1 **Title**

2 **A secreted peptide and its receptors shaping the auxin response pattern and leaf**

3 **margin morphogenesis**

4

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21   **Summary**

22   Secreted peptides mediate intercellular communication [1, 2]. Several secreted peptides

23   in the EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) family regulate

24   morphogenesis of tissues, such as stomata and inflorescences in plants [3-15]. The

25   biological functions of other EPFL family members remain unknown. Here, we show that

26   the *EPFL2* gene is required for growth of leaf teeth. EPFL2 peptide physically interacts

27   with ERECTA (ER) family receptor-kinases and, accordingly, the attenuation of the

28   ER-family activities leads to formation of toothless leaves. During the tooth growth

29   process, responses to the phytohormone auxin are maintained at tips of the teeth to

30   promote their growth [16-19]. In the growing tooth tip of *epfl2* and multiple *er*-family

31   mutants, the auxin response becomes broader. Conversely, overexpression of *EPFL2*

32   diminishes the auxin response, indicating that the EPFL2 signal restricts the auxin

33   response to the tooth tip. Interestingly, the tip-specific auxin response in turn organizes

34   characteristic expression patterns of *ER* family and *EPFL2* by enhancing the *ER*-family

35   expression at the tip while eliminating the *EPFL2* expression from the tip. Our findings

36   identify the novel ligand-receptor pairs promoting the tooth growth, and further reveal a

37   feedback circuit between the peptide-receptor system and auxin response as a mechanism

38   for maintaining proper auxin maxima during leaf margin morphogenesis.

Results and Discussion

***EPFL2* is required for leaf tooth growth**

EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) family peptides represent a group of secreted cysteine-rich peptides [3], which are genetically encoded in diverse land plants including *Arabidopsis thaliana* [20]. Among the eleven *Arabidopsis* EPFLs, EPF1, 2 and EPFL9/STOMAGEN control stomatal patterning [4-11], while EPFL4, 5 and 6 (also known as CHALLAH [CHAL], CHAL-LIKE1 [CLL1], and CLL2) regulate inflorescence development (Figure 1A) [13-15]. However, the biological roles for other EPFLs including EPFL1, 2 and 3, which constitute a subclade, remain unknown (Figure 1A) [20]. To reveal a role for *EPFL2* gene, we analyzed a mutant line that carries a transposon insertion in the *EPFL2* locus (Figure S1A). The full-length *EPFL2* transcripts were not detected in the mutant (Figure S1A), indicating that the mutant is transcriptionally null for *EPFL2*. This mutant was originally isolated in Landsberg *erecta* (L.*er*) accession that carries a loss-of-function mutation in the *ERECTA* (*ER*) gene [21]. Because ER is known as the receptor for some EPFL family peptides, the *epfl2* phenotypes were first analyzed in the L.*er* background that harbors the functional *ER* transgene to exclude a possibility that *epfl2* phenotypes might be modified by the *er* mutation. While *epfl2* did not exhibit obvious growth defects, the mutant leaves showed smooth margin in contrast to wild type leaves, suggesting that *EPFL2* plays a role in leaf serration (Figure S1B and S1C). The phenotype was rescued by the introduction of the wild-type *EPFL2* genomic fragment (Figure S1D). The *epfl2* leaves still exhibited the toothless phenotype after introgression into the Columbia (Col) accession by outcrossing

62   seven times (Figure 1B and 1D). These data indicate that *EPFL2* is required for leaf tooth

63   development.

64   Serration, or saw-like projections of leaf teeth, are initiated as small primordia

65   along the leaf margin during early leaf development (Figure 1C), and eventually grow to

66   dentate structures in mature leaves (Figure 1B). Tooth primordia are initiated normally in

67   young *epfl2* leaves (Figure 1E), indicating that *EPFL2* is required for tooth growth after

68   the initiation. Tooth growth can be promoted either by outgrowth of the tooth primordia

69   or by growth repression of the sinus tissues between the primordia [22]. To test these

70   possibilities, the outlines from the wild type and *epfl2* leaves at a young stage with a size

71   around 2.5 mm^2 were superimposed and compared (Figure 1F). If the tooth outgrowth

72   was reduced in the mutant, positions of mutant tooth tips would be located inward from

73   those of wild type without changes in sinus positions [18]. Conversely, if the growth

74   suppression of sinuses was derepressed in the mutant, tip positions would be well aligned

75   between wild type and the mutant, while positions of mutant sinuses would be shifted

76   outward from those of wild type. The superimposed image between wild type and *epfl2*

77   showed that *epfl2* is classified into the former case. Although the sinus positions are well

78   aligned, the tip positions of *epfl2* were located inward from those of wild type (Figure

79   1F). These results support the hypothesis that *EPFL2* contributes to outgrowth of tooth

80   primordia rather than growth repression of sinus tissues.

81   Next, we analyzed the expression pattern of *EPFL2* using *EPFL2pro::GUS*

82   reporter. The GUS signals were broadly detected in growing leaves and, notably, the

83   signals were excluded from tooth tips and developing veins (Figure 1G). This suggests

84   that the *EPFL2* promotes tooth growth from the peripheral region of the tooth, not at the

85   tooth tip.

86

87   **ERECTA family genes are required for leaf tooth growth**

88   All the EPFLs characterized to date exert their activities through the ER receptor

89   kinase family consisting of ER, ER-LIKE1 (ERL1) and ERL2 [4-6, 10-15]. To address

90   whether ER-family receptors act in leaf tooth development, we first examined the leaf

91   shape of *er*-family mutants. Each of *er*, *erl1* and *erl2* single mutants developed leaf teeth

92   like the wild type (Figure S1E-S1H). Since ER family is known to act in a redundant

93   manner [23], we next examined each combination of *er*-familly double mutants. *er erl1*,

94   *er erl2* and *erl1 erl2* all showed the toothless phenotype resembling that of *epfl2* (Figure

95   1H, 1J and 1L). When young leaves were observed, all of the mutants displayed tooth

96   primordia (Figure 1I, 1K and 1M), indicating that the tooth initiation still occurs in these

97   mutants.

98   Next, we analyzed the shape of growing leaves of the *er*-family double mutants.

99   For this purpose, we focused on *erl1 erl2* rather than *er erl1* and *er erl2*. This is because

100   the *er* mutation affects leaf proportion, such as a ratio of width to length [21, 24], causing

101   misalignments of both tip and sinus positions in superimposed images between wild-type

102   vs. *er erl1* or *er erl2* leaves (Figure S1I). The outlines of growing *erl1 erl2* leaves showed

103   sinus positions well-aligned with those of wild type, while tooth tip positions were

104   located inward from those of wild type (Figure 1N). These results suggest that, like

105   *EPFL2*, *ER* family contributes to outgrowth of tooth primordia rather than growth

106   repression of sinus tissues.

107   We further characterized the expression patterns of *ER*-family genes in growing

108   leaves using *promoter::GUS* reporters [23]. *ERpro::GUS* was expressed broadly

109   throughout young leaves (Figure 1O). *ERL1pro::GUS* signals were also broadly detected,

110   with tooth tips particularly showing strong signals (Figure 1P). Notably, *ERL2pro::GUS*

111   expression was restricted to the tooth tips and some vein precursors (Figure 1Q),

112   contrasting to that of *EPFL2pro::GUS* (Figure 1G). Based on these findings, we conclude

113   that *ER*-family genes redundantly promote leaf tooth morphogenesis at the tooth tips after

114   tooth initiation.

115   The complete absence of three *ER*-family genes conferred severe leaf shape

116   defects, perhaps owing to known defects in the shoot apical meristem and stomatal

117   differentiation [23, 25-27]. Since this made the comparative analysis of leaf margin shape

118   difficult, we focused on the *ER*-family double mutants for further analyses. However, we

119   found that tooth primordia were lost in *er erl1 erl2* triple mutants (Figure S1J and S1K),

120   indicating that *ER*-family may also be involved in the tooth initiation process.

121

122   **EPFL2 peptide and ER-family receptors act as ligand-receptor pairs**

123   Genetic interactions between *EPFL2* and *ER* family were subsequently analyzed.

124   *er erl1 epfl2*, *er erl2 epfl2* and *erl1 erl2 epfl2* triple mutants all exhibited the same

125   toothless phenotype as the parental *epfl2* single and *er-famiy* double mutants (Figure

126   S2A-S2H). To quantify tooth growth phenotypes between wild-type and *epfl2*, we first

127   applied seven different quantification methods: Height vs. Width Ratio of tooth [28],

128   Circularity [29], Aspect Ratio (Length vs. Width Ratio of leaf) [29], Roundness [29],

129   Solidity [29], Bending Engergy [30] and Elliptic Fourier Descriptors (EFD) combined

6

130   with Principal Component Analysis [31]. All of the methods showed with a statistical

131   significance ($p < 0.05$, Welch's *t*-test, n=12) that tooth protrusions in *epfl2* are smaller

132   than those of wild type (Figure S2I-S2O). Among them, the Solidity method captured the

133   difference in the tooth size most significantly according to *F* value (variance between

134   genotypes per variance among individuals within the same genotype). Therefore, we

135   adopted the Solidity method to further compare tooth growth levels among *epfl2*,

136   *er*-family and their multiple mutants (Figure 2A), and defined '1-Solidity' as an index of

137   tooth growth level. All the tested mutants showed significant differences in the tooth

138   growth level from the wild type, while no or only small differences were observed among

139   the mutants (Figure 2B). These quantitative analyses support the notion that *EPFL2* and

140   *ER* family genetically act in the same pathway for the tooth growth.

141   To address whether EPFL2 peptide physically interacts with each ER-family

142   receptor, co-immunoprecipitation experiments were performed. FLAG-tagged EPFL2

143   (EPFL2-FLAG) and each of GFP-tagged ER-family receptors without kinase domains

144   (ERΔK-GFP, ERL1ΔK-GFP and ERL2ΔK-GFP) were co-expressed in *Nicotiana*

145   *benthamiana* leaves. When the receptors were immuno-precipitated using anti-GFP

146   antibody, EPFL2-FLAG was detected in the precipitated fractions (Figure 2C), indicating

147   their physical interaction. On the basis of these genetic and biochemical evidence, we

148   conclude that EPFL2 peptide and ER-family receptors constitute ligand-receptor pairs

149   acting for tooth growth.

150

151   ***EPFL2* and *ER* family negatively regulate auxin responses in leaf margin**

152   Auxin responses are restricted to tips of initiating and growing teeth, which is

153   crucial for tooth development [16-19]. To examine relationships between the *EPFL2*

154   activity and the auxin response, the auxin response reporter *DR5::GFP* was analyzed in

155   *epfl2* and *er*-family mutants (Figure 3). In wild type, the *DR5::GFP* expression is

156   restricted to the tip of growing tooth primordia in a later stage of tooth development

157   (Figure 3A). In an early stage, GFP signals were detected also in vascular cells as well as

158   in the tooth tips (Figure 3F). In *epfl2*, *er erl1*, *er erl2* and *erl1 erl2* mutants, *DR5::GFP*

159   signals spread to the surrounding regions of tooth tips in the later stage (Figure 3B-3E).

160   This phenotype was detected even in the early stage of tooth development: the GFP

161   signals are visible in tip periphery regions along the leaf margin (Figure 3G-3J, dashed

162   rectangles; quantification of GFP fluorescence intensity in the rectangles is shown in 3K).

163   In contrast, the overexpression of *EPFL2* by *CaMV 35S* promoter (*35S::EPFL2*; Figure

164   S3A) reduced the *DR5::GFP* expression at growing tooth tips (Figure 3L-3N, dashed

165   circles; 3O, quantification), showing that the *EPFL2* signal represses the auxin response.

166   These observations indicate that *EPFL2* and *ER* family restrict the auxin response to a

167   small number of cells at the tooth tip during the tooth growth. The ectopic *EPFL2*

168   overexpression conferred a reduction in stomatal density (Figure S3B-S3D), which is

169   mediated by the *ER*-family signaling [26]. This further supports the conclusion that

170   *EPFL2* acts through the *ER*-family (Figure 2).

171

172   **The auxin response organizes the expression patterns of *EPFL2* and *ERL2***

173   The expression pattern of *ERL2pro::GUS* at the tooth tip (Figure 1Q) resembles

174   that of *DR5* reporters (Figure 3A, 3F and 4A). By contrast, the *EPFL2pro::GUS* pattern

175 appears to be inversely correlated with those of *ERL2* and *DR5* reporters (Figure 1G, 1Q

176 and 4A). To examine these relationships, the expression patterns were analyzed after

177 auxin polar transport was chemically perturbed by N-1-naphthylphthalamic acid (NPA).

178 In NPA-treated plants, *ERL2pro::GUS* and *DR5::GUS* similarly exhibited broad

179 expression along the leaf margin except for the basal part (Figure S4A and S4B). On the

180 other hand, *EPFL2pro::GUS* expression was restricted to the basal part (Figure S4C),

181 clearly showing inverse correlation with those of *ERL2pro::GUS* and *DR5::GUS*.

182 Collectively, these results suggest that auxin may induce *ERL2,* while it represses *EPFL2*.

183 To address this, exogenous auxin was applied to *ERL2pro::GUS* and *EPFL2pro::GUS*

184 plants. To avoid complications arising from auxin transport, we used a synthetic auxin

185 2,4-Dichlorophenoxyacetic acid (2,4-D), which is not transported by auxin efflux carriers

186 [32]. Upon the 2,4-D application, the *ERL2pro::GUS* expression was enhanced (Figure

187 S4D and S4E), remarkably resembling the *DR5::GUS* pattern (Figure 4A). By contrast,

188 the 2,4-D application diminished the *EPFL2pro::GUS* expression (Figure 4B and 4C).

189 Consistently, the endogenous *ERL2* transcript level increased within a few hours after the

190 2,4-D application (Figure S4F), while the endogenous *EPFL2* transcript level was

191 diminished (Figure 4D). The inhibitory effect of auxin on the *EPFL2* expression is in

192 accordance with the observation that the *EPFL2* expression is eliminated from tooth tips

193 and vascular tissues (Figure 4B), where the auxin response is the highest (Figure 4A). On

194 the basis of these findings, we propose that the auxin response organizes the

195 mutually-exclusive expression patterns of *ERL2* and *EPFL2*.

196

The auxin-responsive patterning of *ER* family and *EPFL2* promotes the tooth growth

To examine whether the auxin-responsive expression of *ER*-family is sufficient to promote tooth growth, *ER* was expressed under the *DR5* promoter in *er erl1*. As a result, the *DR5*-driven *ER* expression rescued the toothless phenotype of *er erl1* (Figure 4E, 4F, S4G and S4H), demonstrating that the *ER* activity at cells which respond to auxin is sufficient for the tooth growth. We next tested whether the specific expression of *EPFL2* at the peripheral border of growing teeth is sufficient to rescue the tooth growth defects in *epfl2*. For this purpose, *EPFL2* is expressed in *epfl2* mutant under the promoter of *CUP-SHAPED COTYLEDON2* (*CUC2*), which is active at the tooth periphery and repressed by auxin (Figure 4G) [16, 33]. *CUC2pro::EPFL2* rescued the tooth growth defect of *epfl2* (Figure 4H, 4I, S4I and S4J), indicating that the expression of *EPFL2* at the tooth periphery is sufficient to promote the tooth growth. These results highlight the importance of the auxin-responsive patterning of *ER* family and *EPFL2* activities for the tooth growth.

Conclusions and model

We have shown that the EPFL2-ER-family activity represses auxin response. The auxin response in turn represses the *EPFL2* expression and induces the *ERL2* expression. Experimental and computational approaches have been taken to address how auxin response patterns are formed during the tooth initiation step [16-19]. The currently proposed model for the tooth initiation is based on a polar auxin transport to explain the formation of regularly-spaced auxin peaks along leaf margins as well as the formation of

220 a new peak between two existing peaks when the existing peaks are separated apart by

221 tissue growth [16, 34]. In contrast to the tooth initiation process, little has been studied

222 about the molecular basis for the maintenance of a single peak of the auxin response at

223 each tooth tip in the tooth growth step. Our findings establish that the EPFL2-ER-family

224 ligand-receptor pairs restrict the auxin response to the tip of the growing tooth.

225   *CUC2* and its negative regulator *microRNA164* (*miR164*) have been recognized

226 as key regulators of leaf serration [18, 33, 35]. *cuc2-1D*, the *miR164*-resistant dominant

227 allele of *CUC2*, enhances the tooth outgrowth (Figure S4K and S4L) [35]. To address a

228 relationship between CUC2/miR164 and EPFL2-ER-family systems, we constructed

229 *cuc2-1D epfl2* double mutant. *cuc2-1D epfl2* showed obvious teeth even in mature leaves

230 (Figure S4M and S4N), showing that *CUC2* can promote the tooth growth in an

231 *EPFL2*-independent manner. On the other hand, teeth of *cuc2-1D epfl2* were smaller than

232 those of *cuc2-1D* (Figure S4L and S4N), indicating that *EPFL2* is able to act

233 independently of *miR164*. Other transcription factors have been also reported as

234 significant contributors to serration, including BEL1-LIKE HOMEODOMAIN (BLH),

235 KNOTTED1-LIKE   HOMEOBOX   (KNOX),   TEOSINTE

236 BRANCHED/CYCLOIDEA/PCF (TCP) and SQUAMOSA PROMOTER-BINDING

237 PROTEIN LIKE (SPL) proteins [36-38]. It would be interesting to investigate how these

238 factors are orchestrated with EPFL2 and CUC2 to determine the extent of leaf serration.

239   We propose a model that explains how EPFL2 and ER family specify leaf margin

240 morphogenesis (Figure 4J). During tooth growth, the auxin response transiently becomes

241 broader due to proliferation of the tip-located cells showing the auxin response and/or *de*

242 *novo* auxin response at their daughter cells (Figure 4J, left to center). The cells

11

243 responding to auxin cell-autonomously increases the *ER*-family expression (Figure 4J,

244 center). At the same time, since the the *EPFL2* expression is de-repressed in the cells that

245 do not show the auxin response, EPFL2 peptides are produced in the tooth peripheral

246 cells neighboring to the cells responding to auxin at the tooth tip (Figure 4J, center).

247 Secreted EPFL2 peptides are perceived by ER-family proteins in the cells neighboring to

248 the EPFL2-producing cells. This activation of ER-family signaling cell-autonomously

249 suppresses the auxin response (Figure 4J, center to right). This suppression might be

250 mediated by *IAA8/9*, which act redundantly for the leaf tooth growth after initiation like

251 *EPFL2* [39]. These processes continually occur during tooth growth. This circuit enables

252 the highly localized auxin response at the tooth tip and simultaneously keeps the

253 elimination of the *EPFL2* expression from the tip. The feedback regulation between the

254 EPFL2-ER-family system and the auxin response likely represents a novel framework for

255 maintaining auxin responses in growing tissues.

256   The final leaf shape is determined through two distinct processes: the primary

257 morphogenesis that patterns the number and position of primordia of teeth, lobes and

258 leaflets at the initiation step and the secondary morphogenesis that regulates the growth

259 level and direction of developing primordia and surrounding tissues [40]. The impact of

260 the latter process on the leaf shape variation has been shown by the studies using

261 compound-leafed species *Lepidium* [41]. Our findings identify the EPFL2-ER-family

262 system as a regulator of secondary morphogenesis. *EPFL2* belongs to a subclade of

263 *EPFL* family that had not been characterized until this study (Figure 1A), and members

264 of this subclade are well conserved in diverse vascular plants [20]. Further studies on

265   *EPFLs* of this subclade in diverse plant species might provide a novel picture of how the

266   peptide-receptor signaling contributes to a variety of leaf shapes.

267   **Accession numbers**

268   The Arabidopsis Genome Initiative identifiers for the genes referred in this study

269   are as follows: *EPF1* (At2g20875), *EPF2* (At1g34245), *EPFL1* (At5g10310), *EPFL2*

270   (At4g37810), *EPFL3* (AT3G13898), *EPFL4/CLL2* (At4g14723), *EPFL5/CLL1*

271   (At3g22820), *EPFL6/CHAL* (At2g30370), *EPFL7* (AT1G71866), *EPFL8* (AT1G80133),

272   *EPFL9/STOMAGEN* (At4g12970), *ER* (At2g26330), *ERL1* (At5g62230), *ERL2*

273   (At5g07180), *CUC2* (At5g53950).

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275   **Supplemental Information**

276   Supplemental Information includes Supplemental Experimental Procedures, four

277   figures and two tables.

Author contributions

T.T., M.T., K.U.T. and N.U. designed research; T.T., S.O., J.S.L. and N.U. performed research; T.T., S.O., J.S.L. M.A., M.T., K.U.T. and N.U. analyzed and discussed data; T.T., K.U.T. and N.U. wrote the paper

294   **Reference**

295

296   1.   Tavormina, P., De Coninck, B., Nikonorova, N., De Smet, I., and Cammue, B.P.A.

297   (2015). The Plant Peptidome: An Expanding Repertoire of Structural Features and

298   Biological Functions. Plant Cell *27*, 2095-2118.

299   2.   Tameshige, T., Hirakawa, Y., Torii, K.U., and Uchida, N. (2015). Cell walls as a

300   stage for intercellular communication regulating shoot meristem development.

301   Frontiers in Plant Science *6, e324*.

302   3.   Richardson, L.G.L., and Torii, K.U. (2013). Take a deep breath: peptide signalling

303   in stomatal patterning and differentiation. Journal of Experimental Botany *64*,

304   5243-5251.

305   4.   Hara, K., Kajita, R., Torii, K.U., Bergmann, D.C., and Kakimoto, T. (2007). The

306   secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. Genes &

307   Development *21*, 1720-1725.

308   5.   Hara, K., Yokoo, T., Kajita, R., Onishi, T., Yahata, S., Peterson, K.M., Torii, K.U.,

309   and Kakimoto, T. (2009). Epidermal Cell Density is Autoregulated via a Secretory

310   Peptide, EPIDERMAL PATTERNING FACTOR 2 in Arabidopsis Leaves. Plant

311   and Cell Physiology *50*, 1019-1031.

312   6.   Hunt, L., and Gray, J.E. (2009). The Signaling Peptide EPF2 Controls

313   Asymmetric Cell Divisions during Stomatal Development. Current Biology *19*,

314   864-869.

315   7.   Hunt, L., Bailey, K.J., and Gray, J.E. (2010). The signalling peptide EPFL9 is a

316   positive regulator of stomatal development. New Phytologist *186*, 609-614.

317   8.   Sugano, S.S., Shimada, T., Imai, Y., Okawa, K., Tamai, A., Mori, M., and

318   Hara-Nishimura, I. (2010). Stomagen positively regulates stomatal density in

319   Arabidopsis. Nature *463*, 241-U130.

320   9.   Kondo, T., Kajita, R., Miyazaki, A., Hokoyama, M., Nakamura-Miura, T., Mizuno,

321   S., Masuda, Y., Irie, K., Tanaka, Y., Takada, S., et al. (2010). Stomatal Density is

322   Controlled by a Mesophyll-Derived Signaling Molecule. Plant and Cell

323   Physiology *51*, 1-8.

324   10.   Lee, J.S., Kuroha, T., Hnilova, M., Khatayevich, D., Kanaoka, M.M., McAbee,

325   J.M., Sarikaya, M., Tamerler, C., and Torii, K.U. (2012). Direct interaction of

326   ligand-receptor pairs specifying stomatal patterning. Genes & Development *26*,

327   126-136.

328   11.   Lee, J.S., Hnilova, M., Maes, M., Lin, Y.-C.L., Putarjunan, A., Han, S.-K., Avila,

329   J., and Torii, K.U. (2015). Competitive binding of antagonistic peptides fine-tunes

330   stomatal patterning. Nature *522*, 439-443.

331   12.   Niwa, T., Kondo, T., Nishizawa, M., Kajita, R., Kakimoto, T., and Ishiguro, S.

332   (2013). EPIDERMAL PATTERNING FACTOR LIKE5 Peptide Represses

333   Stomatal Development by Inhibiting Meristemoid Maintenance in Arabidopsis

334   thaliana. Bioscience Biotechnology and Biochemistry *77*, 1287-1295.

335   13.   Abrash, E.B., Davies, K.A., and Bergmann, D.C. (2011). Generation of Signaling

336   Specificity in Arabidopsis by Spatially Restricted Buffering of Ligand-Receptor

337   Interactions. Plant Cell *23*, 2864-2879.

338   14.   Uchida, N., Lee, J.S., Horst, R.J., Lai, H.H., Kajita, R., Kakimoto, T., Tasaka, M.,

339   and Torii, K.U. (2012). Regulation of inflorescence architecture by intertissue

17

340   layer ligand-receptor communication between endodermis and phloem.

341   Proceedings of the National Academy of Sciences of the United States of America

342   *109*, 6337-6342.

343 15. Uchida, N., and Tasaka, M. (2013). Regulation of plant vascular stem cells by

344   endodermis-derived EPFL-family peptide hormones and phloem-expressed

345   ERECTA-family receptor kinases. Journal of Experimental Botany *64*,

346   5335-5343.

347 16. Bilsborough, G.D., Runions, A., Barkoulas, M., Jenkins, H.W., Hasson, A.,

348   Galinha, C., Laufs, P., Hay, A., Prusinkiewicz, P., and Tsiantis, M. (2011). Model

349   for the regulation of Arabidopsis thaliana leaf margin development. Proceedings

350   of the National Academy of Sciences of the United States of America *108*,

351   3424-3429.

352 17. Hay, A., Barkoulas, M., and Tsiantis, M. (2006). ASYMMETRIC LEAVES1 and

353   auxin activities converge to repress BREVIPEDICELLUS expression and

354   promote leaf development in Arabidopsis. Development *133*, 3955-3961.

355 18. Kawamura, E., Horiguchi, G., and Tsukaya, H. (2010). Mechanisms of leaf tooth

356   formation in Arabidopsis. Plant Journal *62*, 429-441.

357 19. Kasprzewska, A., Carter, R., Swarup, R., Bennett, M., Monk, N., Hobbs, J.K., and

358   Fleming, A. (2015). Auxin influx importers modulate serration along the leaf

359   margin. Plant Journal *83*, 705-718.

360 20. Takata, N., Yokota, K., Ohki, S., Mori, M., Taniguchi, T., and Kurita, M. (2013).

361   Evolutionary Relationship and Structural Characterization of the EPF/EPFL Gene

362   Family. Plos One *8*, 6.

363 21. Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier,

364   R.F., and Komeda, Y. (1996). The arabidopsis ERECTA gene encodes a putative

365   receptor protein kinase with extracellular leucine-rich repeats. Plant Cell *8*,

366   735-746.

367 22. Malinowski, R., Kasprzewska, A., and Fleming, A.J. (2011). Targeted

368   manipulation of leaf form via local growth repression. Plant Journal *66*, 941-952.

369 23. Shpak, E.D., Berthiaume, C.T., Hill, E.J., and Torii, K.U. (2004). Synergistic

370   interaction of three ERECTA-family receptor-like kinases controls Arabidopsis

371   organ growth and flower development by promoting cell proliferation.

372   Development *131*, 1491-1501.

373 24. Bowman, J. (1994). Arabidopsis: An Atlas of Morphology and Development, 1

374   Edition, (New York: Springer-Verlag).

375 25. Chen, M.K., Wilson, R.L., Palme, K., Ditengou, F.A., and Shpak, E.D. (2013).

376   ERECTA Family Genes Regulate Auxin Transport in the Shoot Apical Meristem

377   and Forming Leaf Primordia. Plant Physiology *162*, 1978-1991.

378 26. Shpak, E.D., McAbee, J.M., Pillitteri, L.J., and Torii, K.U. (2005). Stomatal

379   patterning and differentiation by synergistic interactions of receptor kinases.

380   Science *309*, 290-293.

381 27. Uchida, N., Shimada, M., and Tasaka, M. (2013). ERECTA-Family Receptor

382   Kinases Regulate Stem Cell Homeostasis via Buffering its Cytokinin

383   Responsiveness in the Shoot Apical Meristem. Plant and Cell Physiology *54*,

384   343-351.

385 28. Hasson, A., Plessis, A., Blein, T., Adroher, B., Grigg, S., Tsiantis, M., Boudaoud,

A., Damerval, C., and Laufs, P. (2011). Evolution and Diverse Roles of the CUP-SHAPED COTYLEDON Genes in Arabidopsis Leaf Development. Plant Cell *23*, 54-68.

29. Chitwood, D.H., Ranjan, A., Kumar, R., Ichihashi, Y., Zumstein, K., Headland, L.R., Ostria-Gallardo, E., Aguilar-Martinez, J.A., Bush, S., Carriedo, L., et al. (2014). Resolving Distinct Genetic Regulators of Tomato Leaf Shape within a Heteroblastic and Ontogenetic Context. Plant Cell *26*, 3616-3629.

30. Backhaus, A., Kuwabara, A., Bauch, M., Monk, N., Sanguinetti, G., and Fleming, A. (2010). leafprocessor: a new leaf phenotyping tool using contour bending energy and shape cluster analysis. New Phytologist *187*, 251-261.

31. Chitwood, D.H., Headland, L.R., Kumar, R., Peng, J., Maloof, J.N., and Sinha, N.R. (2012). The Developmental Trajectory of Leaflet Morphology in Wild Tomato Species. Plant Physiology *158*, 1230-1240.

32. Delbarre, A., Muller, P., Imhoff, V., and Guern, J. (1996). Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta *198*, 532-541.

33. Nikovics, K., Blein, T., Peaucelle, A., Ishida, T., Morin, H., Aida, M., and Laufs, P. (2006). The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis. Plant Cell *18*, 2929-2945.

34. Nakamasu, A., Nakayama, H., Nakayama, N., Suematsu, N.J., and Kimura, S. (2014). A Developmental Model for Branching Morphogenesis of Lake Cress Compound Leaf. Plos One *9*, 7.

409   35.   Larue, C.T., Wen, J.Q., and Walker, J.C. (2009). A microRNA-transcription factor

410   module regulates lateral organ size and patterning in Arabidopsis. Plant Journal *58*,

411   450-463.

412   36.   Kumar, R., Kushalappa, K., Godt, D., Pidkowich, M.S., Pastorelli, S., Hepworth,

413   S.R., and Haughn, G.W. (2007). The Arabidopsis BEL1-LIKE HOMEODOMAIN

414   proteins SAW1 and SAW2 act redundantly to regulate KNOX expression spatially

415   in leaf margins. Plant Cell *19*, 2719-2735.

416   37.   Kimura, S., Koenig, D., Kang, J., Yoong, F.Y., and Sinha, N. (2008). Natural

417   variation in leaf morphology results from mutation of a novel KNOX gene.

418   Current Biology *18*, 672-677.

419   38.   Rubio-Somoza, I., Zhou, C.M., Confraria, A., Martinho, C., von Born, P.,

420   Baena-Gonzalez, E., Wang, J.W., and Weigel, D. (2014). Temporal Control of

421   Leaf Complexity by miRNA-Regulated Licensing of Protein Complexes. Current

422   Biology *24*, 2714-2719.

423   39.   Koenig, D., Bayer, E., Kang, J., Kuhlemeier, C., and Sinha, N. (2009). Auxin

424   patterns Solanum lycopersicum leaf morphogenesis. Development *136*,

425   2997-3006.

426   40.   Champagne, C., and Sinha, N. (2004). Compound leaves: equal to the sum of their

427   parts? Development *131*, 4401-4412.

428   41.   Bharathan, G., Goliber, T.E., Moore, C., Kessler, S., Pham, T., and Sinha, N.R.

429   (2002). Homologies in leaf form inferred from KNOXI gene expression during

430   development. Science *296*, 1858-1860.

431

432 **Figure Legends**

433

434 Figure 1. *EPFL2* and *ER* family are required for leaf tooth growth.

435 (A) Neighbor-joining unrooted phylogenetic tree of EPFL members. EPFL2 is

436 highlighted in green. The previously characterized members are indicated by cyan and

437 orange underlines. Scale bar: 0.1 amino acid substitutions per site.

438 (B, D, H, J and L) Mature seventh leaves of wild type (abbreviated as WT in all figures)

439 (B), *epfl2* (D), *er erl1* (H), *er erl2* (J) and *erl1 erl2* (L). Right panels are magnified views

440 of leaf edges. Curled leaf was flattened with incisions indicated by broken lines.

441 (C, E, I, K and M) wild-type (C), *epfl2* (E), *er erl1* (I), *er erl2* (K) and *erl1 erl2* (M)

442 young ninth leaves of 1 mm in length.

443 (F and N) Superimposition of leaf outlines from *epfl2* (F) and *erl1 erl2* (N) with those

444 from wild type. X- and Y-values of each leaf are scaled proportionally so that each leaf

445 size is one and thus the size of half leaves shown in panels is 0.5. The original leaf sizes

446 of *epfl2*, *erl1 erl2* and wild type are 2.82 ± 0.47 mm^2 (mean $\pm$ SEM, n = 10), 2.30 ± 0.39

447 mm^2 (n = 10) and 2.70 ± 0.29 mm^2 (n = 12), respectively. The same wild type data are

448 shown in both panels. The outlines are shown as line plot (left). The positions of each

449 tooth tip (top right) and sinus (bottom right) are shown as scatter plots.

450 (G, O-Q) GUS patterns of *EPFL2pro::GUS* (G), *ERpro::GUS* (O), *ERL1pro::GUS* (P)

451 and *ERL2pro::GUS* (Q) in young sixth leaf of 1 mm in length. The right panels are

452 magnified views.

453 Arrowheads: teeth. Scale bars: B, D, H, J and L, 1 mm; C, E, G, I, K, M and O-Q 100

454 μm.

455

456 Figure 2. Genetic and physical interaction between EPFL2 and ER-family.

457 (A) Black-and-white images of young leaves from multiple mutants of *EPFL2* and *ER*

458 family as well as their parental lines.

459 (B) Tooth growth levels of young leaves from multiple mutants of *EPFL2* and *ER* family

460 and their parental lines. Mean values and the standard errors are shown. n = 12, 12, 13,

461 10, 15, 14, 14, and 12 for wild type, *epfl2*, *erl1 erl2*, *erl1 erl2 epfl2*, *er erl1*, *er erl1 epfl2*,

462 *er erl2* and *er erl2 epfl2*, respectively. Letters indicate significant differences ($p < 0.05$)

463 determined by Tukey's HSD test. See main text and Supplemental Experimental

464 Procedures for the calculation method.

465 (C) Co-immunoprecipitation assays of FLAG-tagged EPFL2 and GFP-tagged

466 kinase-truncated ER-family proteins. The immuno-precipitated (IP) fractions by anti-GFP

467 antibody were separated by SDS-PAGE, and the precipitated proteins were detected by

468 anti-GFP and anti-FLAG antibodies.

469

470 Figure 3. *EPFL2* and *ER* family restrict the auxin response to the tooth tip.

471 (A-J and L-N) Z-projected confocal micrographs of growing teeth in wild type (A, F and

472 L), *epfl2* (B and G), *er erl1* (C and H), *er erl2* (D and I) *erl1 erl2* (E and J) and

473 *35S::EPFL2* (M and N). Auxin responses are indicated by *DR5::GFP* (green and color

474 look-up-tables to the right of I and M). Note that detector settings for GFP fluorescence

475 are different among three groups ('A-E', 'F-J' and 'L-N') according to each dynamic

476 range. The leaf shape is shown by chlorophyll fluorescence (magenta) in A-E or

477 transmitted-light images (gray scale) in F-J and L-N. Growing leaves at the later stage

478   (A-E; sixth or seventh leaves of 14-day-old plants) and the early stage (F-J and L-N;

479   seventh leaves of 13-day-old plants) are used for analysis. Dashed rectangles (F-J) and

480   circles (L-N) indicate the tooth periphery regions and tip regions used to measure the

481   GFP intensity in K and O, respectively. Scale bars: 100 μm

482   (K and O) Bar plots of GFP fluorescence intensity. Mean values per pixel and the

483   standard errors from the indicated regions in F-J (K) and in L-N (O) are shown for each

484   line. n = 10, 13, 15, 16 and 17 for wild type, *epfl2*, *er erl1*, *er erl2* and *erl1 erl2*,

485   respectively (K). n = 9, 5 and 10 for wild type, *35::EPFL2* line #1 and #2, respectively

486   (O). Asterisks indicate significant differences ($p < 0.05$ in Welch's *t*-test) from the

487   wild-type data.

488

489   Figure 4. The auxin-responsive patterning of the *EPFL2* and *ER*-family expression

490   promotes the tooth growth.

491   (A) *DR5::GUS* pattern in a young leaf.

492   (B and C) *EPFL2pro::GUS* patterns in mock- (A) and 2,4-D-treated (B) young leaves.

493   (D) Expression levels of *EPFL2* after 2,4-D treatment measured by real-time RT-PCR.

494   The expression levels were normalized with respect to that of *ACTIN2*. The normalized

495   value at 0 hour was set to 1. Thirty shoot apices were collected as a pool for each sample

496   (n = 4). Asterisks indicate significant differences ($p < 0.05$ in Welch's *t*-test) from the

497   0-hour data. Error bars: standard error of the mean.

498   (E, F, H and I) Mature leaf edges of *er erl1* (E), *er erl1 DR5::ER* (F), *epfl2* (H) and *epfl2*

499   *CUC2pro::EPFL2* (I). See also Figure S4G-S4J for other independent transformant lines.

500   (G) *CUC2pro::GUS* pattern in a young leaf.

501   Arrowheads: teeth. Scale bars: A-C and G, 100 μm; E, F, H and I, 1 mm.

502   (J) A model for the maintenance of the auxin response at the growing tooth tip mediated

503   by the feedback regulation between the EPFL2-ER-family system and the auxin response.

504   See the detailed explanation in main text. Cells responding to auxin, green; EPFL2

505   peptides, blue dots; ER-family receptors, red.

Figure1

Figure 1

A

EPFL2 *EPFL9*

EPFL1

EPFL3 *EPFL5*

EPFL4

EPFL8 *EPFL6*

0.1

EPF1 *EPFL7*

EPF2

━━ : Stomatal regulators
━━ : Inflorescence regulators

B WT

C

D *epfl2*

E

F

WT
epfl2

tip

sinus

G

EPFL2pro::GUS

H *er erl1*

I

J *er erl2*

K

L *erl1 erl2*

M

N

WT
erl1 erl2

tip

sinus

O

ERpro::GUS

P

ERL1pro::GUS

Q

ERL2pro::GUS

Figure2

Figure 2

A

WT *erl1 erl2* *er erl1* *er erl2*

epfl2 *erl1 erl2 epfl2* *er erl1 epfl2* *er erl2 epfl2*

1 mm

B

Tooth growth levels

C

ERΔK-GFP	+	−	−	−	+	−	−
ERL1ΔK-GFP	−	+	−	−	−	+	−
ERL2ΔK-GFP	−	−	+	−	−	−	+
EPFL2-FLAG	−	−	−	+	+	+	+

Input

Anti-GFP ◄ER family-ΔK

Anti-FLAG ◄EPFL2

IP: anti-GFP

Anti-GFP ◄ER family-ΔK

Anti-FLAG ◄EPFL2

Figure3

Figure 3

Figure4

Figure 4

A DR5::GUS

B + mock **C** + 2,4-D
EPFL2pro::GUS

D EPFL2

Expression levels (a.u.)

Hours after 2,4-D treatment

E er erl1

F er erl1 DR5::ER

G CUC2pro::GUS

H epfl2

I epfl2 CUC2pro::EPFL2

J

auxin response

EPFL2 signal

auxin response

growth

repatterning of auxin response

auxin

auxin