  
153 PROTEIN), and compared it to *pTMO7::TMO7-GFP*, and *pTMO7::TMO7-3GFP* in

154 root tips (Fig. 1, Schlereth et al., 2010). The *TMO7* promoter is expressed in  
155 meristematic and lateral root cap cells surrounding the QC and columella cells with a  
156 shootward-declining gradient. While nearly absent, very weak expression can also be  
157 observed in columella cells (Fig. 1A, B). In *pTMO7::TMO7-GFP* plants, we observed  
158 strong fluorescent signals not only in cells with high promoter activity, but also in QC  
159 and columella cells (referred as columella cells hereafter), indicating the rootward  
160 movement of *TMO7-GFP* protein, consistent with the reported movement of *TMO7*  
161 in the *Arabidopsis* embryo (Fig. 1C; Schlereth et al., 2010). In contrast to *TMO7-GFP*,  
162 the expression of *TMO7-3GFP* is highly correlated with the *TMO7* promoter activity  
163 (Fig. 1B, D). Interestingly, the *TMO7-3GFP* protein seems to form aggregates in  
164 several cells, especially in cells above the QC (Fig. 1D). Also, the *TMO7-3GFP*  
165 protein is mostly excluded from the nucleus as low or no fluorescence signal was  
166 detected in the nucleus (Fig. 1D). The absence of fluorescence signal in columella  
167 cells indicates that movement of *TMO7-3GFP* is disrupted; however, weak GFP  
168 fluorescence was detected in cells below the QC in *pTMO7::TMO7-3GFP* roots (Fig.  
169 1D, asterisk mark), which might be due to the weak expression of the *TMO7* promoter.  
170 To distinguish the weak activity of the *TMO7* promoter in columella cells from the  
171 signal derived from protein transport, the per-pixel fluorescence intensity ratio  
172 between columella cells and the rest of meristematic region was quantified (Fig. 1F  
173 and Fig. S1, see detailed description in Experimental Procedures). Consistent with the  
174 qualitative observation, the fluorescence intensity in columella cells is  $28.8 \pm 5.5\%$   
175 (mean  $\pm$  SD, n=19) of the fluorescence in the meristematic region in *pTMO7::n3GFP*  
176 lines. The ratios in *pTMO7::TMO7-GFP* and *pTMO7::TMO7-3GFP* are  $76.9 \pm 9.6\%$   
177 and  $47.5 \pm 7.5\%$  (n=19 and 18; mean $\pm$ SD), respectively (Fig. 1F). These results  
178 indicate that the increase of fluorescence in columella cells in *pTMO7::TMO7-GFP* is  
179 due to the transport of *TMO7-GFP*, which is largely prevented in *pTMO7::TMO7-*  
180 *3GFP*. Importantly, these results show that, as in embryos, *TMO7* protein is mobile in  
181 the post-embryonic root, and that it involves similar constraints.

182         The finding that fusion to 3GFP prohibited transport of *TMO7* suggests that  
183 *TMO7* might, like most other mobile TFs, migrate through plasmodesmata which  
184 have a size restriction (Otero et al., 2016). To further investigate the possible passage  
185 of *TMO7*, we observed the movement of *TMO7-GFP* in the *cals3-2d* mutant, which  
186 over-accumulates callose and prohibits transport via PD (Vaten et al., 2011).

187 Consistent with the notion that TMO7 moves through PD, the movement of TMO7-  
188 GFP was hampered in the primary root of *cals3-2d* mutants (Fig. 1E, F). Based on  
189 these observations we conclude that TMO7 protein moves through PD in the  
190 Arabidopsis root.

191

192 **TMO7 protein sequence, not size, instructs the unidirectional movement into**  
193 **root cap cells**

194 Small proteins (molecule weight lower than 50 KDa) may transport freely through PD  
195 by diffusion (Crawford and Zambryski, 2001; Oparka et al., 1999). Since TMO7 is 93  
196 amino acids long (around 11 KDa), it might thus passively diffuse through PD. To  
197 investigate whether movement is correlated to molecular weight, or rather a  
198 consequence of specific protein features, we focused on other small bHLH proteins.  
199 The Arabidopsis genome encodes four TMO7-like proteins (T7L1-4; De Rybel et al.,  
200 2011), all representing small bHLH TFs (92 to 94 a.a.; Fig. 2A, B). Within the *TMO7*  
201 clade, amino acid sequence is very conserved (up to 45% identity, and 80% similarity;  
202 Fig. 2B). We therefore also selected two other small bHLHs outside the *TMO7* clade,  
203 *AtbHLH138* (AT2G31215, 129 a.a., about 15 KDa) and *AtbHLH151* (AT2G47270,  
204 102 a.a., about 12 KDa) (Fig. 2A, B), to uncouple size from homology. All small  
205 bHLH proteins were expressed as GFP fusion proteins from the *TMO7* promoter, and  
206 we visualized localization (Figure 2C, D) and quantified mobility (Figure 2E).

207 We observed comparable mobility of all proteins within the TMO7-like clade  
208 (Fig. 2D, E). Interestingly, unlike other T7Ls, the bHLH134-GFP protein localized  
209 not only in the nucleus and cytoplasm but also strongly at the cell periphery (Figure  
210 2D). Nonetheless, mobility of bHLH134-GFP was not influenced by this altered  
211 localization (Fig. 2D, E). The unrelated bHLH proteins, bHLH138 and bHLH151,  
212 localize mainly to the nucleus (Figure 2C), consistent with the presence of several  
213 basic residues (K, R – basic residues constitute nuclear localization signals) N-  
214 terminal to the HLH domain (Figure 2B). In addition, bHLH138 also partially  
215 localized in the cytoplasm (Fig. 2C). Yet, despite their small size, no movement of  
216 bHLH138 or bHLH151-GFP could be detected (Fig. 2C, E). Thus, we conclude that  
217 small protein size does not determine TMO7 movement. Rather, the absence of a  
218 clear nuclear localization signal and/or the presence of conserved motifs within the  
219 TMO7 clade proteins may mediate transport through PD.



220 We further explored the transport at the root columella cells. We previously  
221 reported promoter activity of *T7Ls*: *bHLH134*, *bHLH136*, and *bHLH166* are  
222 expressed weakly in the root cap and columella cells while *bHLH161* is expressed  
223 only in the lateral root cap (De Rybel et al., 2011). We therefore generated YFP  
224 fusion protein with the endogenous promoter to explore the direction of transport.  
225 Among these fusion proteins, expression could only be detected for bHLH134-YFP  
226 (not shown) and bHLH166-YFP (Fig 2F). Interestingly, both fusion proteins showed  
227 highest intensity at the very tip of the columella cells with decreasing intensity toward  
228 the shoot, which is comparable to their promoter activity (Fig 2F; De Rybel et al.,  
229 2011). Thus, we conclude that while bHLH134 and 166 proteins are rootward mobile  
230 when expressed in the *TMO7* expression domain, they do not normally move  
231 shootward from their expression site. This finding also suggests that the transport of  
232 *TMO7*-like proteins is unidirectional between meristematic cells and root cap cells.

233

#### 234 **Protein sequence elements and subcellular localization define *TMO7* transport**

235 Given that the *TMO7* protein sequence directs transport, we aimed to map the crucial  
236 region(s) by systematic mutation. We hypothesized that mutations which affect  
237 *TMO7* mobility elements would disrupt transport. We performed a linker-scanning  
238 analysis by replacing regions of seven to nine amino acids with an poly-Alanine  
239 linker of the same length, and thus generated eleven *TMO7* mutants  
240 (*pTMO7::TMO7<sub>m1</sub>*-GFP to *pTMO7::TMO7<sub>m11</sub>*-GFP; Fig. 3A). The relative intensity  
241 of fluorescence in the tip versus meristem was quantified in more than 10 independent  
242 transgenic lines for each mutant (except for *m10*; only 5 T2 lines), and lines showed  
243 some variability in transport (Figure 3C). Yet, transport appeared unaffected in most  
244 mutant versions (Figure 3B, C). We found consistent reduction in movement for three  
245 mutant proteins, *TMO7<sub>m5</sub>*, *TMO7<sub>m8</sub>* and *TMO7<sub>m9</sub>*, similar to the pattern found in  
246 *pTMO7::TMO7*-3GFP (Fig. 3B, C). In addition, *TMO7<sub>m2</sub>*- and *TMO7<sub>m3</sub>*-GFP showed  
247 movement defects in some, but not all transgenic lines (Fig. 3B, C). These results  
248 demonstrate that movement of *TMO7* depends on at least two major and one minor  
249 elements.

250 By comparing the sequence between *TMO7*-like family proteins and the  
251 immobile bHLH138/151 proteins, we found the M5 region to locate within the  
252 conserved HLH domain and with very high conservation between all *TMO7* families.  
253 However, bHLH138 and bHLH151 have limited similarity with the M5 region (Fig



254 2B). The M8/9 regions locate immediately C-terminal to the HLH domain, and this  
255 region is also highly conserved within the TMO7 family, but different from bHLH138  
256 and bHLH151 (Fig 2B). In addition, we also compared the bHLH domain of  
257 GLABRA3 (bHLH1), a known mobile protein that regulates root hair formation  
258 (Bernhardt et al., 2005), with TMO7 and we only found limited similarity at the M5  
259 or M8, 9 regions (data not shown), indicating that tissue-specific transport systems  
260 may operate in the Arabidopsis root. These data suggest that M5 and M8/9 regions  
261 may be specific elements responsible for mobility of TMO7 family proteins.

262 An intuitive hypothesis would be that the *m5* and *m8/9* mutations alter the  
263 secondary or tertiary structure of the TMO7 protein and thus lead to movement  
264 defects. We analysed the possible secondary structure with the SWISS-MODEL  
265 protein-folding tool (<http://swissmodel.expasy.org/interactive>). The predicted TMO7  
266 structure is very similar to the bHLH transcription factor MyoD (Fig S2B; Ma et al.,  
267 1994); however, none of the mutants that disrupt movement is predicted to have a  
268 different secondary structure (Fig. S2A). It is therefore not directly evident how the  
269 mutations would affect TMO7 protein properties, but given that no alternate  
270 secondary structures are predicted, the mutations may also not have dramatic  
271 consequences for protein folding.

272 To further test whether M5, M8 and M9 are *bona fide* mobility elements,  
273 sufficient for driving movement, we inserted these elements into bHLH138 and  
274 bHLH151 to create chimaeric proteins. Since the syntax surrounding M5 and M8/9  
275 may also influence their function, chimaeric proteins were designed based on the  
276 syntax of TMO7. Based on the protein-folding prediction, M5 is localized at the loop  
277 region of HLH domain (Fig. S2B). We replaced the loop sequence of bHLH138 and  
278 151 by the TMO7 M5 sequence to create bHLH138-M5 and bHLH151-M5. Since  
279 there is no predicted secondary structure at the M8/9 region, and because it directly  
280 follows the HLH region in TMO7, we directly inserted the M8/9 region after the  
281 bHLH domain of bHLH138 and 151 to generate bHLH138-M8/9 and bHLH151-  
282 M8/9 respectively. The combination of M5 and M8/9 was also constructed as  
283 bHLH138-M5/8/9 and bHLH151-M5/8/9 (Fig. 4). All the chimeric bHLH138 and  
284 bHLH151 versions except bHLH151-M5-GFP slightly gained movement (Fig. 4B).  
285 However, compared to TMO7, movement of chimaeric bHLH138/151 was limited  
286 (Fig. 4B), perhaps due to strong nuclear localization of bHLH138 and 151 (Fig. 2C  
287 and 4A). It was previously shown that movement of mobile proteins can be altered by

288 localizing the protein to specific subcellular compartments (Crawford and Zambryski,  
289 2000; Daum et al., 2014; Gallagher et al., 2004; Kim et al., 2005; Rodriguez et al.,  
290 2016; Tamaki et al., 2007).

291 However, in contrast to a sole movement-restricting capacity of nuclear  
292 localization, in our linker scanning analysis, we also often observed a lack of nuclear  
293 protein in movement-defective mutants (Fig. 3B and 5A). This suggests that transport  
294 into the nucleus may contribute to TMO7 transport. To test whether strong subcellular  
295 localization interferes with the mobility of the protein, we generated TMO7-GFP  
296 protein with SV40 nucleus localization (NLS) or nuclear export signals (NES) at the  
297 N- or C-terminus of TMO7-GFP, respectively (NLS-TMO7-GFP; TMO7-GFP-NES).  
298 Like in other mobile proteins (Balkunde et al., 2017; Gallagher et al., 2004;  
299 Rodriguez et al., 2016), the nuclear localization signal restricted TMO7 movement.  
300 Surprisingly however, the NES also hindered its mobility (Fig 5B-D), suggesting that  
301 entering as well as leaving the nucleus might be crucial for TMO7 transport.

302 In summary, the TMO7 family contains two major mobile *cis*-elements which  
303 partially endow mobility of bHLH138/151 when transplanted. However, the mobility  
304 strongly depends on subcellular localization; the strong NLS decreases the potential  
305 of direct interaction between TMO7 and PD, while the result of the TMO7-GFP-NES  
306 suggests that the import of TMO7 into nucleus is also critical for its movement.

307

### 308 **Phosphorylation may control TMO7 mobility**

309 Given that specific motifs, as well as residence in the nucleus, seem important for  
310 TMO7 movement, we hypothesized that TMO7 transport involves post-translational  
311 modification. To start exploring this option, we first analysed the potential of  
312 phosphorylation on TMO7 using a prediction server  
313 (<http://www.dabi.temple.edu/disphos/>). Among the 19 Serine, Threonine and  
314 Tyrosine residues, phosphorylation was predicted to occur on 8 (Table S2). Among  
315 these, S39 and S42 are located within the M5 region (Fig. 3A). We therefore  
316 substituted each and both of the Serine residues by Alanines to create  
317 *pTMO7::TMO7-S39A-*, *S42A-* and *S39, 42A-GFP*, respectively. The subcellular  
318 localization of TMO7 was not altered by the mutations, indicating that protein import  
319 into the nucleus is not impaired by the mutation (Fig. 6A, B and C). Interestingly  
320 however, all mutants showed impaired mobility (Fig. 6A-D), which suggests that both  
321 of these potential phosphorylation sites are important for TMO7 transport.

322

323 **Characterization of a stable *tmo7* mutant reveals complex regulatory**  
324 **interactions**

325 To address the importance of TMO7 transport, we first aimed to generate lost-of-  
326 function resources. Previously, we described a mild hypophysis division phenotype,  
327 as well as a low-penetrance rootless seedling defect in lines that had the *TMO7* gene  
328 silenced using RNAi or artificial microRNA expression. No phenotype could be  
329 observed in the available *tmo7-1* and *tmo7-2* insertion mutants (Schlereth et al.,  
330 2010). It is possible that RNAi and amiRNA approaches targeted homologs as well as  
331 *TMO7*, or alternatively it could be that the *tmo7-1* and *tmo7-2* insertion lines may not  
332 represent null alleles. Thus, we generated mutants through CRISPR-Cas9 gene editing  
333 (Tsutsui and Higashiyama, 2016). We designed a short-guide RNA targeting the M5  
334 mobile element and obtained 3 independent mutant alleles (*tmo7-4*, *tmo7-5* and *tmo7-*  
335 *6*), each creating shorter and mutated predicted proteins (Fig. 7A, B). In all CRISPR  
336 *tmo7* alleles, we observed that root length was reduced compare to wild-type plants  
337 (Figure 7C, D). In addition, we consistently found hypophysis division defects in all  
338 three mutant alleles (*tmo7-4* = 12.4%, n= 331; *tmo7-5* = 5.6%, n=302; *tmo7-6* = 5%,  
339 n=357; Col = 1.8%, n= 277; Fig 7E). However, the rootless phenotype that was  
340 observed in 1-7% of seedlings in *amiRTMO7* and *TMO7 RNAi* lines (Schlereth et al.,  
341 2010), was not recovered in the *tmo7-4*, *tmo7-5* and *tmo7-6* alleles (n>1000 seedlings  
342 for *tmo7-4* and *tmo7-5*, n>600 for *tmo7-6*). This suggests that the hypophysis defect  
343 might later be repaired by a yet unknown mechanism in *tmo7-4*, -5, and -6 mutants.

344 One possibility is that the TMO7-like proteins act redundantly with TMO7;  
345 alternatively and additionally, *TMO7-like* genes may be involved in later stages and  
346 help generate an embryonic root following initial defects upon TMO7 depletion. In  
347 either scenario, it would be expected that the expression of *TMO7-like* genes is  
348 misregulated upon *TMO7* gene silencing, for example as an off-target effect. To test  
349 this possibility, we quantified *TMO7Ls* expression in primary roots of TMO7 RNA  
350 suppression lines by quantitative reverse transcription poly-chain reaction (qRT-  
351 PCR). The results confirm that the *TMO7* transcript is down-regulated between 15  
352 and 70% in both *amiRTMO7* and *TMO7 RNAi* lines (Fig. 7F). Furthermore, we  
353 observed complex changes in the regulation of *TMO7* homologues, as different  
354 *TMO7-like* genes are down-regulated in different gene silencing lines, while others  
355 are up-regulated (Fig. 7F). In all *tmo7* CRISPR alleles, *TMO7* and all *TMO7-like*

356 genes were expressed at a level comparative to wild-type (data not shown). These  
357 results are consistent with the notion that redundancy among *TMO7-like* genes may  
358 contribute to embryonic root formation. In RNA suppression lines, multiple homologs  
359 are affected, which may be the cause for the rootless phenotype. Clearly, a higher-  
360 order (CRISPR/Cas9) mutant, knocking out *TMO7-like* genes as well as *TMO7*, will  
361 be required to explore the function of *TMO7* in embryonic root formation.

362

### 363 **TMO7 movement contributes to hypophysis division**

364 Likely functional redundancy with close relatives prevents us from directly testing  
365 whether *TMO7* movement is critical for hypophysis and embryonic root development.  
366 However, we employed the *cals3-2d* mutant to indirectly address this question. The  
367 hyper-accumulation of callose in *cals3-2d* mutant prohibits the movement of proteins,  
368 and we noticed that the mutant (Vaten et al., 2011) shows a strong rootless phenotype,  
369 resembling the *monopteros* (*mp*) (Berleth and Jurgens, 1993) and *TMO7*  
370 *RNAi/amiRTMO7* phenotype (Fig 8A). A detailed analysis of cell division patterns in  
371 the embryo showed a lack of hypophysis division in nearly all *cals3-2d* mutant  
372 embryos (Fig 8C, D; 95%; n>700 embryos). As plasmodesmata were reported to be  
373 involved in auxin transport (Han et al., 2014), and because auxin transport from  
374 embryo to hypophysis promotes root formation (Friml et al., 2003; Weijers et al.,  
375 2006), we first tested if the hypophysis and root defect in the *cals3-2d* mutant is  
376 related to an auxin accumulation problem. We transformed an auxin response  
377 reporter, *pDR5::nGFP*, (Weijers et al., 2006) into the *cals3-2d* background.  
378 Strikingly, the expression of *pDR5::nGFP* in the hypophysis was not affected by  
379 *cals3-2d* (Fig 8B), which suggests that auxin is normally transported to the  
380 hypophysis in the mutant.

381 Due to difficulties with detecting the low *TMO7* protein amounts in the  
382 embryo, we could not determine if *TMO7* movement is affected in the *cals3-2d*  
383 mutant embryo (not shown). However, given that *TMO7* movement in the root of the  
384 *cals3-2d* mutant is impaired (Fig 1E, F), we tested if expression of *TMO7* in the cells  
385 to which it is normally transported (uppermost suspensor cells) would alleviate the  
386 division defect in the *cals3-2d* mutant. Therefore, *TMO7* was expressed from the  
387 suspensor-specific *ARF13* promoter (Rademacher et al., 2012). By counting the  
388 correct division frequency of the hypophysis in both *pARF13::TMO7* and the  
389 *pDR5::nGFP* control, we found a partial rescue of hypophysis division in *cals3-2d* by

390 *pARF13::TMO7* expression (Fig 8E;  $14.4 \pm 7.3\%$  with *pARF13::TMO7* compared to  
391  $6.0 \pm 1.5\%$  with *pDR5::nGFP*; n=7 and 9 lines respectively with at least 90  
392 individuals for each line; p=0.0126 by two-tail student t-test). These results suggest  
393 that the hypophysis division phenotype in the *cals3-2d* mutant is likely caused by PD  
394 obstruction, which prevents *TMO7* movement, but not auxin transport. Our findings  
395 thus indicate that *TMO7* mobility contributes to hypophysis division.

396

## 397 **Discussion**

398

399 In this study, we systematically analysed the mobility of a specific small bHLH  
400 transcription factor family protein. We conclude that transport of *TMO7* is sequence-  
401 dependent, but not primarily determined by protein size. Interestingly, the shuttling  
402 into and out of the nucleus appears to be critical for mobility (Fig. 5). We postulate  
403 that this shuttling into the nucleus labels the protein for transport to other cells. The  
404 importance of nuclear localization for a mobile protein has also been proposed for the  
405 transport of *SHR* and another mobile R3-type MYB-like protein, *CAPRICE* (*CPC*),  
406 which expresses in the non-hair epidermal cells in the roots and moves to the hair  
407 cells to specify the formation of the root hairs (Gallagher and Benfey, 2009;  
408 Gallagher et al., 2004; Kurata et al., 2005). A T289>I point mutation in *SHR*,  
409 prevents the import of *SHR* into the nucleus and its intercellular transport while the  
410 W76A mutation of *CPC* largely reduced its nuclear localization and prevented its  
411 movement (Gallagher et al., 2004; Kurata et al., 2005). The Tyrosine residue 289 of  
412 *SHR* was predicted to be a phosphor-acceptor which functions in dimerization and  
413 proper localization (Gallagher et al., 2004). Consistent with the role of T289 in *SHR*,  
414 the serine residues (S39 and S42) in *TMO7* were also predicted to be phosphorylated.  
415 Replacing the residues by Alanine hampers the intercellular movement, which  
416 suggests that phosphorylation might be a mark for intercellular transport (Fig 6).  
417 However, we cannot rule out the possibility that these mutations act independently of  
418 phosphorylation by e.g. distorting the protein structure or preventing the interaction  
419 with an unknown factor which might be critical for transport, but not for nuclear  
420 import. Further analysis of the post-translational modification status of mobile  
421 proteins will likely provide more insight to intercellular communication mechanisms.

422 We previously showed that *TMO7* is only transported into the hypophysis, but  
423 not into the upper tier of the pro-embryo (Schlereth et al., 2010). Consistent with this

424 directional transport property in the embryo, we found that TMO7 family proteins can  
425 move from the root meristematic region into the root cap, but not in the opposite  
426 direction (Fig 2D, F). In addition, in our *pTMO7::TMO7-3GFP* line, we observed  
427 obvious aggregations in the seedling root which is lacking in *pTMO7::TMO7-GFP*  
428 and other TMO7Ls-GFP fusion lines (Fig 1 and 2). The aggregations seem to be  
429 highly enriched in the meristematic cells closest to the neighbouring QC (Fig .1D). It  
430 is possible that these clusters of proteins are TMO7 with intercellular transport  
431 markers but over the limit size for passing through PD. Interestingly, the stem cell  
432 maintenance transcription factor WUSCHEL HOMEBOX 5 (WOX5) has also been  
433 shown to move from the QC into the columella stem cells, and no shootward transport  
434 was observed (Pi et al., 2015). These data suggest that different regulatory  
435 mechanisms exist to control the rootward and shootward transport between root cap  
436 cells and other meristematic niche cells.

437         The cell walls between the pro-embryo and suspensor and between the upper  
438 and lower tiers in the embryo are generated by the very first and second cell divisions  
439 during embryogenesis (Scheres et al., 1994; ten Hove et al., 2015). The symplastic  
440 transport between suspensor and pro-embryo and within the pro-embryo was shown  
441 to be liberal until the globular stage, as soluble GFP moved from the suspensor to the  
442 whole embryo when expressed from the *AtSUC3* promoter and across the whole  
443 embryo when expressed from the *AtSTM* promoter (Stadler et al., 2005). Interestingly,  
444 the shootward mobility of TMO7 to upper-tier cells is limited in the early embryo,  
445 which suggests the symplastic transport control between the two tiers is established  
446 early in embryogenesis. How and why this control is established, and at which  
447 developmental stage is a very intriguing question.

448         In *tmo7-4*, *-5*, and *-6*, we observed a clear hypophysis phenotype, which is  
449 consistent with the previous finding that *TMO7* is involved in hypophysis  
450 development. Interestingly however, no rootless phenotype was recovered. A possible  
451 scenario is that in the early embryo, TMO7 together with other mobile factors  
452 coordinate the development of hypophysis while other TMO7 family genes control  
453 the later development of QC and root cap. This scenario could explain why,  
454 hypophysis errors in *tmo7* CRISPR mutants do not lead to rootless defects. Indeed,  
455 both *TMO7* gene silencing lines and *tmo7* CRISPR lines give rise to hypophysis  
456 defects but only the gene silencing lines show a low-frequency rootless phenotype, in  
457 which *TMO7* family genes were misregulated (Fig 7F). It has been shown that the



458 *TMO7* family genes are not expressed at least until the late heart stage during  
459 embryogenesis but are expressed in the post-embryonic root in the columella and  
460 lateral root cap (De Rybel et al., 2011). Therefore, the misregulation of *TMO7* family  
461 genes in RNA suppression lines might partially impair a redundancy-based repair  
462 mechanism. A higher-order *TMO7* family mutant might reveal how *TMO7* family  
463 genes help the development of hypophysis and post-embryonic root, and may also  
464 provide insight into the genes and cellular pathways that are controlled by mobile  
465 *TMO7/T7L* proteins in root formation and development.

## 466 **Materials and methods**

467

### 468 ***Plant materials***

469 All seeds were surface sterilized, sown on 1/2 strength MS with 0.01% MES, 1%  
470 sucrose and 0.8% Daishin agar plates, and stratified for 1 day at 4°C in the dark  
471 before they were grown under long-day (16/8 hr) conditions with a constant  
472 temperature of 22°C in a growth room. The *TMO7* reporter lines, *pTMO7::3nGFP*,  
473 *pTMO7::TMO7-GFP* and *pTMO7::TMO7-3GFP* have been previously described  
474 (Schlereth et al., 2010). The *cals3-2d* mutant (Vaten et al., 2011) was kindly provided  
475 by Ykä Helariutta (SLCU, Cambridge).

476

### 477 ***Cloning***

478 All constructs except those for CRISRP-Cas9 gene editing were generated by LIC  
479 cloning methods with pPLV02 vectors as previously described (De Rybel et al.,  
480 2011). For mobility analysis of bHLH138/151, *TMO7* family proteins, and *TMO7*  
481 S39, 42A mutants, a vector containing *TMO7* promoter (*pTMO7* vector) was first  
482 created by amplification of a 2.2kb promoter fragment from *pTMO7-n3GFP* that was  
483 subsequently ligated through *EcoRI* and *BamHI* sites with the pPLV02 vector. The  
484 target genes and GFP were amplified separately with overlapping linkers and further  
485 fused by fusion PCR with primers containing the LIC adaptor. The *TMO7* S39, 42A  
486 mutants were amplified using site direct mutagenesis primers. The 5' and 3'  
487 fragments were further fused together by PCR primers containing the LIC adaptor.  
488 The fused fragments were integrated with the *pTMO7* vector by LIC cloning. For  
489 Alanine-linker scanning, the 2.2kb promoter of *TMO7* plus the 5' fragments before  
490 the mutation site and the 3' fragments after the mutation site plus the GFP tag were



491 separately amplified by PCR. The two fragments were fused by PCR with primers  
492 containing the LIC site and further integrated with pPLV02 by LIC cloning. All  
493 primers are listed in table S1.

494

#### 495 ***Microscopic and expression analysis***

496 For imaging of roots, 5-d-old-seedlings were incubated in 10 mg/mL propidium  
497 iodide solution for 1-2 minutes. GFP and propidium iodide were visualized by  
498 excitation at 488 nm and detection between 500 to 535 nm and 630 to 700 nm,  
499 respectively. For fluorescence ratio analysis, the two regions of interest (ROI) were  
500 selected in the Leica Application Suite (LAS) program as in Fig S1. The QC and three  
501 layers of columella cells were selected as ROI1; the lateral root cap cells and cells in  
502 the stem cell niche (up to the twelfth cortex cell) were selected as ROI2. To prevent  
503 fluorescence intensity variation due to the tilting of the root, fluorescence of PI  
504 staining was used as the reference. The intensity ratio between the two channel of  
505 individual ROI was first calculated, and the final intensity ratio was calculated by  
506 dividing the ratio of ROI1 by ROI2. For the movement analysis in the *cals3-2d*  
507 mutant, the ROIs were selected by morphology, besides the outer most root cap cells,  
508 three layers of cells near the root tip were selected as ROI1, cells within 5 layers of  
509 epidermal cells were selected as the ROI2 region (Fig. S1). For imaging of embryos,  
510 ovules were isolated and mounted in a 4% paraformaldehyde/5% glycerol/PBS  
511 solution including 1.5% SCRI Renaissance Stain 2200 (R2200; Renaissance  
512 Chemicals) for counterstaining of embryos. After applying the coverslip, the embryos  
513 were squeezed out of the ovules, and R2200 and GFP fluorescence were visualized by  
514 excitation at 405 and 488 nm and detection between 430 to 470 and 500 to 535 nm,  
515 respectively. All confocal imaging was performed on a Leica SP5 II system equipped  
516 with hybrid detectors. For differential interference contrast (DIC) microscopy,  
517 dissected ovules were mounted in chloral hydrate solution (chloral hydrate, water, and  
518 glycerol 8:3:1, W/V/V). After incubating overnight at 4° C, samples were investigated  
519 with a Leica DMR microscope equipped with DIC optics.

520

#### 521 ***Phylogenetic and sequences comparison analysis***

522 The phylogenetic relationship of selected bHLH family proteins were analysed by  
523 Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The protein sequences

524 were obtained from TAIR website (<https://www.arabidopsis.org>) with the following  
525 AGI numbers: AT1G19850 (ARF5/MONOPTEROS), AT2G47270 (bHLH151),  
526 AT3G25710 (bHLH32/TMO5), AT2G31215 (bHLH138), AT1G74500  
527 (bHLH135/TMO7), AT2G41130 (bHLH106/TMO5L2), At1g64625 (bHLH157),  
528 AT3G06590 (bHLH148/AIF2), AT2G43060 (bHLH158), AT1G68810  
529 (bHLH30/TMO5L1), AT1G09250 (bHLH149/AIF4), AT3G28857  
530 (bHLH166/TMO7L4), AT3G05800 (bHLH150/AIF1), AT3G47710  
531 (bHLH161/TMO7L1), AT3G17100 (bHLH147/AIF3), AT2G31280 (bHLH155),  
532 AT5G39860 (bHLH136/TMO7L3), and AT3G56770 (bHLH107/TMO5L3).

533

#### 534 ***Quantitative RT-PCR Analysis***

535 Total RNA of Arabidopsis seedling roots was extracted with the RNeasy kit  
536 (QIAGEN). Poly(dT) cDNA was prepared from 1 µg of total RNA with an iScript  
537 cDNA Synthesis Kit (Biorad) and analyzed on a CFX384 Real-Time PCR detection  
538 system (BioRad) with iQ SYBR Green Supermix (BioRad) according to the  
539 manufacturer's instructions. Primer pairs were designed with the Beacon Designer 7.0  
540 (Premier Biosoft International). All individual reactions were done in triplicate with  
541 three biological replicates. Data were analysed with qBase (Hellemans et al., 2007).  
542 Expression levels were normalized to *ACTIN2* (AT3G18780). The oligo-nucleotide  
543 sequences are listed in Table S1.

544

#### 545 ***Statistical analysis***

546 For relative intensity ratio comparison, we took confocal images from homozygous  
547 T3 lines (for *pTMO7::3nGFP*, *pTMO7::TMO7-GFP*, and *pTMO7::TMO7-3GFP*,  
548 *pTMO7::TMO7-GFP/clas3-2d*,  $n=19, 19, 18,$  and  $13$  respectively), homozygous T2  
549 lines (for *TMO7* likes: as least 6 independent lines and 18 to 25 images in total; for  
550 *TMO7* linker-scanning mutants: at least 10 independent lines (beside *m10*, 5  
551 independent lines, 11 images) and 15 images in total; for NLS and NES analysis: at 6  
552 independent lines, 17 and 19 images; for *TMO7-S39A* and *S42A*,  $n= 6$  and  $7$   
553 independent lines, 13 and 16 images ), and T1 lines (for *bHLH138/151-GFP*,  $n= 7$   
554 and  $13$ ; *bHLH138-M5*, *-M8/9*, *-M5/8/9*,  $n= 10, 7,$  and  $4$ ; *bHLH151-M5*, *-M8/9*, *-*  
555 *M5/8/9*,  $n=10, 15,$  and  $4$ ; for *TMO7-S39,42A*,  $n=7$  independent lines, respectively).  
556 Images were taken and the ROI1/ROI2 ratio were calculated as described above. The  
557 data were box-plotted and analysed by one way ANOVA between every two data

558 sets, the same label was give when  $p > 0.05$ . Note that we use the same  
559 *pTMO7::3nGFP*, *pTMO7::TMO7-GFP*, and *pTMO7::TMO7-3GFP* data set as  
560 references in all figures and *pTMO7::bHLH138-* and *bHLH151-GFP* data set in Fig. 4  
561 as references for clarity.

562 For CRISPR *tmo7* mutant root length analysis, seedling images were taken by  
563 scanning 7 days after germinating on 1/2MS-agar plate. The root length was analysed  
564 by ImageJ (<https://imagej.net>). Student t-test was used to analyse the significance.

565

### 566 **CRISPR-Cas9 *tmo7* mutant generation**

567 *tmo7* CRISPR constructs were designed as previously described (Tsutsui and  
568 Higashiyama, 2016) with minor modification. The short guidance RNA (sgRNA)  
569 sequence was designed in the reverse primer to amplify the U6 promoter and the  
570 forward primer to amplify the sgRNA scaffold. The two fragments were fused  
571 together using the sgRNA sequence as the overlapping region and amplified with  
572 primers with adaptor sequence complementary to pKIR1.1 vector. The fused fragment  
573 was further integrated with the pKIR1.1 by SLiCE cloning method (Zhang et al.,  
574 2014). After transformation, the red T1 Arabidopsis seeds were selected under the  
575 Leica M205 FA microscope equipped with epifluorescence. The T1 inflorescence  
576 apices were collected for genotyping (primers are listed in Table S1). The sequencing  
577 results were analysed using web-tool TIDE (<https://tide.nki.nl>). The T2 generation of  
578 the transformants with high genome modification probability were harvested and non-  
579 fluorescent seeds were grown for genotyping analysis. Homozygous T3 were selected  
580 for embryonic and rootless phenotype analyses.

581

### 582 **Acknowledgement**

583

584 We thank Ykä Helariutta for generously sharing the *cals3-2d* mutant, Maritza van  
585 Dop, Colette ten Hove, Margo Smit, Prasad Vadepalli and Jos Wendrich for  
586 constructive feedback on the manuscript and members of the Weijers group for  
587 fruitful discussions.

588

### 589 **Competing interest**

590

591 No competing interests are declared

592

593 **Author contributions**

594

595 K.J.L., B.D.R and D.W. designed the experiments. H.v.M. and B.D.R. performed the  
596 *cals3-2d* rescue analysis. K.J.L. performed the rest of experiments. K.J.L and D.W  
597 wrote the manuscript with input from B.D.R..

598

599

600

601

602 **Funding**

603

604 This work was supported by a fellowship from the Ministry of Science and  
605 Technology of the Republic of China (grant no. 103-2917-I-564-021) to K.J.L.; by a  
606 grant from the European Research Council (ERC; StG CELLPATTERN; contract  
607 number 281573) to D.W. and by grants from the Netherlands Organization for  
608 Scientific Research (NWO; VIDI grant 842.06.012) and The Research Foundation -  
609 Flanders (FWO; Odysseus II G0D0515N) to B.D.R..

610

611

612

613 **References**

614

615 **Balkunde, R., Kitagawa, M., Xu, X. M., Wang, J. and Jackson, D.** (2017).  
616 SHOOT MERISTEMLESS trafficking controls axillary meristem formation,  
617 meristem size and organ boundaries in Arabidopsis. *The Plant Journal*.

618 **Berleth, T. and Jurgens, G.** (1993). The Role of the Monopteros Gene in Organizing  
619 the Basal Body Region of the Arabidopsis Embryo. *Development* **118**, 575-  
620 587.

621 **Bernhardt, C., Zhao, M., Gonzalez, A., Lloyd, A. and Schiefelbein, J.** (2005). The  
622 bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit  
623 that controls cell patterning in the Arabidopsis root epidermis. *Development*  
624 **132**, 291-298.

625 **Burch-Smith, T. M. and Zambryski, P. C.** (2012). Plasmodesmata paradigm shift:  
626 regulation from without versus within. *Annu Rev Plant Biol* **63**, 239-260.

627 **Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis,**  
628 **A., Farrona, S., Gissot, L., Turnbull, C., et al.** (2007). FT protein movement  
629 contributes to long-distance signaling in floral induction of Arabidopsis.  
630 *Science* **316**, 1030-1033.

631 **Crawford, K. M. and Zambryski, P. C.** (2000). Subcellular localization determines  
632 the availability of non-targeted proteins to plasmodesmatal transport. *Curr*  
633 *Biol* **10**, 1032-1040.

634 ---- (2001). Non-targeted and targeted protein movement through plasmodesmata in  
635 leaves in different developmental and physiological states. *Plant Physiol* **125**,  
636 1802-1812.

637 **Daum, G., Medzihradzky, A., Suzaki, T. and Lohmann, J. U.** (2014). A  
638 mechanistic framework for noncell autonomous stem cell induction in  
639 Arabidopsis. *Proc Natl Acad Sci U S A* **111**, 14619-14624.

640 **De Rybel, B., van den Berg, W., Lokerse, A., Liao, C. Y., van Mourik, H., Moller,**  
641 **B., Peris, C. L. and Weijers, D.** (2011). A versatile set of ligation-  
642 independent cloning vectors for functional studies in plants. *Plant Physiol*  
643 **156**, 1292-1299.

644 **Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa,**  
645 **R. and Jurgens, G.** (2003). Efflux-dependent auxin gradients establish the  
646 apical-basal axis of Arabidopsis. *Nature* **426**, 147-153.

647 **Gallagher, K. L. and Benfey, P. N.** (2009). Both the conserved GRAS domain and  
648 nuclear localization are required for SHORT-ROOT movement. *Plant J* **57**,  
649 785-797.

650 **Gallagher, K. L., Paquette, A. J., Nakajima, K. and Benfey, P. N.** (2004).  
651 Mechanisms regulating SHORT-ROOT intercellular movement. *Curr Biol* **14**,  
652 1847-1851.

653 **Gallagher, K. L., Sozzani, R. and Lee, C. M.** (2014). Intercellular protein  
654 movement: deciphering the language of development. *Annu Rev Cell Dev Biol*  
655 **30**, 207-233.

656 **Han, X., Hyun, T. K., Zhang, M., Kumar, R., Koh, E. J., Kang, B. H., Lucas, W.**  
657 **J. and Kim, J. Y.** (2014). Auxin-callose-mediated plasmodesmal gating is  
658 essential for tropic auxin gradient formation and signaling. *Dev Cell* **28**, 132-  
659 146.

- 660 **Hardtke, C. S. and Berleth, T.** (1998). The Arabidopsis gene MONOPTEROS  
661 encodes a transcription factor mediating embryo axis formation and vascular  
662 development. *Embo J* **17**, 1405-1411.
- 663 **Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G.,**  
664 **Hauser, M. T. and Benfey, P. N.** (2000). The SHORT-ROOT gene controls  
665 radial patterning of the Arabidopsis root through radial signaling. *Cell* **101**,  
666 555-567.
- 667 **Kim, J. Y., Rim, Y., Wang, J. and Jackson, D.** (2005). A novel cell-to-cell  
668 trafficking assay indicates that the KNOX homeodomain is necessary and  
669 sufficient for intercellular protein and mRNA trafficking. *Genes Dev* **19**, 788-  
670 793.
- 671 **Kim, J. Y., Yuan, Z. and Jackson, D.** (2003). Developmental regulation and  
672 significance of KNOX protein trafficking in Arabidopsis. *Development* **130**,  
673 4351-4362.
- 674 **Kurata, T., Ishida, T., Kawabata-Awai, C., Noguchi, M., Hattori, S., Sano, R.,**  
675 **Nagasaka, R., Tominaga, R., Koshino-Kimura, Y., Kato, T., et al.** (2005).  
676 Cell-to-cell movement of the CAPRICE protein in Arabidopsis root epidermal  
677 cell differentiation. *Development* **132**, 5387-5398.
- 678 **Long, J. A., Moan, E. I., Medford, J. I. and Barton, M. K.** (1996). A member of  
679 the KNOTTED class of homeodomain proteins encoded by the STM gene of  
680 Arabidopsis. *Nature* **379**, 66-69.
- 681 **Long, Y., Scheres, B. and Blilou, I.** (2015). The logic of communication: roles for  
682 mobile transcription factors in plants. *J Exp Bot* **66**, 1133-1144.
- 683 **Lucas, W. J., Bouche-Pillon, S., Jackson, D. P., Nguyen, L., Baker, L., Ding, B.**  
684 **and Hake, S.** (1995). Selective trafficking of KNOTTED1 homeodomain  
685 protein and its mRNA through plasmodesmata. *Science* **270**, 1980-1983.
- 686 **Ma, P. C., Rould, M. A., Weintraub, H. and Pabo, C. O.** (1994). Crystal structure  
687 of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and  
688 implications for transcriptional activation. *Cell* **77**, 451-459.
- 689 **Nakajima, K., Sena, G., Nawy, T. and Benfey, P. N.** (2001). Intercellular  
690 movement of the putative transcription factor SHR in root patterning. *Nature*  
691 **413**, 307-311.
- 692 **Oparka, K. J., Roberts, A. G., Boevink, P., Santa Cruz, S., Roberts, I., Pradel, K.**  
693 **S., Imlau, A., Kotlizky, G., Sauer, N. and Epel, B.** (1999). Simple, but not  
694 branched, plasmodesmata allow the nonspecific trafficking of proteins in  
695 developing tobacco leaves. *Cell* **97**, 743-754.
- 696 **Otero, S., Helariutta, Y. and Benitez-Alfonso, Y.** (2016). Symplastic  
697 communication in organ formation and tissue patterning. *Curr Opin Plant Biol*  
698 **29**, 21-28.
- 699 **Pi, L., Aichinger, E., van der Graaff, E., Llavata-Peris, C. I., Weijers, D., Hennig,**  
700 **L., Groot, E. and Laux, T.** (2015). Organizer-Derived WOX5 Signal  
701 Maintains Root Columella Stem Cells through Chromatin-Mediated  
702 Repression of CDF4 Expression. *Dev Cell* **33**, 576-588.
- 703 **Rademacher, E. H., Lokerse, A. S., Schlereth, A., Llavata-Peris, C. I., Bayer, M.,**  
704 **Kientz, M., Freire Rios, A., Borst, J. W., Lukowitz, W., Jurgens, G., et al.**  
705 (2012). Different auxin response machineries control distinct cell fates in the  
706 early plant embryo. *Dev Cell* **22**, 211-222.
- 707 **Rodriguez, K., Perales, M., Snipes, S., Yadav, R. K., Diaz-Mendoza, M. and**  
708 **Reddy, G. V.** (2016). DNA-dependent homodimerization, sub-cellular



- 709 partitioning, and protein destabilization control WUSCHEL levels and spatial  
710 patterning. *Proc Natl Acad Sci U S A* **113**, E6307-E6315.
- 711 **Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C.**  
712 **and Weisbeek, P.** (1994). Embryonic Origin of the Arabidopsis Primary Root  
713 and Root-Meristem Initials. *Development* **120**, 2475-2487.
- 714 **Schlereth, A., Moller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E. H.,**  
715 **Schmid, M., Jurgens, G. and Weijers, D.** (2010). MONOPTEROS controls  
716 embryonic root initiation by regulating a mobile transcription factor. *Nature*  
717 **464**, 913-916.
- 718 **Sessions, A., Yanofsky, M. F. and Weigel, D.** (2000). Cell-cell signaling and  
719 movement by the floral transcription factors LEAFY and APETALA1.  
720 *Science* **289**, 779-782.
- 721 **Stadler, R., Lauterbach, C. and Sauer, N.** (2005). Cell-to-cell movement of green  
722 fluorescent protein reveals post-phloem transport in the outer integument and  
723 identifies symplastic domains in Arabidopsis seeds and embryos. *Plant*  
724 *Physiol* **139**, 701-712.
- 725 **Tamaki, S., Matsuo, S., Wong, H. L., Yokoi, S. and Shimamoto, K.** (2007). Hd3a  
726 protein is a mobile flowering signal in rice. *Science* **316**, 1033-1036.
- 727 **ten Hove, C. A., Lu, K. J. and Weijers, D.** (2015). Building a plant: cell fate  
728 specification in the early Arabidopsis embryo. *Development* **142**, 420-430.
- 729 **Tsutsui, H. and Higashiyama, T.** (2016). pKAMA-ITACHI vectors for highly  
730 efficient CRISPR/Cas9-mediated gene knockout in Arabidopsis thaliana.  
731 *Plant and Cell Physiology* **58**, 46-56.
- 732 **Vaten, A., Dettmer, J., Wu, S., Stierhof, Y. D., Miyashima, S., Yadav, S. R.,**  
733 **Roberts, C. J., Campilho, A., Bulone, V., Lichtenberger, R., et al.** (2011).  
734 Callose biosynthesis regulates symplastic trafficking during root development.  
735 *Dev Cell* **21**, 1144-1155.
- 736 **Vollbrecht, E., Veit, B., Sinha, N. and Hake, S.** (1991). The developmental gene  
737 Knotted-1 is a member of a maize homeobox gene family. *Nature* **350**, 241-  
738 243.
- 739 **Weijers, D., Schlereth, A., Ehrismann, J. S., Schwank, G., Kientz, M. and**  
740 **Jurgens, G.** (2006). Auxin triggers transient local signaling for cell  
741 specification in Arabidopsis embryogenesis. *Dev Cell* **10**, 265-270.
- 742 **Welch, D., Hassan, H., Blilou, I., Immink, R., Heidstra, R. and Scheres, B.**  
743 (2007). Arabidopsis JACKDAW and MAGPIE zinc finger proteins delimit  
744 asymmetric cell division and stabilize tissue boundaries by restricting  
745 SHORT-ROOT action. *Genes Dev* **21**, 2196-2204.
- 746 **Wu, X., Dinneny, J. R., Crawford, K. M., Rhee, Y., Citovsky, V., Zambryski, P.**  
747 **C. and Weigel, D.** (2003). Modes of intercellular transcription factor  
748 movement in the Arabidopsis apex. *Development* **130**, 3735-3745.
- 749 **Yadav, R. K., Perales, M., Gruel, J., Girke, T., Jonsson, H. and Reddy, G. V.**  
750 (2011). WUSCHEL protein movement mediates stem cell homeostasis in the  
751 Arabidopsis shoot apex. *Genes Dev* **25**, 2025-2030.
- 752 **Zambryski, P. and Crawford, K.** (2000). Plasmodesmata: gatekeepers for cell-to-  
753 cell transport of developmental signals in plants. *Annu Rev Cell Dev Biol* **16**,  
754 393-421.
- 755 **Zhang, Y., Werling, U. and Edelman, W.** (2014). Seamless Ligation Cloning  
756 Extract (SLiCE) cloning method. *Methods Mol Biol* **1116**, 235-244.
- 757

758



759

760

761 **Figure legends**

762

763 **Fig 1. TMO7 moves through plasmodesmata into root cap cells in the**

764 **Arabidopsis primary root.** (A) Schematic image of Arabidopsis root structure.

765 Different tissues are colour coded according to the colour legend. (B-E) 5-day-old

766 root Fluorescence images in median plane of p*TMO7*::n3GFP (B), p*TMO7*::TMO7-

767 GFP (C), p*TMO7*::TMO7-3GFP (D) and p*TMO7*::TMO7-GFP in *cals3-2d* (E); right

768 panels show the false colour image of the left panel. (F) Statistical analysis of the

769 fluorescence ratio between columella cells and stem cell niches regions of different

770 transgenic plants, n≥13 roots per genotype, sample size is indicated in materials and

771 methods. Significant differences by one-way ANOVA test are indicated by letters

772 above bars (p<0.05). \* marks the regions that shows highest movement differences.

773 Scale bar = 25µm.

774

775 **Fig 2. TMO7 family proteins carry intrinsic features required for transport.** (A)

776 Phylogenetic analysis of selected bHLH proteins in Arabidopsis based on the full-

777 length protein sequence. Branch lengths indicate phylogenetic distances (see scale

778 bar: fraction of deviations). Green rectangle indicates the location of bHLH138 and

779 bHLH151. ARF5/MP is used as an unrelated reference. (B) Protein sequences

780 comparison within the TMO7 family and between TMO7, bHLH138, and bHLH151.

781 The schematic figure indicates the location of HLH domain and the mobile *cis*-

782 elements in TMO7 protein in red. (C-E) Mobility analysis of bHLH138, 151 and

783 TMO7-Like proteins. Confocal images of bHLH138-GFP, bHLH151-GFP (C), and

784 bHLH134-, bHLH136-, bHLH161-, and bHLH166-GFP expressed by the *TMO7*

785 promoter (D), and the fluorescence ratio analysis (E) Samples size is indicated in

786 materials and methods. Significant differences by one-way ANOVA test are indicated

787 by letters above bars (p<0.05). (F) Confocal images of *pbHLH166*::3nGFP and

788 *pbHLH166*::bHLH166-GFP, note that no fluorescence was detected outside the

789 promoter active regions. Scale bar = 25µm.

790

791 **Fig 3. TMO7 contains two major and one minor mobility motifs.** (A) Schematic

792 figure indicating the position of each mutant, those indicated above the sequence are

793 mobile defective mutants. (B) Confocal images of each linker-scanning mutant. Note

794 that the QC and columella region in m2, m5, m8 and m9 region shows reduced or no  
795 fluorescence. (C) Fluorescence ratio analysis of linker-scanning mutants, sample size  
796 is indicated in materials and methods. Significant differences by one-way ANOVA  
797 test are indicated by letters above bars ( $p < 0.05$ ). Scale bar = 25 $\mu$ m.

798

799 **Fig 4. Transferability of TMO7 mobility motifs.** (A) Confocal images of  
800 bHLH138, bHLH151 with M5 and M5, 8, 9 insertions. (B) Fluorescence intensity  
801 analysis of bHLH138-M5, -M8,9, -M5, 8, 9-GFP and bHLH151-M5, -M8,9, -M5, 8,  
802 9-GFP. Sample size is indicated in materials and methods. Significant differences by  
803 one-way ANOVA test are indicated by letters above bars ( $p < 0.05$ ). Note that the  
804 images have been enhanced for visualization. Scale bar = 25 $\mu$ m.

805

806 **Fig 5. Nuclear localization and exclusion signals restrict the movement of TMO7.**  
807 (A) Confocal image of the pTMO7::TMO7m5-GFP and pTMO7::TMO7m9-GFP root  
808 tip. Arrows indicate nucleus without fluorescence. (B and C) Confocal images of  
809 pTMO7::NLS-TMO7-GFP (B) and pTMO7::TMO7-GFP-NES (C), note that  
810 fluorescence signal is absent in the root tip regions in both images. (D) Fluorescence  
811 intensity analysis of pTMO7::NLS-TMO7-GFP and pTMO7::TMO7-GFP-NES,  
812 sample size is indicated in materials and methods. Significant differences by one-way  
813 ANOVA test are indicated by letters above bars ( $p < 0.05$ ). Scale bar = 25 $\mu$ m.

814

815 **Fig 6. Potential phosphorylation sites contribute to TMO7 movement .** (A-C)  
816 Confocal images of pTMO7::TMO7S39A-, S42A-, and S39, 42A-GFP. (D)  
817 Fluorescence intensity analysis of pTMO7::TMO7S39A-, S42A-, and S39, 42A-GFP,  
818 sample size is indicated in materials and methods. Significant differences by one-way  
819 ANOVA test are indicated by letters above bars ( $p < 0.05$ ). Note that the expression  
820 intensity of the TMO7 mutants is relatively lower compared to the pTMO7::TMO7-  
821 GFP line, however, the statistical analysis still indicates the reduction of mobility in  
822 all the mutants. Note that the images were modified for visualization. Significant  
823 differences by one-way ANOVA test are indicated by letters above bars ( $p < 0.05$ ).  
824 Scale bar = 25 $\mu$ m.

825

826 **Fig 7. Characterization of CRISPR-Cas9 *tmo7* mutants.** (A) Schematic figure  
827 indicates the sgRNA targeting region and the mutated amino acid sequences (in  
828 green) of *tmo7-4*, *tmo7-5*, and *tmo7-6*. (B) Sequencing traces of Col, *tmo7-4*, *tmo7-5*  
829 and *tmo7-6*. Orange line indicates sgRNA target sequence, purple line indicates the  
830 protospacer adjacent motif (PAM) in Col. The red line indicates the inserted  
831 nucleotide in *tmo7-4* and *tmo7-6*, the blue line indicates the deletion region in *tmo7-5*.  
832 (C) Root structure of Col, *tmo7-4*, *tmo7-5* and *tmo7-6*. Scale bar= 1cm. (D) Average  
833 root length comparison between Col and CRISPR *tmo7* lines, significance by student  
834 t-test, \*\*\* $p < 0.01$ . (E) Differential interference contrast (DIC) images of hypophysis  
835 division phenotype observed in *tmo7-4*, *tmo7-5*, and *tmo7-6*. Scale bar= 1cm. (F)  
836 Expression level of *TMO7* family genes in RNA suppression lines by qRT-PCR  
837 analysis, relative expression level compared with endogenous control, *ACTIN2*.  
838 Quantified with three biological repeats and error bar indicates standard error of  
839 mean.

840

841 **Fig 8. Reintroduction of *TMO7* into the *cals3-2d* suspensor partially**  
842 **complements the hypophysis division phenotype.** (A) Rootless phenotype of *cals3-*  
843 *2d* mutant. Scale bar = 100 $\mu$ m. (B) Expression of p*DR5*::nGFP in a *cals3-2d* embryo.  
844 Note that the accumulation of GFP signal is in the upper-most suspensor cell, and still  
845 no division has occurred in heart stage (insert). Scale bar = 5 $\mu$ m. (C and D) DIC  
846 image of a wild-type early-globular stage embryo (C) and a *Cals3-2d* late-globular  
847 stage embryo (D). Scale bar = 10 $\mu$ m. Note that the division that normally occurs in  
848 WT (asterisk mark in C) is absent in *cals3-2d* embryo (asterisk mark in D). (E)  
849 Statistical analysis of wild-type division frequency in p*DR5*::nGFP/*cals3-2d* (n=7  
850 independent lines, at least 100 embryos per line) and p*TMO7*::*TMO7*-GFP/*cals3-2d*  
851 (n=9 independent lines, at least 90 embryos per line) embryos. \*\*: 0.01 <  $p < 0.05$ ,  
852 student t-test.

853

Fig 1. Lu *et al.*

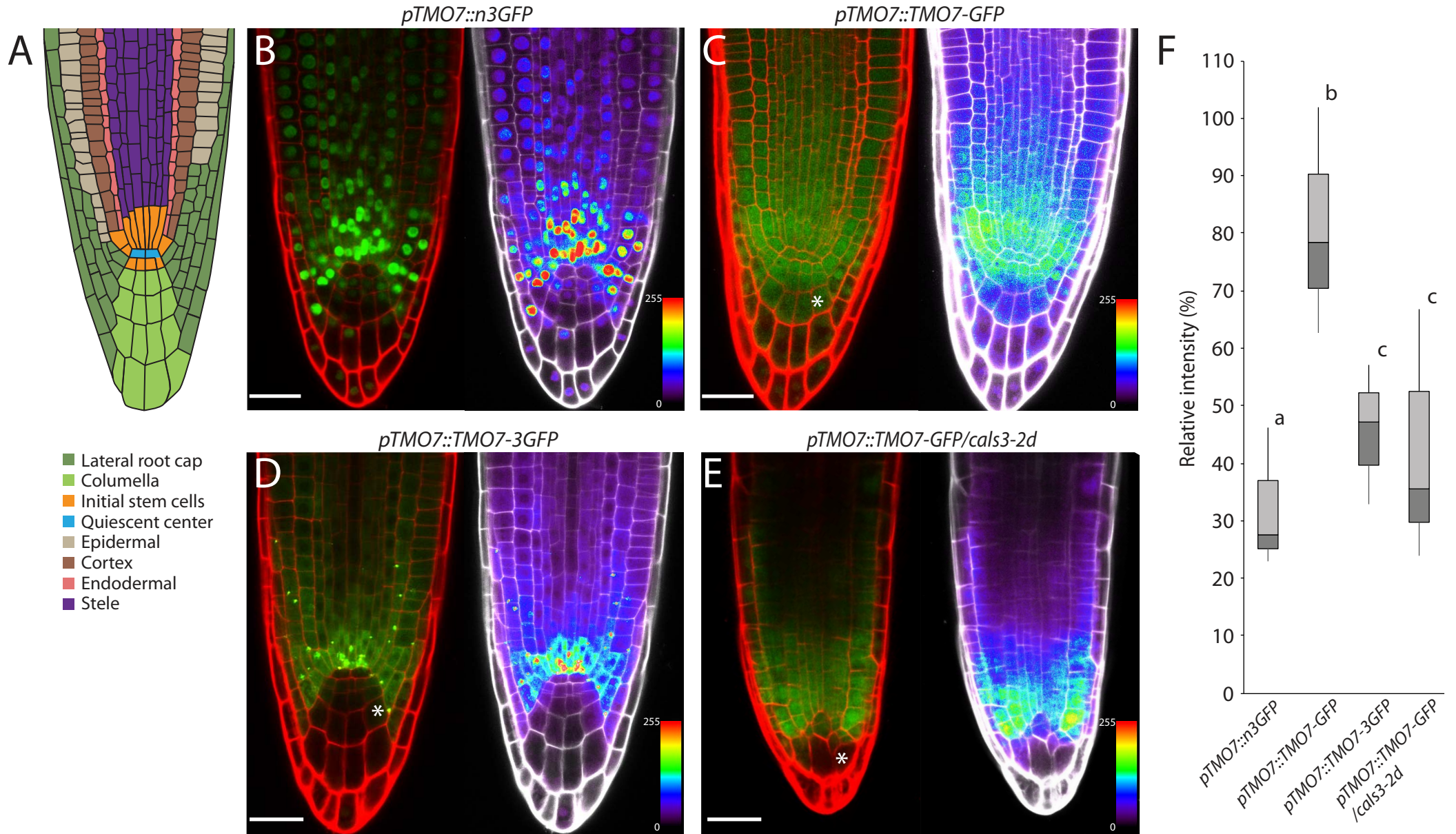






Fig 3. TMO7 contains three mobile *cis*-elemsnts

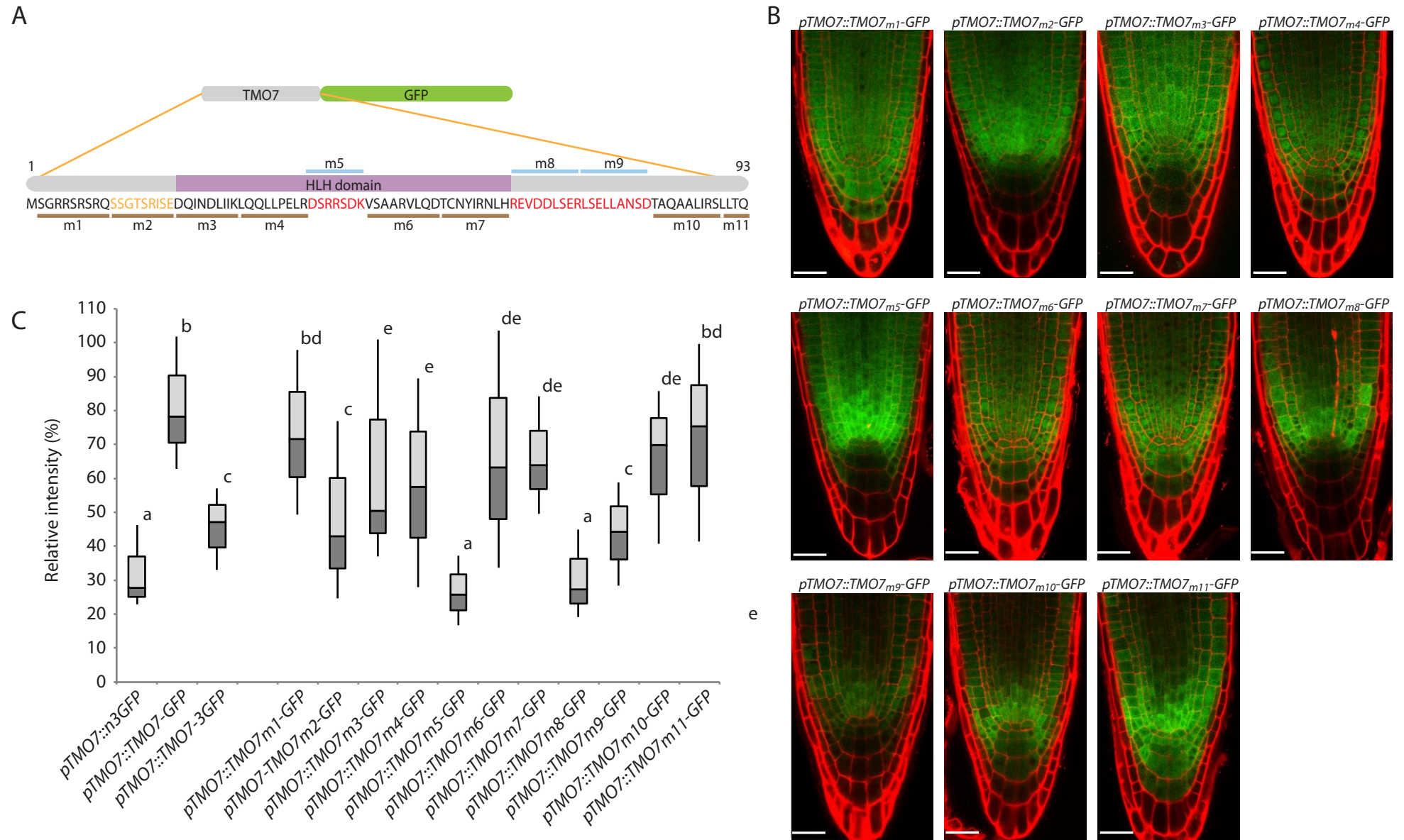




Fig 4 Lu et al.

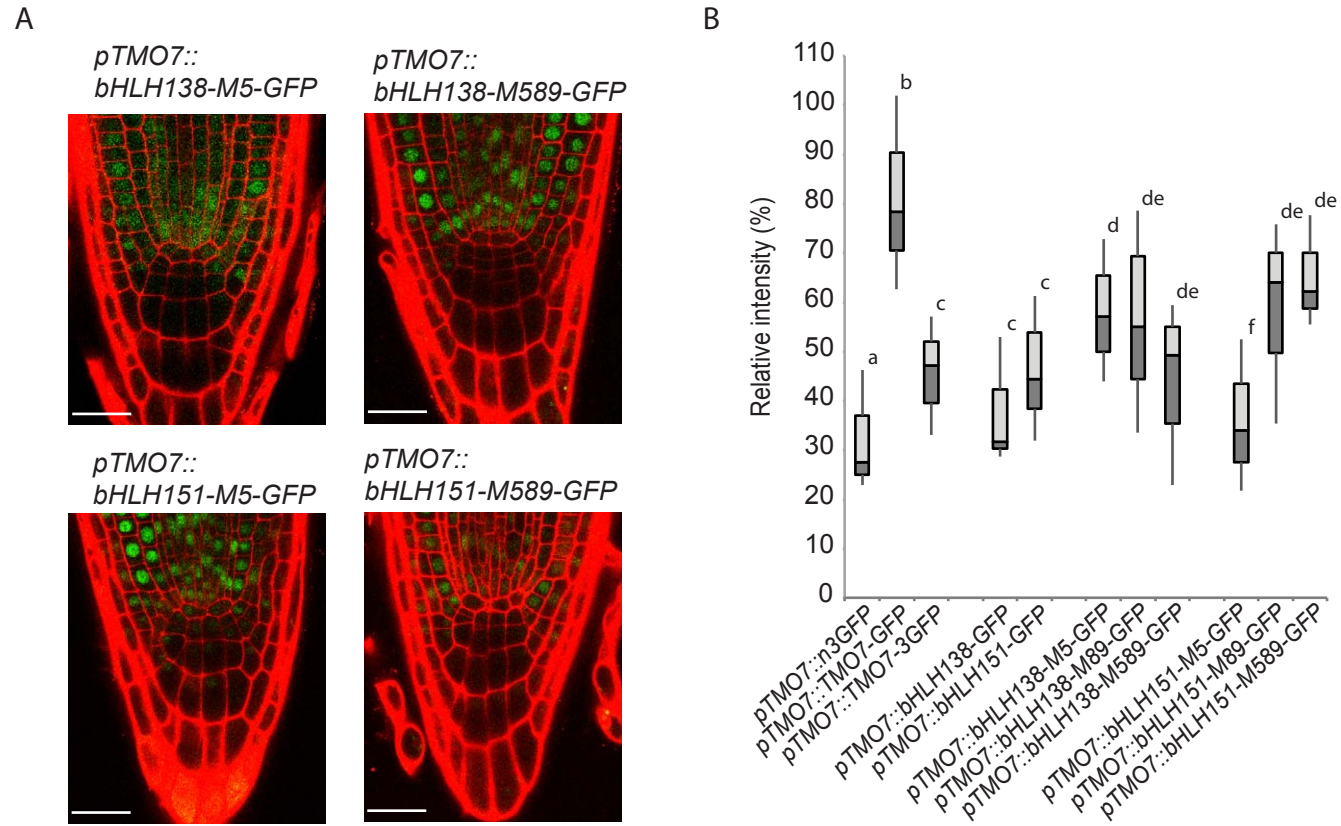


Fig 5. Lu et al.

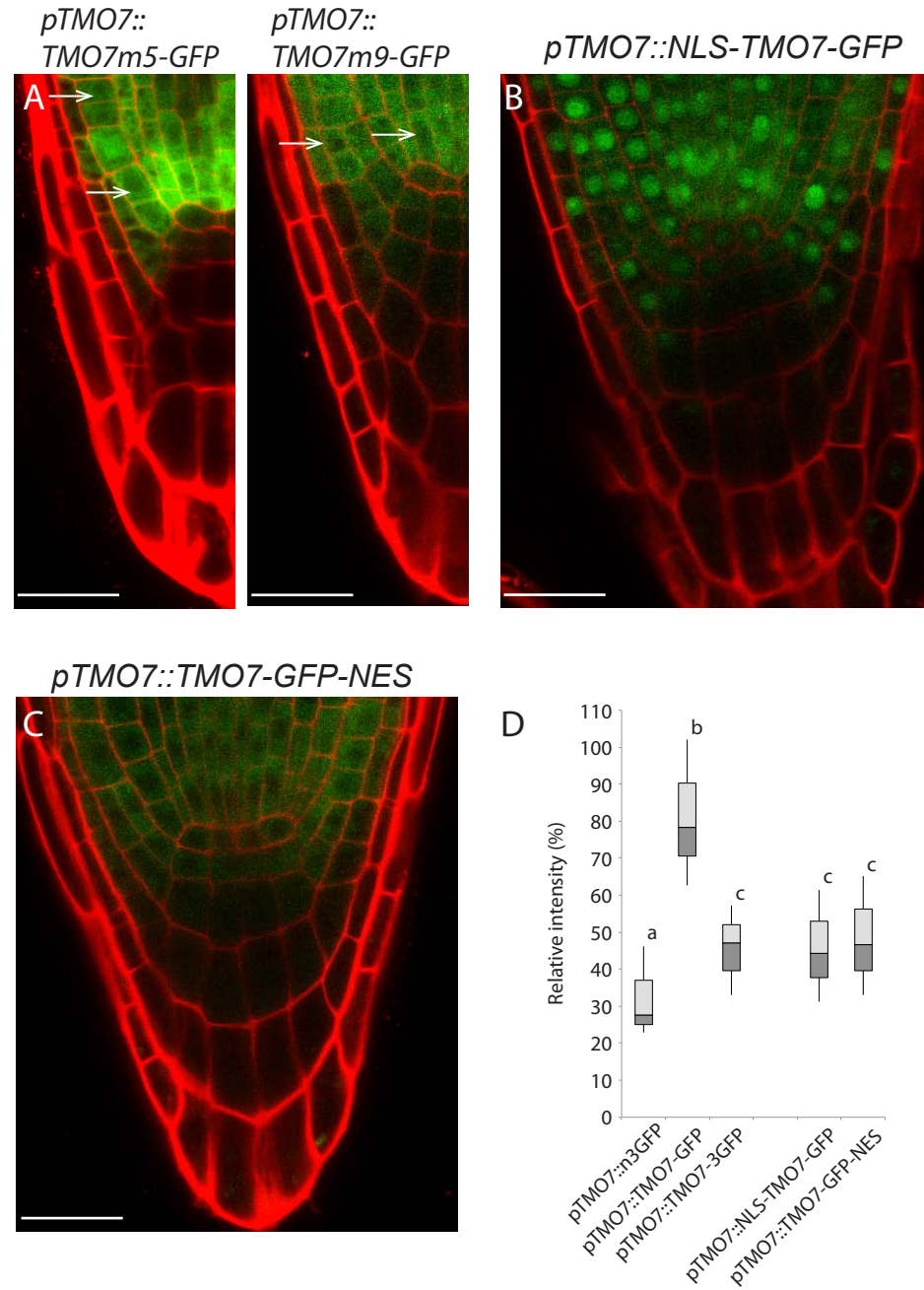


Fig 6. Lu et al

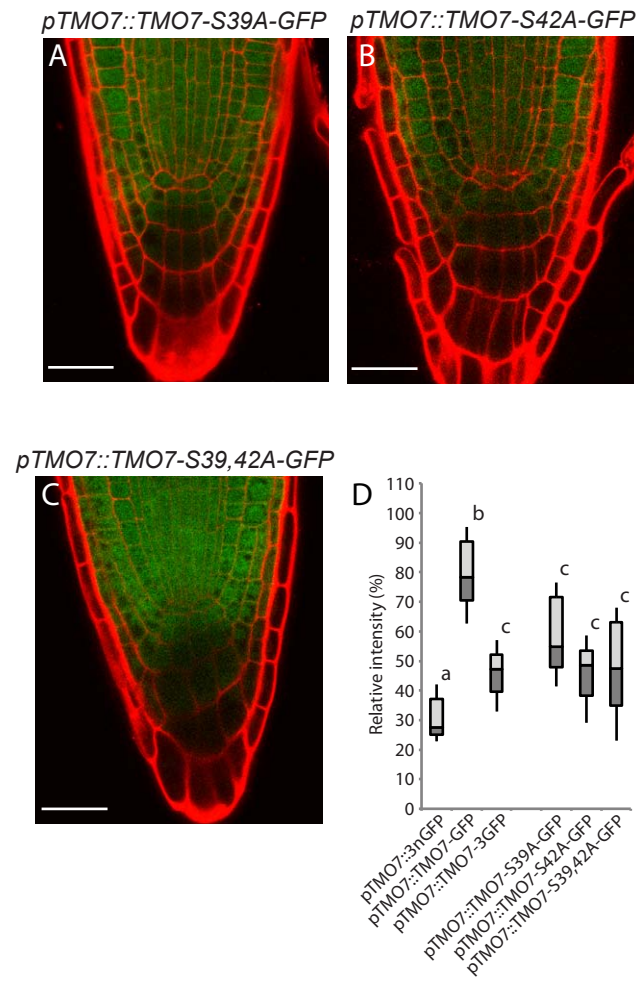


Fig 7. Lu et al.

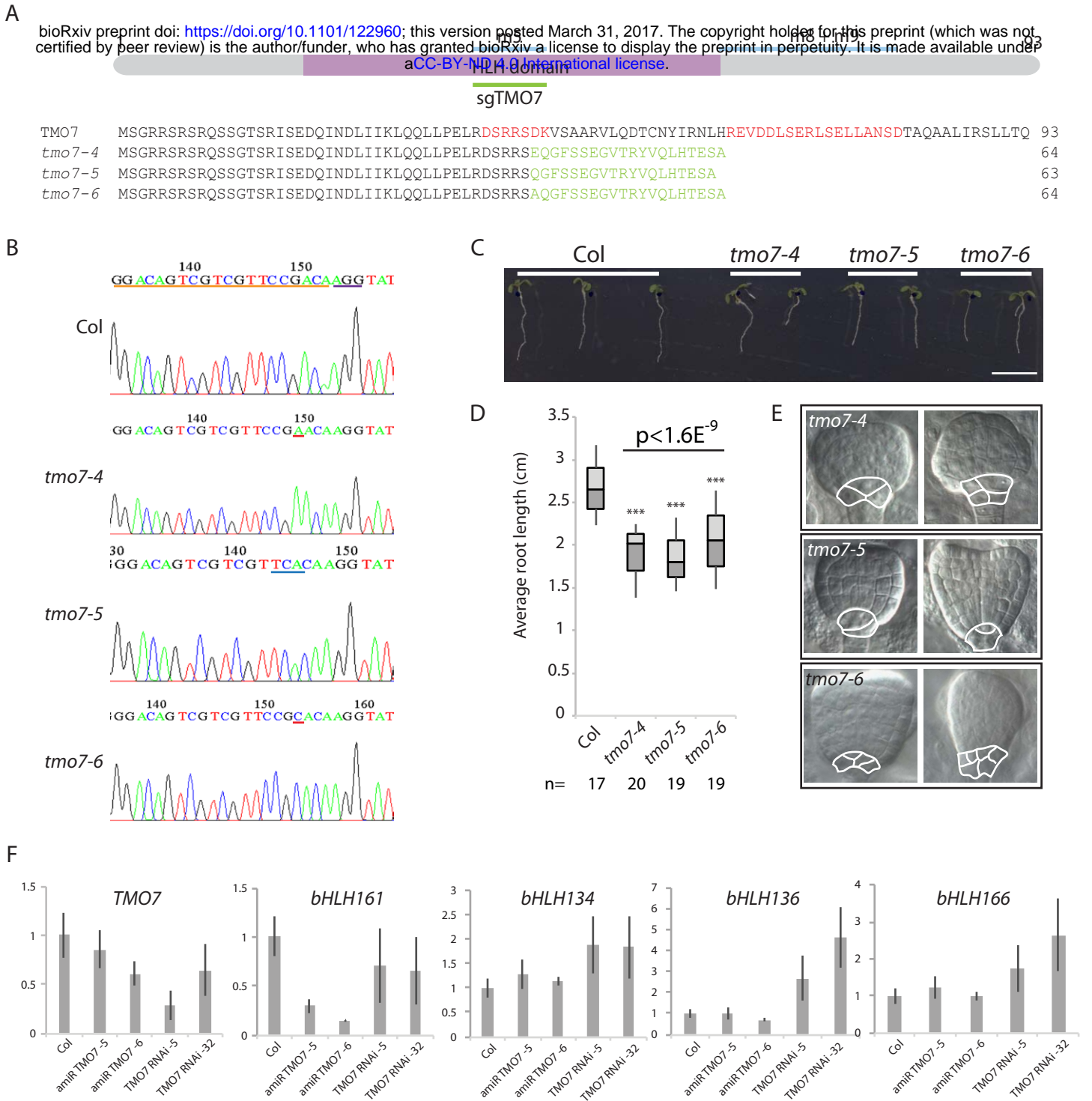


Fig 8. Lu et al.

