

Age, wound size and position of injury – dependent vascular regeneration assay in growing leaves

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30 **ABSTRACT**

31 **Background**

32 Recurring damage to aerial organs of plants necessitates their prompt repair, particularly their
33 vasculature. While vascular regeneration assay in aerial plant parts such as stem and
34 inflorescence stalk are well established, those on leaf vasculature remained unexplored.
35 Recently we established a new vascular regeneration assay in growing leaf and discovered the
36 underlying molecular mechanism.

37 **Results**

38 Here we describe the detailed stepwise method of incision and the regeneration assay used for
39 studying the leaf vascular regeneration. By using a combination of micro-surgical
40 perturbations, brightfield microscopy and other experimental approaches, our new findings
41 show that the regeneration efficiency decreases with aging of the leaf, and increases with the
42 nearness of the wound towards the proximal end of the leaf.

43 **Conclusion**

44 This easy-to-master vascular regeneration assay is an efficient and rapid method to study the
45 mechanism of vascular regeneration in growing leaves. It can be readily adapted for other plant
46 species and can be combined with cellular and molecular biology techniques.

47 **Keywords:** Arabidopsis regeneration, leaf incision, vascular regeneration, wound size, age
48 dependent

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58 INTRODUCTION

59 Due to their sessile nature, plants are frequently subjected to injuries caused by biotic and
60 abiotic factors. These injuries when left unattended can compromise the plant immunity,
61 growth and even survival (Hwang, Yu, and Lai 2017; Radhakrishnan et al. 2020). In order to
62 overcome the adversities of wounding, plants evolved a remarkable repertoire of regenerative
63 responses ranging from, wound healing in the form of local cell proliferation to complete
64 replacement of amputated organs, such as root tip regeneration (Ikeuchi et al. 2016;
65 Shanmukhan et al. 2020). Although numerous studies have probed the mechanisms underlying
66 several regenerative responses in plants, investigation regarding regeneration potential of aerial
67 organs are limited (Durgaprasad et al. 2019; Iwase et al. 2011; Kareem et al. 2015). Thus,
68 despite their higher susceptibility to injuries than underground organs, there is a dearth of
69 information on regeneration in aerial organs of plants, particularly, in the leaves. Although
70 leaves play a crucial role in plant physiology, their regeneration potential has hardly been
71 investigated (Kuchen et al. 2012; Radhakrishnan et al. 2020).

72 Leaves possess an elaborate network of vascular tissue with a central midvein that transports
73 substances to-and-fro between the main plant body. Damages to the midvein calls for prompt
74 repair, failing which the transport of substances, and consequently the growth of the leaf and
75 its adjacent branch are impaired (Radhakrishnan et al. 2020; Sachs and Hassidim 1996).
76 Recently, a new vascular incision assay in leaf was developed to study the wound repair and
77 tissue restoration in response to injury. The assay revealed that the mechanically disconnected
78 parental stands are reunited by regenerating vascular tissue that bypasses the site of injury. The
79 assay was instrumental in understanding the molecular mechanism underlying vascular
80 regeneration in aerial organs growing in normal developmental context. Upon injury a coherent
81 feed-forward loop comprising of cell fate determinants, PLETHORA (PLT) and CUP-
82 SHAPED COTYLEDON2 (CUC2) activate the local auxin biosynthesis leading to vascular

83 regeneration in growing aerial organs (Radhakrishnan et al. 2020). Here we show that, in
84 addition to the extent of the injury, regenerative ability of the leaf vasculature is determined by
85 age of the leaf explant, and position of the injury along the proximo-distal axis of the leaf blade.
86 This easy-to-master, reproducible assay can be performed using readily available laboratory
87 supplies. The convenience of performing real time confocal imaging and other molecular
88 techniques such as quantitative real time PCR using the injured leaves makes the assay valuable
89 in studying the molecular players and mechanisms regulating wound induced response and
90 regeneration in the normal developmental context. The method will also be useful in studying
91 the interplay between mechanisms of vein patterning during development and that of vein
92 regeneration.

93 **MATERIALS**

94 **Equipment**

95 *Equipment for in vitro culture*

- 96 • Laminar Air Flow chamber (LAF)
- 97 • Sterile pipette-tips (200 µl and 1 ml)
- 98 • Micro pipettes
- 99 • 1.5 ml micro-centrifuge tubes
- 100 • Sterile disposable square Petri plates, size: 120 mm × 120 mm (Himedia PW050-1)
- 101 • Clingfilm (Himedia Phytawrap)
- 102 • Plant growth chamber (Percival AR-100L3).

103

104 *Equipment for incision and sample collection*

- 105 • Fine pointed tweezer (Dumont tweezer, Style 5)

- 106 • Sterile razor blade
- 107 • Forceps
- 108 • Microscissors- Vannas scissor straight (Ted Pella, 1340)
- 109 • Gloves
- 110 • Face mask
- 111 • 70% ethanol
- 112 • 35mm round petriplate

113

114 *Equipment for microscopy*

- 115 • Steriozoom microscope (Zeissstemi 2000) for incision and sample collection
- 116 • Confocal laser scanning microscope (Leica TCS SP5 II) for brightfield imaging
- 117 • Microscope slides (Labtech)
- 118 • Microscope cover glass 22×22mm (Corning 2850-22)
- 119 • Watercolour brush (with small bristles)

120

121 **Reagents for Seed sterilization**

- 122 • Seeds of wildtype (Columbia) *Arabidopsis thaliana*
- 123 • 20% sodium hypochlorite
- 124 • 70% ethanol
- 125 • Autoclaved Milli-Q water.

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129 **Murashige and Skoog (MS) medium preparation**

130 To prepare 1L (Half-strength) MS medium, add 2.165 g MS salt (Sigma Aldrich, M5524) and
131 10 g sucrose (Sigma Aldrich, S0389) to about 850ml Milli-Q water. Adjust pH to 5.7 with 1 N
132 KOH and make up the volume to 1L. Add 8 g plant Agar (Sigma Aldrich, A7921). Autoclave
133 the medium (121 °C for 20 min) and cool it to about 45–50 °C. Add 1 ml of 100 mg/ ml filter-
134 sterilized Ampicillin (final concentration in medium- 100 µg/ml) to 1L medium and pour 50 ml
135 into each sterile square Petriplate (Himedia, PW050) within the LAF. Allow to cool and
136 solidify.

137

138 **Reagents for decolourising sample**

- 139 • 15% ethanol
- 140 • 50% ethanol
- 141 • 70% ethanol
- 142 • 96% ethanol
- 143 • 100% ethanol
- 144 • Glycerol (Sigma Aldrich, G5516)
- 145 • Chloral hydrate (Sigma Aldrich, 23100)
- 146 • Milli-Q water

147 Preparation of clearing solution: Dissolve 8g chloral hydrate in 3ml water. Vortex the solution
148 until chloral hydrate is completely dissolved. Add 1ml glycerol to the solution.

149 Note: The solution has to be freshly prepared for clearing the leaf samples. It is also used for
150 mounting samples for brightfield confocal microscopy.

151

152

153 **METHOD**

154 **Seed sterilization**

155 Seed sterilization should to be performed within LAF under sterile conditions. The work space
156 and tools (Micro pipette, tip boxes, reagent bottles) required for the procedure have to be
157 thoroughly wiped with 70% ethanol and UV irradiated. Prior to commencement of *in vitro*
158 culture, hands should be washed using soap and wiped with 70% ethanol. A liquid surface
159 sterilization protocol for seeds is described here.

160 1. Aliquot the required number of wildtype seeds in 1.5ml microcentrifuge tube.

161 Note: For efficient sterilization do not take more than 300 seeds per tube and remove any
162 debris such as parts of siliques left over from seed collection.

163 2. Add 1ml 70% ethanol. Agitate the contents by inverting the tube for 2-3 minutes.

164 Note: Prior to centrifugation, ensure that the centrifuge and rotor surfaces are clean. Avoid
165 touching inside the lid of the microcentrifuge tube while opening and closing to minimize
166 contamination.

167 3. Brief spin the tube at 6000rpm and carefully discard the ethanol without losing any seeds.

168 4. Add 1ml 20% sodium hypochlorite and shake the contents for 2-3 minutes. Repeat step 3.

169 5. Wash the seeds 5-7 times using 1ml sterile autoclaved Milli-Q water.

170 6. Stratify the seed in 1ml sterile autoclaved Milli-Q water for two days at 4°C.

171 7. Plate the seeds on half strength MS medium in rows with at least 0.5cm gap between
172 consecutive seeds.

173 Note: For the ease of incision, avoid placing the seeds very close to each other.

174 8. Incubate the petriplates vertically in growth chamber under 45 $\mu\text{mol}/\text{m}^2/\text{s}$ continuous white
175 light at 22°C and 70% relative humidity.

176

177

178 **Leaf Incision**

179

180 1. Leaf incision can be performed on work bench after adopting necessary measures to
181 minimize contamination. Wear gloves and face mask during the procedure. Prior to the
182 incision, wipe the surface of the dissection microscope (Zeiss stemi 2000) and gloved hands
183 with 70% ethanol. The tweezer for incision should be dipped in 70% ethanol and left to dry
184 for a few minutes before incision. Opening the plate on multiple days for incision will
185 increase the incidence of contamination.

186 Note: Due to the fragile nature of the tweezer tips, sterilization techniques that may damage
187 it or make it blunt are not recommended.

188 2. The plate containing seedlings is opened under a dissection microscope to confirm the age
189 of the seedling and to perform incision in 5dpg (days post germination) old seedlings.

190 Note: Due to the asynchronous nature of seed germination, all the seedlings may not be of
191 the same age. To maintain consistency, only injure plants which are at the desirable
192 developmental stage. Move aside the uninjured plants to distinguish them from injured ones.
193 Age of the seedlings is important as older seedling display reduced regeneration efficiency
194 while very young leaves are extensively damaged during the procedure. The appropriate age
195 of incision is between 4-6dpg (Figure1).

196

197 3. Out of the two leaves belonging to the first pair (true leaves), the leaf that faces the lid of
198 the petriplate is chosen for incision due to the ease of access. Using the sharp tip of the
199 tweezer an incision is made on the lower abaxial surface of the leaf belonging to the first
200 pair. The incision is carefully performed at the junction between petiole and basal end of
201 the lamina (Fig. 1A). This ensures that the injury occurs just above the first lateral vein
202 (counted from base of the leaf) where the regeneration efficiency is highest in comparison

203 with other positions along the proximo-distal axis of the midvein (Fig. 2). The incision
204 should be performed with just enough force so that it punctures the vascular tissue located
205 close to the abaxial surface of the leaf without piercing out through the adaxial surface.
206 This is important as extensive damages are not repaired (Radhakrishnan et al. 2020)(Fig.
207 1P).

208 Note: Care should be taken, not to inflict multiple damages on the leaves. Therefore, it is
209 not advisable to perform incision in both leaves of the first pair. During incision, a 200ul
210 sterile microtip or sterile forceps can be used to restrict the movement of the plant.
211 However, avoid touching the damaged leaves as it can inflict further damage.

212

213 4. After incision, the plates are closed and incubated vertically under continuous light
214 condition at 22°C in growth chamber.

215

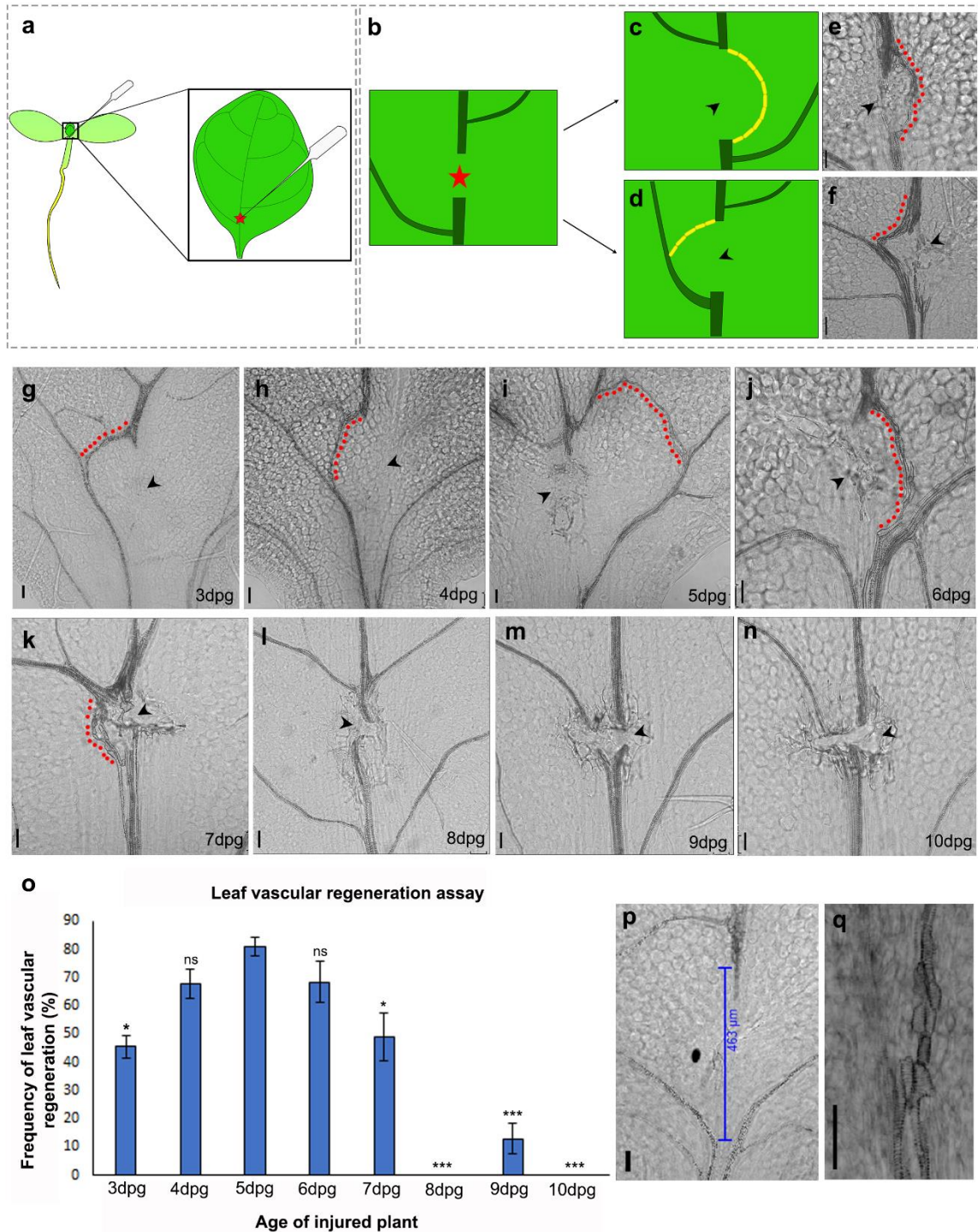
216 5. Four days post incision (dpi), the injured leaf is carefully cut at the petiole using a Vannas
217 scissors and placed in 15 % ethanol in small round petriplates (35mm).

218 Note: Around 20-30 leaves can be treated using 2-3ml ethanol in 35mm petriplates during
219 the decolourising procedure. Alternatively, 6 well plates can be used when handling
220 multiple samples.

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224

225 **Figure 1: Leaf vascular regeneration upon midvein injury depends on the age of the**
226 **injured leaf**

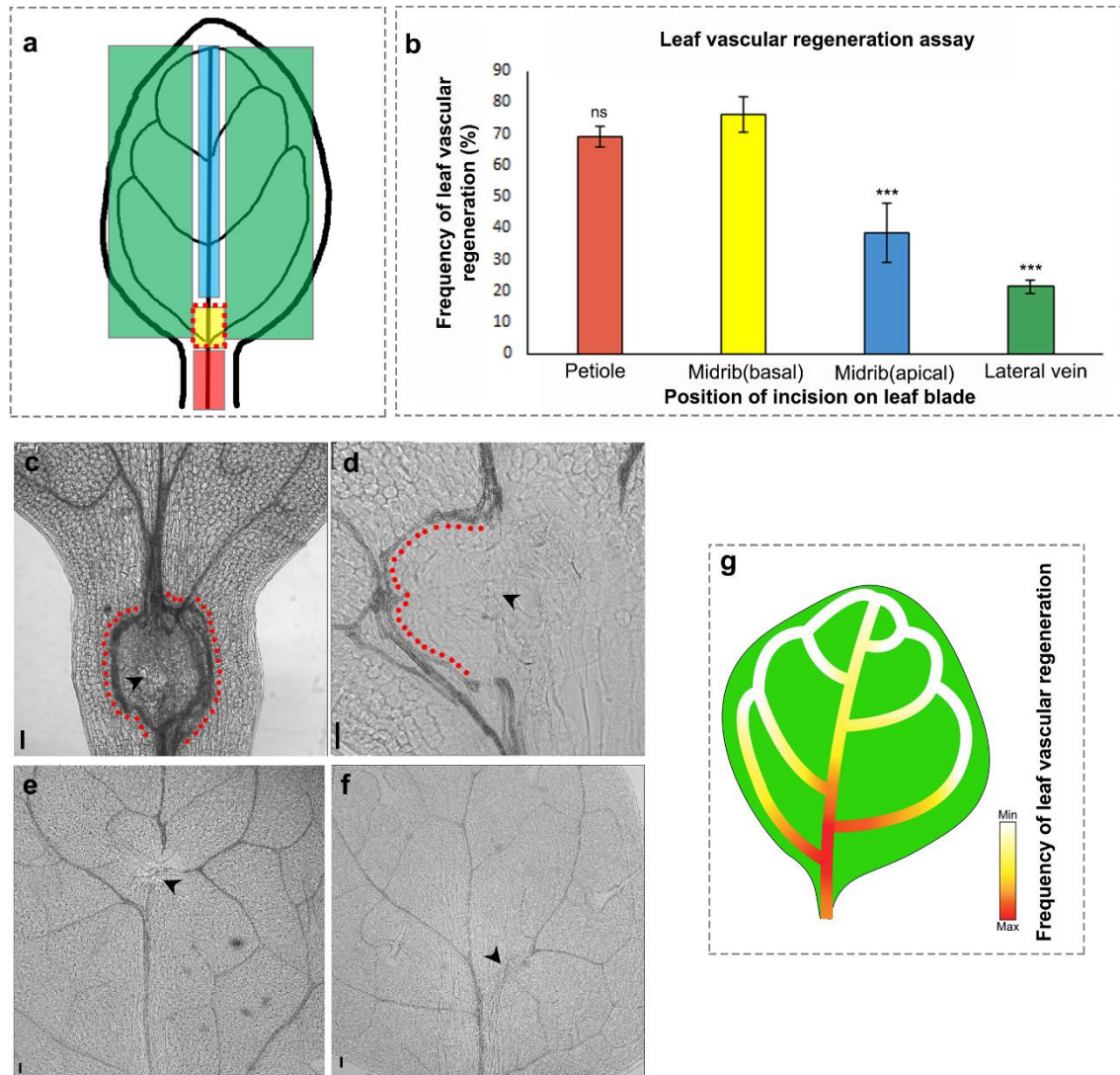
227 (a-b) Illustration depicting the location of incision for effective vascular regeneration. Red star
228 represents site of injury. (c,e) Schematic and brightfield image show the regenerating

229 vasculature re-uniting the disconnected parental stands forming a D-loop bypassing the site of
230 injury. (d,f) Illustration and brightfield image show the regenerating vasculature connecting to
231 the nearest lateral vein. Yellow blocks in the (c) and (e) represent end-to-end connected xylem
232 elements. (g-n) Regeneration response in leaves injured at 3dpg (g)(* $P=0.016$, $n=24$), 4dpg
233 (h)($P=0.426$,not significant (ns), $n=45$), 5dpg (i)($n=20$), 6dpg (j)($P=0.605$,not significant (ns),
234 $n=40$), 7dpg (k)(* $P=0.03$, $n=34$),8dpg (l)(*** $P=1.6 \times 10^{-12}$, $n=43$), 9dpg (m)(*** $P=2.405 \times 10^{-$
235 9 , $n=52$), and 10dpg (n) (*** $P=4.8 \times 10^{-08}$, $n=21$). Statistical analysis by Pearson's χ^2 test. Note
236 that the 3-7dpg leaves are capable of reconnecting their disconnected vasculature but the
237 regeneration efficiency declines with progressive aging of leaves. The regenerating vasculature
238 is indicated by red dots. Black arrowheads indicate the site of injury. (o) Graph depicts
239 frequency of leaf vascular regeneration in leaves injured at different ages. (p) Extensive
240 damage creates a gap exceeding $400\mu\text{m}$ between parental vascular strands, as a result no
241 vascular regeneration is observed. (q)End-to-end attached xylem elements of regenerated
242 vascular strand. Scale bars represent: $50\mu\text{m}$

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247 **Figure 2: Vascular regeneration in leaf depends on the position of injury in the leaf.**

248 (a) Schematic depicting positions of incision on the leaf blade and petiole. (b) The frequency
249 of vascular regeneration at different positions (represented by coloured boxes in (a)) of the leaf
250 are represented by the same colour bars in the graph (b) Petiole (n=21, $P=0.95$, not significant
251 [ns]), basal correct position (n=45), midvein upper end (n=42, $***P=0.0009$), lateral vein
252 (n=21, $***P=0.0002$). (c-f) Images show the incision to vasculature in petiole (c), base of
253 midvein (d), apical region of midvein (e), and lateral vein (f). Note the multiple strand
254 formation upon injury in petiole (c). Red dots indicate regenerated vascular strand and black
255 arrowheads represent site of incision. Scale bars represent: 50 μ m. (g) Gradient represents the

256 efficiency of vascular regeneration along the leaf blade with maximum regeneration
257 (represented by red) at the base of mid vein. Lateral veins and distal end of midvein exhibit
258 reduced regeneration frequency.

259

260 **Decolourising and clearing samples**

261 1. The sample is placed in 2-3ml of 15% ethanol for 15 minutes.

262 Note: Initially the leaves float on the surface of the solvent. Gently submerge the leaves
263 using a paint brush with small bristles.

264 2. The ethanol is drained using micropipette with care being taken not to damage the samples.

265 3. Similarly, the samples are treated with 50%, 70% and 96% ethanol consecutively for 15
266 minutes each. After discarding 96% ethanol, the leaves are incubated for 12 hours in 100%
267 ethanol for dehydrating the tissue and to remove chlorophyll pigmentation.

268 4. After discarding the ethanol, the samples are consecutively incubated for 15 minutes each
269 in 96%, 70%, 50% and finally 15% ethanol to rehydrate the samples.

270 5. After discarding the ethanol as mentioned in step 2, freshly prepared clearing solution
271 (preparation described in materials section) containing chloral hydrate is added to the
272 sample. The samples are incubated in the clearing solution for atleast 3 hours prior to
273 mounting the slides for brightfield imaging.

274 Note: Increasing the duration of clearing can enhance the contrast during brightfield
275 imaging to some extent.

276 **Slide preparation**

277 Using a small paint brush, each cleared leaf is placed on a clean slide with the adaxial surface
278 of the leaf facing upward. The brush can be used to gently tease open any curled leaves without
279 inflicting any further damage. The coverslip is mounted over the sample, taking care not to

280 create any bubble. Multiple leaves (6-8) can be placed under a single coverslip. The clearing
281 solution is used for mounting the samples.

282

283 **Brightfield Imaging**

284 The regeneration of vascular strands in the cleared samples can be assessed using brightfield
285 mode in fluorescent or confocal microscope. However, confocal microscope is recommended
286 to acquire high resolution images of the regenerating xylem elements. The settings described
287 here are for Leica TCS SP5 II inverted microscope. Argon laser or DPSS 561 can be used for
288 the imaging at a laser power of 30%, scan speed:200Hz, line average: 2 and pixel
289 format:1024x1024. Newly formed vascular strands display distinct morphology characterised
290 by end-to-end connected xylem elements (Fig1. E,F,J,K,Q).When the regenerating vein re-
291 united the cut ends of the midvein forming a D-loop (Fig1. C, E) or connected either of the cut
292 ends to a lateral vein (Fig1. D,F), the outcomes were scored as successful regeneration. To
293 maintain consistency in the methodology, incisions made in locations other than junction of
294 first lateral vein were not scored while studying age dependency of regeneration. Additionally,
295 only incisions creating a gap less than 400 μ m between detached parental strands were scored.

296 **Statistical Analysis**

297 Statistical analysis was performed using R software. The collected data was statistically
298 analysed by Pearson's χ^2 squared test.

299 **RESULTS AND DISCUSSION**

300 Although the regeneration ability of plants have been widely investigated, leaves are seldom
301 studied for their regeneration and local wound repair ability (Kuchen et al. 2012;
302 Radhakrishnan et al. 2020). We describe a detailed stepwise method of a novel leaf vascular

303 regeneration assay which can be used to study regeneration of the midvein in response to local
304 injury. Our studies have previously shown that a mechanical disconnection of midvein, creating
305 a gap of under 400 μ m (measured after sample clearing) can be bridged by regenerating vascular
306 strand (Radhakrishnan et al. 2020). While the injured vascular tissue degenerates, the newly
307 synthesized vasculature can either reunite the disconnected strands, or connect the cut end to
308 the nearest lateral vein (Fig. 1B-F). Either way, the reconnection ensures restoration of leaf
309 vascular network, and transport between leaf and rest of the plant body. However, extensive
310 damage generating a gap larger than 400 μ m cannot be repaired, thereby denying functional
311 restoration of leaf vascular tissue (Radhakrishnan et al. 2020)(Fig.1P). Here, the wound-size
312 dependency of vascular regeneration was recapitulated *in silico* by implementing a
313 computational model based on canalisation hypothesis of vein formation in leaf(Rolland-Lagan
314 and Prusinkiewicz 2005). According to the canalisation hypothesis, positive feedback between
315 auxin flux and PIN1 polarization leads to channelized auxin flow that, in turn, promotes the
316 differentiation of vascular tissue(Tsvi Sachs 1991). Consistent with our previous experimental
317 observations, the computational model demonstrate that the formation of a new vascular strand
318 is indeed dependent on the size of the opening (mimicking wound induced gap) created in a
319 matrix of cells (resembling leaf blade). Equations governing the mathematical model and other
320 relevant details are presented in the supplementary information (Supplementary1,
321 Supplementary Videos). Our result indicates that in addition to higher animal cells and
322 unicellular *Dictyostelium*, wound size sensitivity of the repair process is conserved in plants as
323 well (Pervin et al. 2018).

324 Having substantiated the wound-size dependency of vascular regeneration, we next
325 investigated whether the regeneration response is dependent on the age of the wounded plant.
326 In many higher animals progressive aging is associated with reduced the regeneration ability
327 (Yun 2015). To probe how age regulates the regeneration response in leaves, we performed the

328 incision in plants of ages ranging from 3dpg to 10dpg. Performing incisions on the miniscule
329 3dpg leaves were tedious and often damaged the leaves excessively. Upon comparison, 3dpg
330 plants showed lower regeneration efficiency than 5dpg plants (Fig. 1G,1O). Plants belonging
331 to the age group of 4-6dpg displayed highest regeneration efficiency, making it the optimum
332 age to study the vascular regeneration in leaves (Fig.1H-J,1O). Although it is easier to perform
333 incision in older and larger leaves, the regeneration efficiency declined steeply, with 10dpg
334 leaves completely failing to regenerate (Fig.1K-O). It is important to note that even when injury
335 induced gap was lesser than 400 μ m, vascular regeneration was impeded in these older leaves
336 (Fig.1L-N). Thus our data suggests that vascular regeneration efficiency reduces with increase
337 in the age of the injured plant.

338 Regeneration studies in plants and animals have demonstrated that the competence to
339 regenerate in response to injuries can vary even within a specific organ (Durgaprasad et al.
340 2019; Morgan 1902). So we next examined how the position of incision on the growing leaf
341 influenced the vein regeneration efficiency. To analyse this, we made incisions at different
342 positions along the leaf blade, namely, the petiole of the leaf, the basal end (proximal to the
343 plant body axis) of the midvein, the apical end (distal to the plant body axis) of the midvein
344 and on the lateral veins (Fig. 2A, 2C-F). The highest regeneration frequency was recorded at
345 the basal end of the midvein, particularly, between the first and second lateral vein (Fig. 2B,
346 2D). The petiole also showed similar regeneration efficiency upon incision and often led to
347 formation of multiple strands in response to injury (Fig. 2B, 2C). However, since the leaf is
348 excised at the petiole during sample collection, the incision site and regenerated vascular strand
349 is occasionally damaged leading to loss of valuable samples. Additionally, since incisions
350 performed in the petiole leads to multiple stand formation instead of single stand regeneration,
351 it will be more appropriate to make injuries in leaf blade, as the study involves following a
352 single regenerating strand in real-time to study recognition, communication and reunion of

353 vascular strands. Upon injuring other positions, we observed that the regeneration efficiency
354 drastically declined towards the apical regions of the midvein and in the lateral veins (Fig. 2B,
355 2E-G).

356 Collectively, our data demonstrates that the leaf vascular regeneration is sensitive to the size
357 of the wound, the age of the injured leaf and the position of incision on the leaf.

358 **CONCLUSION**

359 Our study reveals that 4-6dpg leaves respond most efficiently to smaller wounds (400 μ m or
360 less in size) that are inflicted at the junction of first lateral vein at the proximal end of the leaf
361 blade. While adopting this assay to study regeneration in other plant species, we recommend
362 standardization of the method with respect to the above mentioned criteria.

363 The assay will be helpful in exploring the mechanisms underlying regeneration of vascular
364 tissue in growing leaves. To begin with, the assay may prove tedious, however with repeated
365 practice; this method can be performed deftly and rapidly in large number of samples. The
366 short duration of the experiment (experimental data can be collected 5 days post injury) and
367 the dispensability of specialized equipment make it amenable to the larger scientific
368 community. Our method, revealing the dependency on leaf age and wound position during
369 vascular regeneration, can be used in combination with other cell biology and molecular
370 biology techniques with little or no standardisation, thereby adding to its utility.

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