1 **Research paper**

2 **Title:** Live-cell imaging of early events following pollen perception in self-incompatible

3 *Arabidopsis thaliana*

4

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31   **Highlight:** A live imaging system coupled with self-incompatible Arabidopsis lines highlight the

32   role of stigmatic cells in controlling pollen hydration and in reacting to pollen tube intrusion by

33   remodeling actin cytoskeleton.

34

35   **Abstract:** Early events occurring at the surface of the female organ are critical for plant

36   reproduction, especially in species with a dry stigma. Following landing on the stigmatic papilla

37   cells, the pollen hydrates and germinates a tube, which penetrates the cell wall and grows

38   towards the ovules to convey the male gametes to the embryo sac. In self-incompatible (SI)

39   species within the Brassicaceae, these processes are blocked when the stigma encounters an

40   incompatible pollen. Here, based on the generation of SI-Arabidopsis lines and by setting up a

41   live imaging system, we showed that control of pollen hydration has a central role in pollen

42   selectivity. The faster pollen pumps water from the papilla during an initial period of 10

43   minutes, the faster it germinates. Furthermore, we found that the SI barriers act to block the

44   proper hydration of incompatible pollen and when hydration is promoted by high humidity, an

45   additional control prevents pollen tube penetration into the stigmatic wall. In papilla cells, actin

46   bundles focalize at the contact site with the compatible pollen but not with the incompatible

47   one, raising the possibility that stigmatic cells react to the mechanical pressure applied by the

48   invading growing tube.

49

50   **Keywords :** actin, Arabidopsis, compatible and incompatible pollinations, hydration threshold,

51   mechanical pressure, pollen-stigma interaction.

52

Introduction

53

54 Flowers of Brassicaceae species have a dry stigma, highly discriminatory with early control of

55 pollen capture following pollination (Dickinson, 1995). The stigma consists of a dome of flask-

56 shaped epidermal cells (papillae). Once pollen grains land on papillar cells, only those

57 recognized as compatible are accepted, whereas undesirable pollen grains (i.e., from unrelated

58 species or self-pollen in self-incompatible species) are rejected (reviewed in Doucet *et al.* 2016).

59 Pollen grains need to hydrate on the stigma so as to permit the emergence of a pollen tube that

60 penetrates the papilla cell wall and then grows within the pistil tissues (reviewed in Chapman

61 and Goring 2010). *In vitro* assays showed that growing through the stigma is required for pollen

62 tubes to efficiently target the ovules (Palanivelu and Preuss, 2006). Likewise, genetic ablation of

63 the stigmatic papillae prevents normal pollen hydration or germination (Tung *et al.*, 2005). Early

64 events of the pollen-stigma interaction are thus crucial steps for successful fertilization.

65 Most of the Brassicaceae species have a self-incompatibility (SI) system, which allows the stigma

66 to reject self-pollen grains and hence to prevent self-fertilization (de Nettancourt, 2001). This

67 self/non-self recognition mechanism, genetically controlled by a single multiallelic locus, the *S*-

68 locus, depends on a receptor-ligand interaction (Ivanov *et al.*, 2010). The ligand, the *S*-locus

69 Cysteine Rich protein (SCR/SP11), is present in the material contained in the cavities of the

70 pollen cell wall (Schopfer *et al.*, 1999; Takayama *et al.*, 2000), flows out onto the papilla surface

71 and interacts with the *S*-locus Receptor Kinase (SRK) (Stein *et al.*, 1991; Iwano *et al.*, 2003),

72 localized to the plasma membrane of stigmatic papillae. This interaction induces SRK

73 phosphorylation and activation of the signaling cascade leading to self-pollen rejection (Cabrillac

74 *et al.*, 2001). Incompatible pollen grains fail to hydrate properly and do not germinate or only

75 germinate a short pollen tube whose growth is arrested on papilla cells (Dickinson, 1995; Hiroi

76 *et al.*, 2013). Although *Arabidopsis thaliana* is self-fertile, transgenic SI *A. thaliana* lines were

77 generated by reintroducing SCR/SP11 and SRK gene pairs from its close SI relative *A. lyrata*

78 (Nasrallah and Nasrallah, 2002; Nasrallah *et al.*, 2004). SI Arabidopsis lines have opened the way

79 for thorough analysis of pollen-stigma recognition mechanisms through the use of the genetic

80 tools and mutant collections available for this model plant species.

81 Although they have been studied for many years, the early steps leading to pollen acceptance or

82 rejection decision are still not clearly characterized. In this work, based on the generation of

83 new SI Arabidopsis lines, we revisited the cellular aspects of pollen-stigma interaction by setting

84 up a live imaging system to monitor pollen hydration and germination following compatible as

85 well as incompatible pollinations. We quantified the degree of pollen hydration required for

86 pollen activation and highlighted a late SI control restricting penetration of stigmatic cell wall by

87 the pollen tube. Furthermore, we monitored the dynamics of actin network during compatible

88 and incompatible pollination and found that remodeling of actin cables is triggered in stigmatic

89 cells only when pollen-stigma interaction is engaged in compatibility.

90

91 **Materials and methods**

92

93 **Plant growth conditions**

94 *A. thaliana* Col-0, C24, *A. lyrata ssp petraea* haplotype *S14* originating from the Czech Republic

95 (seeds kindly provided by Dr Pierre Saumitou-Laprade, Université de Lille F59655 Villeneuve

96 d'Ascq cedex, France) and all transgenic plants were grown in growth chambers under a long-

97 day cycle of 16-h light/8-h dark at 21°C/19°C with a relative humidity around 60 %. *A. lyrata*

98 plants were propagated by cuttings every 3 months.

99

100 ***A. lyrata SCR14* and *SRK14* gene cloning, plasmid construction, plant transformation and gene**

101 **expression**

102 The *AlSRK14* genomic sequence spanning the coding region from the start to the stop codons

103 (3,620 kb) and a fragment of 4,081 kb containing the *AlSCR14* gene, 1,844 kb of the 5' upstream

104 region and 817 bp of the 3' downstream region were amplified from genomic DNA of *A. lyrata*

105 containing the *S14* locus. Subsequent cloning steps and transgenic lines produced in this work

106 are described in Supplementary protocol S1. Sequences of *AlSRK14* and *AlSCR14* transgenes

107 introduced in *A. thaliana* have been submitted to GENBANK (*pSLR1-gAlSRK14*: accession

108 number MH680585, *pgAlSCR14*: accession number MH680584).

109   *AlSRK14* expression level in stigma of transgenic plants was determined as described in

110   Supplementary protocol S2.

111

112   **Pollination assays**

113   Flower buds at the end of developmental stage 12 (Smyth *et al.*, 1990) were emasculated and

114   18 hours later stigmas that had reached developmental stage 13 or Early 14 (14E) were

115   manually pollinated with mature pollen. Six hours after pollination, stigmas were fixed in acetic

116   acid 10 %, ethanol 50 % (v/v) and stained with aniline Blue (Kho and Baër, 1968) for pollen tube

117   counting. In a second series of pollination assays, stage 12 flower buds were emasculated and

118   42h (stage 15) or 66 h (stage 16) later, stigmas were pollinated with fresh mature pollen for 6h

119   followed by fixation and aniline blue staining. In a third experiment, stigmas were pollinated at

120   stage 13-14E and collected after six, 24, 48 or 72 hours followed by fixation and aniline blue

121   staining. Pollination assays were carried out on at least three stigmas and repeated at least at

122   two different dates. Pollination was considered as incompatible when less than five pollen tubes

123   were counted in the stigma (Kitashiba *et al.*, 2011).

124

125   **Dual pollination: semi *in vivo* assay**

126   Stage 12 flower bud was emasculated and 18 hours later (stage 13-14E), pistil was cut

127   transversally in the middle of the ovary and placed vertically in a half-cut, perforated PCR tube

128   whose base was introduced in a solid agar medium. Pollen was deposited by gently touching the

129   stigma with a mature anther. Incompatible pollen was deposited first, followed (within less than

130   1 minute) by deposition of compatible pollen, which defined the timing starting point (T0) for

131   monitoring pollen behavior. A cover slip was delicately applied on the surface of the pollinated

132   stigma for confocal imaging. The system was maintained throughout the experiment at 21°C

133   and under 45 % relative humidity. To increase the relative humidity at the vicinity of the

134   pollinated stigma, pieces of solid agar medium were added around the mounted pistil. For cell

135   surface labelling, after 35 minutes in high humidity conditions, pollinated stigma was incubated

136   in FM™ 4-64 Dye (*N*-3-Triethylammoniumpropyl-4-6-4-Diethylamino Phenyl Hexatrienyl

137   Pyridinium Dibromide, Life Technologies T3166, 8.23 μM) for five minutes and subsequently

138   washed in 1/2 Murashige and Skoog basal medium containing 10 % (w/v) of sucrose, before

139   mounting between slide and coverslip in this medium.

140

141   **Confocal microscopy and actin fluorescence intensity**

142   Images were acquired with a SP8 laser-scanning confocal microscope (Leica) using a 25x

143   objective (numeric aperture 0.95, water immersion). Detailed acquisition settings are provided

144   in Supplementary protocol S3. Change in fluorescence intensity in papillae, beneath the pollen

145   grain, around the emerging pollen tube or along the growing tube was estimated by eyes.

146

147   **Measurement of pollen hydration rate**

148   Because the increase of water content in pollen grain is correlated with change of its shape

149   from ellipsoid to spheroid (Zuberi and Dickinson, 1985; Hiroi *et al.*, 2013; Wang *et al.*, 2016), for

150   each pollen grain, we recorded every two minutes the ratio between the long and the wide axis

151   (L/W) to estimate the pollen hydration rate until germination. Slopes were determined by linear

152   regression of the hydration curve based on pollen ratio evolution during the first 10 minutes

153   period (5 data points), using the curve f = ax + b. The volume of the pollen grain was estimated

154   using the formula for a prolate spheroid: $4/3 * \pi * L/2 *(W/2)^2$, where L = length of the longest

155   axis and W = width of the pollen grain.

156

157   **Seed set**

158   Flower buds at stage 12 were emasculated and pollinated with mature pollen 18 h later. 21 days

159   after pollination, mature siliques were opened to count the number of seeds.

160

161   **Statistic analysis**

162   For each experiment (except the initial selection of SRK and SCR transgenic plants), at least two

163   independent biological replicates (performed at different dates) were carried out. After

164   checking that the mean and variability of the samples were homogenous between replicates,

165   data were pooled to apply a statistical test. Graphs and statistics were obtained with R studio or

166   Excel softwares. We tested the sample distribution with a Shapiro-Wilk test and applied a

167   Student-test to compare the mean of two given data sets. To compare multiple samples with a

168   single test, we performed an ANOVA. In this study, p-values < 0.05 were considered statistically

169   significant.

170

171   **Results**

172

173   **Generation of transgenic lines designed for live cell imaging**

174   We generated transgenic plants for *AlSRK14* in Col-0 background as this accession is more

175   suitable for crossing with available fluorescent marker lines. We used the *Brassica oleracea SLR1*

176   (*S-locus-related gene 1*) promoter to express the *Al*SRK14 receptor in stigmatic cells. Among the

177   12 transgenic lines we generated, we isolated four independent lines possessing a unique

178   insertion and homozygous for *AlSRK14*. Stage 13-14E stigmas from these four lines rejected

179   pollen from the original *A. lyrata S14* plant (less than five pollen tubes/stigma) whereas *A. lyrata*

180   pollen produced numerous pollen tubes on Col-0 stigmas (Supplementary Fig S1A, B, C). We

181   quantified the expression level of the *AlSRK14* transgene in stage 13-14E stigmas of two of the

182   strongest transgenic plants (lines #10 and #18, Supplementary protocol S2) and found that the

183   relative expression was two times lower than that detected in *A. lyrata S14* stigmas but still

184   sufficient to induce SI response (Supplementary Fig S1D). We selected the transgenic line #10

185   for further analysis and named it Col-0/*AlSRK14.* In spite of several attempts to introduce

186   *AlSCR14* in Col-0, we did not succeed in generating transgenic pollen that could be rejected by

187   stigmas of Col-0/*AlSRK14*. As an alternative, we used the C24 accession, this accession exhibiting

188   a strong SI response when transformed with *A. lyrata SRK/SCR* genes (Nasrallah et al., 2004; Liu

189   et al., 2007). We first transformed C24 plants with *AlSRK14* to generate a female tester capable

190   of rejecting *A. lyrata S14* pollen grains (named C24/*AlSRK14* line #14; Supplementary Fig S1C).

191   Then, among the 18 transgenic lines transformed with *AlSCR14* in C24 background, we isolated

192   five independent lines with unique insertion and homozygous for *AlSCR14*. Pollen from each of

193   these lines was rejected on C24/*AlSRK14* line #14 mature stigmas (Supplementary Fig S1E). We

194   selected transgenic line #4 for further analysis, which was named C24/*AlSCR14.*

195   To facilitate live-cell monitoring of pollen tube growth on the stigma, we introduced Lifeact

196   fused to Venus to label stigma papillae of Col-0/*AlSRK14 (*Col-0/SRK14 + Act:Venus), TURQUOISE

197   protein to monitor compatible pollen of C24 (C24/TURQ) and RFP for incompatible pollen of

198   C24/*AlSCR14* (C24/SCR14 + RFP) (Fig. 1A). We found that the presence of fluorescent proteins

199   did not alter the compatibility (ANOVA test p-value = 0.23), as well as the SI response (T-test p-

200   value = 0.15) assessed by pollen tube counting (Fig. 1B). Despite the strong pollen rejection

201   response following incompatible cross (female Col-0/SRK14 + Act:Venus x male C24/SCR14 +

202   RFP), some seeds developed in siliques (Fig. 1C). A comparable number of seeds were counted

203   in siliques derived from an incompatible cross between non-labeled pollen and stigmas (mean

204   values of 18.35 versus 19.04, T-test p-value = 0.82, Fig. 1C). However, the incompatible crosses

205   exhibited a significantly reduced seed set compared with the compatible ones (T-test p-value =

206   2.2E-16 between the four compatible crosses cumulated and the two incompatible crosses

207   cumulated, Fig. 1C). Together, our data show that we succeeded in restoring SI system in

208   *A.thaliana* using a new *A. lyrata* haplotype *S14* and that a mature stigma recognizes and rejects

209   incompatible pollen independently of the accession background. Futhermore, expressing a

210   fluorescent protein in addition to the SI determinant does not modify completion of the SI

211   response.

212

213   **SI response is maintained for about two days and then partially breaks down**

214   As some seeds could develop in siliques following incompatible crosses (Fig. 1C), we

215   investigated how persistent the SI response was during stigma development. To this end, we

216   first carried out pollination experiments using compatible (C24/TURQ) or incompatible

217   (C24/*AlSCR14*) pollen grains deposited on Col-0/SRK14 + Act:Venus stigmas at different pistil

218   developmental stages (Fig. 2A). When incompatible pollen grains were deposited on stage-15

219   stigmas, almost no pollen tube bypassed the stigmatic barrier (mean of 1.85 tube/stigma, graph

220   Fig. 2A), whereas some pollen tubes succeeded in penetrating stage-16 stigmas (mean of 13.2

221   tubes/stigma, graph Fig. 2A). By contrast, when compatible pollinations were performed on

222   stigmas at stages 15 or 16, pollen tubes abundantly grew in the stigma. In a second set of

223   experiments, we examined how long incompatible pollen grains deposited on stage 13-14E

224   stigmas could stay inhibited after 6, 24, 48 or 72 hours of contact with papilla cells (Fig. 2B). We

225   observed a breakdown of the SI barrier 48h and 72h after pollen deposition, when stigmas

226   reached stage 16 and stage 17, respectively. However, the number of pollen tubes overpassing

227   SI barrier (mean of 9.9 tubes/stigma and 7.3 tubes/stigma, respectively, graph Fig. 2B) was

228   much less than the above 50 tubes/stigma counted in compatible situations (Graphs Fig. 2A, B).

229   Together, these experiments show that a partial breakdown of the SI response occurs late

230   during pistil ageing and that a strong SI phenotype is maintained during a large time-window of

231   floral development, from stage 13 (anthesis) to stage 15 (stigma extended above long anthers),

232   that is to say slightly less than two days (Smyth *et al.*, 1990). Thus the SI pollination partners we

233   generated are suitable for developing a live-cell imaging to monitor pollen behavior at the

234   surface of mature stigmas (stage 13-14E) without risking weakening of the SI barrier.

235

236   **Design of a semi *in vivo* assay that allows live cell imaging of early pollination events**

237   To study the very early cellular changes that occur following pollen-stigma interaction, we

238   designed a device that maintains stigma alive for at least one hour and allows cell-live imaging

239   under confocal microscopy (Fig 3A). In this system, compatible and incompatible pollen can be

240   separately deposited on the same stigma and their behavior tracked in the same experimental

241   conditions (Fig 3B, Supplementary video S1). To test whether this setup permitted the

242   maintenance of compatibility/incompatibility responses, we performed five independent

243   experiments where a Col0/SRK14+Act:Venus stigma was dual-pollinated with C24/SCR14+RFP

244   (incompatible) and C24/TURQ (compatible) pollen grains. We found that the percentage of

245   germination for compatible pollen varied from 58 % to 81 %, with a mean value of 74 % (n=106,

246   Fig. 3C). Emission of a pollen tube occurred on average 20 minutes after pollen deposition (Fig.

247   3C). On the contrary, germination of incompatible pollen was a rare event, occurring for only 1.3

248   % of the tracked grains (n=75, Fig. 3C). Thus, our semi *in vivo* system allows live cell imaging of

249   pollinated stigmas while preserving the highly regulated SI process.

250

251   **Actin dynamics in stigmatic cells during pollination**

252   Because remodeling of the actin cytoskeleton in the papilla cells has been reported following

253   pollination in Brassica (Iwano *et al.*, 2007), we decided to monitor actin dynamics over time.

254   Before pollination, we found the actin cytoskeleton network to be composed of bundles mainly

255   oriented along the longitudinal axis of the stigmatic cell (Fig. 4A). Upon compatible pollination, a

256   clear accumulation of actin that focalized at the contact site with the compatible pollen was

257   observed in 92 % of the pollinated papillae (22/24, Fig. 4B). For 42% of the stigmatic cells

258   (10/24), actin fluorescence increased beneath the pollen grain just before germination. For 50%

259   of the cells, actin focalized when a clear emergence of the tube (minimum 1 μm) was visible

260   (8%, 2/24) or when the tube started to grow on the stigma surface (42%, 10/24) (Fig. 4B, C,

261   Supplementary video S2, supplementary Fig S3). At later stages of pollen tube progression, actin

262   focalization was observed all along the tube path in 83% (20/24) of the monitored papilla cells

263   (Fig. 4D, Supplementary video S3). Interestingly, when several pollen germinated on the same

264   papilla, actin focalization was observed below each tube (Fig.4E). By contrast, on the seven dual-

265   pollinated stigmas we tracked over time, we never detected any actin focalization beneath the

266   incompatible pollen grains (Supplementary video S4). All together, our live-cell imaging analysis

267   shows that the actin cytoskeleton polymerizes in the stigmatic cell following deposition of

268   compatible but not incompatible pollen grains. Actin accumulation at the pollen contact

269   occurred around the germination process and goes on throughout the elongation of the pollen

270   tube.

271

272   **Kinetics of compatible pollen hydration is divided into two phases**

273   Next, we compared the kinetics of pollen hydration and germination following dual pollination,

274   using the L/W ratio to estimate the pollen hydration level (Zuberi and Dickinson, 1985). At

275   maturity, the mean L/W ratio of pollen grains was close to two (Fig. 5A, Supplementary table

276   S1). We measured the pollen ratio every two minutes, starting two minutes after pollen

277   deposition (Fig. 5B, Supplementary Fig S2). Among the 69 compatible pollen grains we tracked,

278   58 germinated in the course of the experiment whereas 11 never did. We found that during the

279   first 10 minutes (preceding germination, Supplementary table S2), the mean pollen ratio

280   dramatically decreased indicating that grains got roundish (solid blue curve Fig. 5B).

281   Interestingly, over the same time period, the pollen volume increased (3455 μm^3 at 2 min and

282   4974 μm^3 at 10 min) strongly supporting that pollen swelling is linked to water uptake and

10

283   strengthens the use of the L/W ratio as a proxy for pollen hydration. During the following 20

284   minutes, water uptake almost stopped; indeed, there was no significant difference between the

285   12 min and 30 min L/W ratios (T-test p-value = 0.71). We found that germination started when

286   the pollen ratio was below 1.4 (solid blue curve Fig. 5B). Among the 58 pollen grains that

287   germinated during the course of the experiment, a huge majority (57/58) reached the 1.4 ratio

288   before germination; only one germinated pollen grain never reached this ratio. For the 11

289   compatible pollen grains that never germinated (dashed blue curve Fig. 5B), the hydration curve

290   shows no rapid hydration phase during the first 10 minutes and the mean ratio never reached

291   the value of 1.4. Taken together, our results show that in Arabidopsis, water transport toward

292   the pollen grain follows a bi-phasic kinetics: first, an initial phase characterized by a high pollen

293   hydration rate required to reach a certain degree of water content in the pollen (ratio 1.4),

294   followed by a second phase where the hydration rate dramatically decreases and pollen

295   germination occurs.

296

297   **Variability in pollen germination time depends on the hydration rate during the early**

298   **interaction phase**

299   In our experimental conditions, pollen germination was not synchronous, starting 12 minutes

300   after pollen contact with the stigma and spreading over the 32 minutes of the experiment

301   (Supplementary table S2). We suspected that different hydration rates of pollen grains might be

302   the cause of this variability. To test this hypothesis, we classified the 69 compatible pollen grains

303   according to their germination capability and categorized them into four classes: those that

304   germinated early (from 12 to 18 minutes, n = 19), intermediate (from 20 to 24 minutes, n = 22),

305   late (from 26 to 32 minutes, n = 17), and those that did not germinate (n = 11), and we

306   compared their hydration rate during the rapid hydration phase from two to 10 minutes (Fig.

307   5C). At two minutes, no significant differences were found between the L/W ratios of pollen for

308   each classes (1.85/1.87/1.85/1.96, respectively; ANOVA test p-value = 0.13, Fig. 5C). At 10

309   minutes, pollen that germinated late were significantly less hydrated (higher L/W ratio) than

310   those that germinated early or intermediate (T-test p-value = 2.43E-04 and 1.64E-03,

311   respectively, Fig. 5C), though their hydration degree was significantly higher than non

11

312   germinated pollen (T-test p-value = 3.62E-02, Fig. 5C). We calculated the slopes of the hydration

313   curves by linear regression, as previously described (Wang *et al.*, 2016), and found that early

314   and intermediate germinating pollen grains had the highest hydration rates compared with late

315   and non germinating pollen grains (Fig 5C). Non germinating pollen exhibited a very slow

316   hydration rate (slope - 0.043) associated with a poor water uptake during the first 10 minutes,

317   deduced from the high pollen L/W ratio at 10 minutes (mean of 1.77). Our data show that most

318   of the tracked compatible pollen germinated within 32 minutes and that the germination time

319   relates to the capacity of the pollen to take water during an initial short period of 10 minutes.

320   The faster the pollen takes water from the stigma, the quicker it germinates.

321

322   **Incompatible pollen grains do not fully hydrate and pollen tubes do not penetrate the**

323   **stigmatic cell wall**

324   Next, we examined the hydration rate of incompatible pollen on the dual-pollinated stigmas. Of

325   the 53 incompatible grains we monitored, none germinated during the time course of the

326   experiment. Futhermore, most of them (n = 43) never exhibited a L/W ratio below the threshold

327   value of 1.4 (solid red line, Fig. 6A) and only hydrated poorly compared with compatible grains

328   (solid blue curve, Fig. 6A). The mean hydration curve for these 43 incompatible grains showed a

329   constant slope during the 32 minutes of the experiment. By contrast, the 10 remaining

330   incompatible grains reached a L/W ratio below 1.4 with a mean hydration curve (dashed red

331   line, Fig. 6A) resembling that of compatible pollen. In addition, their hydration rate within the

332   first 10 minutes was similar to late germinating compatible pollen (slope value of - 0.074 versus

333   - 0.089, respectively, Fig. 6B), and hence hydrated as efficiently as some compatible grains.

334   As high humidity can stimulate pollen hydration and promote incompatible pollen germination

335   (Carter and McNeilly, 1976; Ockendon, 1978; Zuberi and Dickinson, 1985), we checked the

336   effect of high humidity (100 %) on pollen behavior in a modified semi *in vivo* system

337   (Supplementary Fig S3). We found that compatible pollen germination was slightly stimulated

338   compared with standard conditions (88 % germination versus 74 % and mean germination time

339   of 18.55 minutes versus 20.38 minutes, respectively; Supplementary Fig S5). Moreover, high

340   humidity conditions did not dramatically affect actin reorganization as we detected a clear

341 focalization of actin beneath the pollen grain/tube in 75 % of the pollinated papillae (33/44)

342 (Fig. 7A, Supplementary Fig S5, Supplementary video S5). Following incompatible pollination at

343 high humidity, we observed that germination of incompatible pollen was strongly stimulated,

344 varying from 18 % to 58 % depending on the experiment (mean of 35 %, Supplementary Fig S6)

345 compared with the 1.3 % of germination in standard conditions. In addition, among the 46

346 grains monitored, 46 % reached a L/W ratio of 1.4 within 10 minutes (Supplementary table S3),

347 whereas only 4 % reached this value in standard conditions. However, it took more time for

348 incompatible pollen tubes to emerge compared with compatible tubes (24.87 minutes versus

349 18.55 minutes, Supplementary Fig S6), and the tube seemed blocked at the papilla surface, the

350 pollen being detached from the papilla (Fig. 7B, Supplementary video S6). The growth rate of

351 incompatible tube was 1.05 μm/minute, whereas a compatible tube extended much more

352 rapidly in both standard and high humidity conditions (2.41 μm/minute and 1.91 μm/minute,

353 respectively, Fig. 7C). Growth kinetics shows that during the first three minutes, elongation rate

354 of both compatible and incompatible tubes was comparable, but then compatible tubes

355 extended rapidly whereas incompatible tubes slowed down (Fig. 7C). These observations

356 suggest that the incompatible tube may not be able to penetrate the papilla cell wall. To test

357 this hypothesis, we stained papilla cells with the amphiphilic styryl dye FM4-64. Contrary to

358 most plant cells where the dye labels the plasma membrane (PM) and the vesicular network

359 (Grebe *et al.*, 2003; Jaillais *et al.*, 2007; Jelínková *et al.*, 2010), we noticed that it remained at the

360 surface of the papilla as it did not colocalize with the actin or the PM canonical marker LTI6b

361 (Supplementary Fig S6). This labelling is probably due to the interaction of the dye with the

362 hydrophobic cuticle layer of the stigmatic cell. While the compatible pollen tube clearly

363 penetrated the FM4-64-labeled layer and grew in close contact with the papilla cytoplasm

364 identified by the actin fluorescence (9 tubes/9, Fig 7D), the incompatible tube was never

365 observed under the FM4-64 layer and stayed outside the papilla (11 tubes/11, Fig 7D).

366 Additionally, at the contact site with the short abnormal incompatible tube, we rarely observed

367 focalization of actin in the papilla, contrary to the compatible situation (Fig. 7B, Supplementary

368 Fig S5 and video S6). Taken together, our results show that incompatible grains generally fail to

369 fully hydrate, and when pollen hydration and germination are promoted by high relative

13

370 humidity, the SI reaction efficiently blocks tube penetration into the papilla cell wall, the tube

371 remaining outside the papilla surface without triggering actin focalization at the contact site

372 with the stigmatic cell.

373

374 **Discussion**

375 Here, we set up a live imaging system with a fast-scanning confocal microscope to

376 simultaneously monitor compatible and incompatible Arabidopsis pollen behavior on the same

377 single stigma and provide a thorough analysis of the dynamics of the cellular events following

378 pollen perception.

379

380 *AlSCR14* and *AlSRK14* SI Arabidopsis lines

381 The two Arabidopsis lines we generated, one that expressed the female determinant (*Al*SRK14)

382 and the second the male determinant (*Al*SCR14) were self-fertile but exhibited a strong SI

383 response when crossed. This SI response was maintained during flower development for about

384 two days, only weakening at late stages of stigma development where few pollen tubes grew in

385 pistil tissues and ultimately led to seed set. Nevertheless, the seed yield remained much below

386 the one reported for selfed Col-0 Arabidopsis (19 seeds per silique versus 62 seeds per silique).

387 Thus, these SRK14 and SCR14 transformants almost fully recapitulate the SI phenotype of the

388 natural SI Brassicaceae, the strength of the response being however less persistent compared

389 with strictly SI lines. Previous work reported a strong SI phenotype, although over a narrow

390 window, in stigmas at stage 13 and early stage 14 in Col-0 lines expressing the cognate *A. lyr*ata

391 *SCRb-SRKb* gene pair (the so-called Col-0/*AlSb* line), whereas older flowers fully accepted self-

392 pollen (Nasrallah, 2002; Nasrallah *et al.*, 2004). By contrast, in another study, the same *SCRb-*

393 *SRKb* gene pair was shown to be unable to confer SI in Col-0, even in stage-13 flowers, the

394 transgenic plants being fully fertile with a seed set similar to selfed Col-0 plants (Indriolo *et al.*,

395 2014). In this latter study, a role for the E3-ubiquitin ligase ARC1 in promoting SI was

396 demonstrated as only Col-0 co-expressing the *A. lyrata* or *B. oleracea* ARC1 with the *SCRb-SRKb*

397 pair acquired the capacity to reject self-pollen. Interestingly, the Col-0/*AlSRK14* lines generated

398 in our study express a strong SI response in mature stigmas without the need of co-expressing

14

399 *AlARC1*, the stigmas remaining partially self-incompatible at later flower developmental stages.

400 Earlier work showed that the variability of SI phenotypes observed between Arabidopsis

401 transformants was mainly due to *SRK* transcript levels (Nasrallah and Nasrallah, 2014). It has

402 been reported that transient SI phenotype and self-fertility of Col-0/*AlSb* transformants were

403 caused by modifier loci harbored in the Col-0 genome (Liu *et al.*, 2007; Boggs *et al.*, 2009). One

404 of these loci, *PUB8*, encoding an ARM-repeat and U-box protein, regulates *SRK* transcript levels

405 and is responsible for the pseudo-self compatibility observed in old flowers of Col-0/*AlSb* (Liu *et*

406 *al.*, 2007). Contrary to the Col-0/*AlSb* transformants mentioned above, in our study, *AlSRK14*

407 expression was driven by the *SLR1* promoter region that does not contain the *SRK* 5' and 3'

408 regulatory sequences. As a consequence, it is likely that transcription of *AlSRK14* gene and/or

409 stability of the transcripts escape the regulation of Col-0 SI modifiers, leading to the

410 accumulation of AlSRK14 protein to a level sufficient to maintain a partial pollen rejection

411 response during flower development. The weakening of SI response we observed in late

412 developmental stages was expected as it is known that *SLR1* promoter activity is

413 developmentally regulated, decreasing with flower ageing (Lalonde *et al.*, 1989), and hence

414 leading to lower abundance of *SRK14* transcript levels.

415 Though we attempted to transform Col-0 with *AlSCR14*, we failed to obtain transformants

416 whose pollen grains were rejected on *AlSRK14*-expressing stigmas. In SI Arabidopsis species, a

417 dominance hierarchy between *SCR* alleles has been reported, which is regulated by small RNAs

418 (sRNAs) (Durand *et al.*, 2014). We may propose that the inability to introduce a functional

419 *AlSCR14* in Col-0 might reside in the presence of vestigial sRNAs targeting the recessive *AlSCR14*

420 allele, while these sRNAs would be absent in the C24 background.

421

422 **Pollen behavior**

423 We observed that around 20 minutes after interaction with the stigma papillae, the compatible

424 pollen germinates a tube, which is consistent with the germination time reported in Arabidopsis

425 after *in vivo* pollination (Kandasamy *et al.*, 1994; Iwano, 2004). Once emerged, the pollen tube

426 extends at a rate of 2.41 μm/minute, which is significantly faster than tube elongation *in vitro*

427 (around 1 μm/minute; Iwano *et al.*, 2004; Yang *et al.*, 2017) and consistent with growth rates

15

428 reported in other *in vivo* systems (Iwano, 2004; Cheung *et al.*, 2010). Futhermore, we found that

429 tube growth rate is not constant, exhibiting a slow growth phase whithin the first three minutes

430 after tube emergence, followed by a second phase of more rapid growth. A bi-phasic growth

431 kinetics was already reported for Arabidopsis pollen tube growing *in vitro*, except that the

432 timing was much longer; the transition from germination to a rapid growth phase requiring 30

433 minutes (Vogler *et al.*, 2015). While compatible pollen grains promptly achieve germination,

434 incompatible grains deposited on the same stigma, and in the close vicinity of compatible ones,

435 follow a complete different destiny. Indeed, incompatible pollen grains exhibit an extremely low

436 germination rate (1.3 %), which is in agreement with the few pollen tubes we detected after

437 aniline blue staining. Thus, our semi *in vivo* system recapitulates what happens in nature when

438 pollen from various origins lands on the same stigma, accepting compatible whereas rejecting

439 incompatible pollen grains.

440 We found that almost immediately after landing on the stigma, compatible pollen starts to

441 hydrate and within 10 minutes is almost fully hydrated. By contrast, during the same time

442 period, the L/W ratio of incompatible grains only poorly evolves. Thus, inhibition of the

443 incompatible pollen acts very early, within the first minutes following stigma contact, blocking

444 pollen hydration. As previously described, hydration appears as the first check point controlling

445 pollen rejection in SI species with dry stigma (Zuberi and Dickinson, 1985; Dickinson, 1995;

446 Samuel *et al.*, 2009; Hiroi *et al.*, 2013; Safavian and Goring, 2013; Wang *et al.*, 2016). In this

447 study, the hydration threshold required to trigger germination was found to correspond to a

448 pollen L/W ratio below 1.4. Pollen turgor pressure has been proposed to be the main driving

449 force for germination (Hiroi *et al.*, 2013; Vogler *et al.*, 2015; Wang *et al.*, 2016). Our result

450 suggest that the optimal pollen turgor pressure corresponds to a 1.4 L/W ratio which is reached

451 after 10 minutes of pollen deposition.

452 Although 19 % (10/53) of the incompatible grains exhibit hydration features similar to the

453 compatible ones, they, however, never germinate. Thus, a second check point acts to ensure

454 that undesirable pollen grains that escape the hydration control are properly inhibited, blocking

455 their germination. When hydration of incompatible pollen is artificially boosted in a saturated

456 humidity environment, a significant proportion of the grains bypasses the second checkpoint

16

457 and germinates. However, pollen tube growth is slow and does not reach the second phase of

458 rapid elongation observed with compatible tubes. Futhermore, tubes grow outside the papilla

459 cell, unable to penetrate the cell wall. Production of a short incompatible tube, whose growth is

460 arrested in papilla cells, has been described in some *S*-haplotypes of SI Brassica species (

461 Elleman and Dickinson, 1994; Dickinson, 1995). Thus, our data highlight this late control of SI

462 reaction that prevents penetration of the stigmatic cell wall by incompatible tubes.

463 The current SI model in the Brassicaceae proposes that the signaling cascade triggered by SRK

464 activation disrupts the delivery of stigmatic secretory vesicles required for compatible pollen

465 acceptance (reviewed in Jany *et al.*, 2019). The content of these vesicles is currently unknown

466 but regarding the three inhibition levels described in this study and others (Zuberi and

467 Dickinson, 1985; Haasen *et al.*, 2010) we may postulate that stigmatic vesicles transport mainly

468 water to hydrate the pollen grain and trigger germination as well as enzymatic activities to

469 prepare the stigmatic cell wall for pollen tube penetration.

470

471 **Actin cytoskeleton**

472 In Brassica, remodeling of the actin cytoskeleton architecture has been described at the site of

473 pollen-stigma interaction (Iwano *et al.*, 2007). However, these results remain controversial, as a

474 previous work suggested that actin filaments do not show any rearrangement (Dearnaley *et al.*,

475 1999). Using an actin marker line, we show that a clear reorganization of actin occurs at the

476 contact site with compatible pollen. However, while actin bundles appear in Brassica papillae as

477 soon as pollen hydration starts and remain visible during the entire pollen hydration process

478 (Iwano *et al.*, 2007), in Arabidopsis, actin reorganization starts later, from the end of pollen

479 hydration (just before germination) or following pollen tube emergence. One explanation for

480 these discrepancies could be that SI mechanism in Brassica and Arabidopsis does not rely on

481 rigorously similar cellular processes as previously proposed (Kitashiba *et al.*, 2011).

482 Alternatively, these discrepancies could be due to technical reasons: (i) we used stable

483 transformed Arabidopis lines instead of transient expression in *Brassica* papillae, (ii) we

484 performed massive pollination of the stigmatic cells compared with deposition of a single

485 *Brassica* pollen by micromanipulation, and (iii) we used the Lifeact peptide instead of the mouse

17

486   Talin protein to label F-actin because this short peptide does not affect actin dynamics *in vivo*

487   (Era *et al.*, 2009). Nevertheless, whatever the species, actin focalization appears as a hallmark of

488   the compatible reaction as it is never detected in incompatible pollination. Interestingly,

489   Hardham et al. (2008) showed that touching the surface of the *A. thaliana* cotyledon epidermal

490   cells with a microneedle induced a rapid actin focalization around the contact point, leading the

491   authors to propose that actin reorganization is triggered by detection of mechanical pressure.

492   Likewise, reorganization of actin microfilaments was observed in many plant-pathogen

493   interactions, where actin cables accumulate at the contact site with the pathogen (Takemoto,

494   2004 and references therein). Altogether, we may hypothetize that reorganization of actin is

495   triggered in stigmatic cells that sense the mechanical pressure produced by the pollen

496   grain/tube at the site of cell wall penetration. In accordance with this, we generally did not

497   detect modification of the actin architecture at the contact site with incompatible pollen tubes

498   that were generated in high humidity conditions and that could not penetrate the stigmatic cell

499   wall. Alternatively, the pollen grain, when engaged in compatibility, could also initiate a

500   signaling cascade leading to the actin polymerization at the contact site with the germinating

501   grain and/or the growing pollen tube. Nevertheless, the focalization of actin is consistent with

502   the well characterized role of actin network in vesicular delivery to the plasma membrane

503   (Szymanski and Staiger, 2018) induced upon compatible pollination.

504

505   Taking together, using fast scanning confocal microscopy, our observations underline the

506   suitability of our system to capture very dynamic events occurring within minutes.Notably, we

507   identified a hydration threshold (L/W < 1.4) that pollen grains must reach to acquire the critical

508   water content required for supporting pollen germination. In addition, we monitored the

509   dynamics of actin network during compatible and incompatible responses and found that

510   focalization of actin cables was triggered when pollen-stigma interaction is engaged in

511   compatibility. This actin reorganization might be elicited in response to the mechanical pressure

512   exerted by pollen grain/tube or alternatively by a specific factor delivered by the pollen . Finally,

513   our semi in vivo system provides a particularly well-suited system to test putative components

514   involved in the acceptance or rejection of pollen grains through the analysis of Arabidopsis

18

515   mutants as well as transgenic lines and will make possible the deciphering of the early events

516   controlling sexual reproduction.

517

518   **Supplementary data**

519   **Protocol S1-S3**: supporting material

520   **Figure S1**: *AlSRK14* and *AlSCR14* Transgenic plant selection

521   **Video S1**: Dual pollination with compatible and incompatible pollen deposited on the same

522   stigma

523   **Video S2**: Actin rearrangement at the pollen contact site

524   **Video S3**: Actin rearrangement along the pollen tube path

525   **Video S4**: Actin behavior in stigmatic cells in contact with incompatible pollen

526   **Table S1**: L/W ratio of pollen grains coming out of mature anthers

527   **Figure S2**: Dynamics of actin focalization following compatible pollination

528   **Figure S3:** Hydration kinetics of compatible and incompatible pollens in the semi *in vivo* system

529   **Table S2**: Germination of compatible pollen tracked during experiments described in figure 5

530   **Figure S4**: Standard and high humidity assays

531   **Figure S5**: Behavior of compatible pollen in high humidity conditions

532   **Video S5**: Compatible pollen germination and pollen tube growth in high humidity conditions

533   **Figure S6**: Behavior of incompatible pollen in high humidity conditions

534   **Table S3**: L/W ratio of incompatible pollen in high humidity conditions 10 minutes after pollen

535   deposition

536   **Video S6**: Incompatible pollen germination and pollen tube growth in high humidity conditions

537   **Figure S7**: FM4-64 labelling of stigmatic cells

538

546

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660

661

662   **Figure Legends**

663

664   **Fig. 1.** Generation of SI Arabidopsis lines. (A) The Col-0 transgenic line expressing *AlSRK14* was

665   transformed with the fusion protein Act:Venus under the SLR1 promoter to label stigmas (green

666   fluorescence), whereas wild-type C24 or transgenic C24 expressing *AlSCR14* were transformed

667   with the TURQUOISE protein (TURQ, blue fluorescence) or the RFP protein (red fluorescence)

668   under pollen promoters, respectively. Bar = 20 μm. (B) SI phenotype of generated lines. Stage

669   13-14E stigmas were pollinated with mature pollen and pollen tubes present in stigmas were

670   counted after aniline blue staining. In all crosses, the first name refers to the female partner and

671   the second to the male partner (example: female Col0 x male C24/Turq) (C) Seed set. Stage 13-

672   14E stigmas were pollinated with mature pollen, after 21 days silique were dissected and seeds

673   counted. n = number of pollinated stigmas. Mean (black diamond) of 3 independent

674   experiments. Black dots are extreme values. Experiments separated by a dashed line were

675   performed at different dates. Error bars indicate Standard Error of the Mean (SEM).

676

677   **Fig. 2.** Dynamics of the SI response in Col-0 background. (A) SI phenotype following pollination

678   of stigmas at two developmental stages. Flower buds at the end of developmental stage 12

679   were emasculated and pollinated with mature pollen 42h (stigma stage 15) or 66h (stigma stage

680   16) after emasculation (upper panel) and pollen tubes in stigmas were counted 6h after

681   pollination. Pollen tube count after aniline blue staining of stigmas (bottom graph). (B)

682   Maintenance of SI reaction over time. Flower buds at the end of stage 12 were emasculated,

683   pollinated with mature pollen 18h later (stage13-14) and harvested 6, 24, 48 or 72 hours after

684   pollination (upper panel). Pollen tube count after aniline blue staining of stigmas (bottom

685   graph). Beyond 100 pollen tubes per stigma, exact number was not determined and given a

686   value of 100 (no mean, no error bar for compatible crosses). In all crosses, the first name refers

687   to the female partner and the second to the male partner. n = number of pollinated stigmas.

688   Blue dots correspond to individual pollinated stigma; when several stigmas have the same

689   value, the stigma number is indicated in bracket. Mean (black diamond) of two independent

690   experiments. Black dots are extreme values. Error bars indicate SEM.

24

691 **Fig. 3.** Set up of the semi *in vivo* assay. (A) Upper part of the pistil was cut (white line) and stuck

692 in a solid agar medium. To control humidity level, a small chamber was made with the bottom

693 quarter of a PCR tube whose the base was pierced with a needle and placed upside down on the

694 medium. Stigma was pollinated and a coverslip was gently applied for live imaging. Bar = 500

695 μm (B) Dual pollination on a single stigma. Stigma (Col-0/SRK14+Act:Venus, green fluorescence)

696 was pollinated first with incompatible pollen (C24/SCR14+RFP, red fluorescence) and

697 immediately after with compatible pollen (C24/TURQ, blue fluorescence), and pollen behavior

698 monitored under confocal microscopy for 32 minutes. Image is a Z-projection (two minutes

699 after pollen deposition). Bar = 50 μm. (C) Pollen germination features extracted from 5 dual-

700 pollinated stigmas. Only one incompatible grain germinated (germination time = 28 minutes).

701 SEM stands for Standard Error of the Mean

702

703 **Fig. 4**. Actin reorganization at the contact site with the compatible pollen grain and pollen tube.

704 (A) Visualization of actin bundles in unpollinated stigmatic cells expressing the Act:Venus marker

705 (Col-0/SRK14+Act:Venus, green fluorescence). Image is a Z-projection. (B) Dynamics of actin

706 focalization following compatible pollination. Act:Venus stigmas were dual-pollinated in the

707 semi *in vivo* system and a Z-stack was taken every minute during 32 minutes. Twenty four

708 Papillae in contact with a compatible pollen grain (blue fluorescence) were selected on 14

709 stigmas and first appearance of actin fluorescence (focalization) was recovered. Timing of actin

710 focalization was defined regarding the pollen development stage. (C) Actin reorganization in

711 papillae just before pollen germination. (D) Actin reorganization in papillae during germination

712 and pollen tube growth. Indicated time corresponds to time after pollen deposition. Images are

713 Z-projections. (E) Mutiple pollen germination on a single papilla. Image is a Z-projection. Arrows

714 show the focalization of actin fluorescence. Bar = 10 μm.

715

716 **Fig. 5.** Kinetics of compatible pollen hydration following dual pollination. (A) During hydration,

717 pollen shape changes from an ovoid towards a round shape given by the Length/Width ratio

718 (L/W ratio). (B) Evolution of the L/W ratio during a compatible interaction. Among the 69

719 tracked pollen grains, 58 germinated during the experiment (solid blue line), whereas 11 failed

720   to germinate (dashed blue line). (C) Kinetics of pollen hydration related to rate of germination.

721   Pollen grains were classified by their germination time in four categories: early, intermediate,

722   late and non germinated. Slopes were calculated by linear regression based on pollen hydration

723   curve during the first 10 minutes. n = number of pollen grains. Mean of five independent

724   experiments. Error bars indicate SEM.

725

726   **Fig. 6.** Kinetics of incompatible pollen hydration following dual pollination. (A) Evolution of the

727   L/W ratio during an incompatible interaction. Among the 53 tracked pollen grains, 43 exhibited

728   a L/W ratio that never reached a ratio below 1.4 (solid red line) whereas 10 had a ratio below

729   1.4 (dashed red line). The solid blue line that indicates hydration dynamics of compatibe pollen

730   was reproduced from Fig. 5B to facilitate the comparison. (B) Hydration rates during the first 10

731   minutes of incompatible pollen with a L/W ratio >=1.4 (solid red line), incompatible pollen with

732   a L/W ratio <1.4 (dashed red line) and compatible pollen that germinated late (solid blue line,

733   reproduced from Fig. 5C). Slopes were calculated by linear regression. n = number of pollen

734   grains. Mean of 5 independent experiments, error bars indicate SEM.

735

736   **Fig. 7.** Pollen germination in high humidity conditions. (A) Compatible pollen (blue fluorescence)

737   was deposited on an Act:Venus stigma and incubated in high humidity conditions. (B)

738   Incompatible pollen (red fluorescence) was deposited on an Act:Venus stigma and incubated in

739   high humidity conditions. A Z-stack was taken every minute after pollen deposition. Images are

740   Z-projections. Bar = 10 μm. (C) Growth rate of pollen tubes in standard and high humidity

741   conditions. Pollen tube length was measured at each time point from germination (0 on the

742   horizontal axe) during 6 minutes, for compatible pollen in standard conditions (light blue line),

743   in high humidity conditions (dark blue line) or for incompatible pollen in high humidity

744   conditions (red line). n = number of tracked pollen grains on minimum 6 different stigmas, error

745   bars indicate SEM. (D) 35 minutes after incubation in high humidity conditions, stigmas were

746   stained with FM4-64 . A Z-stack was processed with image J to generate a Z-projection.

747   Compatible pollen tube (white star) was detected beneath the FM4-64 labelled layer (red

748   fluorescence surrounding the papilla cell), whereas incompatible tube (yellow star) was outside.
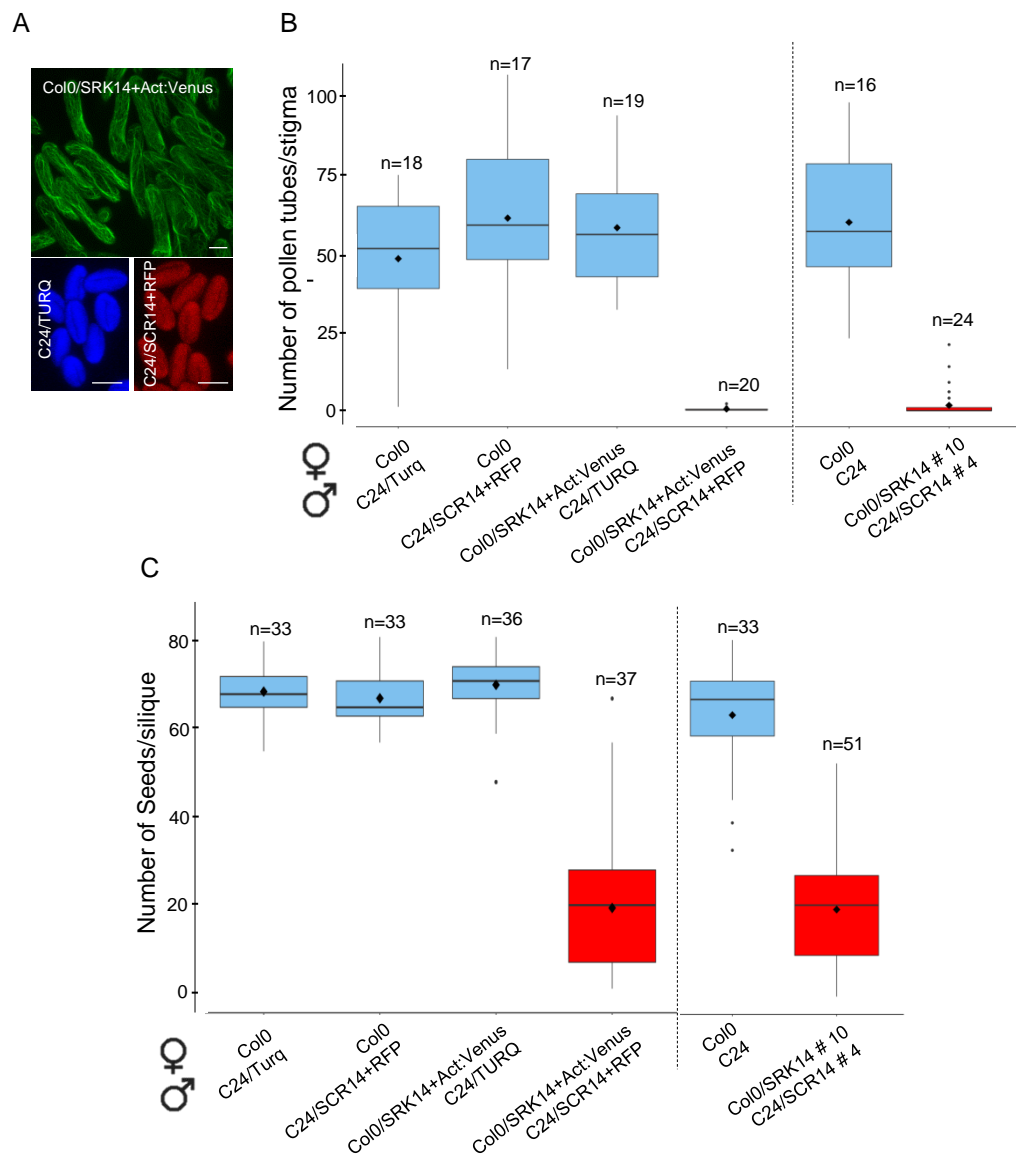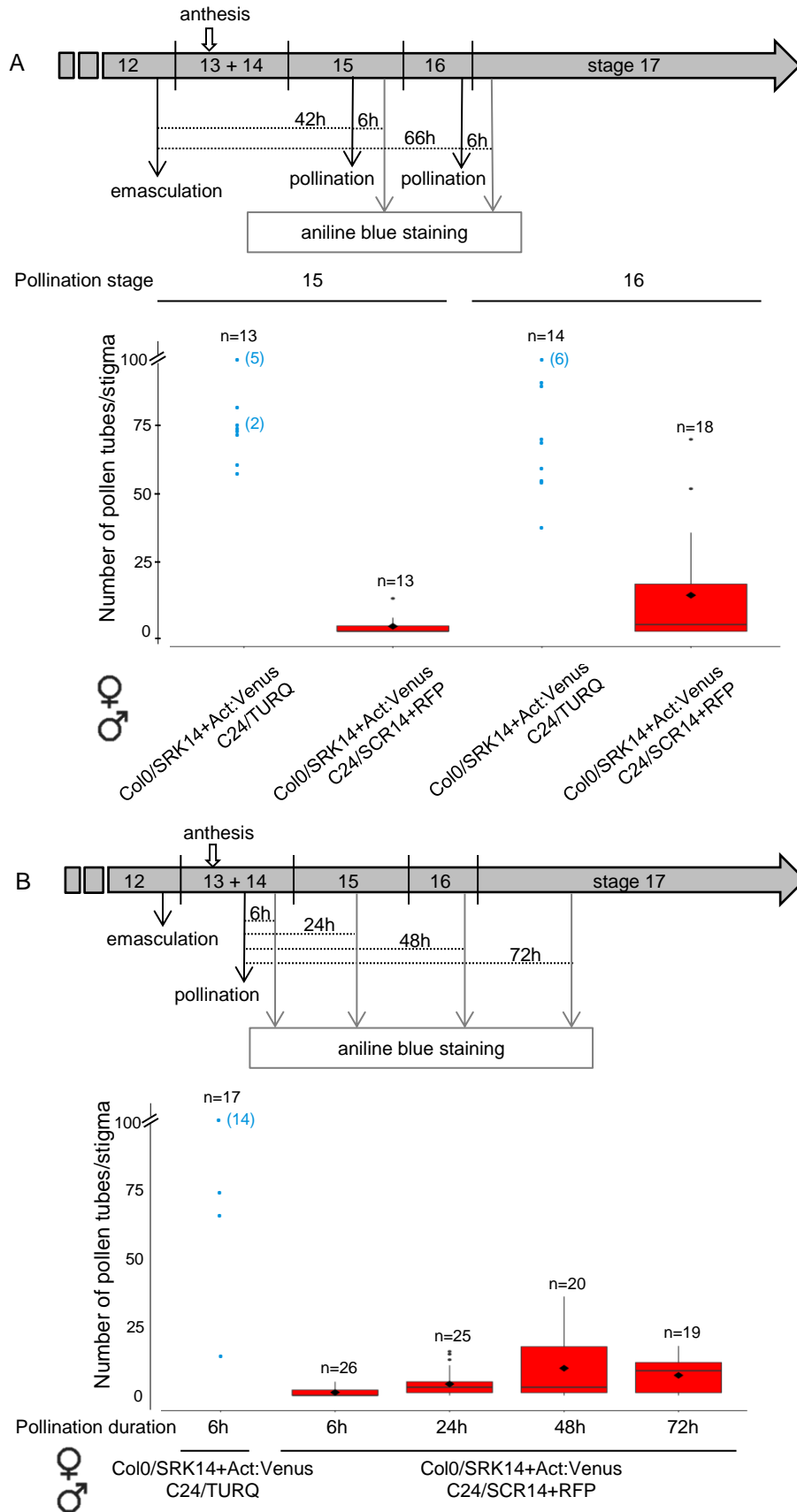
749   Bar = 10 μm.

Figure 1

A

Col0/SRK14+Act:Venus

C24/TURQ

C24/SCR14+RFP

B

Number of pollen tubes/stigma

n=18
n=17
n=19
n=20

n=16
n=24

♀♂

Col0
C24/Turq

Col0
C24/SCR14+RFP

Col0/SRK14+Act:Venus
C24/TURQ

Col0/SRK14+Act:Venus
C24/SCR14+RFP

Col0
C24

Col0/SRK14 # 10
C24/SCR14 # 4

C

Number of Seeds/silique

n=33
n=33
n=36
n=37

n=33
n=51

♀♂

Col0
C24/Turq

Col0
C24/SCR14+RFP

Col0/SRK14+Act:Venus
C24/TURQ

Col0/SRK14+Act:Venus
C24/SCR14+RFP

Col0
C24

Col0/SRK14 # 10
C24/SCR14 # 4

Figure 2

Figure 3

A

water
cut pistil
coverslip
air
grease plug
slide
pierced PCR tube
solid agar medium

B

C

	germination			pollen number
	%	time (min)	SEM	
compatible	74	20.38	0.55	106
incompatible	1.3	-	-	75

Figure 4

A

B

first appearance of actin focalization in papilla	number of papilla
before germination	10/24 (42%)
concomitant with pollen tube emergence	2/24 (8%)
after germination	10/24 (42%)
no actin focalization	2/24 (8%)

C

17 min

18 min

19 min : germination

D

19 min : germination

22 min

24 min

E

Figure 5

Figure 6

A

B

Figure 7

A — Compatible

germination   + 3 min   + 6 min   + 11 min

B — Incompatible

C

Mean pollen tube length (µm) vs Time after pollen germination (min)

- incompatible pollen in humid conditions
- compatible pollen in standard conditions
- compatible pollen in humid conditions

tube growth: 2.41 µm/min   n=14

tube growth: 1.91 µm/min   n=36

tube growth: 1.05 µm/min   n=14

D — Compatible / Incompatible