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A molecular framework for proximal secondary vein branching in the *Arabidopsis thaliana* embryo.

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Summary

- The establishment of a closed vascular network in foliar organs is achieved through the coordinated specification of newly recruited procambial cells, their proliferation and elongation. An important, yet poorly understood component of this process, is secondary vein branching; a mechanism employed in *Arabidopsis thaliana* cotyledons to extend vascular tissues throughout the organ's surface by secondary vein formation.
- To investigate the underlying molecular mechanism in vein branching, we analyzed at a single-cell level the discontinuous vein network of *cotyledon vascular pattern 2 (cvp2) cvp2-like 1 (cvl1)*. Utilizing live-cell imaging and genetic approaches we uncovered two distinct branching mechanisms during embryogenesis.
- Similar to wild type, distal veins in *cvp2 cvl1* embryos emerged from the bifurcation of cell files contained in the midvein. However, the branching events giving rise to proximal veins are absent in this mutant. Restoration of proximal branching in *cvp2 cvl1* cotyledons could be achieved by increasing *OCTOPUS* dosage as well as by silencing of *RECEPTOR LIKE PROTEIN KINASE 2 (RPK2)* expression. The RPK2-mediated restriction of proximal branching is auxin and CLE-independent.
- Our work defines a genetic network conferring plasticity to *Arabidopsis* embryos to adapt the spatial configuration of vascular tissues to organ growth.

1 Introduction

2 The appearance of a continuous vascular network in plants, as means of water
3 and nutrient exchange among organs, greatly contributed to their conquering of a wide
4 range of terrestrial ecosystems (Lucas et al. 2013; Agusti and Blazquez 2020). The
5 evolution of plants is characterized by the selection of a spatial arrangement of
6 vascular strands (vascular patterns) that maximize their overall functionality (Lucas et
7 al. 2013). In *Arabidopsis thaliana* (*Arabidopsis*), the vascular pattern of foliar organs is
8 generated and maintained through the formation of continuous procambial cell files;
9 these are further organized in vascular bundles comprising the conductive tissues
10 phloem and xylem (Lavania et al. 2021). While in most species the patterning of leaf
11 vascular tissues exhibits a high degree of plasticity (Scarpella 2017), the robust and
12 reproducible patterns of the vein network in *Arabidopsis* cotyledons offer an ideal
13 model to identify the positional and molecular cues underlying this process. The
14 vascular network in these organs includes a single primary vein (midvein) that extends
15 along the central part of this organ, and ensures the connection to the stem's vascular
16 system (Scarpella 2017). A pair of secondary veins diverge from the midvein and
17 extend toward the cotyledon margins as this organ expands laterally due to the
18 proliferation of plate meristematic cells (Fig. 10) (Scarpella 2017; Tsukaya 2021).
19 These vascular cells are surrounded by mesophyll cells, which have been proposed to
20 limit vein propagation in *Arabidopsis* leaves through their differentiation, terminating
21 the vein path (Scarpella et al. 2004). Similar to vascular cells, mesophyll cells derive
22 from ground meristem (GM) cells located in the subepidermal layer of cotyledons
23 (Lavania et al. 2021). During the specification of GM cells into either mesophyll or
24 vascular cells, positional cues determine the acquisition of both identities and thus cell
25 types (Lavania et al. 2021). The widely accepted model for cotyledon/leaf vascular cell
26 specification, the auxin canalization model, supports that a directional auxin flow acts
27 as a pre-pattern and reinforces vascular cell identity delineating an incipient vascular
28 path along the cells that have been most exposed to the auxin flow (Scarpella et al.
29 2006; Lavania et al. 2021). In particular, specific subepidermal GM cells of the
30 cotyledon will acquire pre-procambial identity (Scarpella 2017), which is associated
31 with the expression of auxin-related genes such as *MONOPTEROS* (*MP*) or
32 *ARABIDOPSIS THALIANA HOMEBOX 8* (*ATHB8*). The subsequent elongation of
33 pre-procambial cells results in their transition to procambial cells (Lavania et al. 2021).

34 While auxin has been considered the necessary and sufficient signal for vein
35 formation (Lavana et al. 2021), the molecular regulators underpinning the branching
36 of vascular tissues have been poorly explored. Furthermore, the auxin canalization
37 model itself cannot solely explain the appearance of an intermittent vascular network
38 in vein mutants such as *cotyledon vascular pattern 2 (cvp2) cvp2-like 1 (cvl1)* (Carland
39 and Nelson 2009). In particular, the simultaneous depletion of phosphatidylinositol 4,5-
40 biphosphate [PtdIns(4,5)P₂] phosphatases CVP2 and its homologous CVL1 results in
41 a reduction in vascular complexity and the appearance of off-path vascular islands
42 (Carland and Nelson 2009). Initially, CVP2 and CVL1 were suggested to generate
43 PtdIns4P, which is required to activate ARF-GAP SCARFACE (SCF) (Naramoto et al.
44 2009). While the discontinuous vascular network observed in *scf* was related to its role
45 in controlling PIN1 endocytosis (Naramoto et al. 2009), further studies have
46 demonstrated a wider range of subcellular activities such as polarity establishment,
47 which CVP2 and CVL1 modulate. In particular, augmented PtdIns(4,5)P₂ levels
48 enhanced subcellular trafficking towards the vacuole, a process that has been
49 associated with the misspecification of root protophloem and companion cells (Gujas
50 et al. 2020; Rodriguez-Villalon et al. 2015). The root vascular phenotypes associated
51 with the skewed ratio of PtdIns4P and PtdIns(4,5)P₂ can be rescued by introducing
52 *receptor protein like kinase 2 (rpk2)* mutation into a *cvp2 cvl1* background (Gujas et
53 al., 2020). *RPK2* and its homolog *RECEPTOR PROTEIN LIKE KINASE 1 (RPK1)* were
54 first described during embryogenesis to control protodermal identity, as evident by the
55 expanded vascular domain found in *rpk1 rpk2* globular embryos (Nodine et al. 2007).

56 The morphological complexity of vascular tissues has hindered thus far our
57 understanding of the sequence of events resulting in the establishment of the
58 embryonic vein pattern. Here, we revise the directionality of cotyledon vein formation
59 during embryogenesis. We observed that two different mechanisms initiate the
60 branching of distal secondary veins (the secondary veins located in the distal region of
61 the cotyledon) and proximal secondary veins (the secondary veins that emerge from
62 the distal veins in the proximal region of the cotyledon), and that the progression of
63 both types of secondary veins exhibit opposite directionality. While distal branching
64 involves the bifurcation of the cell files comprised in the midvein, proximal secondary
65 veins arise from the branching of distal veins by a poorly understood mechanism.
66 Although distal branching involves PIN1 function, polar auxin transport seems not to
67 control vascular cell fate commitment, as revealed by the continuous vascular network

68 observed in high order *pin* mutants. Furthermore, we report that both *CVP2* and *CVL1*
69 are necessary to promote proximal branching, which is counteracted by the activity of
70 *RPK2*. Our work demonstrated that silencing *RPK2* expression partially restores *cvp2*
71 *cvl1* cotyledon vein network complexity by promoting vein branching, which is
72 necessary to create proximal secondary veins. Through transcriptomic profiling and
73 genetic assays, we found that a reduced activity of *RPK2* restores the impaired
74 cotyledon vein pattern of *cvp2 cvl1* embryos independent of auxin and vascular-
75 specific CLAVATA SURROUNDING EMBRYO (CLE) peptides. By expressing *RPK2*
76 at the cotyledon margins, plants establish a vein network boundary by which veins do
77 not extend towards this area. Additionally we showed that the positive vascular
78 regulator *OCTOPUS* (*OPS*) promotes proximal branching. Together, our work
79 supports a genetic network by which the positive regulation of vein branching by *CVP2*,
80 *CVL1* and *OPS* is limited by *RPK2*, modulating the vein pattern to organ outgrowth and
81 in turn, maximizing the functionality of vascular tissues.

82 **Materials and methods**

83 **Plant material and growth conditions**

84 *Arabidopsis* ecotype Columbia-0 was used as a wild-type control in all cases with the
85 exception of the experiments related to *pin1-3*, *pin1-5* and *clv3-7*, in which Ler was
86 used as a control, respectively. Seeds of *pin1-3*, *pin1-5*, *pin1,3,6,4,7,8* and *PIN1::PIN1-*
87 *GFP* were kindly provided by Dr. Friml (Institute of Science and Technology, Austria),
88 Dr. Miguel Perez-Amador (Institut of Plant Molecular Biology of Valencia, Spain) and
89 Dr. Scarpella (University of Alberta) whereas seeds of *aux1-21 lax1* were provided by
90 Dr. Fankhauser (University of Lausanne). *cle45.cr2* and *clv3* loss-of-function mutants
91 were provided by Dr. Takashi Ishida (Kumamoto Health Science University, Japan)
92 and Dr. Hamant (ENS Lyon), respectively. The transgenic lines *SHR::SHR-GFP* and
93 *MP::MP-GFP* were provided by Dr. Vermeer and Dr. Weijers, respectively.
94 *CVP2::NLS-3xVENUS*, *CVL1::NLS-3xVENUS*, *CVP2::GUS* and *CVL1::GUS* have
95 been previously described (Rodriguez-Villalon et al. 2014; Rodriguez-Villalon et al.
96 2015; Gujas et al. 2020; Carland and Nelson 2009) . Likewise, *rpk2*, *cvp2 cvl1*,
97 *amiRPK2 cvp2 cvl1* and *ops* were reported elsewhere (Gujas et al. 2020). *MP::MP-*
98 *GFP*, *SHR::SHR-GFP*, *DR5::NLS-VENUS*, *PIN1::PIN1::GFP*, *AUX1::AUX1-YFP* and
99 *OPS::OPS-GFP* translational fusion reporter lines in distinct genotypes were obtained
100 by crossing these lines with the indicated loss-of-function mutants or have been

101 previously published (Rodriguez-Villalon et al. 2015). Seeds were surface-sterilized,
102 stratified at 4°C and grown vertically on 0.5x MS plates under standard continuous-
103 light growth conditions. Seedlings were transferred to soil and grown in 5x5 cm pots in
104 peat-based compost medium in a walk-in chamber at constant 23°C, 65% humidity in
105 a 16h photoperiod and light intensity of 250µmol photons m⁻² sec⁻¹ until flowering when
106 siliques were collected to extract embryos.

107 **Cloning and plant transformation**

108 All constructs were generated using double or triple Multi-Site Gateway system
109 following the handbook instructions. To clone *ABI3* promoter, 2kb genomic DNA region
110 was PCR-amplified and introduced via pDNRP4-P1r (Invitrogen) to generate pENTRY-
111 *ABI3*. *CLV3* and *CLE45* were amplified using the following primers *CLV3_XmaI_F*
112 “TAACCCGGGATGGATTCTGAAGAGTTTTCTGC” *CLV3_SpeI_R*
113 “CGCCACTAGTTCAAGGGAGCTGAAAGTTGT” *CLE45_XmaI*
114 “TAACCCGGGATGTTGGGTTCCAGTACAAGA” *CLE45_SpeI_R*
115 *CGCCACTAGTTTAAGAAAATGGCTGAGCTTTGT*” and the PCR products were
116 cloned into pENTRY vectors by standard procedures and further recombine together
117 with pENTRY-*ABI3* into the destination vector pEDO 097. *BAM3* promoter (2139 bp)
118 was amplified using the following primers: p*BAM3_attB4_F*: GGGG ACA ACT TTG
119 TAT AGA AAA GTT GCC CTGCTTCCCTAGTTTATCTAATAAATCTGATG and
120 p*BAM3_attB1r_R*: GGGG AC TGC TTT TTT GTA CAA ACT TGG
121 TGTAACATCAGAAAAATAAAAACAAAATTTGTCC and fused to *NLS-3xVENUS*
122 construct as previously described (Gujas et al., 2020). Transgenic plants were
123 generated using floral-dip transformation techniques as previously described (Gujas et
124 al. 2017).

125 **Confocal microscopy**

126 Mature embryos were dissected from seed coats and stained utilizing the Modified
127 Pseudo Schiff Propidium Iodide (mPS-PI) method according to (Truernit et al. 2008) and
128 then imaged using a ZEISS LSM 780 confocal microscope. Embryos in globular stage
129 and onwards were visualized after being dissected from siliques and fixed in 75%
130 ethanol and 25% acetic anhydride for 24hours at 4 degrees Celsius. After the fixing
131 step, embryos were washed, incubated in 1% period acid and rinsed with water
132 following incubation in Schiff's reagent and Propidium Iodide. Finally, embryos were

133 mounted on glass slides in chloral hydrated (Sigma-Aldrich 302-17-0). Embryos with
134 fluorescent reporters were imaged using Renaissance staining SR2200 as previously
135 described (Smit et al. 2020). Pictures showing the localization of protein in the roots
136 were obtained using a two-photon laser Leica SP8 microscope. 6-day-old roots were
137 stained for 5 min in a 10 µg/ml aqueous solution of propidium. For esthetical reasons,
138 images were rotated and displayed on a matching background. All image processing
139 was performed using ImageJ software. Procambial cell file width was measured on
140 individual procambial cells belonging to the midvein using ImageJ and represented as
141 a mean before and after distal branching. Midvein width was measured as the total
142 width of all procambial cell files comprising the midvein before and after distal
143 branching. Intensity ratios to assess polar protein distribution in procambial and
144 protophloem cells were quantified using ImageJ by measuring mean intensity for each
145 region of interest (ROI). Basal, apical and lateral membranes' signals were normalized
146 by the total ROI area. The mean of all cells quantified is represented as the intensity
147 ratio of each protein in the corresponding genotype.

148 **mRNA sequencing of Arabidopsis embryos and data analysis.**

149 50 torpedo staged embryos of each genetic background, WT, *cvp2 cvl1* and
150 *pRPK2::amiRPK2 cvp2 cvl1* were manually dissected out of the ovule with needles
151 and immediately placed in TRIzol reagent (Ambion) and grounded with a sterile pestle.
152 Two or three biological replicates (50 embryos) were collected and analyzed. All
153 samples were frozen in TRIzol and kept at -80°C until RNA extraction. RNA extraction
154 was completed by incubating samples at 60°C for 30 minutes and then purified
155 according to (Palovaara et al. 2017). The resulting RNA was cleaned up and
156 concentrated using the RNeasy MinElute Cleanup Kit Qiagen (74204) according to
157 (Palovaara et al. 2017). Samples were eluted with 14µL RNase-Free water and stored
158 at -80°C. Isolated RNA displayed a RNA Integrity Number (RIN) ranging from 6.8 to
159 7.7. mRNA-seq libraries were generated with the Smart-seq2 kit (Agilent) and
160 subsequently sequenced in an Illumina NovaSeq 6000 in the Functional Genomics
161 Center Zurich (FGCZ). Data have been stored on the Gene Expression Omnibus
162 (GEO) with accession number GSE178241. Quality validation was carried on using
163 FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), after which
164 adapter sequences were removed using Trimmomatic (v0.39, PMID: 24695404) with
165 options SE -phred33 ILLUMINACLIP:trimmed/NexteraPE-PE.fa:2:30:10

166 SLIDINGWINDOW:4:15 MINLEN:50. Trimmed reads were then aligned onto
167 Arabidopsis thaliana TAIR10 Ensembl genome and genes annotation (retrieved from
168 igenome) using HISAT2 (v2.2.1 ; PMID: 31375807) with options -k 10 --max-intronlen
169 1000--known-splicesite-infileTAIR10_splicesites.txt after applying
170 hisat2_extract_splice_sites to TAIR10 Ensembl genes annotation. Reads count table
171 for annotated genes was generated using the featureCounts function (v2.0.1) from the
172 Subread package (PMID: 24227677) with options -O -M -T 10—largest Overlap--
173 minOverlap 10 --primary. Differential analysis was performed using DESeq2 (v1.22.2,
174 PMID: 25516281). Genes with a p-adjusted value (padj) lower than 0.05 were
175 considered as differentially expressed.

176 **Quantitative reverse transcription-PCR (RT-qPCR)**

177 Total RNA was isolated with a RNeasy Plant Mini Kit (QIAGEN) according to the
178 manufacturer's instructions. cDNA was prepared from 2µg of total RNA with
179 ThermoScientific RevertAID First Strand cDNA Synthesis Kit following manufacturer's
180 instructions. Resulting cDNA was diluted 1:10 in ddH₂O and 2 µL of the resulting
181 dilution were used in the PCR reaction. qPCR was prepared using KAPA SYBR FAST
182 qPCR mix. All reactions were performed in triplicates and expression levels were
183 normalized to those of *PDF2*. Primers sequences are shown below: *PDF2*_Fw: 5'-
184 TAACGTGGCCAAAATGATGC-3'; *PDF2*_Rv: 5'-GTTCTCCACAACCGCTTGGT-3'
185 (Czechowski et al. 2005); *AUX1*_Fw: 5'-GGATGGGCTAGTGTAAC-3'; *AUX1*_Rv: 5'-
186 TGACTCGATCTCTCAAAG-3' (Dindas et al. 2018); *PIN1*_Fw: 5'-
187 ACAAACGACGCAGGCTAAG-3', *PIN1*_Rv: 5'-AGCTGGCATTTCATGTTCC-3'
188 (Heisler et al. 2005); *OPS*_Fw: 5'-GACAGGTCTAGTAGCTCCATGAGG-3'; *OPS*_Rv:
189 5'-AGCTTTGGCTCGTCCATATCCG-3'. *OPS* primers were designed using
190 QuantPrime.

191 **Histology and GUS staining**

192 Developing cotyledons were imaged at 7 or 8 days as indicated. Cotyledons and leaves
193 were fixed with 3:1 ethanol: acetic acid, dehydrated in 80% ethanol, and then 100%
194 treated with 10% sodium hydroxide for 1hour at 37°C and mounted in 50% glycerol.
195 Black and white images were taken and brightness and contrast were adjusted using
196 ImageJ. To visualize GUS staining, we used a staining buffer as previously described
197 in (Carland and Nelson 2004) with 2mM 5-bromo-4-chloro-3indol-b-D-glucuronide and
198 1mM potassium ferricyanide and ferrocyanide. We considered branching points (BP)

199 as lateral/secondary veins bifurcated from both midvein and distal secondary veins
200 (see model shown in Fig.1O).

201 **Seedling treatments**

202 To perform NPA and CLE treatments, 4-day-old seedlings grown in MS were
203 transferred to a media supplemented with or without NPA (10 μ M), CLV3 (5nM), CLE45
204 (20nM), CLE25 (100nM) and CLE26 (150nM) for 5 days. The quantification of the root
205 sensitivity to NPA treatment was performed by measuring the length of the primary
206 root using ImageJ.

207

208 **Results**

209 **Two distinct branching mechanisms control cotyledon vein network formation** 210 **in torpedo stage embryos**

211 To gain further insight into the contribution of procambial cell identity acquisition
212 and cell proliferation in cotyledon vein network complexity, we first sought to
213 characterize the cotyledon vascular ontogeny during embryogenesis. The emergence
214 of procambial cell files can be detected by confocal microscopy based on their
215 characteristic narrow and elongated morphology. Confocal microscopy analysis of
216 Renaissance stained embryos revealed the appearance of procambial cell files at early
217 torpedo stage, when a midvein could be detected within the cotyledons (Fig. 1A-C).
218 While the complete establishment of the vein network was detectable in late torpedo
219 stage, elongated narrow cells diverging from the midvein could be observed at earlier
220 developmental time points (what we termed intermediate torpedo stage) (Fig. 1D, E).
221 To further corroborate these observations, we monitored the auxin efflux PIN1 protein,
222 whose distribution in torpedo embryos is restricted to procambial and protodermal cells
223 (Fig. 1F). We observed a progressive formation of the midvein concomitant with the
224 base-to-tip appearance of distal secondary veins (Fig. 1F-I, O). The morphological
225 progressive directionality of the distal secondary veins was corroborated through
226 monitoring auxin response by means of *DR5::NLS-VENUS* and by the gene
227 expression analysis of *MP* (pre/procambial) as well as the root phloem regulator
228 *BARELY ANY MERISTEM 3 (BAM3)* (Fig. 1J-N,O) (Scarpella et al. 2004; Przemeck
229 et al. 1996; Rodriguez-Villalon et al. 2014). Contrary to the base-to-tip growth of the
230 distal secondary veins, PIN1 tagged with a green fluorescence protein (GFP) polarly

231 accumulates at the basal membrane of the vein network procambial cells as previously
232 reported (Fig. 1G,H) (Scarpella et al. 2006). Since the distal vascular strand is
233 extended in a base-to-tip manner, the polar accumulation of PIN1 at the basal
234 membrane appears to act as a reinforcement identity mechanism in cells already
235 committed to vascular cell fate. To assess the origin of the procambial cell files
236 resulting in distal secondary vein formation we analysed the region near the base of
237 the cotyledon. Here we observed that the number of cell files that constitutes the
238 midvein is higher before distal secondary vein formation vs right after (Fig. 1P-S). In
239 contrast, the number of cell files comprising the proximal and distal veins appears to
240 be similar (Fig. 1P-U). These observations indicate that while distal secondary veins
241 directly diverge from the cell files comprised in the midvein, the branching of proximal
242 veins appears to be different and follow a yet-to-be described mechanism.

243 **In *cvp2 cvl1* mutants GM cells fail to commit to procambial cell identity**

244 To better understand the spatio-temporal arrangement of cotyledon vascular
245 formation during embryogenesis, we decided to exploit the discontinuous *cvp2 cvl1*
246 cotyledon venation pattern as a model. Consistent with previous studies (Carland and
247 Nelson 2009), 7-day old *cvp2 cvl1* cotyledons exhibit a discontinuous and more
248 simplified cotyledon vein network (Fig. 2A-C) (Carland and Nelson 2009). Yet, the
249 midvein was always morphologically intact based on cotyledon clearing visualization
250 techniques (Fig. 2B). These vascular defects originated during embryogenesis, as
251 manifested by the analysis of cotyledons in mature embryos stained by pseudo-schiff-
252 propidium iodide (mPS-PI) (Fig. 2D-G). At this developmental stage, procambial cells
253 can be observed as elongated, narrow elements while surrounding mesophyll cells are
254 rather spherical (Fig. 2E, G). The latter morphology can be detected in between
255 vascular islands, indicating that GM cells in *cvp2 cvl1* fail to commit to procambial cell
256 identity (Fig. 2G) and become mesophyll cells instead. This notion was corroborated
257 by the lack of *ATHB8* expression in the spherical cells flanking *cvp2 cvl1* vascular
258 islands, whereas a continuous expression of this gene within the cotyledon vein
259 network can be detected in wild type embryos (Fig. 2H-K). Since *CVP2* and *CVL1* are
260 expressed in the vein domain from globular stage onwards (Supporting Information
261 Fig. S2, Fig. 2L-P), we decided next to evaluate the vascular identity domains in early
262 embryonic stages in *cvp2 cvl1*. In globular embryos, the expression of *SHORT ROOT*
263 (*SHR*) or *MP* marks the onset of vascular formation and delineates the separation of

264 the future ground tissues (De Rybel et al. 2016; Scarpella 2017). At this particular
265 stage, none of the vascular markers analyzed showed an aberrant expression domain
266 in *cvp2 cvl1* embryos with the exception of *MP* in globular embryos, whose expression
267 appears very weak (Supporting Information Fig. S2F-M). Together, these results
268 indicate that the cotyledon vein network discontinuities in *cvp2 cvl1* are not due to
269 impaired vascular identity domains during the early stages of embryogenesis and must
270 occur in later embryonic developmental stages.

271 **CVP2 and CVL1 activities are required to modulate cell division and proximal** 272 **branching**

273 Matching the embryonic stages relevant to their expression, we observed a
274 plethora of aberrant morphologies in *cvp2 cvl1* double mutant embryos (Supporting
275 Information Fig. S3). While some embryos resembled wild type (phenotype A), several
276 globular embryos exhibited an excessive number of divisions in the suspensor and
277 hypophysis (phenotype B, Supporting Information Fig. S3). Moreover, cells in the
278 globular embryo of *cvp2 cvl1* display aberrations regarding the number and the
279 orientation of their divisions (Supporting Information Fig. S3). We observed a high
280 embryo abortion rate in *cvp2 cvl1* siliques (ca. 50%), which we believe is partially
281 contributed by aberrant divisions observed mainly in the hypophysis, since an
282 abnormal suspensor development has been reported to be lethal (ten Hove et al.
283 2015). Although subsequent developmental stages appear morphologically similar to
284 wild type, we cannot exclude the appearance of mild division defects occurring within
285 the vascular domain due to the technical limitations imposed by working with torpedo
286 stage embryos (Supporting Information Fig. S3). However, we observed that proximal
287 branching was absent in the cotyledon vein network of *cvp2 cvl1* (Fig. 2Q-T) even if
288 *CVP2* expression can be detected at the BPs (Fig. 2N-P). In the event of secondary
289 vein formation within the proximal cotyledon region, these procambial cell files
290 appeared to be connected to the basal end of the midvein but without a clear apical
291 branching site (Fig. 2R, S). Indeed, these incipient cell files propagate in a base-to-tip
292 manner (Fig. 2S). Taken together, the presence of an intact midvein and the lack of
293 proximal secondary vein branching imply that the activities of *CVP2* and *CVL1* are
294 required to initiate proximal secondary veins at the proximal branching point.

295 **PIN1 polar distribution is not altered in *cvp2 cvl1***

296 To gain further insight into the vein network branching defects observed in *cvp2*
297 *cvl1* embryonic cotyledons, we decided first to assess the role of auxin and its PIN1-
298 mediated transport. Confocal microscopy analysis of PIN1-GFP in *cvp2 cvl1* torpedo
299 embryos showed a normal basal polarization of the auxin carrier in the distal secondary
300 veins (Fig. 3A-D'), which we confirmed by the quantification of PIN1 accumulation in
301 the basal membrane in comparison to a lateral membrane (Fig. 3E) Similarly, analysis
302 of *PIN1-GFP* in root protophloem cells, where PIN1 is polarly accumulated at the basal
303 membrane, did not show any abnormal distribution of this auxin efflux carrier (Fig. 3F-
304 J'). Previous reports have suggested that PIN1 activation requires the function of
305 PIP5K1 and PIP5K2 (Marhava et al. 2020), which catalyze the inverse enzymatic
306 reaction as that of CVP2 and CVL1 (Gujas and Rodriguez-Villalon 2016). To determine
307 the extent to which the vascular phenotypes observed in *cvp2 cvl1* cotyledons may be
308 due to a perturbed PIN1 activity, we decided to analyze the vascular phenotypes of
309 *pin1* seedlings. A plethora of cotyledon defects have been described in distinct *pin1*
310 mutants, including fused cotyledons and aberrant morphologies (Friml et al. 2003). To
311 overcome the impact of defective organogenesis in the analysis of vascular patterning,
312 we decided to focus on separated cotyledons from the loss-of-function *pin1-3* and *pin1-5*
313 mutants. We consistently observed an increased distal branching in *pin1* single
314 mutants while the continuity of the vascular strands appeared intact (Fig. 4A-C, G-I).
315 Likewise, the simultaneous depletion of other vascular PIN carriers such as *PIN3 PIN4*
316 *PIN6 PIN7* and *PIN8* (*pin1,3,4,6,7,8*) did not result in a discontinuous vein pattern (Fig.
317 4E, H, I), although the duplication of the midvein and subsequent bifurcation in distal
318 veins could be found in these mutants (Fig. 4E). These observations indicated that the
319 polar auxin transport represses distal branching. An increase in PIN1 dosage by
320 introducing *PIN1::PIN1-GFP* (Yanagisawa et al., 2021) (Supplemental Fig. S1) in *cvp2*
321 *cvl1* did not aggravate the vascular phenotype of the double mutant (Fig. 3K-N),
322 consistent with our findings showing that *cvp2 cvl1* exhibits defects in proximal
323 branching yet is able to form distal secondary vein branching points (Fig. 2Q, S). To
324 identify the origin of vascular identity failure observed in *cvp2 cvl1* embryonic
325 cotyledons, we decided to analyze auxin response. The deficient activity of the auxin
326 receptors TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX
327 (TIR1/AFB) suppress the formation of secondary veins whereas the midvein appears
328 still intact (Fig.4 F), a phenotype consistent with previous publications (Mazur et al.
329 2020). To further investigate whether the inability of *cvp2 cvl1* cells to perceive auxin

330 could explain the appearance of a discontinuous vascular network in this mutant, we
331 decided to monitor the distribution of *DR5::NLS-VENUS*, a widely used auxin response
332 biosensor. Confocal microscopy analysis of embryonic cotyledons showed a positive
333 correlation among cells exhibiting vascular morphology and *DR5* expression (Fig. 4J-
334 M). However, no signal was detected in the cells flanking vascular island (Fig. 4L-M).
335 Taken together, our observations suggest that while auxin cues may be necessary to
336 establish continuous secondary veins in *cvp2 cvl1* embryos, the polar transport of this
337 hormone contributes to regulate the distal branching of secondary veins but not their
338 continuity.

339 **Silencing of *RPK2* expression rescues the proximal branching defects of** 340 ***cvp2 cvl1* embryonic cotyledons**

341 Considering that auxin transport itself cannot solely explain the vascular defects
342 observed in *cvp2 cvl1*, we decided to explore cotyledon vascular ontogeny in plants
343 with a deficient activity of *RPK2*. We have previously reported that silencing of *RPK2*
344 expression in *cvp2 cvl1* (*amiRPK2*) restores the continuity of the root protophloem
345 strands in this mutant (Gujas et al. 2020). Notably, a partial silencing of *RPK2*
346 expression did not rescue the discontinuous secondary veins of *cvp2 cvl1* (Fig. 5 A, B,
347 D, E). Instead, an increased number of branching points could be observed in these
348 lines (Fig. 5A, B, D-G, K, K'). In light of these results, we decided to further elucidate
349 the potential role of *RPK2* in cotyledon vein patterning. Examination of *rpk2-2* vascular
350 pattern revealed the occasional appearance of additional proximal branching points
351 within the embryonic cotyledon vein network, even if a complete/closed additional
352 aerole was rarely observed (Fig. 5C, F, G). Furthermore, divisions giving rise to a
353 bifurcated vein path could also be occasionally detected in mPS-PI stained *rpk2-2*
354 embryos (Fig. 5H-K'). Consistent with previous studies, *RPK2* expression can be
355 mainly detected in the protoderm and in some cells of its adjacent cell file (Fig. 5L, L')
356 (Nodine et al. 2007). Confocal microscopy analysis of *RPK2* expression in *cvp2 cvl1*
357 however, revealed a slightly broader expression pattern towards the inner cell layers,
358 with its greatest expression at the tip of the cotyledon (Fig. 5M, M'). By repressing the
359 branching of proximal secondary veins, *RPK2* modulates vein complexity while
360 preventing the extension of procambial cell files into the cotyledon margin area.

361 **The partial *RPK2*-mediated restoration of *cvp2 cvl1* vascular phenotype seems** 362 **to be *PIN1*-independent**

363 To gain further insight into the mechanisms by which RPK2 modulates vein
364 patterning we decided to monitor the transcript profiles of wild-type, *cvp2 cvl1* and
365 *amiRPK2 cvp2 cvl1* embryos between the early torpedo and bent cotyledon stages.
366 Both mutants showed hundreds of differentially expressed genes (DEGs) when
367 compared to wild type (782 in the case of *cvp2 cvl1*, 423 in the case of *amiRPK2 cvp2*
368 *cvl1*). Remarkably, silencing *RPK2* expression resulted in a partial complementation at
369 the transcriptomic level of the *cvp2 cvl1* defects (Fig. 6A-C), as evident for instance by
370 the repression of the augmented transcript levels of *ALTERED PHLOEM (APL)* (Bonke
371 et al. 2003) and *SISTER APL (SAPL)* (Ross-Elliott et al. 2017) of *cvp2 cvl1* embryos
372 (Fig. 6D). Consistent with this result, the induced expression of At2g28810 (Furuta et
373 al. 2014) or the callose biosynthetic enzyme *CALS7* (Vaten et al. 2011) -known targets
374 of *APL*- got reverted to a wild-type situation in *amiRPK2 cvp2 cvl1* (Fig. 6D). Moreover,
375 the expression of genes associated with mesophyll identity such as *CHLOROPHYLL*
376 *A/B BINDING PROTEIN 3 (CAB3)* (Mitra et al. 1989) is not affected in *cvp2 cvl1*
377 embryos (Fig. 6E), excluding an altered genetic program of mesophyll cells as
378 responsible for *cvp2 cvl1* procambial cells' misspecification. Surprisingly, our
379 transcriptomic analysis did not reveal altered expression levels neither in auxin
380 biosynthetic nor auxin related genes in *cvp2 cvl1* embryos (Fig. 6F), with the exception
381 of the *AUXIN TRANSPORTER 1 (AUX1)* and *AUXIN TRANSPORTER-LIKE PROTEIN*
382 *1 (LAX1)* (Swarup and Bhosale 2019). Contrary to *cvp2 cvl1*, cotyledons of *aux1* and
383 *lax1* null mutants exhibit a continuous vein network, even when both genes are
384 simultaneously knocked-out (Fig. 6G, J-N). Introgression of *AUX1-GFP* transgene in
385 *cvp2 cvl1* did not rescue the vascular phenotype of this mutant (Fig. 6G-I, M, N,
386 Supporting Information Fig.1), inferring that *rpk2*-mediated rescue of *cvp2 cvl1* is not
387 directly due to the modulation of auxin influx and its biosynthetic pathways. To exclude
388 a potential regulation of PIN1-mediated auxin distribution by RPK2 as the restoring
389 mechanism of *cvp2 cvl1* defects, we performed chemical treatments using a widely
390 used auxin transport inhibitor, 1-naphtylphthalamic acid (NPA), in leaves. These
391 organs form *de novo* during the post-embryonic growth of the plant. The establishment
392 of the vascular strands in leaves is believed to follow a similar molecular regulation as
393 cotyledons, even if the directionality of the secondary strands differs (Lavania et al.
394 2021). Under NPA treatment, the acropetal transport of auxin that directs the growth
395 of the midvein is altered, resulting in the duplicated formation of midveins (Scarpella et
396 al. 2006). Additionally, secondary vein formation is slightly affected whereas tertiary

397 vein formation could appear suppressed (Scarpella et al. 2006). Similar to wild type
398 and *cvp2 cvl1* plants, NPA-treated *rpk2* leaves appeared very affected in terms of vein
399 complexity (Supporting Information Fig. S4A,F). Moreover, root growth inhibition of
400 *rpk2* roots subjected to NPA treatments was very similar to the one observed in wild
401 type and *cvp2 cvl1* plants (Supporting Information Fig.S4 L). These observations imply
402 that RPK2 activity is not necessary to integrate auxin polar transport cues into the
403 establishment of the vein pattern, despite the rescue of the *cvp2 cvl1* leaf vein pattern
404 through silencing *RPK2* expression (Supporting Information Fig. S4G-I). Consistent
405 with these results, live cell image analysis of PIN1-GFP localization in *rpk2* mutants
406 did not reveal any defective PIN1 localization in this genetic background. Given the
407 reduced offspring of *rpk2*, we decided to focus in the root stele to have a clear
408 assessment of PIN1 polarity. Within the stele, PIN1 polarly accumulates at the basal
409 cell membrane. The polar PIN1 distribution in *rpk2* roots (Supporting Information Fig.
410 S4J-K') together with the unaltered response of this mutant to NPA treatments
411 suggests that RPK2 activity in modulating vein emergence is independent of PIN1-
412 mediated auxin transport.

413 **RPK2-mediated suppression of proximal branching is independent of CLE** 414 **peptides**

415 To further explore the underpinning molecular mechanisms of RPK2-mediated
416 repression of secondary proximal veins, we analyzed the potential involvement of CLE
417 peptides in controlling this process. *rpk2* loss-of-function mutant is resistant to the root
418 growth-inhibition effect of a vast array of CLE peptides, including CLAVATA 3 (CLV3)
419 and CLE45 (Gujas et al. 2020). In particular, RPK2 has been shown to perceive CLV3
420 peptide, negatively regulating root and shoot apical meristem cell proliferation (Racolta
421 et al. 2018). Moreover, we have previously described that the *rpk2*-mediated
422 restoration of a continuous root protophloem strand in *cvp2 cvl1* involves CLE45
423 (Gujas et al. 2020). To investigate the potential role of both peptides in orchestrating
424 cotyledon vascular patterning during embryogenesis, we cleared cotyledons of *clv3*
425 and *cle45* mutant seedlings (Yamaguchi et al. 2017). Neither *cle45* nor *clv3* cotyledons
426 showed any perturbation in their vein pattern or vein network complexity (Supporting
427 Information Fig. S5A-D, G), suggesting that they are either not involved in regulating
428 cotyledon vein networks or that there is a high redundancy within this family of
429 peptides. An overexpression of *CLE45* results in lethality (Depuydt et al. 2013). Thus,

430 we aimed at increasing *CLE45* and *CLV3* expression specifically during
431 embryogenesis. To this aim we generated transgenic lines expressing *CLE45* and
432 *CLV3* under the *ABSCISIC ACID INSENSITIVE 3 (ABI3)* promoter. *ABI3* is broadly
433 expressed in the embryo, beginning at the globular stage until embryo maturation and
434 ceases to be expressed after germination (To et al. 2006). Consistent with the previous
435 results (Supporting Information Fig. S5), analysis of 8-day old *ABI3::CLE45* seedlings
436 did not reveal any vascular defects in the cotyledons, implying that RPK2-mediated
437 regulation of vascular patterning is independent of *CLE45* (Supporting Information Fig.
438 S5E, G). In contrast, a discontinuous vascular network in 25% of *ABI3::CLV3* seedlings
439 was detected, even if this frequency is 5% in wild type plants (Supporting Information
440 Fig. S5F, G). This feature was associated with reduced proximal branching and, in
441 turn, vascular complexity (Supporting Information Fig. S5F, G). Our results indicated
442 that despite this peptide having the ability to suppress proximal vein branching, its
443 restricted expression at the shoot apical meristem (Brand et al. 2002) most likely
444 excludes it as the ligand responsible for RPK2-mediated control of proximal branching.

445 **OCTOPUS promotes proximal branching in embryonic cotyledons**

446 Another factor involved in the regulation of procambial cell division in embryonic
447 cotyledons is the plasma membrane-associated protein OCTOPUS (OPS) (Truernit et
448 al. 2012). In *ops* embryos, a reduced number of cell files in distal secondary veins can
449 be detected as the procambial cells fail to periclinally divide (Roschttardt et al. 2014).
450 Live-cell imaging analysis of OPS distribution revealed a broader expression domain
451 than that of the future vein path in intermediate torpedo stage embryos (Fig. 7I-I') and
452 a non-polar cellular distribution in cells not within the vein path (Fig. 7J, J'). In contrast,
453 OPS appears polarly distributed in the apical cell membrane of procambial cells in
454 intermediate torpedo stage embryos and in mature embryos once the vein network has
455 achieved its final complexity (Fig. 7K-L',M). OPS distribution can also be detected in
456 the non-procambial cells adjacent to the branching point and close to the cotyledon
457 margin of intermediate torpedo embryos, near the *RPK2* expression domain (Fig. 7I-
458 J). Interestingly, an increase in OPS protein dosage by introducing the hyperactive
459 GFP-tagged OPS protein (Supporting Information Fig. S1) (Breda et al. 2017)
460 increased the number of proximal branching points in wild type plants (Fig. 7A, C, F).
461 Likewise, an increase in branching points was observed in *cvp2 cvl1* as well as an
462 overall more continuous cotyledon vein network, even though vein pattern

463 discontinuities still persisted (Fig. 7A-H). These results confirmed that *CVP2* and *CVL1*
464 are necessary to establish the proximal branching of secondary veins, a process
465 enhanced by the positive vascular regulator *OPS* and counteracted by *RPK2*.

466 Discussion

467 ***CVP2* and *CVL1* regulate the specification of provascular cells and proximal** 468 **branching**

469 Within a multicellular organ, the self-establishment of tissue patterns such as
470 vascular tissues requires the coordinated activity of oriented cell divisions and cell fate
471 commitment (Lavania et al. 2021). Our data reveal that *CVP2* and *CVL1* contribute to
472 the regulation of vein patterning by modulating vascular specification and the
473 branching points of proximal secondary veins (Fig. 2). On the one hand, *cvp2 cvl1*
474 cotyledons exhibit a discontinuous vein network because of GM cell misspecification
475 into mesophyll instead of pro-vascular cells (Fig. 2). A defective vascular identity
476 perfectly matches with the lack of auxin activity in these cells, as revealed by auxin
477 biosensors (Fig. 4L, M). Yet, the lack of this hormone in these cells does not correlate
478 with a significant different auxin transcriptional response in *cvp2 cvl1* embryos at an
479 earlier developmental stage, the torpedo stage (Fig. 6). Moreover, our analysis of
480 vascular identity domains in *cvp2 cvl1* globular and heart stage embryos did not reveal
481 defects in the establishment of vascular vs ground tissue domains over embryogenesis
482 (Supporting Information Fig. S2). Thus, an alternative explanation consistent with *cvp2*
483 *cvl1* phenotypes is that a premature differentiation of mesophyll cells terminates vein
484 propagation, a phenomenon that has been described in leaves (Scarpella et al. 2004).
485 However, further experiments are required to elucidate whether *CVP2* and *CVL1*
486 participate in repressing mesophyll differentiation, even if the transcripts associated to
487 mesophyll identity did not appear significantly altered in *cvp2 cvl1* embryos as evident
488 by our transcriptomic profiling (Fig. 6E). Alternatively, the deficient phloem-mediated
489 sugar transport in *cvp2 cvl1* cotyledons (Rodriguez-Villalon et al. 2015; Carland and
490 Nelson 2009) may inhibit the regulation of GM specification into procambial cells.
491 Further studies are required to assess whether sucrose intervenes in the conversion
492 of GM into either mesophyll or procambial cells and whether a perturbed sucrose
493 distribution may translate in the appearance of vascular islands in *cvp2 cvl1*. While
494 these disconnected vascular islands can be frequently observed in the *cvp2 cvl1*
495 cotyledon vein network, the midvein always appears intact (Fig. 2). In cotyledons, the

496 midvein originates directly from a pool of cells located in the vicinity of the shoot apical
497 meristem, which start to elongate and divide parallel to the proximo-distal axis of
498 cotyledon growth at the torpedo stage (Nelson and Dengler 1997). This process is
499 mostly regulated by auxin signalling factors such as *ARF6/CULLIN1* or
500 *ARF5/MONOPTEROS* (Scarpella et al. 2004; Scarpella et al. 2006), implying that the
501 activity of these factors remains unaffected by the loss of *CVP2* and *CVL1* during
502 embryogenesis until early torpedo stage. Several vascular-associated genes appeared
503 miss-expressed in *cvp2 cvl1* embryos, even if *RPK2* silencing only reverted *APL* (and
504 *APL*- downstream target genes) as well as *SAPL* expression (Fig. 6). On the other
505 hand, the reduced vein network complexity described in *cvp2 cvl1* cotyledons reflects
506 the inability of proximal secondary veins to initiate branching. Together with the
507 aberrant divisions detected in the suspensor and hypophysis (Supporting Information
508 Fig. S3), these observations infer that the activity of both *CVP2* and *CVL1* may be
509 required to a certain extent, to control the orientation of cell divisions. Interestingly,
510 closer examination at the branching point in wild type plants revealed a periclinal
511 division of a distal vein cell next to another cell harbouring either a vascular marker or
512 *DR5* (Supplemental Information Fig. S6). While it appears possible that a periclinal
513 division precedes the formation of a vascular cells from which the incipient proximal
514 secondary vein will extend, we cannot exclude at this stage that a plate meristematic
515 cell adjacent to the vascular cells is recruited at the branching point to give rise to the
516 new vein. Another interesting aspect of this process is whether auxin is involved in the
517 coordination of proximal vein branching. Genetic blockage of polar auxin transport by
518 depleting the activity of PIN transporter results in an elevated number of distal veins
519 with additional distal branching points (Fig. 4). While these results indicated that polar
520 auxin cues contribute to repress distal branching, they cannot explain the reduced
521 cotyledon vein network complexity observed in *cvp2 cvl1* nor its intermittent cotyledon
522 vein pattern. Our observations show that PIN1 polarity appears unaltered in *cvp2 cvl1*
523 veins (Fig. 3). Additionally, genetic increase of PIN1 dosage does not enhance the
524 branching defects observed in *cvp2 cvl1* (Fig. 3), indicating that at least another
525 mechanism independent of PIN1 must be responsible for this phenotype. Previous
526 studies have shown that regardless of the absence of auxin transport, the remnant
527 auxin signalling is sufficient to guide the recruitment of new procambial cells into
528 vascular cell files (Verna et al. 2019). However, auxin response in *cvp2 cvl1* cotyledons
529 by means of *DR5* distribution appears discontinuous in the vein path. Hence, it appears

530 possible that an auxin-independent mechanism preceding *PIN1* expression is required
531 to modulate vascular cell identity acquisition and in turn, the future vein path.

532 ***RPK2* constrains proximal secondary vein branching**

533 In globular embryos, *RPK2* is expressed in the outermost layer that gives rise
534 to the ground tissues, an expression pattern that is maintained during the following
535 developmental stages of embryogenesis (Fig. 5) (Nodine et al. 2007). Previous studies
536 have suggested that *RPK2* is required to exclude vascular identity from the
537 protodermis of globular embryos (Nodine et al. 2007). Here, we provide evidence that
538 *RPK2* activity is not only necessary to exclude vascular identity in the future epidermis
539 but also necessary to modulate the branching of secondary veins (Fig. 5). The activity
540 of *RPK2* in controlling the development of cortical and endodermal cells have been
541 mostly explained by the perception of CLE 17 (Racolta et al. 2018). Within the root
542 stele, CLE45 sensing by *RPK2* confers developmental plasticity to companion and
543 protophloem cells to safeguard phloem functionality by re-establishing a correct
544 phloem pattern in case the original one fails to form (Gujas et al. 2020). Yet, our
545 analysis implies that the suppression of cotyledon proximal branching by *RPK2* is
546 independent of vascular-specific CLE peptides (Supporting Information Fig. S5),
547 inferring a differential mechanism between vascular formation in the shoot and the root.
548 Our results indicate that the negative *RPK2*-mediated control of vein patterning is
549 independent of auxin. Yet, plant cells need to integrate PAT cues and *RPK2* signalling
550 to coordinate the establishment and maintenance of vascular tissues. Considering that
551 *rpk2* sensitivity to the blockage of PAT by NPA is not perturbed (Supporting Information
552 Fig. S4), additional factors other than *RPK2* may act as a hub to integrate polar auxin
553 transport cues with *RPK2*-mediated signalling. *RPK2* expression ends at the cell file
554 delineating the border between the cotyledon margin and the vein cells, in a region
555 where the positive vascular regulator OPS is localized before the establishment of a
556 closed vein network (Fig. 5L, L', 7I-K). While future studies will elucidate whether the
557 mutually exclusive presence of OPS and *RPK2* is required to trigger proximal vein
558 branching, it appears possible that OPS contributes to integrate PAT cues with *RPK2*
559 signalling by means of its interaction with VASCULATURE COMPLEXITY AND
560 CONNECTIVITY (VCC) (Roschztardt et al. 2014). The latter contributes to spatio-
561 temporally modulate PAT during cotyledon vein formation by delivering *PIN1* to the
562 vacuole for protein degradation (Yanagisawa et al. 2021). Together, our results

563 revealed a molecular genetic framework by which plants at the late stages of
564 embryogenesis modulate the tissue complexity of their vascular networks. Although
565 the function of *RPK2* is conserved in cylindrical and foliar organs, its regulation appears
566 to be tissue specific, comprising unique molecular mechanisms to optimize the
567 functionality of vascular tissues to the constantly changing shape of the organs to
568 which they belong.

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577

578 **Author contributions**

579 AR-V, EK and NB-T designed the research experiments. EK, NB-T, AS performed
580 experiments and data analysis. AS and BG generated genetic material. AR-V, EK and
581 NB-T wrote the manuscript.

582

583 **Declaration of interests**

584 The authors declare no competing interests.

585

586 **Figure legends**

587 **Figure 1. Vein progression and branching in torpedo embryos. A-B)**
588 Representative pictures of mPS-PI cell wall-stained embryos in late transition stage
589 (A) and late heart stage (B). **C-D)** Analysis by confocal microscopy of extracted
590 embryos from ovules and stained with the cell wall dye SR2200 Renaissance. (C)
591 Embryo in transition between heart and early torpedo in which the future midvein is
592 marked by a white arrow. (D) Cotyledon of early torpedo stage in which the secondary
593 vein formation can be detected. Note that in D, based on the elongated morphology of
594 procambial cells, secondary vein formation is initiated from the midvein and
595 progressing upwards towards the top of the cotyledon (marked by white arrows). **E)**

596 Cotyledon of mature embryo in which the cotyledon vein network is completed. **F-I)**
597 Representative images of *PIN1::PIN1-GFP* early and late torpedo stage embryos
598 stained with SR2200 as a cell wall counter stain showing the progression of midvein
599 or primary vein (F), distal secondary vein (G-H) and proximal secondary vein formation
600 (I). Dashed red arrows represent the directionality of the forming veins. **J-K)**
601 Cotyledons from early torpedo stage embryos harbouring *DR5::NLS-VENUS* showing
602 the progression of distal secondary veins (J) as well as the initiation of the proximal
603 secondary veins (K). **L-N)** Early torpedo stage embryos harbouring *MP::MP-GFP* (L),
604 and *BAM3::NLS-3XVENUS* (M, N) showing cotyledon proximal vein formation
605 occurring in a tip-to-base manner (n=25/27), except in N, in which proximal veins also
606 proceeds in a base-to-tip manner (n=2/27). **O)** Scheme representing the proposed
607 branching sites of distal and proximal secondary veins. Distal branching points 1 and
608 2 are represented by the red dots and the direction of vein formation is represented by
609 the white arrows. Proximal secondary vein branching, branching points 3 and 4 (red
610 dots) and the direction of vein formation (white arrows) are represented in the lower
611 panels. Note that distal vs proximal secondary vein formation occurs normally in
612 opposing directions. The rare appearance of proximal veins in base-to-tip manner is
613 represented by a dashed white arrow. **P-R)** Representative images of proximal and
614 distal branching in embryonic cotyledons stained with mPS-PI and visualized by
615 confocal microscopy. ADB: after distal branching; BDB: before distal branching. **S-U)**
616 Quantification of the frequency of appearance of the indicated number of cell files (S),
617 average midvein width (T) and average midvein cell file width (U) in the region marked
618 as ADB and BDB. Note that the difference represented in (T) are not due to differences
619 in the width of procambial cells in these regions, as indicated in (U). Scale bars
620 represent 20µm in A-D, J, L, N and O and 50µm in E, H, I, K, P.

621 **Figure 2. Cotyledon vein defects of *cvp2 cvl1*.** **A, B)** Cleared 7-day-old cotyledons
622 of WT and *cvp2 cvl1* imaged with a stereomicroscope in bright field on a black
623 background. **C)** Quantification of the frequency of ground meristem cells surrounding
624 a disconnected vascular islands (gap frequency) and vein complexity in WT and *cvp2*
625 *cvl1* cotyledons. n= 20-40 for each genotype. This quantification is part of a bigger
626 experiment which is fully represented in Figure 4G. **D-G)** Confocal microscopy
627 analysis of the vein pattern of mature embryonic cotyledons (vein network at its
628 complete stage) of WT and *cvp2 cvl1* having undergone mPS-PI staining. E and G are
629 magnifications of D and F. White arrows indicate vein gaps. Number of embryonic

630 cotyledons exhibiting gaps is indicated in E and G. **H-K) *ATHB8::GUS*** expression in
631 WT and *cvp2 cvl1* mature embryonic cotyledons. (I, K) Magnifications of the veins
632 displayed in H and J respectively. **L-P)** Expression pattern of *CVL1::GUS* and
633 *CVP2::GUS* in embryos. Magnification of an embryonic cotyledon (N) displaying *CVP2*
634 expression in the proximal branching points is shown in (O,P). **Q-S)** mPSI-PI staining
635 of mature embryonic cotyledons of WT and *cvp2 cvl1* showing distal vs proximal
636 branching. Note the two phenotypes observed in *cvp2 cvl1*, when there is a third
637 branching event (R) or not (S). **T)** Quantification of the frequency of the reduced vein
638 complexity observed in each genotype. n= 30. BP: branching points, counted as the
639 initiation (even if not completed) of a new secondary vein. Scale bars represent 50µm
640 in D, F, L, O, P 100 µm in N, and 1 inch in H-K.

641 **Figure 3. PIN1 polar distribution is not affected in *cvp2 cvl1*. A-D')** Confocal
642 microscopy analysis of early torpedo stage embryos of the indicated genotypes stained
643 with SR2200 Renaissance showing PIN1-GFP distribution in distal secondary veins as
644 they are progressively forming upwards. Magnifications of the region squared in red in
645 A) (B, B') and, in C) (D, D') are displayed. **E-F)** Quantification of the polar distribution
646 of PIN1-GFP in procambial and protophloem cells as ratio of GFP signal detected in
647 the basal membrane (BM) versus the lateral membrane (LM). A polarity index bigger
648 than 1 is considered a polar distribution. **G-J')** 6-day-old roots harbouring *PIN1::PIN1-*
649 *GFP* in WT and *cvp2 cvl1* background showing PIN1 localization and strong basal
650 polarization in the protophloem strand. Magnification of protophloem differentiating
651 cells in WT (G, H') and *cvp2 cvl1* (I, J'). Scale bars represent 50µm (A-D'), 20µm (G-
652 J') and 200 µm (K-L).

653 **Figure 4. PIN-mediated auxin transport is not involved in modulating proximal**
654 **branching in embryonic cotyledons. A-G)** Representative images of 7-day-old
655 cleared cotyledons of the indicated genotypes. Note that *pin1* single mutants are in a
656 Ler background. Magnification of the midvein region where distal branching occurs in
657 *pin1-5* is displayed in G. **H, I)** Quantification of branching (H) and gap (vascular
658 discontinuities) (I) frequency in the indicated genotypes. n= 30-52 for each genotype.
659 BP: branching points, counted as the initiation (even if not completed) of a new
660 secondary vein. **J-M)** Auxin distribution analyzed by *DR5* expression in WT (J, K) and
661 *cvp2 cvl1* (L, M) embryonic cotyledons counterstained with SR2200 Renaissance. K
662 and M displayed GFP signal. Scale bars in J-M represent 20 µm and in A-F 200 µm.

663 **Figure 5. Silencing of *RPK2* expression rescues the branching defects of *cvp2***
664 ***cvl1*. A-D)** Analysis of the continuity and complexity of cotyledon vein network in 7-
665 day-old seedlings of the indicated genetic backgrounds. **E-G)** Quantification of gap
666 (E), vein complexity (F) and branching (G) frequency observed in the cotyledons of the
667 plants depicted in A-D. n= 23-50 for each genotype. **H-K')** mPS-PI stained embryos
668 displaying the vein pattern of the indicated genotypes. H', I', J' and K' represent a
669 magnification of the squared region represented in H, I, J and K respectively. Yellows
670 arrows mark proximal branching while the red arrow marks the lack of proximal
671 branching. **L-M')** Confocal microscopy analysis of *RPK2* expression in the cotyledons
672 of torpedo embryos of the indicated genotypes stained with Renaissance. L' and M'
673 show only GFP signal. Scale bars represent 200 μ m in A-D, 20 μ m in H- M'.

674 **Figure 6. Differential gene expression analysis among WT, *cvp2 cvl1* and**
675 ***amiRPK2 cvp2 cvl1*. A-B)** Venn diagram showing the overlap of upregulated (A) and
676 down-regulated (B) genes in *cvp2 cvl1* with the DEGs in *amiRPK2 cvp2 cvl1*. **C)** MA
677 plot showing the log₂ fold change (LFC) of each gene over the mean of normalized
678 counts. **D-F)** Heatmaps show enrichment (LFC) of genes with known roles in vascular
679 development (D), with known expression in mesophyll cells (E) and involved in auxin
680 biosynthesis, signalling and transport (F). * represents p-value <0.05, ** represent p-
681 value <0.01 and *** represent p-value <0.001. **G-L)** Bright-field images of 7-day-old
682 cotyledons of the indicated genotypes. Scale bars represent 200 μ m. **M-N)**
683 Quantification of gap and branching frequency of the vein network phenotypes
684 observed in the cotyledons represented in G-L. n= 21-24 for each genotype. BP:
685 branching points, counted as the initiation (even if not completed) of a new secondary
686 vein.

687 **Figure 7. OCTOPUS promotes proximal branching in WT and *cvp2 cvl1***
688 **embryonic cotyledons. A-D)** Representative images of cleared 8-day-old cotyledons
689 of the indicated genotypes imaged with a stereomicroscope in bright field on a black
690 background. White arrows mark vein gaps and yellow arrows indicate additional
691 proximal branching sites. Scale bars represent 200 μ m. **E-F)** Quantification of gap and
692 branching frequency of the vein network phenotypes observed in the cotyledons
693 represented in A-J. n= 29-35 for each genotype. BP: branching points, counted as the
694 initiation (even if not completed) of a new secondary vein. **G-H)** mPS-PI stained
695 embryos visualized by confocal microscopy of the indicated genotypes. Scale bars

696 represent 20 μm . **I-L'**) Visualization of OPS distribution in early (I-J') and late (K-L')
697 torpedo stage embryos stained with Renaissance. J and L represents a magnification
698 of the branching region represented in I and K. In I', J', K' and L' only the GFP signal
699 is shown. Scale bars represent 50 μm in, I, I', K, K' and 20 μm in J, J', L, L'. **M)**
700 Quantification of OPS polarity in cells from early and late torpedo stages as means of
701 the ratio of GFP signal between the apical and lateral membrane.

702 **Supporting Information Fig Supplemental 1.** Transcriptional profiling of the
703 transgenic lines used in the current study.

704 **Supporting Information Fig. Supplemental 2.** Analysis of *CVP2* and *CVL1*
705 expression and vascular identity domains in *cvp2 cvl1* embryos.

706 **Supporting Information Fig. Supplemental 3.** *cvp2 cvl1* embryos exhibit aberrant
707 divisions.

708 **Supporting Information Fig. Supplemental 4.** Sensitivity to PIN1-mediated auxin
709 transport is not disturbed in *rpk2*.

710 **Supporting Information Fig. Supplemental 5.** RPK2 modulation of vascular
711 branching is independent of vascular-specific CLE peptides.

712 **Supporting Information Fig. Supplemental 6.** A periclinal division occurs at the
713 branching point.

714

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Figure 1

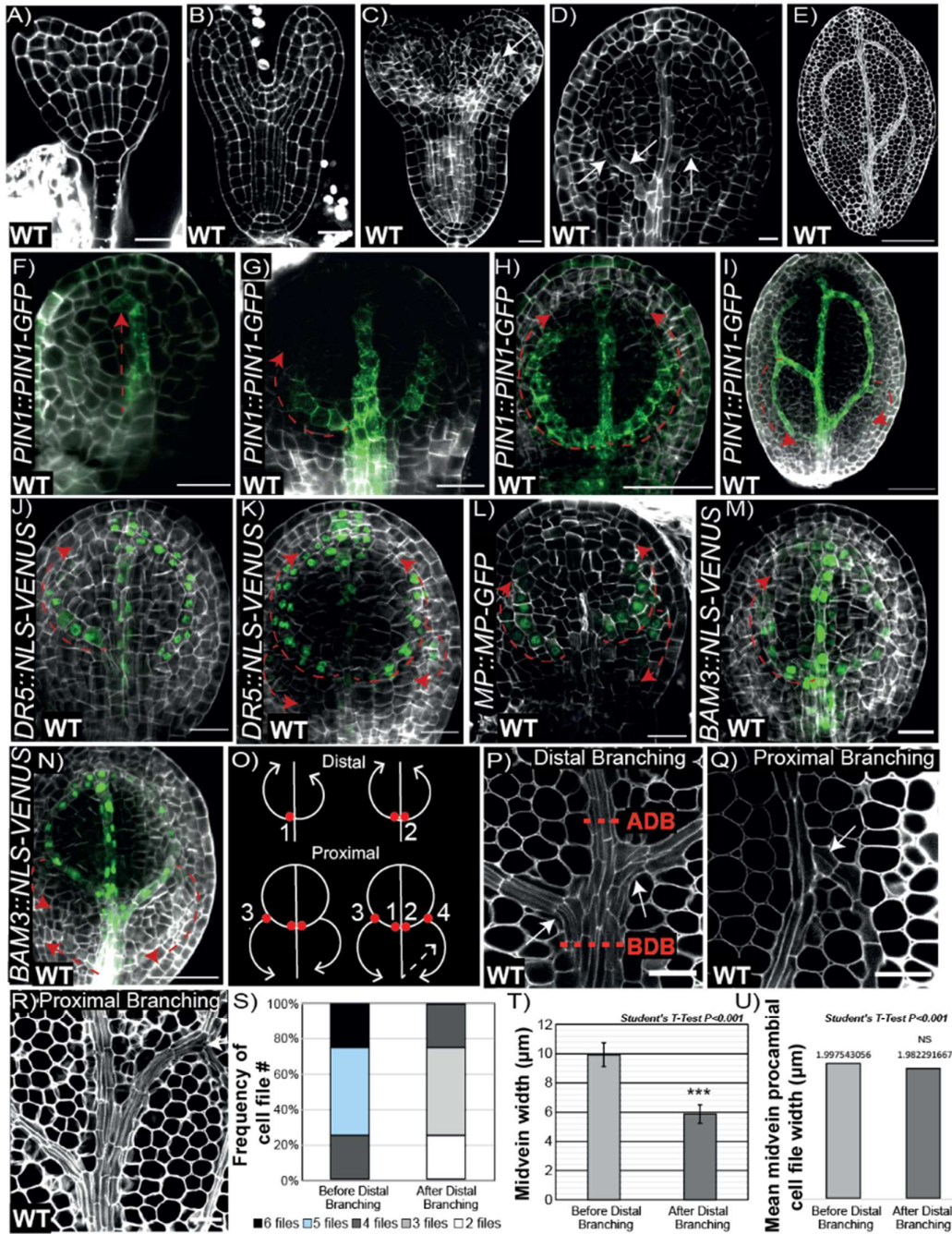


Figure 1. Vein progression and branching in torpedo embryos. A-B) Representative pictures of mPS-PI cell wall-stained embryos in late transition stage (A) and late heart stage (B). **C-D)** Analysis by confocal microscopy of extracted embryos from ovules and stained with the cell wall dye SR2200 Renaissance. (C) Embryo in transition between heart and early torpedo in which the future midvein is marked by a white arrow. (D) Cotyledon of early torpedo stage in which the secondary vein formation can be detected. Note that in D, based on the elongated morphology of procambial cells, secondary vein formation is initiated from the midvein and progressing upwards towards the top of the cotyledon (marked by white arrows). **E)** Cotyledon of mature embryo in which the cotyledon vein network is completed. **F-I)** Representative images of *PIN1::PIN1-GFP* early and late torpedo stage embryos stained with SR2200 as a cell wall counter stain showing the progression of midvein or primary vein (F), distal secondary vein (G-H) and proximal secondary vein formation (I). Dashed red arrows represent the directionality of the forming veins. **J-K)** Cotyledons from early torpedo stage embryos harbouring *DR5::NLS-VENUS* showing the progression of distal secondary veins (J) as well as the initiation of the proximal secondary veins (K). **L-N)** Early torpedo stage embryos harbouring *MP::MP-GFP* (L), and *BAM3::NLS-3XVENUS* (M, N) showing cotyledon proximal vein formation occurring in a tip-to-base manner (n=25/27), except in N, in which proximal veins also proceeds in a base-to-tip manner (n=2/27). **O)** Scheme representing the proposed branching sites of distal and proximal secondary veins. Distal branching points 1 and 2 are represented by the red dots and the direction of vein formation is represented by the white arrows. Proximal secondary vein branching, branching points 3 and 4 (red dots) and the direction of vein formation (white arrows) are represented in the lower panels. Note that distal vs proximal secondary vein formation occurs normally in opposing directions. The rare appearance of proximal veins in base-to-tip manner is represented by a dashed white arrow. **P-R)** Representative images of proximal and distal branching in embryonic cotyledons stained with mPS-PI and visualized by confocal microscopy. ADB: after distal branching; BDB: before distal branching. **S-U)** Quantification of the frequency of appearance of the indicated number of cell files (S), average midvein width (T) and average midvein cell file width (U) in the region marked as ADB and BDB. Note that the difference represented in (T) are not due to differences in the width of procambial cells in these regions, as indicated in (U). Scale bars represent 20µm in A-D, J, L, N and O and 50µm in E, H, I, K, P.

Figure 2

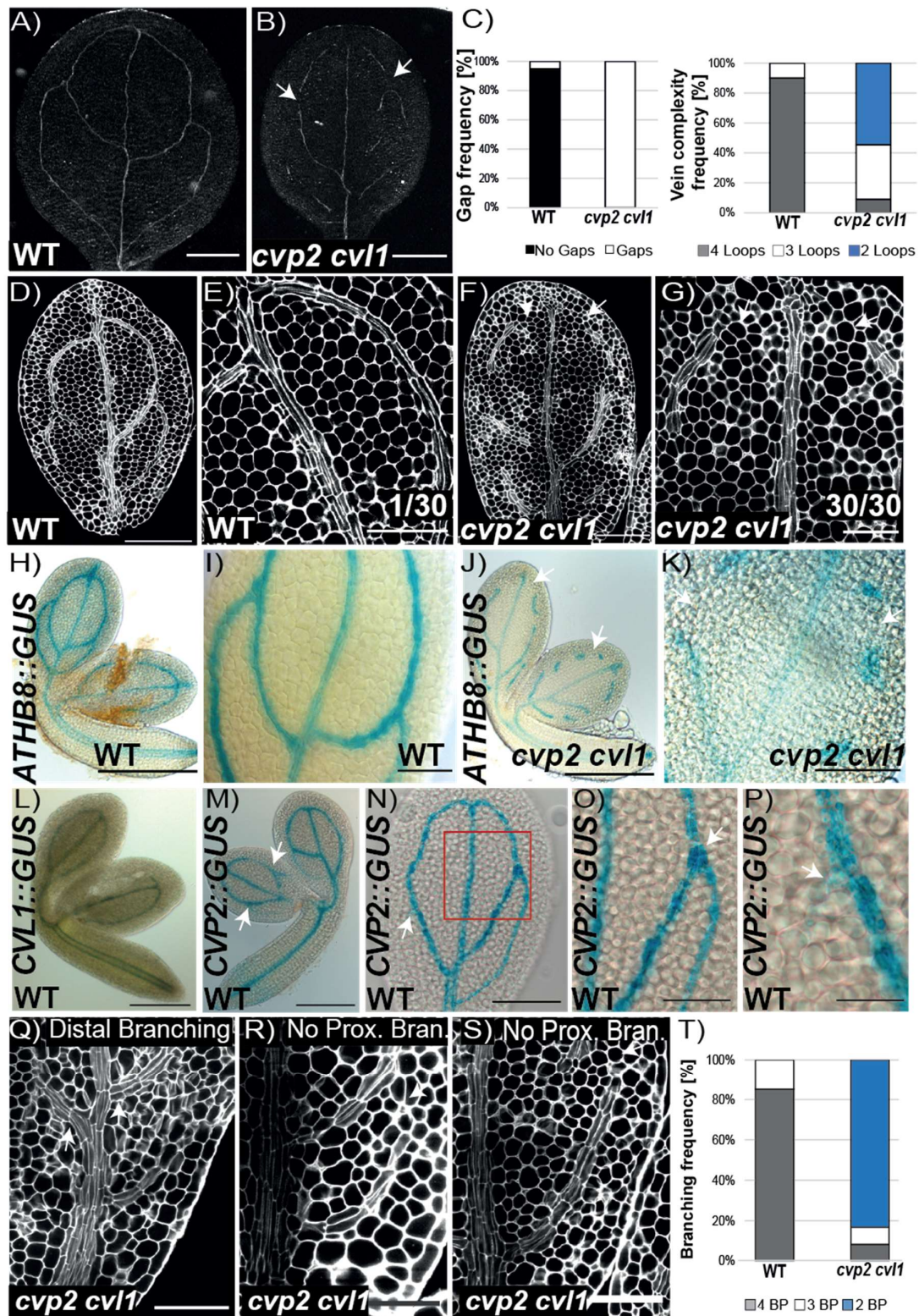


Figure 2. Cotyledon vein defects of *cvp2 cvl1*. **A, B)** Cleared 7-day-old cotyledons of WT and *cvp2 cvl1* imaged with a stereomicroscope in bright field on a black background. **C)** Quantification of the frequency of ground meristem cells surrounding a disconnected vascular islands (gap frequency) and vein complexity in WT and *cvp2 cvl1* cotyledons. n= 20-40 for each genotype. This quantification is part of a bigger experiment which is fully represented in Figure 4G. **D-G)** Confocal microscopy analysis of the vein pattern of mature embryonic cotyledons (vein network at its complete stage) of WT and *cvp2 cvl1* having undergone mPS-PI staining. E and G are magnifications of D and F. White arrows indicate vein gaps. Number of embryonic cotyledons exhibiting gaps is indicated in E and G. **H-K)** *ATHB8::GUS* expression in WT and *cvp2 cvl1* mature embryonic cotyledons. (I, K) Magnifications of the veins displayed in H and J respectively. **L-P)** Expression pattern of *CVL1::GUS* and *CVP2::GUS* in embryos. Magnification of an embryonic cotyledon (N) displaying *CVP2* expression in the proximal branching points is shown in (O,P). **Q-S)** mPSI-PI staining of mature embryonic cotyledons of WT and *cvp2 cvl1* showing distal vs proximal branching. Note the two phenotypes observed in *cvp2 cvl1*, when there is a third branching event (R) or not (S). **T)** Quantification of the frequency of the reduced vein complexity observed in each genotype. n= 30. BP: branching points, counted as the initiation (even if not completed) of a new secondary vein. Scale bars represent 50µm in D, F, L, O, P 100 µm in N, and 1 inch in H-K.

Figure 3

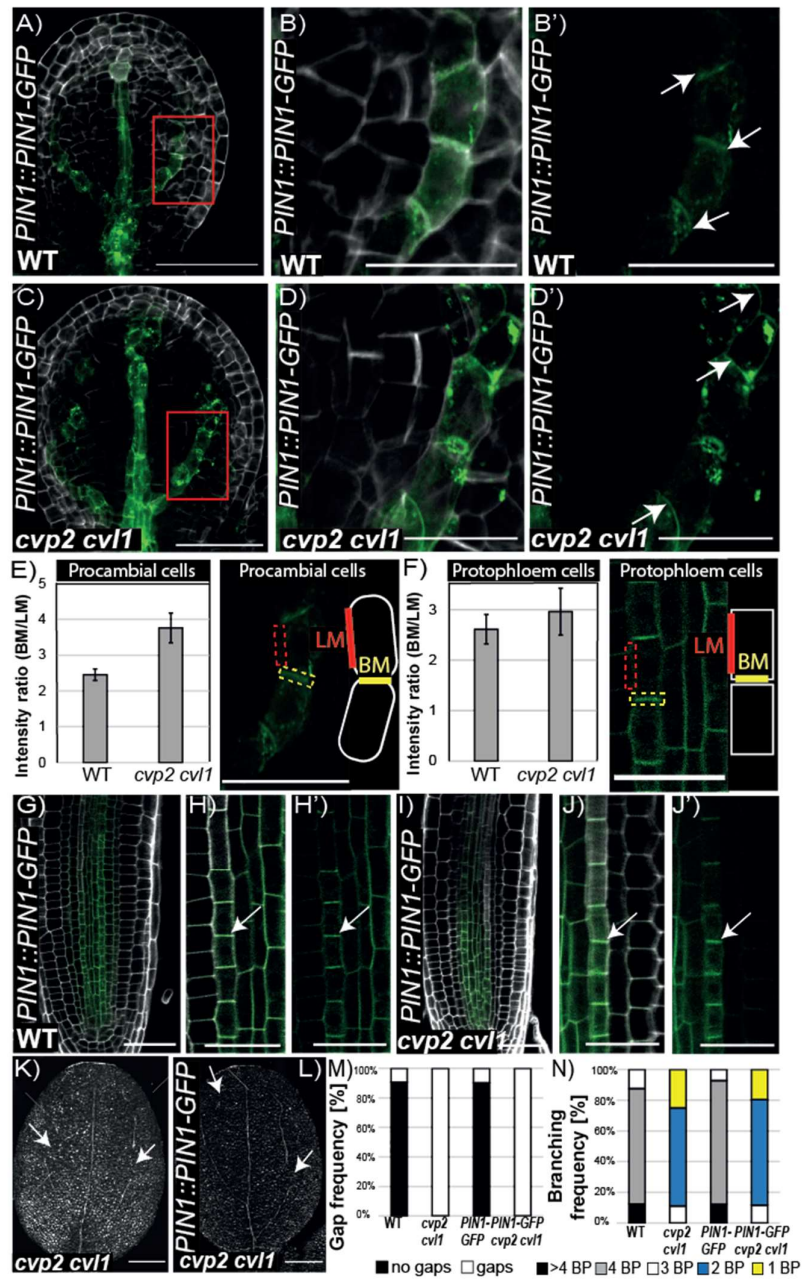


Figure 3. PIN1 polar distribution is not affected in *cvp2 cvl1*. A-D') Confocal microscopy analysis of early torpedo stage embryos of the indicated genotypes stained with SR2200 Renaissance showing PIN1-GFP distribution in distal secondary veins as they are progressively forming upwards. Magnifications of the region squared in red in A) (B, B') and, in C) (D, D') are displayed. **E-F)** Quantification of the polar distribution of PIN1-GFP in procambial and protophloem cells as ratio of GFP signal detected in the basal membrane (BM) versus the lateral membrane (LM). A polarity index bigger than 1 is considered a polar distribution. **G-J')** 6-day-old roots harbouring *PIN1::PIN1-GFP* in WT and *cvp2 cvl1* background showing PIN1 localization and strong basal polarization in the protophloem strand. Magnification of protophloem differentiating cells in WT (G, H') and *cvp2 cvl1* (I, J'). Scale bars represent 50 μ m (A-D'), 20 μ m (G- J') and 200 μ m (K-L).

Figure 4

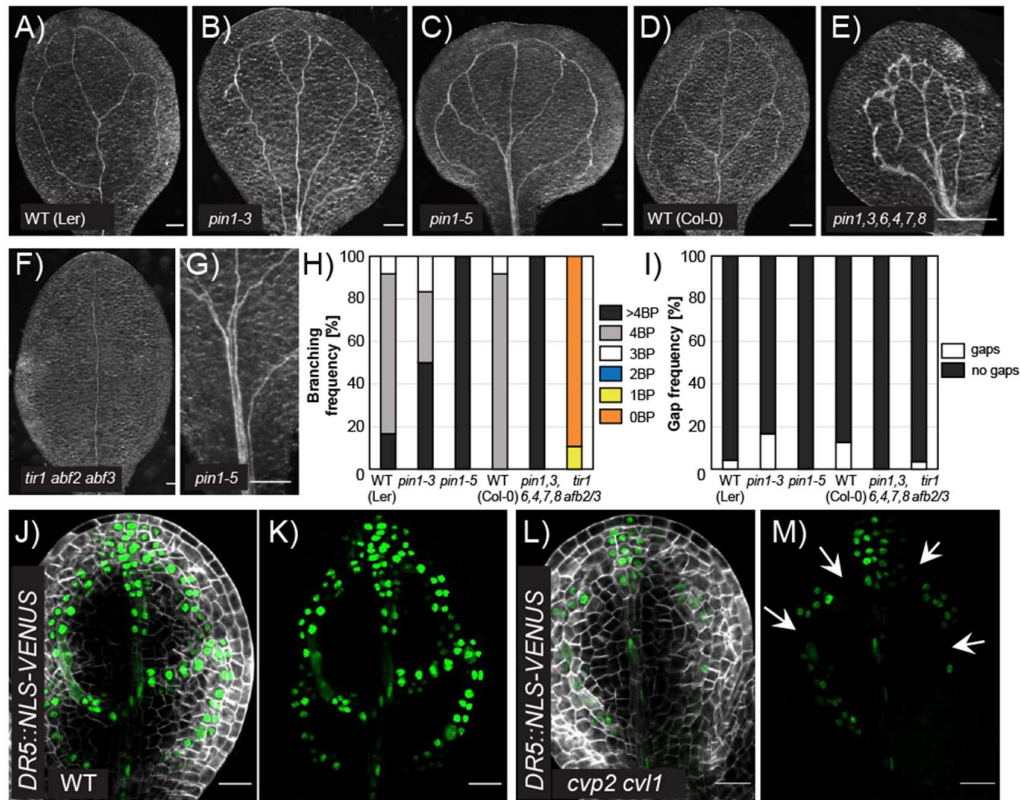


Figure 4. PIN-mediated auxin transport is not involved in modulating proximal branching in embryonic cotyledons. A-G) Representative images of 7-day-old cleared cotyledons of the indicated genotypes. Note that *pin1* single mutants are in a *Ler* background. Magnification of the midvein region where distal branching occurs in *pin1-5* is displayed in G. **H, I)** Quantification of branching (H) and gap (vascular discontinuities) (I) frequency in the indicated genotypes. n= 30-52 for each genotype. BP: branching points, counted as the initiation (even if not completed) of a new secondary vein. **J-M)** Auxin distribution analyzed by *DR5::NLS-VENUS* expression in WT (J, K) and *cvp2 cvl1* (L, M) embryonic cotyledons counterstained with SR2200 Renaissance. K and M displayed GFP signal. Scale bars in J-M represent 20 µm and in A-F 200 µm.

Figure 5

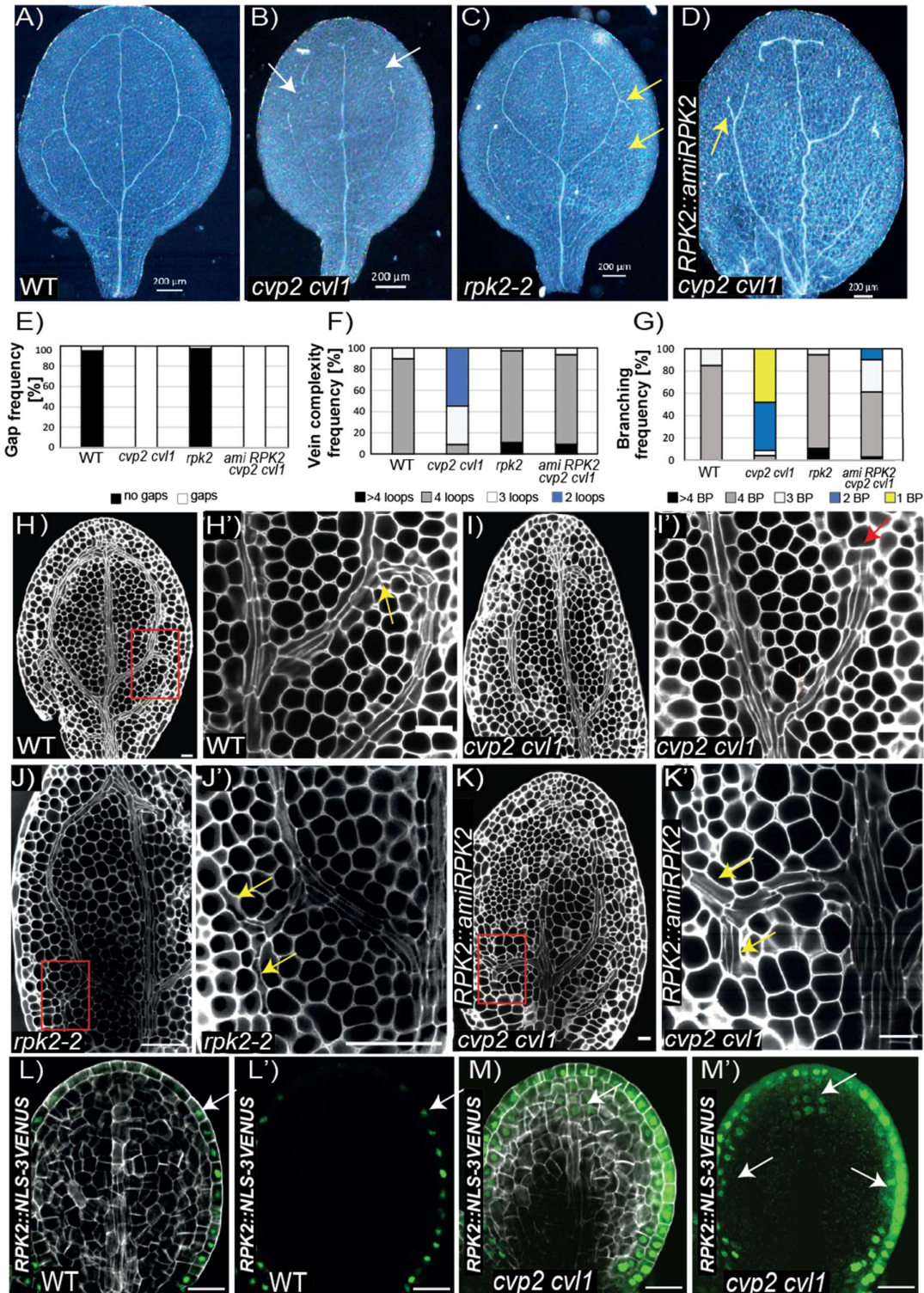


Figure 5. Silencing of *RPK2* expression rescues the branching defects of *cvp2 cvl1*. **A-D)** Analysis of the continuity and complexity of cotyledon vein network in 7-day-old seedlings of the indicated genetic backgrounds. **E-G)** Quantification of gap (E), vein complexity (F) and branching (G) frequency observed in the cotyledons of the plants depicted in A-D. n= 23-50 for each genotype. **H-K')** mPS-PI stained embryos displaying the vein pattern of the indicated genotypes. H', I', J' and K' represent a magnification of the squared region represented in H, I, J and K respectively. Yellow arrows mark proximal branching while the red arrow marks the lack of proximal branching. **L-M')** Confocal microscopy analysis of *RPK2* expression in the cotyledons of torpedo embryos of the indicated genotypes stained with Renaissance. L' and M' show only GFP signal. Scale bars represent 200 μm in A-D, 20 μm in H- M'.

Figure 6

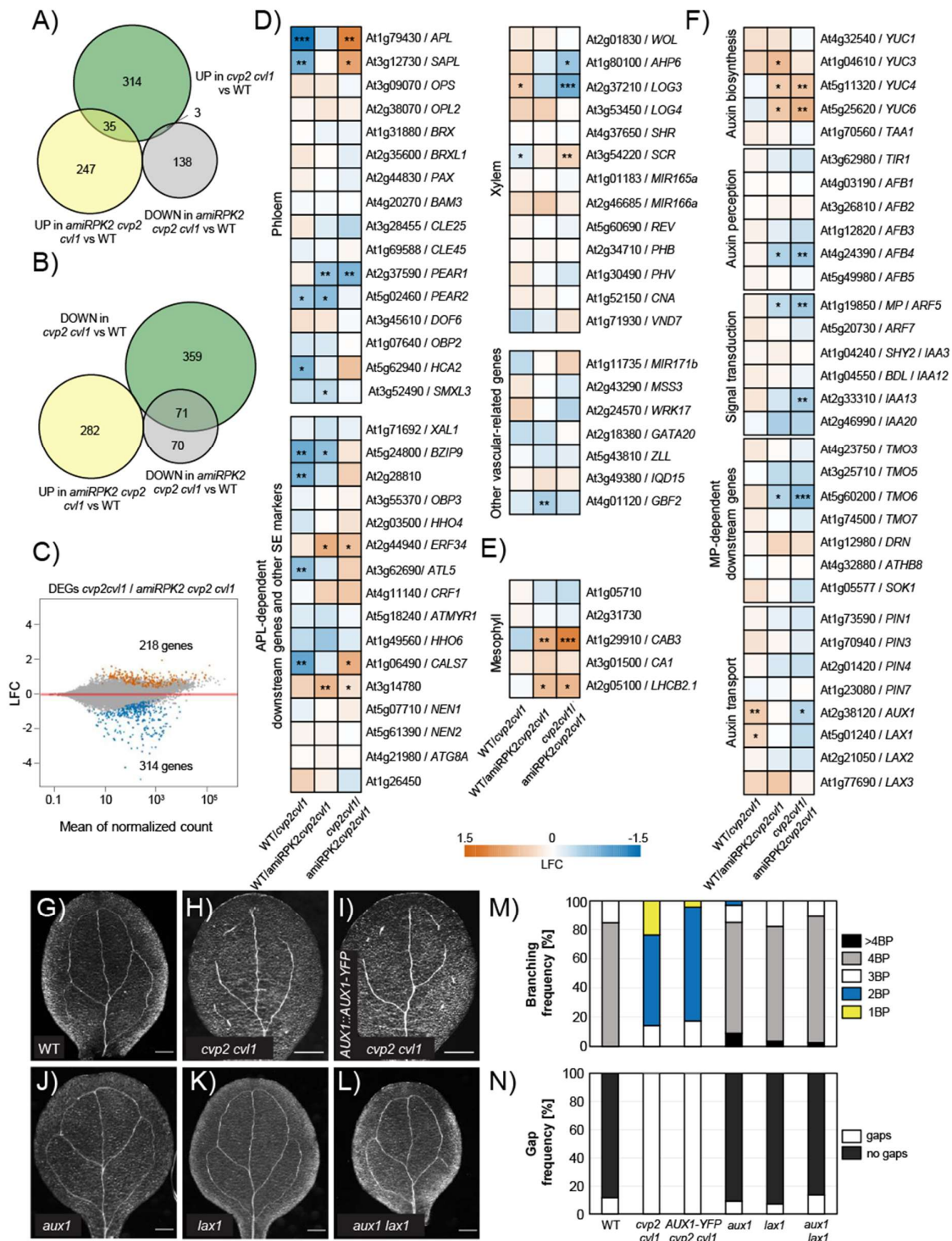


Figure 6. Differential gene expression analysis among WT, *cvp2 cvl1* and *amiRPK2 cvp2 cvl1*. **A-B)** Venn diagram showing the overlap of upregulated (A) and down-regulated (B) genes in *cvp2 cvl1* with the DEGs in *amiRPK2 cvp2 cvl1*. **C)** MA plot showing the log₂ fold change (LFC) of each gene over the mean of normalized counts. **D-F)** Heatmaps show enrichment (LFC) of genes with known roles in vascular development (D), with known expression in mesophyll cells (E) and involved in auxin biosynthesis, signalling and transport (F). * represents p-value <0.05, ** represent p-value <0.01 and *** represent p-value <0.001. **G-L)** Bright-field images of 7-day-old cotyledons of the indicated genotypes. Scale bars represent 200µm. **M-N)** Quantification of gap and branching frequency of the vein network phenotypes observed in the cotyledons represented in G-L. n= 21-24 for each genotype. BP: branching points, counted as the initiation (even if not completed) of a new secondary vein.

Figure 7

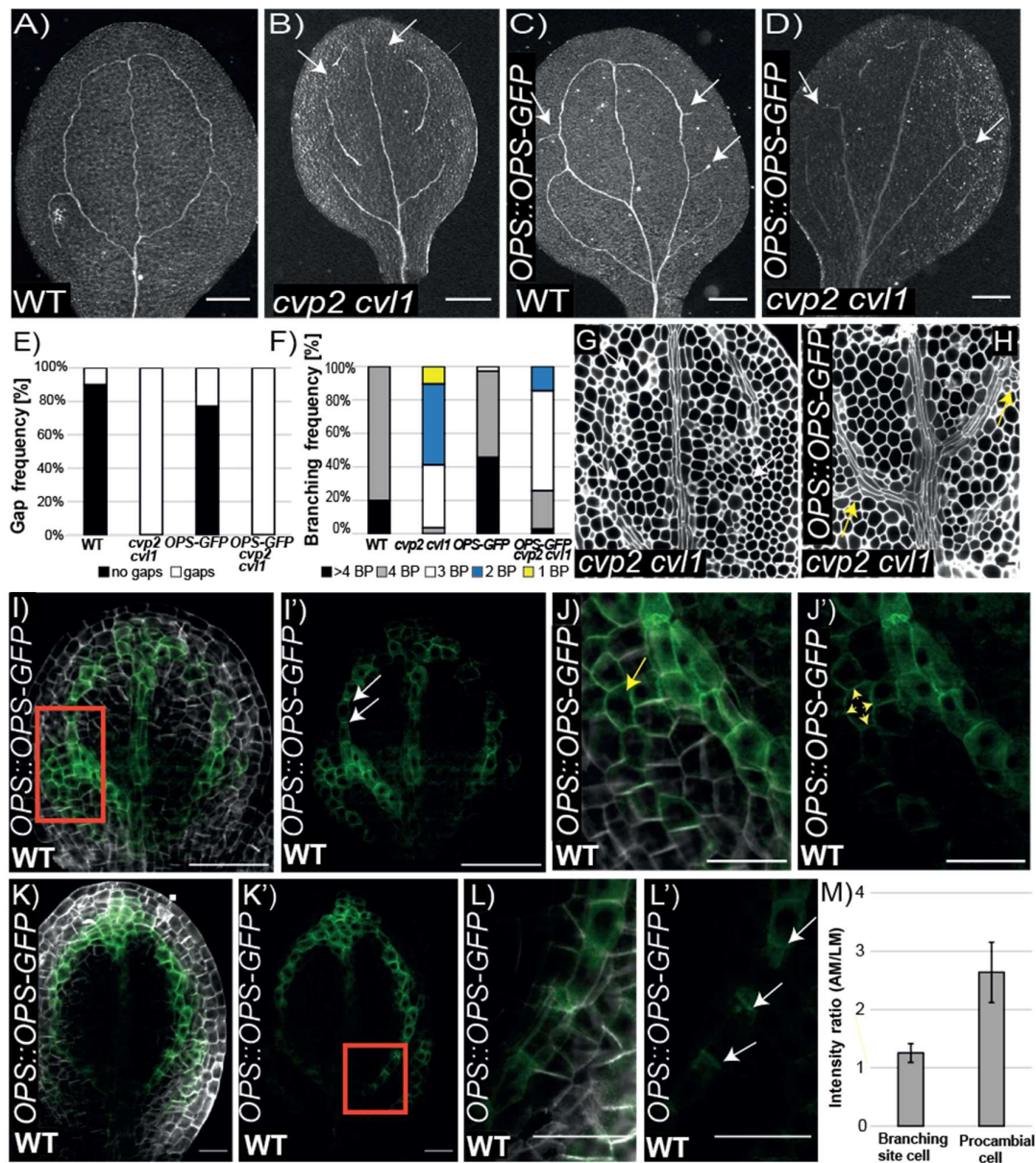


Figure 7. OCTOPUS promotes proximal branching in WT and *cvp2 cvl1* embryonic cotyledons. **A-D)** Representative images of cleared 8-day-old cotyledons of the indicated genotypes imaged with a stereomicroscope in bright field on a black background. White arrows mark vein gaps and yellow arrows indicate additional proximal branching sites. Scale bars represent 200 μ m. **E-F)** Quantification of gap and branching frequency of the vein network phenotypes observed in the cotyledons represented in A-J. n= 29-35 for each genotype. BP: branching points, counted as the initiation (even if not completed) of a new secondary vein. **G-H)** mPS-PI stained embryos visualized by confocal microscopy of the indicated genotypes. Scale bars represent 20 μ m. **I-L')** Visualization of OPS distribution in early (I-J') and late (K-L') torpedo stage embryos stained with Renaissance. J and L represents a magnification of the branching region represented in I and K. In I', J', K' and L' only the GFP signal is shown. Scale bars represent 50 μ m in I, I', K, K' and 20 μ m in J, J', L, L'. **M)** Quantification of OPS polarity in cells from early and late torpedo stages as means of the ratio of GFP signal between the apical and lateral membrane.

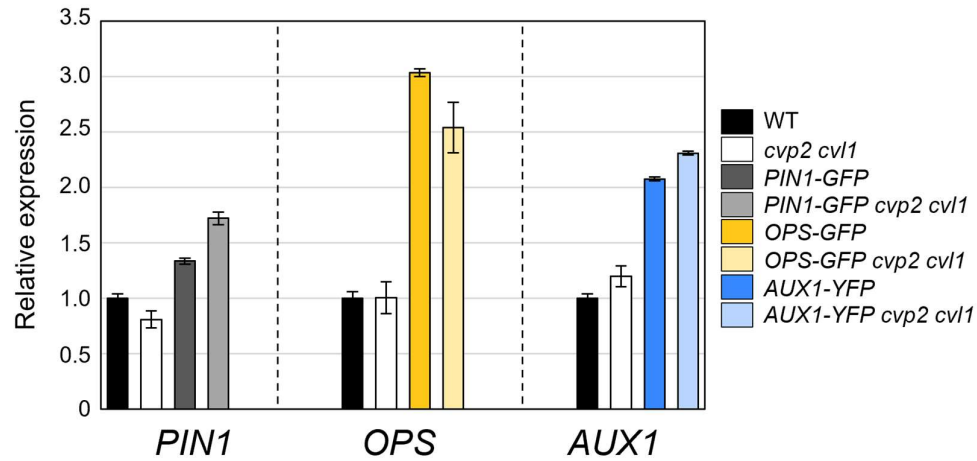


Fig. S1. Transcriptional profiling of the transgenic lines used in the current study. Levels of *PIN1*, *OPS* and *AUX1* normalized expression in 7-day-old seedlings of the indicated genotypes in comparison to wild type. Values represent the mean of 3 technical replicates and error bars represent the standard deviation of these replicates.

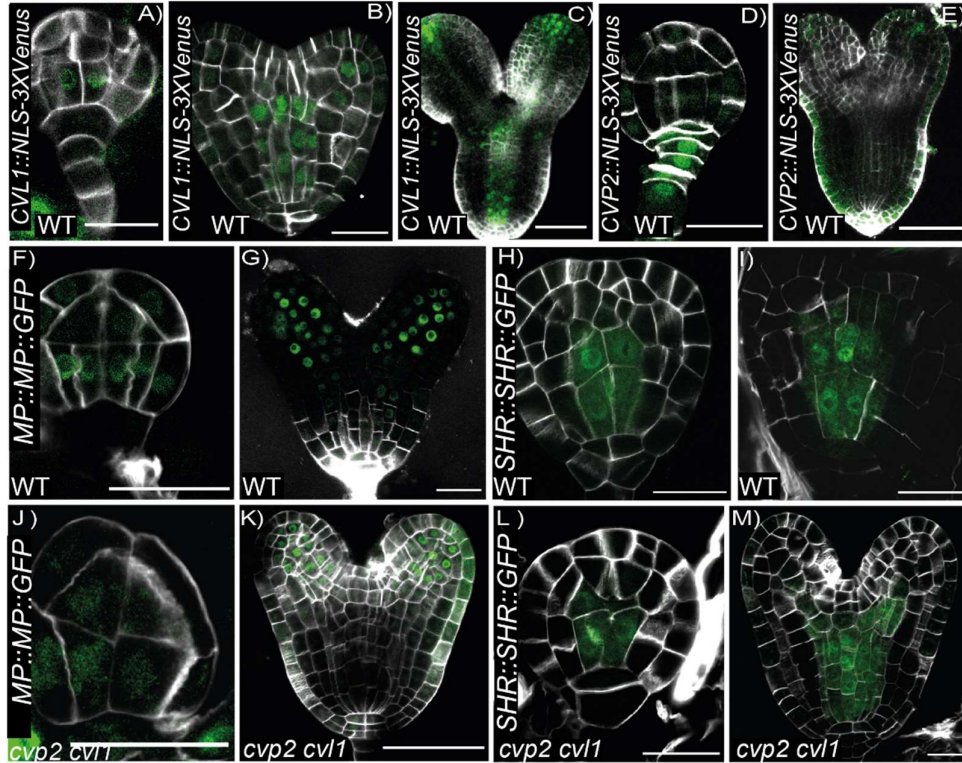


Fig. S2. Analysis of *CVP2* and *CVL1* expression and vascular identity domains in *cvp2 cvl1* embryos. A-E Expression pattern of the indicated genes in early globular (A, D), heart (B, E) and torpedo (C) developmental stages. Renaissance SR2200 staining highlights patterning divisions by labelling plant cell walls. Scale bars represent 50µm in A, C, D, E and 20µm in B. **(F-I)** Expression pattern of the indicated vascular genes in globular (F, H, J, L) and heart stage (G, I, K, M) embryos of WT and *cvp2 cvl1*. Scale bars represent 20µm in (G, I, K, M) and 50µm in (F, H, J, L).

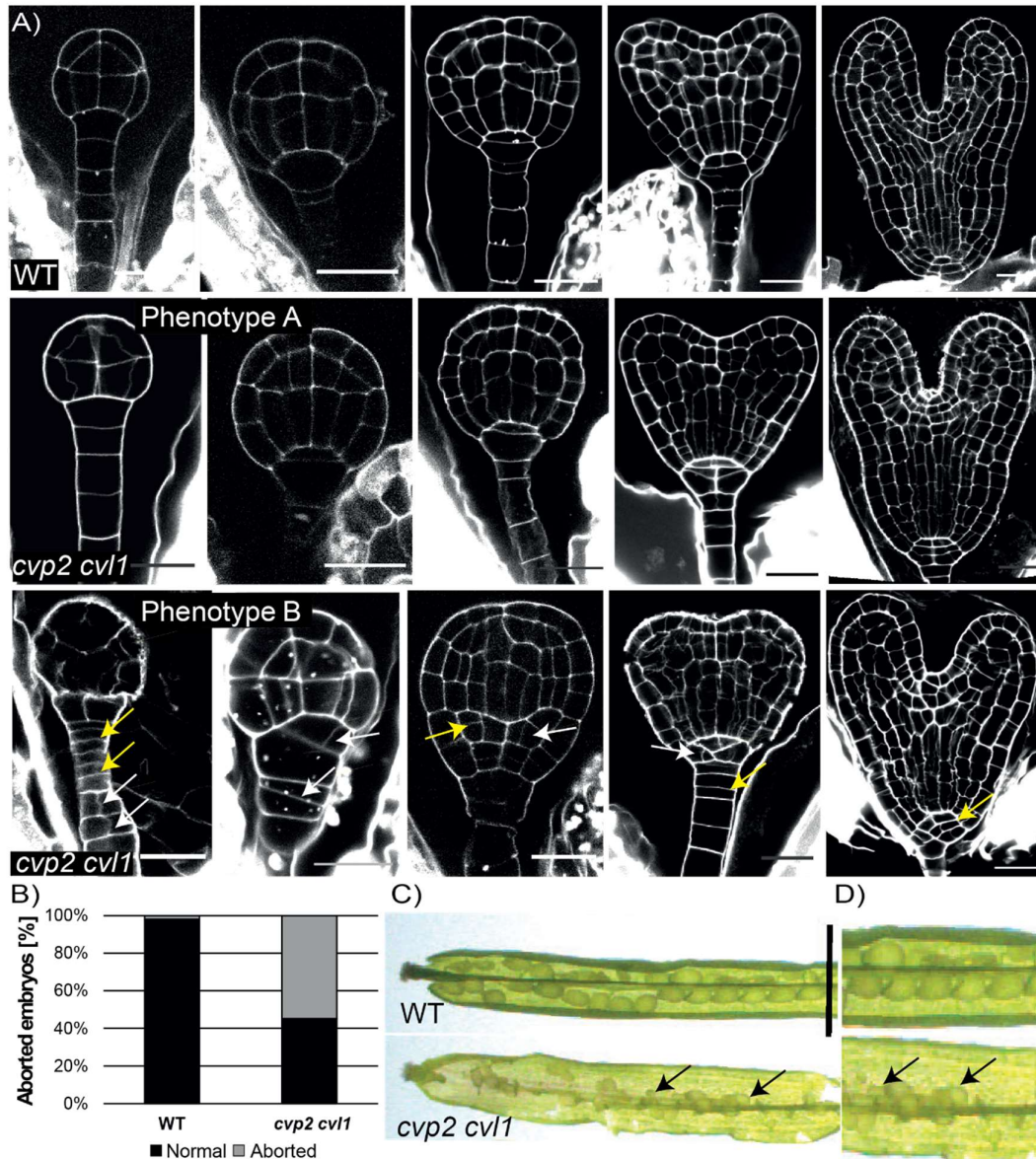


Fig. S3. *cvp2 cvl1* embryos exhibit aberrant divisions. A) Embryo morphology of WT compared to *cvp2 cvl1* embryos using mSP-PI on ovules taken from green siliques and imaged using confocal microscopy. *cvp2 cvl1* shows aberrant divisions at all stages of embryogenesis but most abundantly during the globular stage. Yellow arrows point to aberrant divisions, and scale bars are 20µm or 50µm. **B)** Graphical representation of the percent of aborted embryos occurring in *cvp2 cvl1* vs. WT siliques numbered 2 and 3 counted from the apical meristem (containing globular stage embryos). **C)** Image of a dissected WT silique as compared to *cvp2 cvl1* under bright field using a stereomicroscope. Scale bar represents 1inch.

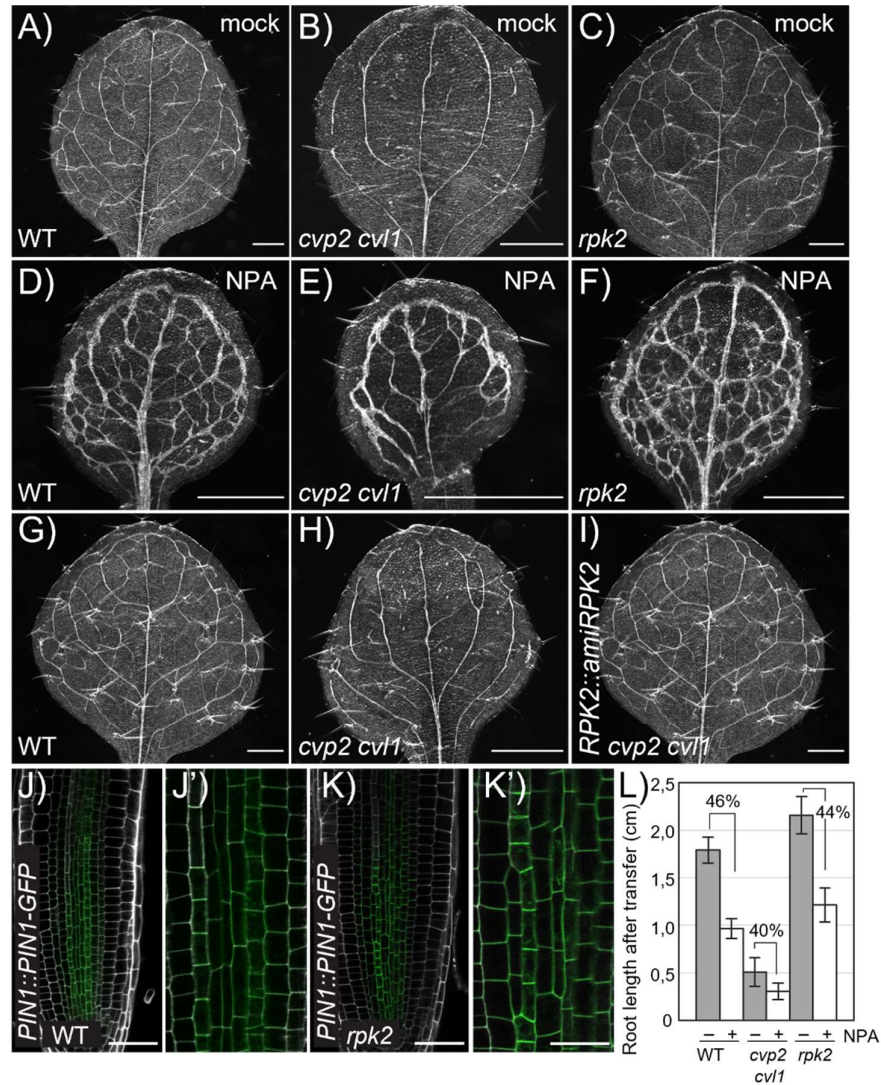


Fig. S4. Sensitivity to PIN1-mediated auxin transport is not disturbed in *rpk2*. **A-F)** Analysis of cleared leaves of seedlings grown for 4 days (until clear emergence of cotyledons could be detected) transferred to a media supplemented with 10 μ M NPA or mock conditions for 5 days. n= 19-35 for each genotype. Scale bars: 500 μ m. **G-I)** Cleared leaves of the indicated genotypes imaged with a stereomicroscope in bright field on a black background. n= 14-21 for each genotype. **J-K')** Confocal microscopy analysis of *PIN1::PIN1-GFP* distribution in cells of the root stele of WT and *rpk2-2* seedlings. J' and K' represent magnification of the region shown in J and K. Scale bars represent 50 μ m in J,K and 20 μ m in J' and K'. **L)** Root length of seedlings grown as described in A-F) were measured. Note that root length was measured after the treatment with NPA or mock. The root length of seedlings treated with mock were set to 100% and the % shown in the graph represents the % of inhibition of root length by NPA. n=39-56.

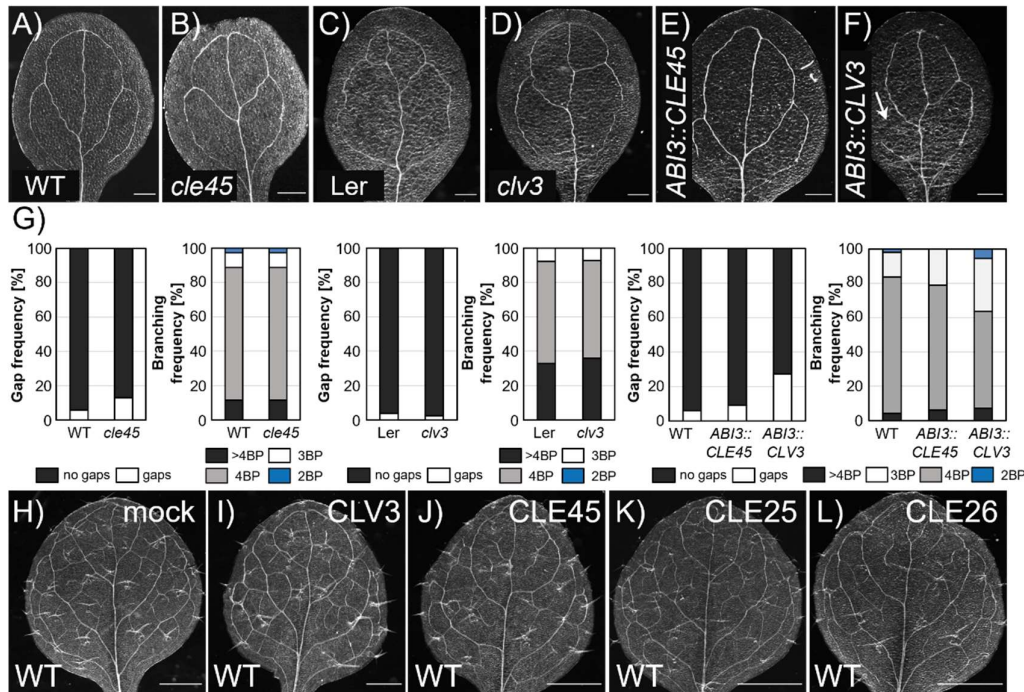


Fig. S5. RPK2 modulation of vascular branching is independent of vascular-specific CLE peptides. **A-F)** Analysis of the continuity and complexity of cotyledon vein network in 8-day-old seedlings of the indicated genetic backgrounds. Arrow in F indicates defects in proximal branching in *pABI3::CVL3*. Scale bars represent 200 μ m. **G)** Quantification of the gap and branching frequency in the cotyledons analyzed in A-F. n = 17-55 for each genotype. **H-L)** Analysis of the vein pattern in cleared leaves of 9-day-old seedlings transferred to a medium supplemented with the indicated CLE peptides once the emergence of the cotyledons could be detected. n = 16-46 for each genotype. Scale bars represent 500 μ m.

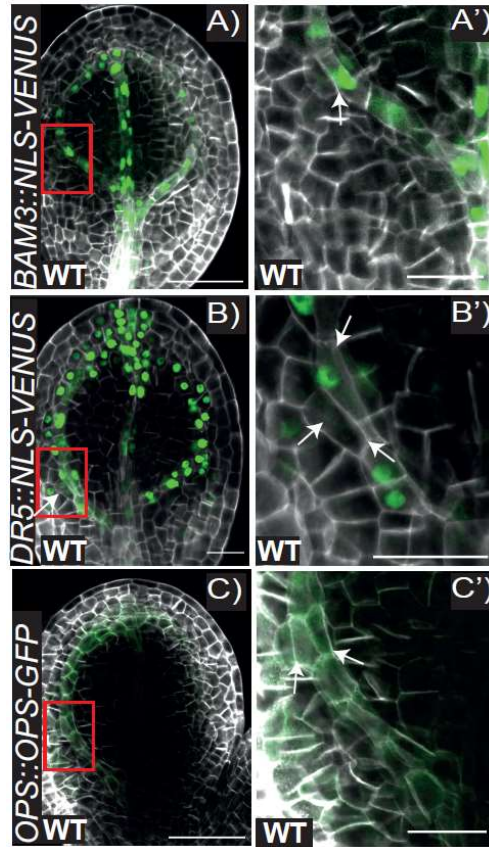


Fig. S6. A periclinal division occurs at the branching point. Representative pictures of early torpedo stage embryos harbouring *BAM3::NLS-3xVENUS* (A), *DR5::NLS-VENUS* (B) and *OPS::OPS-GFP* (C). Embryonic cotyledons were stained with Renaissance stain SR2200 and visualized by confocal microscopy. Magnification of the branching region squared in A), B) and C) is shown in A'), B') and C'), respectively. White arrows indicate nuclei in the cells having undergone a periclinal cell division at the branching point. Scale bars represent 50 μ m in A,C and 20 μ m A'-C',B.