Linking gene expression to unilateral pollen-pistil reproductive barriers

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Abstract

Unilateral incompatibility (UI) is an asymmetric reproductive barrier that unidirectionally prevents gene flow between species and/or populations. UI is characterized by a compatible interaction between partners in one direction, but in the reciprocal cross fertilization fails, generally due to pollen tube rejection by the pistil. Although UI has long been observed in crosses between different species, the underlying molecular mechanisms are only beginning to be characterized. The wild tomato relative *Solanum habrochaites* provides a unique study system to investigate the molecular basis of this reproductive barrier, as populations within the species exhibit both interspecific and interpopulation UI. Here we used a transcriptomic approach to identify genes in both pollen and pistil tissues that may be probable key players in UI. We confirmed UI at the pollen-pistil level between a self-incompatible population and a self-compatible population of *S. habrochaites*. A comparison of gene expression between pollinated styles exhibiting the incompatibility response and unpollinated controls revealed only a small number of differentially expressed transcripts. Many more differences in transcript profiles were identified between UI-competent versus UI-compromised reproductive tissues. A number of intriguing candidate genes were highly differentially expressed, including a putative pollen arabinogalactan protein, a stylar Kunitz family protease inhibitor, and a stylar peptide hormone Rapid Alkalinization Factor. Our data also provide transcriptomic evidence that fundamental processes including reactive oxygen species signaling are likely key in UI pollen-pistil interactions between both populations and species. Our transcriptomic analysis highlighted specific genes, including those in ROS signaling pathways that warrant further study in investigations of UI. To our knowledge, this is the first report to identify candidate genes involved in unilateral barriers between populations of the same species.

2

24 **Keywords**

Intraspecific reproductive barriers; interpopulation interactions; plant mating system; pollen-pistil transcriptomes; self-incompatibility; *Solanum habrochaites*; unilateral incompatibility; wild tomato species

25 **Background**

26   Reproductive barriers are critical for maintaining species integrity, and their emergence

27 between populations is associated with decreased gene flow and increased genetic divergence,

28 ultimately leading to speciation. In a number of plant families, including the Solanaceae, a

29 unidirectional post-mating prezygotic barrier termed unilateral incompatibility (UI) occurs at the

30 level of pollen-pistil interactions. UI has most often been studied between genera or species [1-

31 5], but there is also evidence of UI between populations of the same species [1, 6-9].

32   The unidirectionality of UI in crosses between species has been linked to plant mating

33 system and specifically to the self-incompatibility response. Self-incompatible (SI) species are

34 obligate outcrossers, whereas self-compatible (SC) species are capable of producing offspring

35 through self-pollination. Gametophytic SI is the most common system of incompatibility in

36 flowering plants [10] and in many families it is dependent on the activity of pistil-expressed *S*-

37 locus ribonucleases, or S-RNases [11, 12]. Distinct from the sporophytic SI of the Brassicaseae

38 in which pollen is rejected at the stigma surface [13, 14], in gametophytic SI growing pollen

39 tubes are actively rejected within the style [11, 15-17]. This type of S-RNase-based SI is

40 genetically determined by the polymorphic *S*-locus, which harbors pistil- (S-RNase) and pollen-

41 (S-locus F-box) expressed factors that are required for the specificity of the SI response [18-21].

42 Additional factors have also been implicated in SI, although they do not determine specificity.

3

43   These include pistil-expressed HT-proteins and 120K glycoproteins [22-24], as well as pollen-

44   expressed components of the E3 ubiquitin ligase complex (Cullin1 and SKP1) [15, 25-29].

45   UI generally follows the SI x SC rule, wherein pollen of SC species is unable to fertilize

46   SI ovules, but the reciprocal cross is compatible [4]. Because of this link between SI and UI,

47   initial studies suggested pleiotropic effects of the *S*-locus on interspecific incompatibilities. In

48   fact, more recent studies have shown that the proteins S-RNase, HT-protein, S-locus F-box 23

49   and Cullin1 all function in both the SI and UI response, demonstrating mechanistic overlap

50   between these two types of reproductive barriers [26, 28, 30-33]. However, it is also clear that

51   genetic factors other than those involved in SI function in UI.

52   Crosses between SC species or populations (many of which do not express S-RNase)

53   exhibit unexpected incompatibilities [1-4, 7, 31, 34, 35]. This suggests that there are at least two

54   mechanisms underlying UI, one of which is S-RNase based and the other of which is S-RNase

55   independent. Most SC species exhibit full crossability with other SC species; however only

56   recently evolved SC populations of SI species exhibit UI [2, 4]. This suggests that the

57   mechanistic factors underlying S-RNase independent UI are also present in populations that

58   contain S-RNase. However, the number and type of factors involved in UI remains unclear and

59   most models suggest roles for multiple UI genes of both large and small effect [2, 5, 8, 36].

60   The tomato clade (*Solanum* section *Lycopersicon*) offers ample opportunities to further

61   understand and identify the genes involved in UI [37]. This small clade has recently diverged

62   from a common SI ancestor [38, 39] over the course of ~2.5 million years [40]. The clade

63   harbors six SC species, and seven SI species, three of which contain both SI and SC populations

64   [41]. A number of studies have examined both the physiological aspects [1, 5, 42, 43] and

65   genetic basis [26-28, 30, 31, 33] of UI between members of the clade. In addition, a small

66   number of studies have assessed UI between SI and SC populations within a species [1, 6-9, 43].

67   The wild tomato *Solanum habrochaites* is an ideal species in which to study both

68   interspecific and interpopulation UI within the context of recently diverged populations. *S.*

69   *habrochaites* has undergone at least two independent transitions from the ancestral SI state to SC

70   at both the northern and southern species range margins [6,7, 9, 44]. The loss of SI at the

71   northern range margin is correlated with the loss of pistil-side SI factor S-RNase [6, 31, 45];

72   whereas southern *S. habrochaites* SC populations express an S-RNase protein with little or no

73   RNase activity [31]. The genetic structure of *S. habrochaites* is consistent with the spread of

74   populations both north and south from a central origin, with central SI populations showing the

75   highest levels of diversity [44, 46]. Interestingly, the types and strengths of reproductive barriers

76   displayed by a number of populations also follow this same latitudinal axis of divergence [6, 44].

77   Previous studies have demonstrated variation in the strength of both intra- and

78   interspecific UI in different *S. habrochaites* populations [1, 6-9, 31, 35]. For instance, a central

79   *S. habrochaites* SI population (LA1777) rejects pollen tubes from a subgroup of northern *S.*

80   *habrochaites* SC populations, including LA0407; whereas LA0407 and other SC populations

81   accept LA1777 pollen tubes [1, 6].

82   In interspecific interactions, when either SC *S. lycopersicum* or SC *S. neorickii* (as

83   female) is crossed with *S. habrochaites* (as male), crosses result in fruit [47, 48]. In the

84   reciprocal cross, pistils of SI and southern SC populations of *S. habrochaites* rapidly reject

85   interspecific SC pollen after a few millimeters of growth through the style, demonstrating a

86   classic UI response [1, 6, 31]. However, interspecific pollen tube rejection is delayed in many

87   northern SC populations of *S. habrochaites*, [1, 6, 31], and one unique SC population (LA1223)

5

88 is unable to reject both *S. lycopersicum* and *S. neorickii* pollen tubes [6]. LA1223 is the only

89 known *S. habrochaites* population that does not accumulate the pistil HT-protein [6, 31], which

90 may explain this extraordinary lack of UI at the pollen-pistil level.

91 Although elegant mapping, biochemical and transgenic studies have revealed that SI

92 factors are important players in UI [12, 23, 26, 28, 30, 31, 33, 49], the full suite of molecular

93 factors involved in the complex UI response are only beginning to be characterized. The

94 evidence for both interspecific and interpopulation UI in *S. habrochaites* suggests that factors

95 other than those involved in SI are involved in UI. This is consistent with transgenic studies in

96 tomato showing that together S-RNase and HT-protein can act to reject pollen from only a subset

97 SC species, and even then pollen tube rejection is delayed [33].

98 Transcriptome profiling can provide a broad view of the factors involved in pollen-pistil

99 interactions and represents a powerful tool for candidate gene identification. Studies in

100 *Arabidopsis* have identified large numbers of genes that are specific to pollen or pistil, as well as

101 those that are specifically induced upon the interaction between the two in compatible

102 pollinations [50-53]. However, very few studies have investigated pollen-pistil transcriptomes in

103 response to UI. In a recent study, Pease et al. [54] identified a number of interspecific UI

104 candidate genes by comparing stylar transcriptomes of UI-competent *S. pennellii* and UI-

105 deficient *S. lycopersicum*, two species which likely diverged over 2 mya [40]. Their analysis

106 identified a large number of loci that varied in expression between styles of these two species,

107 and highlighted the importance of HT-protein in UI [54].

108 Here, we used a similar transcriptomics approach to broadly characterize the molecular

109 factors involved in UI in *S. habrochaites* at the level of pollen-pistil interactions. The

110 populations selected for study have recently diverged (< 0.8 mya, [40]), and exhibit differences

6

111   in both interspecific and interpopulation reproductive barriers. To our knowledge, this is the first

112   report using transcriptomics to identify novel candidate genes associated with reproductive

113   barriers between diverging populations within a species.

114

115   **Results**

116   ***Pollen-pistil interactions in SI LA1777 and SC LA0407***

117   Using pollen tube growth assays, we confirmed that all LA1777 (SI) individuals used in

118   RNA-seq experiments rejected both self-pollen tubes and those of LA0407, but accepted pollen

119   from other LA1777 individuals (intrapopulation). Further, we confirmed that each LA0407 (SC)

120   individual accepted pollen tubes from LA1777, self, and intrapopulation crosses.

121   In compatible crosses, pollen tubes generally reach the ovary within 24 hours [1, 31].

122   However, incompatible crosses may differ in the timing of pollen tube rejection [1]. To identify

123   the post-pollination time-point at which pollen tube rejection occurs in the incompatible cross

124   between LA1777 (female) and LA0407 (male), pistils were pollinated and harvested over a time

125   course. Pistils of LA1777 were actively rejecting pollen of LA0407 at 12 hours post-pollination,

126   and full rejection occurred by 48 hours (Fig. 1). In an attempt to capture changes in gene

127   expression that would correspond to active pollen tube rejection, we chose to harvest stylar tissue

128   16 hours post-pollination for subsequent RNA-seq experiments. At this point, incompatible

129   pollen tubes will have traversed ~20% of the style length (Fig. 1), whereas compatible pollen

130   tubes will have traversed ~60% of style length [31].

131

132 *Differential gene expression between UI-competent and compromised populations*

133   We sampled both pollen and stylar tissue to better characterize differences in

134 transcriptomes between UI-competent LA1777 and UI-compromised LA0407 (Fig. 2). Styles

135 were either unpollinated (UP) or subject to three different treatments including self,

136 intrapopulation and interpopulation pollination (Fig. 2). We analyzed a total of 33,055 genes,

137 out of which 7,358 (22.3%) were specific to pollen (that is, they were upregulated in pollen with

138 respect to all styles), and 4,793 (14.5%) were specific to styles. Additionally, we found 1,492

139 genes that were highly upregulated only in tissues of LA1777 and 1,382 genes highly

140 upregulated only in LA0407 (4.5% and 4.2% of genes analyzed, respectively).

141

142 *Pollination type significantly influences a small number of genes*

143   We expected that stylar transcriptomes would exhibit differences due to the type of

144 pollination and whether or not a pollination was compatible. We performed three separate

145 pairwise comparisons to look at differential gene expression in unpollinated (UP) LA1777 styles

146 compared to different pollination types (intrapopulation, self and interpopulation). Pairwise

147 comparisons between pollinated and UP LA1777 styles revealed only a small number of

148 differentially expressed genes, all of which were expressed at very low levels (<0.3 counts per

149 million (cpm)), Additional File 1, Table S1 (self vs UP); Additional File 1 Table S2

150 (intrapopulation vs UP) and Additional File 1, Table S3 (interpopulation vs UP)). In all

151 pollination treatments we identified pollination-induced differential expression of a number of

152 hypothetical proteins, RNA-directed DNA polymerases and retrovirus-related reverse

153 transcriptases. However, only three genes showed similar regulation among all pollination

154   treatments: a hypothetical protein (Sopen05g010690) and two genes that have high homology to

155   a Copia-like retrotransposon (Sopen01g019140 and Sopen02g008750).

156   Five genes were similarly differentially expressed in incompatible crosses with LA1777

157   as female (self and interpopulation crosses, Additional File 1, Table S1 and Table S3) versus

158   compatible crosses (intrapopulation, Additional File 1, Table S2), three of which encoded

159   hypothetical proteins. In addition, a cystathionine beta synthase (CBS) domain-containing

160   protein (Sopen06g019460) was downregulated 3-fold in incompatible crosses. It has been

161   proposed that CBS proteins are redox regulators involved in the modification of cell wall

162   composition, and in *Arabidopsis*, changes in CBS expression can reduce self-fertility [55]. A

163   homolog of *Defective meristem silencing 3* (DMS3, Sopen03g019410), a gene that is involved in

164   silencing and epigenetic modification, was also downregulated 3-fold in incompatible crosses

165   [56].

166   Thirty two genes that were differentially regulated between interpopulation pollination

167   and UP styles (Additional File 1, Table S3), but not in other treatments (self vs UP, Additional

168   File 1, Table S1 or intrapopulation vs UP, Additional File 1, Table S2).Interesting candidates

169   included a Ras-related GTPase (Sopen04g023040, Rab3), a pollen specific calcium binding

170   annexin (Sopen04g003390), and a K^+ transporter (Sopen11g006300) were upregulated ~3-fold in

171   interpopulation pollinations. Another candidate gene, upregulated 16-fold in interpopulation

172   pollinations encodes a H_2O_2 transporter (Sopen10g033580, PIP2). Each of these genes are

173   potentially involved in reactive oxygen species (ROS) signaling during pollen-pistil interactions

174   [57-61], and their upregulation in interpopulation pollinations may alter ROS signaling, resulting

175   in the dysfunctional pollen tube growth characterized by UI. Further, in interpopulation

176   pollinations, an endonuclease was upregulated over 18-fold, and a DNase over 2.9 fold.

177 Intriguingly, in *Pyrus pyrifolia* and *Papaver rhoeas*, the disruption of ROS signaling in

178 incompatible (self) pollen tubes leads to depolymerization of the actin cytoskeleton and an

179 increase in nuclear DNA degradation [62, 63].

180

181 ***Differential gene expression related to UI-competence in styles***

182 Although a number of potentially interesting candidates involved in UI were identified

183 from our pairwise comparisons, they were expressed at relatively low levels, often showed only

184 low fold-changes, and had relatively high p-values ($p < 0.05$). A principal components analysis

185 (PCA) of all stylar treatments showed that most of the variation between samples could be

186 explained by source population, and that there was no consistent grouping of styles due to

187 pollination treatment (Additional File 2, Fig. S1). The fact that few significant differences were

188 detected between UP styles and pollination treatments may reflect results of previous studies

189 indicating that the genes involved in UI-competence are expressed in styles regardless of

190 pollination status [31, 37, 54]. In other words, the UI-competent styles appear 'primed' to reject

191 incompatible pollen, as large changes in gene expression are not seen between UP styles and

192 those pollinated with compatible versus incompatible pollen parents. Because of these results,

193 we also performed an analysis in which all stylar treatments within a population (UP, pollinated

194 with self, intrapopulation and interpopulation pollen) were pooled to capture differential

195 expression between UI-competent (LA1777) versus UI-compromised (LA0407) styles.

196 We identified 179 genes that were significantly upregulated in UI-competent versus

197 compromised styles, and 179 that were significantly downregulated (Additional File 1, Table S4

198 and Table S5).   However, we focused our interest predominantly on genes that showed a mean

199   expression level over 3 normalized cpm and were upregulated over 10-fold in UI-competent

200   versus compromised styles. The top 25 genes showing the largest upregulation in UI-competent

201   styles are shown in Table 1.

202   Within the top 25 genes showing the highest upregulation in UI-competent versus

203   compromised styles, we found ten that are involved in oxidation-reduction reactions (Table 1).

204   These included three Cytochrome P450 genes (Sopen02g037430, Sopen06g021780,

205   Sopen07g028940), an Alkenal Reductase (Sopen12g006330), a Glutathione S-Transferase

206   (Sopen12g026980), an NAD(P)H-Dehydrogenase (Sopen05g003640), and an NAD(P)-

207   Oxidoreductase (Sopen12g021490). In addition, we identified two genes involved in early steps

208   of flavonoid biosynthesis: Chalcone Synthase 2 (Sopen05g032070) and Chalcone-Flavanone

209   Isomerase (Sopen05g030780). Finally, we discovered that the gene showing the highest

210   upregulation in UI-competent styles (hypothetical protein, Sopen12g017530) contains a heavy

211   metal-associated domain (Additional File 2, Fig. S2), suggesting that it also may be involved in

212   oxidation-reduction reactions.

213   In UI-competent styles, we also identified three upregulated genes that are putatively

214   involved in defense response. These included an Endo-Chitinase involved in jasmonic acid and

215   ethylene signaling (Sopen02g027710) that was highly expressed (182 cpm) and upregulated over

216   20-fold, a Beta-Glucosidase (Sopen11g004500) and a Disease Resistance Protein

217   (Sopen10g025030) (Table 1). These genes are of interest, as many molecular components

218   involved in plant-pathogen interactions show significant overlap with those involved in pollen

219   tube growth and guidance [64].

220   One gene of particular interest that was highly upregulated in UI-competent styles is the

221   hypothetical protein Sopen02g033850 (Table 1), which shows 80% amino acid identity to the

222   peptide hormone Rapid ALkalinization Factor (RALF) from the wild potato, *S. chacoense*.

223   Small secreted peptides including RALFs are involved in a wide variety of plant functions

224   including development and immunity [65], and a pollen-specific RALF from *S. lycopersicum*

225   (SlPRALF) was found to negatively regulate pollen tube elongation *in vitro* [66]. We also

226   identified a protein involved in oligo-peptide transport (Sopen05g001950) which was

227   upregulated >28-fold in UI-competent styles and may be involved in the transport/secretion of

228   peptide hormones such as RALFs.

229   Another gene of interest that was highly upregulated in UI-competent versus

230   compromised styles was a Kunitz family protease inhibitor (Table 1). In *Nicotiana*, the Kunitz

231   family protease inhibitor, NaStep, is highly expressed in the pistils of SI species and is thought to

232   stabilize HT-proteins, although the mechanistic basis of this interaction remains unknown [67].

233   The NaStep protein is taken up by both compatible and incompatible pollen tubes, and the

234   transgenic suppression of *NaStep* in SI *Nicotiana* species compromises rejection of both self- and

235   some types of interspecific pollen tubes [67]. The reduced expression of this gene in UI-

236   compromised LA0407 may reflect this population's lack of ability to reject some types of

237   interspecific pollen tubes [1, 6].

238   Although we were most interested in genes showing the highest upregulation in UI-

239   competent styles, we also analyzed genes that are highly downregulated. We found that three of

240   the top 25 most highly downregulated genes in UI-competent styles were involved in oxidation-

241   reduction reactions, and one was putatively involved in defense response (Additional File 1,

242   Table S5). Notably, five of the top 25 genes downregulated in UI-competent styles are

243   putatively involved in cell wall modification (Additional File 1, Table S5). These include two

244   Pectin Lyases (Sopen12g009500 and Sopen01g038750), a Glucosyltransferase

245 (Sopen00g008620) and two Glycosyl Hydrolases (Sopen04g034210 and Sopen07g001240), all

246 of which were upregulated over 13-fold. Eight additional genes involved in cell wall

247 modification were downregulated in UI-competent styles, although to lesser extents (Additional

248 File 1, Table S5).

249   In addition to our analysis of genes that are highly up- or downregulated in UI-competent

250 versus compromised styles, we investigated the expression of 33 *a priori* candidates (Additional

251 File 1, Table S6) based on reports in the literature of stylar genes that may be involved in UI [26,

252 28, 31, 54]. These candidates included genes that were identified in a recent study comparing

253 stylar transcriptomes between UI-competent *S. pennellii* 0716 and the cultivated tomato *S.*

254 *lycopersicum* M82 which shows no UI response [54]. Only three of the 33 *a priori* candidates

255 showed over a 2-fold increase in styles of UI-competent LA1777 versus UI-compromised

256 LA0407 (Additional File 1, Table S6). These included a Pectin-Methylesterase Inhibitor

257 (Sopen04g027820, upregulated 2.4-fold) and two Glucosyltransferases (Sopen08g002330 and

258 Sopen08g002350, both upregulated over 8-fold); however all were expressed at low levels, < 1

259 cpm. HT-protein, a small asparagine-rich protein required for S-RNase-based UI [33] was

260 highly expressed in styles from both populations and was downregulated slightly in LA1777

261 (HT-A, 1.3-fold). Interestingly HT-B transcript was expressed in both populations, although

262 neither population accumulates functional protein due to an early stop codon in the transcript

263 [31]. Each individual of LA1777 harbored two unique S-RNase alleles as expected (data not

264 shown), whereas LA0407 did not express S-RNase transcript, as has been documented in

265 previous studies [31].

266

267 ***Differential gene expression related to UI-competence in pollen***

13

268   As coordinated interactions between pollen and pistil are required for successful

269   fertilization, we also compared pollen transcriptomes between populations. Pollen tubes of

270   LA1777 reach ovaries of all SI and SC species within the tomato clade [1]. However, LA0407

271   pollen tubes are rejected by all SI species, including by SI *S. habrochaites* LA1777 [1]. The

272   inability of LA0407 pollen to traverse the styles of LA1777 and other SI *Solanum* species

273   suggests that it has lost a pollen factor(s) required for S-RNase resistance. For our analysis, we

274   therefore considered LA1777 pollen to be UI-competent, and LA0407 pollen to be UI-

275   compromised.

276   We identified 90 genes that were upregulated in UI-competent pollen (Additional File 1,

277   Table S7) and 99 that were downregulated (Additional File 1, Table S8). Ten of the top 25 genes

278   upregulated in UI-competent versus UI-compromised pollen were annotated as hypothetical

279   proteins (Table 2; Additional File 1, Table S7). Upon further analysis, the hypothetical gene

280   with the highest upregulation in UI-competent pollen (Sopen12g014190) likely encodes an

281   arabinogalactan protein (AGP) (Additional File 2, Fig. S2). In *Arabidopsis*, pollen AGPs are

282   required for proper pollen tube development and growth [68-70], and pistil AGPs are known to

283   stimulate pollen tube growth [69-72]. We also identified a Rab-GTPase (Rab4A,

284   Sopen01g033860) that was upregulated nearly 200-fold in UI-competent pollen. Pollen specific

285   proteins from this family have been found to promote pollen tube tip growth and play a role in

286   the ability of the pollen tube to sense directional cues [73, 74].

287   Our analysis of pollen also identified differentially expressed genes that may be involved

288   in protein degradation pathways (Table 2; Additional File 1, Table S7 and Table S8). These

289   genes are of interest, as components of a pollen SCF (SKP- Cullin-F-box) E3 ubiquitin ligase

290   complex have been implicated in both SI and UI, and are potentially involved in detoxifying

291   pistil side factors such as S-RNases through the proteasomal degradation pathway [11, 15, 21,

292   26, 27, 29, 75]. We found two F-box/Skp-2 –like genes (Sopen05g003280 and

293   Sopen12g022970) that were upregulated over 150-fold in UI-competent pollen (Table 2).

294   Another gene involved in protein degradation, an aspartyl protease (Sopen01g032940) was

295   highly upregulated 189-fold in UI-competent pollen (Table 2). No previously identified SLFs

296   [28] were significantly differentially regulated, nor was the pollen UI factor Cullin1 [26]

297   (Additional File 1, Table S9).

298   We identified two protein kinases that were upregulated over 50-fold in UI-competent

299   pollen, one of which encodes a calcium binding serine/threonine wall-associated kinase

300   (Sopen10g028190), and the other a cysteine-rich receptor like kinase (RLK) (Sopen02g013470)

301   (Table 2). Because RLKs have a proven role in pollen tube growth [76], these genes are of

302   potential interest. Further, pollen-expressed RLKs are involved in reception of peptide and

303   hormone signals from the pistil [77-79].

304   Genes involved in transcriptional regulation are likely to be important components of

305   pollen tube growth. We identified two types of transcription factors known to play key roles in

306   stress response [80, 81] that were highly upregulated in UI-competent pollen: a NAC-domain

307   containing protein (Sopen03g020850) and a bZIP family protein (Sopen12g006170) that has

308   previously been localized to pollen (Table 2; Additional File 1, Table S7).

309   An analysis of genes that were highly downregulated in UI-competent pollen identified

310   an F-box Protein (Sopen11g004020) and a Cysteine-rich RLK (Sopen05g014070) that were

311   downregulated over 45- and 100-fold respectively (Supplemental Table S8). Genes showing

312   over 100-fold decreases in UI-competent pollen also included two Histone Deacetylases

313   (HDACs, Sopen11g004040 and Sopen11g004050; Additional File 1, Table S8). The proper

314   function of HDACs has been linked to successful pollen tube germination and tip growth in

315   *Picea willsoni* [82].

316

UI competence in additional S. habrochaites populations

318   Our initial analyses comparing transcriptomes of LA1777 and LA0407 reproductive

319   tissues led to some intriguing candidates that might be integral to UI. In an attempt to further

320   narrow down candidate gene and to expand our analysis to a broader range of *S. habrochaites*

321   populations, we performed a second RNA-seq experiment that included UP styles and pollen

322   from *S. habrochaites* populations with selected phenotypes (Table 3; Additional File 1, Table

323   S10).

324   First we confirmed that the UI phenotypes of the individuals tested reflected previous

325   results [6] (phenotypes summarized in Table 3). A PCA of all samples shows that much of the

326   sample variation (1st PC) can be explained by tissue type, whereas a smaller percentage of

327   variation (2nd PC) is explained by source population and potentially mating system (Fig. 3).

328   Interestingly, the additional populations selected for transcriptome analysis, which lie between

329   LA1777 and LA0407 geographically, cluster between these two populations in the PCA.

330   For each gene, we trained a linear discriminant function for UI-competence on the

331   expression values for LA1777 and LA0407, and then classified gene expression in other

332   populations as UI-competent or UI-compromised. We took into account the UI patterns of these

333   populations with LA0407, as well as previously reported results on interspecific UI ([6],

334   summarized in Table 3). Because styles of LA2119 are unique in that they accept

335   interpopulation LA0407 pollen while rejecting interspecific pollen, we interpret the information

16

336   in Table 4 to reflect the variation in UI phenotype of LA2119 styles (i.e., genes that are up in

337   LA2119 are top candidates in interspecific UI, whereas genes that are down in LA2119 are top

338   candidates in interpopulation UI).

339   The top candidates for interspecific UI-competence in styles identified in the linear

340   discriminant analysis (LDA) included the hypothetical protein Sopen01g011020, that is highly

341   expressed and upregulated >100-fold (Table 4). Six genes potentially involved in oxidation-

342   reduction reactions were identified including the NAD(P)-linked Oxidoreductase

343   (Sopen12g021490), a Cytochrome P450 (Sopen07g030750), a Lipid Oxidoreductase

344   (Sopen06g026260), a Heavy Metal Transport Protein (Sopen05g028380), a Peroxisomal

345   Membrane Protein (Sopen03g005470), and the Photosystem I Reaction Center (a Ferredoxin

346   Oxidoreductase, Sopen12g006800).

347   Top candidates for interpopulation UI-competence in styles identified in the LDA

348   included two potentially involved in ROS signaling (Table 4). A DELLA-like transcription

349   factor (Sopen01g0311700) which is responsive to gibberellins [83] and participates in ROS

350   signaling by regulating ROS accumulation [84], was upregulated 11-fold. A Hemoglobin

351   Protein (Sopen08g021970) containing redox active transition metals was upregulated 3-fold.

352   Other interpopulation UI candidates included the cell wall synthesis protein Alpha-1,4-Glucan-

353   Protein Synthase (Sopen05g006860), an Alpha/beta Hydrolase family protein

354   (Sopen11g005210), a P-Glycoprotein ABC Transporter-like protein (Sopen08g025270) and an

355   F-box-LRR Protein (Sopen12g006260).

356   The LDA of pollen genes was more straightforward in that LA2119 and LA1264 were

357   expected to be UI-competent (i.e., similar to LA1777) whereas LA1223 is UI-compromised and

358   may be missing pollen factor(s) required to traverse SI styles. As shown in Table 5, using the

359   LDA we identified 22 genes that were upregulated in UI-competent pollen. Two of these encode

360   F-box proteins that are both putatively involved in the ubiquitin ligase complex

361   (Sopen03g040880 and Sopen01g027170) and were both upregulated over 25-fold. In addition, a

362   RAPTOR/KOG kinase homolog that is associated with the CUL4 RING ubiquitin ligase

363   complex (Sopen10g027930) was upregulated 12-fold in UI-competent pollen, and an annexin-

364   like calcium binding protein (Sopen05g030530) putatively involved in calcium signaling and

365   polysaccharide transport was upregulated over 4-fold. Two transcription factors were also

366   identified in the analysis: Sopen12g027920 encodes a suppressor of FRI1 that may act to recruit

367   histone H3 methyltransferases and Sopen11g020250 encodes a zinc finger transcription factor.

368   **Discussion**

369   Although UI is widespread in plant families, the underlying molecular basis of this

370   unidirectional reproductive barrier is not well understood. Here, using a transcriptomic

371   approach, we identified genes in both pollen and stylar tissues that represent strong candidates

372   for involvement in UI. Overall, our analyses identified a large number of differentially

373   expressed genes that are involved in oxidation-reduction reactions and ROS signaling. Oxidative

374   stress responses are involved in a variety of plant physiological processes, including

375   reproduction [57, 59, 85-87]. The production of ROS in plants generally results in one of two

376   outcomes: adaptation to stress or programmed cell death [85]. The balanced interplay between

377   pollen and pistil ROS production can display either of these results: signaling and detoxification

378   are required for successful fertilization (adaptation to stress) and can also function in the

379   incompatible (SI) response (cell death of incompatible pollen tubes). For example, in the

380   Papaveraceae family ROS are recognized as key regulators of programmed cell death in

381   incompatible (self) pollen tubes [63, 88] and recent studies of Rosaceae family member *Pyrus*

18

382 *pyrifolia* have demonstrated a link between ROS accumulation, Ca^{2+} signaling, calmodulin levels

383 and actin filament depolymerization during self-pollen tube rejection [62, 89]. One of the first

384 indications that ROS might be involved in pollen tube growth and cross-compatibilities in the

385 Solanaceae came from histochemical staining in styles *Petunia hybrid,* which demonstrated that

386 peroxidase activity is found in unpollinated styles and decreases following compatible

387 pollinations, but remains high during incompatible self-pollinations [90]. This pattern is also

388 observed in an analysis of cytochrome P450 (CYP51G1-Sc) in the wild potato *S. chacoense,*

389 where in compatible pollinations, mRNA levels of CYP51G1-Sc declines, but in incompatible

390 (self) pollinations, levels of this cytochrome remain stable.

391   In our pairwise comparisons investigating changes between UP and pollinated styles, we

392 found increases in ROS pathway members in incompatible interpopulation pollinations

393 (Additional File 1, Table S3) but not incompatible self-pollinations (Additional File 1, Table S1)

394 or compatible intrapopulation pollinations (Additional File 1, Table S2). For instance, an H_2O_2

395 transporter was upregulated over 16-fold only in incompatible interpopulation pollinations.

396 These transporters generally pump reactive H_2O_2 into the apoplast, an acidic environment with

397 low numbers of ROS scavengers, resulting in oxidative stress [85]. A K^+ channel and a Ca^{2+}-

398 binding annexin protein, both of which were annotated as being pollen-expressed were also

399 upregulated in interpopulation interactions, as was a Rab-GTPase. All three of these proteins can

400 play important roles in ROS generation and signaling. In pollen tubes, annexins may provide an

401 important link between Ca^{2+}, the membrane and the cytoskeleton [91]. Further, many annexins

402 form Ca^{2+} channels which are vital not only for the oscillating Ca^{2+} influx associated with pollen

403 tube tip growth, but also for facilitating ROS signaling, cell elongation and cell wall remodeling

404 [85, 92]. Interestingly, SKOR K^+ channels like the one identified in our analysis can also act as

19

405 ROS-activated Ca^{2+} channels [92]. Small GTPases, including Rabs, increase NADP(H)-oxidase

406 activity in a Ca^{2+}-dependent manner [58, 93], and their proper function is required for pollen

407 tube growth [58, 60, 73, 74, 94]. For example, the overexpression of both active and mutant

408 forms of the RAB11 protein leads to the inhibition of pollen tube growth in *Nicotiana* [74]

409 suggesting that the correct balance of multiple Rabs is required for effective pollen tube growth.

410 In UI-competent styles, ten of the top 25 most highly upregulated genes are putatively

411 involved in ROS generation and/or signaling, including an NADP(H)-oxidase and multiple

412 cytochrome P450s (Table 1). We also identified two highly upregulated gene candidates in the

413 flavonoid pathway, which could produce flavonoid compounds to act as pro- or anti-oxidants

414 (Table 1). In our subsequent analysis using transcriptome data from additional *S. habrochaites*

415 populations, we found that six of the nine candidate stylar genes that may be involved in

416 interspecific UI were ROS pathway genes (Table 4). Surprisingly, only two ROS-linked genes

417 were upregulated in interpopulation UI-competent styles, one of which encodes a DELLA-like

418 transcription factor that inhibits ROS accumulation and restrains cell expansion [83, 84]. In sum,

419 these results suggest a dynamic interplay between pollen and pistil that must be held in a tight

420 balance for pollen tubes to successfully grow through styles to reach the ovary.

421 The generation of ROS is linked to cell expansion, growth, cell wall cross linking and

422 callose deposition [95]. Pollen tube walls consist of a number of polymers (callose, cellulose

423 and pectin) that are highly cross-linked to each other; however the mechanisms by which cell

424 wall modification is regulated remains unclear [96-98]. Studies using microarray analysis have

425 found an upregulation of cell-wall modification genes in pollinated versus unpollinated styles

426 [51]. However, few have specifically investigated specificity to the SI or UI response (but see

427 Pease et al. [54]). Using electron microscopy, de Nettancourt et al [99] found differences in

20

428 callose deposition between SI and UI crosses in *S. peruvianum* wherein SI crosses showed large

429 levels of callose deposition at the pollen tube tips, but interspecific crosses did not [99]. Our

430 pairwise comparisons in UP versus pollinated LA1777 styles did not reveal any genes involved

431 in cell wall modification. However, we did identify a number of differentially expressed genes

432 involved in cell wall modification in our larger analysis of stylar tissue (Additional File l, Table

433 S4 and Table S5), most of which were highly downregulated in UI-competent styles.

434   One of the most intriguing gene candidates from our analysis of stylar tissue included a

435 putative RALF peptide hormone (Sopen02g033850) that was upregulated over 22-fold in UI-

436 competent styles. Peptide hormone signaling is involved in numerous processes during pollen-

437 pistil interactions, from pollen hydration to fertilization [77]. A tomato RALF has been shown to

438 reduce pollen tube growth during specific windows of development [66], and therefore a stylar-

439 secreted RALF could play a role in the rejection of interspecific pollen tubes. Another

440 interesting style-expressed candidate to pursue is the Kunitz-like Protease Inhibitor that was

441 upregulated in UI-competent styles. The *Nicotiana* NaStep protein from this family is required

442 for the rejection of some (but not all) interspecific pollen [67], and this protease inhibitor may

443 play a similar role in *S. habrochaites* UI. Finally, the most highly-upregulated stylar gene

444 identified in UI-competent styles encodes a putative prenylated heavy-metal binding protein

445 (Additional File 2, Fig. S2), that may be worthy of further investigation. Other proteins of this

446 type have been identified in a variety of tissues, and the few that have been characterized have

447 been implicated in stress response [100].

448   In UI-competent pollen, we identified a highly upregulated putative AGP

449 (Sopen12g014190) (Table 2) that contains a signal peptide, a hydrophobic C-terminal domain,

450 eight dipeptides that are found in known AGPs and is composed of > 35% Pro/Ala/Ser/Thr

21

451 (PAST) amino acids: a defining characteristic of AGPs (Additional File 2, Fig. S2; [70, 101]). In

452 *Arabidopsis,* pollen AGPs are required for pollen tube growth, and may be involved in complex

453 signaling cascades [68-70], and pollen AGPs have been localized to pollen tube tips in some

454 species [102]. Another gene that warrants further study is a Rab-GTPase (Sopen01g033860) that

455 was upregulated nearly 200-fold in UI-competent pollen. It will be interesting to see if

456 increasing the expression of these genes in UI-compromised pollen is able to increase rates of

457 pollen tube growth through SI styles.

458 ### *Conclusions*

459   Our analyses revealed differentially expressed genes that may contribute to reproductive

460 incompatibility between populations of *S. habrochaites*. This work represents an important first

461 step in understanding how unilateral barriers might arise between populations, and how they are

462 maintained during speciation. The variability in UI responses between *S. habrochaites*

463 populations provides an exciting opportunity in which to further analyze these candidate genes

464 and link them to specific UI phenotypes.

465

466 ### **Materials and Methods**

467 **Solanum habrochaites** *plant material and growth*

468   *Solanum habrochaites* (S. Knapp & D. M. Spooner) is a wild relative of tomato that

469 demonstrates variability in mating system [41, 44], as well as interspecific [31, 35] and

470 interpopulation [1, 6-8] cross-compatibilities. Seeds from the *S. habrochaites* accessions

471 (referred to hereafter as populations) used in this study (Table 3) were acquired from the C.M.

472 Rick Tomato Genetic Resource Center (TGRC) at University of California, Davis

473   (www.tgrc.ucdavis.edu, [103]), sterilized according to recommendations from the TGRC, grown

474   under greenhouse conditions as previously described [1] for approximately 3 weeks, and

475   transplanted in covered outdoor agricultural field plots at Colorado State University. Plants used

476   in the study were randomized within a single block, and remained large and healthy throughout

477   the experiment. Specific populations were chosen based on reproductive characteristics as

478   described in Broz et al., 2016 [6], see Table 3 for more information.

479

480   ***Pollen tube growth phenotypes***

481   Pollen tube growth through the style was assessed for self, intrapopulation and reciprocal

482   interpopulation crosses of *S. habrochaites* individuals, although for LA0407, self and

483   intrapopulation crosses consistently resulted in fruit-set, so pollen tube growth was not assessed

484   for every individual. For all crosses, buds were emasculated 1 day prior to anthesis (day -1),

485   hand-pollinated 24 hours later (day 0, budbreak), and harvested into fixative (1:3 acetoethanol)

486   48 h after pollination. For crosses between LA1777 (female) and LA0407 (male), styles were

487   harvested at various time points after pollination (12, 24 and 48 h) to determine the time at which

488   pollen tube rejection occurred. Pollinations were typically performed in the late afternoon and

489   collected the following morning. However, pollen tube growth through styles was similar for

490   pollinations performed in both the morning and the afternoon (data not shown).

491   Pollinations were covered with mesh bags to prevent unintended pollen deposition by

492   pollinators. Pollen tube growth was assessed using fluorescence microscopy as previously

493   described [31], and the length of styles and the point in the style at which no more than three

494   pollen tubes passed were measured using ImageJ 1.47v (http://rsb.info.nih.gov/ij/; [104]).

495

Tissue collection, RNA extraction and library construction

496

497   The primary RNA-seq experiment was performed to identify genes involved in

498 interpopulation pollen tube rejection observed in crosses between LA1777 females and LA0407

499 males. Samples consisted of unpollinated styles, pollinated styles (self-pollination,

500 intrapopulation pollination, or interpopulation pollination), and pollen (Fig. 1; Additional File 1,

501 Table S10) – resulting in five treatment/tissue types for each individual. Three individuals from

502 each population were used as biological replicates, resulting in a total of 30 libraries. In a

503 follow-up experiment designed to narrow the list of genes involved in interpopulation

504 interactions, RNA samples from between one and three individuals were pooled at an equimolar

505 ratio before library creation (Additional File 1, Table S10).

506   For all style samples, flowers were emasculated and pollinated as described above for

507 pollen tube growth experiments and harvested 16 hrs post-pollination (the approximate time at

508 which pollen tube rejection occurred in interpopulation crosses). Unpollinated controls

509 underwent the same treatment (emasculation at day -1), except they were left unpollinated. For

510 each individual plant, approximately 30 styles were collected for each treatment and pooled

511 before RNA extraction. To minimize variation due to environmental conditions, all treatments

512 were conducted on the same days and harvested at approximately the same time of day. Styles

513 (including stigmas) were harvested directly into RNALater (Qiagen) and stored at 4º C for one

514 week, after which styles were blotted dry and immediately frozen at -80º C until processing.

515 Approximately 100 mg of pollen was harvested from each individual plant and immediately

516 frozen at -80º C.

517   Tissues were ground using the Tissue-lyser (Qiagen), RNA was extracted using the

518 Qiagen RNeasy Plant mini-kit and brought to a final concentration of 70-200 ng/uL. A subset of

24

519 RNA samples was checked for quality by agarose gel electrophoresis and visualization by

520 ethidium bromide staining. Sample quality was further evaluated using the Agilent 2200 RNA

521 TapeStation system before library creation. Stranded, paired-end libraries of total RNA were

522 generated for each sample using Illumina Truseq Stranded mRNA sample preparation kits.

523 Libraries were pooled and distributed evenly across two lanes of Illumina HiSeq™ 2000

524 (Illumina Inc., San Diego, CA, USA). RNA quality control, library preparation, and pooling

525 were performed by the Indiana University Center for Genomics and Bioinformatics. The raw

526 transcriptome data is available on the NCBI SRA database (BioProject SRP069274).

527

528 ***RNA-seq read processing and mapping***

529 Prior to mapping and assembly, reads were trimmed and filtered using the SHEAR

530 program (http://www.github.com/jbpease/shear; [54]). Briefly, SHEAR first uses the Scythe

531 algorithm (https://github.com/vsbuffalo/scythe; [105]) to remove adapters from the 3′ end, and

532 then filters low quality reads (mean Q<10), reads with >7 ambiguous bases (N's), reads < 50 bp,

533 and repetitive reads with mutual information score > 0.5. The program then trims reads on both

534 ends by removing low quality bases (Q<20), poly-A or poly-T runs of n ≥ 12, and ambiguous

535 bases. The appearance of AGATC at the 3′ end was also removed as we presumed it was an

536 adapter fragment. We removed both reads in a pair if either one of them failed the filters. On

537 average, 2.9% of all reads failed to pass the filter (min 2.5%, max 3.4%). The full command and

538 parameters used for SHEAR can be found in Additional File 2, Method S1.

539 We mapped RNA-seq reads to the *Solanum pennellii* reference genome using the STAR

540 spliced aligner with default parameters [106]. The reference genome sequence and the genome

541 annotation (Spenn v2.0) were downloaded from solgenomics.net [107]. On average across

25

542   libraries, 81% of reads mapped uniquely to the reference genome. We counted reads mapped to

543   the reference gene annotation and to unannotated putative S-locus F-box (SLF) genes [28] (a

544   total of 48938 genic regions) using featureCounts v1.4.5-p1 [108]. A total of 663185500 read

545   pairs were counted (72% of raw reads). For genes annotated as 'hypothetical' in Spenn v2.0

546   [107], further sequence alignments were carried out using NCBI BLAST searches

547   (https://blast.ncbi.nlm.nih.gov; [109]).

548

549   ***Differential gene expression analysis***

550   For all tests of differential expression we used linear models implemented by the *limma*

551   package [110, 111] and modules from the edgeR package [112] in R [113]. First, we normalized

552   and transformed the raw reads with *voom,* a weighted transformation based on the expected

553   relationship between expression mean and variance [111]. We computed *t*-statistics on the

554   transformed expression values for each gene using an empirical Bayes adjustment of standard

555   errors with the *eBayes* function [110].

556   We initially searched for differential expression among stylar tissues by carrying out

557   separate pairwise comparisons. Specifically, we compared the following pairs of style treatments

558   in LA1777: intrapopulation-pollinated (compatible) against unpollinated (UP) styles, self-

559   pollinated (incompatible) against UP styles and interpopulation (incompatible) against UP styles.

560   We visualized the genome-wide patterns of expression through a PCA of the normalized

561   mean read counts per gene (cpm reads in the library) with the *prcomp* function (implemented in

562   R; [113]). The PCA showed that there were negligible differences at the genome-wide scale

563   among all style treatments within a population (Fig. 3; Additional File 2, Fig. S1). Because of

564   these high consistencies in their gene expression profiles, all style treatments (UP, self-, intra-

565 and interpopulation pollinated) within a population were considered identical in our linear

566 models that focus on the differences between UI-competent (LA1777) and UI-compromised

567 (LA0407) styles.

568   We identified genes that are differentially expressed in UI-competent tissues using a

569 linear model with a single fixed effect (collinear with the population of origin) for pollen (n=6)

570 and styles (n = 24) separately. From these models, we took genes as differentially expressed if

571 they showed large differences in expression (> 3-fold change), with statistical significance at a

572 false discovery rate (FDR) of 5%. Further, we considered only tissue-specific genes: we required

573 genes to have a significant (FDR < 5%) tissue effect in a general linear model $Y \sim P + T + e$

574 (where P is a population effect, T is a tissue effect, and e is the error term). For stylar-side

575 factors, we included genes as differentially expressed if they were upregulated in styles with

576 respect to pollen, and vice versa for pollen-side factors. This last filter ensured that differences in

577 styles were unlikely to be contributed by pollen in the styles of pollinated samples.

578   A number of *a priori* pollen and pistil UI candidate genes were selected based on

579 information from previous publications [23, 26, 28, 31, 54]. Some of the SLF genes have not yet

580 been annotated in the *S. pennellii* genome, so we used locus numbers and sequences from Li and

581 Chetelat (2015) [28] to find these genes in our dataset. We identified expression levels of all *a*

582 *priori* candidates, and carried out statistical tests similar to the ones described above to determine

583 whether they were differentially expressed.

584

591

595
596 *Authors' contributions*

AB performed RNA extraction, analyzed and interpreted data and wrote the manuscript with editing contributions from all authors. RG performed all bioinfomatics and statistical analyses of the data. AR designed and performed crossing experiments and collected tissue. YB performed crossing experiments and collected tissue. MH designed experiments, oversaw bioinformatics experiments and assisted in data analysis. PB conceived and designed experiments, collected tissue and interpreted data. All authors read and approved the manuscript.

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Table 1. Top-25 upregulated genes in styles of LA1777 vs. LA0407.

Gene	p value	Mean expression	Fold change	Annotation
Sopen12g017530.1	1.9E-20	3.8	1451.4	[HP] Hypothetical protein; Contains DUF pfam0403, heavy metal-associated
Sopen02g037430.1	4.1E-17	4.2	163.8	Cytochrome P450; family 81, subfamily D
Sopen05g032070.1	3.8E-14	9.2	154.9	Chalcone Synthase 2; flavonoid biosynthesis
Sopen06g021780.1	5.1E-13	3.9	116.2	Cytochrome P450; family 71, subfamily B
Sopen01g011020.1	4.0E-21	75.9	108.4	Hypothetical protein
Sopen12g006330.1	8.4E-15	22.2	58.6	2-Alkenal Reductase; involved in response to oxidative stress
Sopen08g021280.1	3.4E-11	8.4	51.8	Chlorophyll a-b Binding Protein 3C
Sopen12g026980.1	5.7E-14	4.0	36.6	Glutathione S-Transferase; involved in stress response
Sopen05g003640.1	2.8E-19	4.3	35.6	NAD(P)H Dehydrogenase Subunit; in chloroplast, PsbQ-like 2
Sopen09g003470.1	6.1E-06	10.3	32.1	Threonine Dehydratase; isoleucine biosynthesis, plastid
Sopen04g002110.1	4.9E-12	5.2	29.6	Programmed Cell Death 6-Interacting Protein; endosomal
Sopen05g001950.1	2.6E-17	4.3	28.7	Major facilitator superfamily; oligopeptide transport protein
Sopen07g028940.1	4.8E-11	4.5	26.7	Cytochrome P450; family 72, subfamily A
Sopen02g033850.1	1.7E-10	10.6	22.0	[HP] Hypothetical protein; 80% identity to *Solanum chacoense* RALF-like5
Sopen02g027710.1	6.0E-09	182.2	20.2	Acidic Endochitinase; involved in defense response
Sopen02g022730.1	2.5E-11	5.4	19.6	Dormancy-Associated Auxin-Repressed small protein
Sopen02g021850.1	2.2E-11	3.4	18.9	Transcription factor; bHLH30-like DNA binding superfamily
Sopen12g021490.1	7.0E-18	10.2	17.4	NAD(P)-linked Oxidoreductase superfamily protein; involved in stress response
Sopen10g025030.1	3.1E-09	4.9	14.9	Dirigent-Protein Like 22; involved in defense response
Sopen11g004500.1	2.8E-08	3.8	14.4	Beta Glucosidase 42 (BGLU42)
Sopen12g006340.1	6.6E-11	5.2	14.4	Transferase, HXXXD-type acyl-transferase family protein
Sopen01g047950.1	1.6E-04	17.7	14.0	ABC Transporter G family member 11-like; cutin or wax export, stress response
Sopen10g001450.1	8.8E-18	3.2	13.4	Hypothetical protein, chloroplast
Sopen03g029620.1	1.0E-04	6.8	12.8	Kunitz family trypsin and protease inhibitor (similar to Miraculin)
Sopen05g030780.1	3.7E-12	18.8	12.8	Chalcone-Flavanone Isomerase family protein

Gene annotation from Spenn_v2.0 was enhanced, when possible, for genes noted as hypothetical proteins (marked as [HP]).

Table 2. Top-25 upregulated genes in pollen of LA1777 vs. LA0407.

Gene	p value	Mean expression	Fold change	Annotation
Sopen12g014190.1	9.3E-05	26.2	2922.0	[HP] Putative GPI-anchored AGP
Sopen01g010170.1	4.1E-04	3.7	1138.1	Hypothetical protein
Sopen12g018710.1	1.1E-05	2.7	598.0	KH Domain RNA-Binding Protein
Sopen06g022970.1	7.5E-05	2.7	591.3	Isoflavone-7-O-Methyltransferase 9; flavonol biosynthesis
Sopen05g003280.1	3.0E-04	5.2	397.0	F-box Protein CPR1/30; negative regulator of defense response
Sopen09g012310.1	7.7E-04	1.9	318.1	Ribonuclease H-like superfamily protein; possible non-LTR retrotransposon family (LINE)
Sopen10g022570.1	2.7E-05	1.9	287.3	Hypothetical protein
Sopen08g010210.1	7.1E-04	1.8	277.0	[HP] Possible retroelement
Sopen01g033860.1	2.2E-04	1.5	198.4	RabA4 subfamily of Rab GTPases; promotes tip growth of pollen tubes
Sopen01g032940.1	3.6E-04	1.5	189.8	Aspartyl Protease family protein, CDR1-like
Sopen12g018720.1	2.0E-04	1.4	168.0	Hypothetical protein
Sopen12g022970.1	6.9E-04	1.3	154.6	F-box/RNI superfamily; plant-specific FBD domain
Sopen08g010180.1	6.2E-04	1.2	130.0	Hypothetical protein
Sopen03g005740.1	1.5E-04	1.1	105.8	Acyl Activating Enzyme, Benzoate-CoA Ligase
Sopen03g020850.1	5.5E-04	1.1	98.6	Possible transcription factor; VND-interacting 1, NAC domain
Sopen12g027860.1	5.6E-04	1.0	86.2	[HP] possible non-coding RNA
Sopen05g018880.1	1.6E-04	1.5	84.7	Hypothetical protein
Sopen10g022630.1	7.8E-04	0.9	74.6	[HP] Non-coding RNA
Sopen10g023190.1	2.1E-04	0.9	71.5	[HP] Possible LTR retrotransposon
Sopen06g021510.1	5.4E-05	0.9	71.4	Zeatin O-Glucosyltransferase-Like
Sopen12g006170.1	4.6E-06	9.5	70.0	Basic Leucine Zipper 34-Like, transcription factor
Sopen03g007850.1	1.7E-04	0.8	57.8	Tetratricopeptide Repeat (TPR)-Like superfamily protein; pre-mRNA splicing factor SYF1
Sopen10g028190.1	3.2E-04	1.1	52.6	Wall-Associated Receptor Kinase (WAK2-like), Ca^{2+} binding, cell wall expansion
Sopen02g013470.1	6.6E-04	1.1	50.4	Cysteine-Rich RLK
Sopen10g022640.1	5.4E-04	0.8	48.8	[HP] Non-LTR retrotransposon family (LINE)

Gene annotation from Spenn_v2.0 was enhanced, when possible, for genes noted as hypothetical proteins (marked as [HP]).

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Table 3. *Solanum habrochaites* populations used in this study.

Country	Province or Department	Population/ Accession[a]	Mating system[b]	Interpopulation UI[c]		Interspecific UI[c]	
				Pollen accepted by LA1777	Accepts pollen of LA0407	Accepts pollen of *S. neorickii*	Accepts pollen of *S. lycopersicum*
Ecuador	Guayas	**LA0407**	SC	I	SC	C	I
Ecuador	Chimborazo	**LA1223**	SC	I*	C	C	C
Ecuador	Chimborazo	**LA1264**	SC	C	C	I	I
Ecuador	Loja	**LA2119**	SC	C	C	I	I
Ecuador	Loja	**LA2098**	SI/SC	C	I	I	I
Peru	Ancash	**LA1777**	SI	SI	I	I	I

[a] all populations and passport information was acquired from the Tomato Genetics Resource Center (TGRC, tgrc.ucdavis.edu, [103]) at the University of California, Davis.

[b] mating system was verified experimentally, and is consistent with data from the TGRC, Rick *et al.* 1979 and Broz *et al.* 2016 [6, 44].

[c] interpopulation and interspecific UI as reported in Broz et al., 2016 [6].

*pollen of the LA1223 individual used in this experiment was not accepted by LA1777, Broz et al., 2016 [6] reports variation in individual LA1223 phenotypes for this cross

SC, self-compatible; SI, self-incompatible; C, compatible; I, incompatible

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**Table 4.** Upregulated genes in styles of LA1777 vs. LA0407 that show concordant expression patterns.

Interspecific UI (upregulated in LA2119)

Gene	p value	Mean expression	Fold change	Annotation
Sopen01g011020.1	4.0E-21	75.9	108.4	Hypothetical protein
Sopen12g021490.1	7.0E-18	10.2	17.4	NAD(P)-linked Oxidoreductase
Sopen07g030750.1	1.0E-13	4.7	6.1	Cytochrome P450 (CYP72A15)
Sopen06g026260.1	5.5E-17	39.4	4.7	Oxidoreductase, involved in lipid metabolic process
Sopen05g028380.1	2.0E-08	7.9	3.6	Heavy metal transport/detoxification superfamily protein
Sopen10g033870.1	1.4E-10	12.7	3.3	RING/U-box superfamily protein
Sopen12g006800.1	3.2E-13	73.7	3.1	Photosystem II, Light-harvesting Chlorophyll B-binding protein
Sopen06g019380.1	1.6E-14	108.4	3.1	Photosystem I Reaction Center Subunit II
Sopen03g005470.1	1.2E-13	6.9	3.0	Peroxisomal Membrane 22 kDa (Mpv17/PMP22) family protein

Interpopulation UI (downregulated in LA2119)

Gene	p value	Mean expression	Fold change	Annotation
Sopen10g001450.1	8.8E-18	3.2	13.4	Unknown chloroplast stroma protein
Sopen01g031170.1	5.3E-06	9.9	11.0	Transcription factor GRAS, DELLA-like protein
Sopen05g006860.1	7.4E-14	74.6	6.5	Alpha-1,4-Glucan-Protein Synthase, involved in cell wall biogenesis
Sopen11g005210.1	6.8E-19	33.1	5.3	Alpha/beta-Hydrolases superfamily protein, Serine Hydrolase
Sopen11g028080.1	7.3E-16	5.2	4.2	DHBP, RibB-like; 3,4-Dihydroxyl-2-Butanone 4-Phosphate Synthase
Sopen08g025270.1	7.9E-12	54.7	4.1	ABC-transporter-like, P-Glycoprotein 2 (PGP2)
Sopen03g006340.1	5.2E-13	19.1	3.6	Unknown function (DUF827)
Sopen07g027150.1	3.4E-10	131.8	3.5	Phosphoenolpyruvate Carboxylase (PEPC)
Sopen12g001030.1	1.1E-08	9.2	3.4	RING/U-box superfamily protein
Sopen04g034310.1	1.4E-09	2.8	3.4	HXXXD-type Acyl-transferase family protein
Sopen11g001350.1	2.6E-11	11.0	3.1	Plastid-lipid Associated Protein PAP / fibrillin family protein
Sopen08g021970.1	2.8E-13	17.8	3.1	Hemoglobin Protein 3 (GLB3)
Sopen12g006260.1	5.1E-13	3.9	3.1	F-box/LRR Protein, MAX2

A linear discriminant function was trained on the expression values of LA1777 and LA0407, and then used to classify other accessions as UI or non-UI.
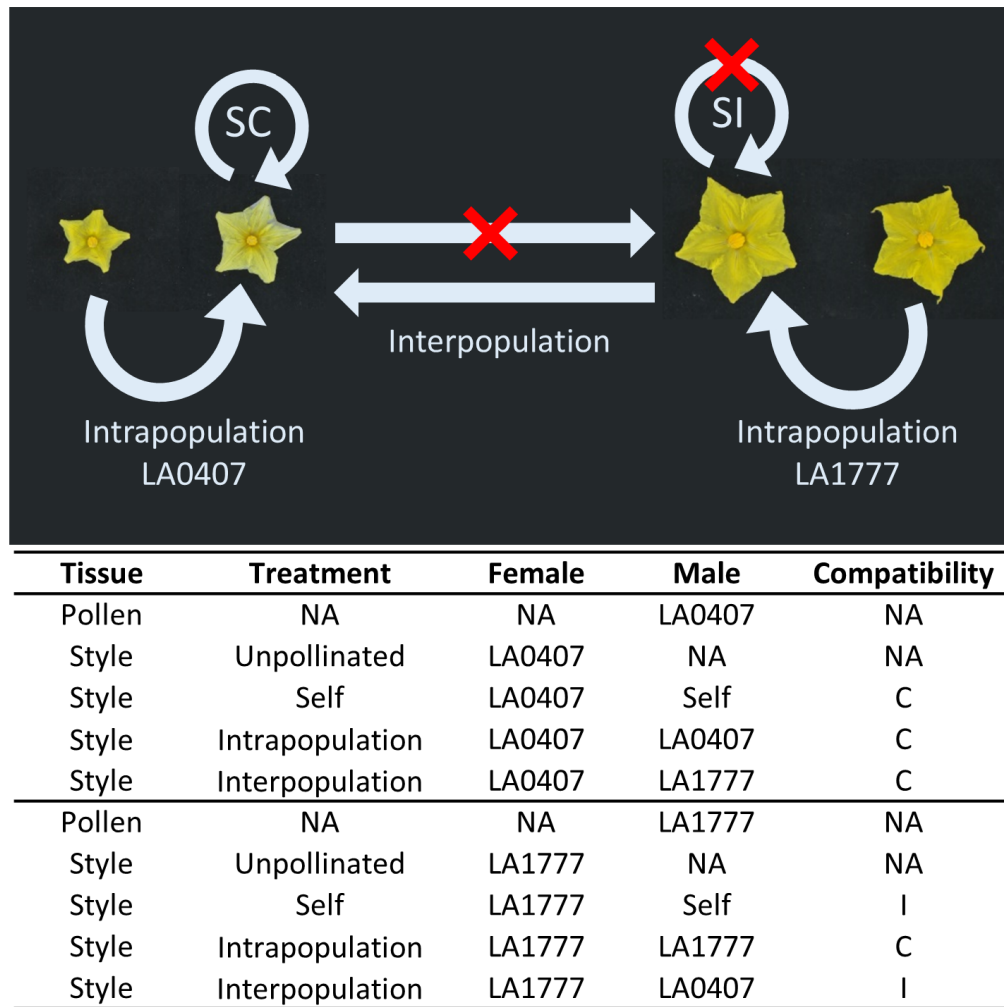
600

Table 5. Upregulated genes in pollen of LA1777 vs. LA0407 that show concordant expression patterns.

| Gene	p value	Mean expression	Fold change	Annotation
Sopen05g018880.1	1.58E-04	1.5	84.7	Hypothetical protein
Sopen03g040880.1	8.68E-06	0.7	36.0	RNI-like, F-box, ubiquitin-protein ligase activity
Sopen01g027170.1	7.75E-05	0.6	28.5	RNI-like, F-box, Skp2-like
Sopen02g038110.1	7.81E-06	0.6	24.9	Cytochrome P450 724B1
Sopen02g036410.1	1.18E-05	64.8	16.7	Putative Hexokinase
Sopen12g026740.1	3.26E-04	114.7	11.9	Hypothetical protein
Sopen10g027930.1	4.98E-04	5.0	11.9	RAPTOR/KOG homolog located in CUL4 RING ubiquitin ligase complex
Sopen01g008220.1	1.05E-04	1.9	9.3	Hypothetical protein
Sopen05g029540.1	2.80E-04	0.4	8.8	RNA-directed DNA polymerase
Sopen01g028020.1	5.26E-05	3.4	7.3	Hypothetical protein
Sopen12g027920.1	4.39E-05	0.3	7.0	Zinc Finger Transcription Factor SUF4, involved in histone methylation
Sopen12g004180.1	3.29E-04	112.9	4.6	Oligouridylate Binding Protein 1B
Sopen01g005900.1	1.36E-04	111.6	4.5	Hypothetical protein
Sopen05g030530.1	3.09E-04	20.8	4.5	Annexin, Ca^{2+}-binding protein
Sopen03g024730.1	4.76E-05	5.5	3.9	DNA Helicase
Sopen03g027800.1	2.34E-04	10.9	3.8	Exostosin
Sopen02g038520.1	6.46E-04	247.3	3.7	Lysine-Histidine Transporter (LHT1)
Sopen11g020250.1	4.01E-04	5397.5	3.7	GATA Type Zinc Finger Transcription Factor
Sopen06g022340.1	4.85E-04	6.5	3.5	Disease resistance/zinc finger/chromosome condensation-like region domain containing protein
Sopen07g015260.1	3.61E-04	108.5	3.2	Vacuolar Proton ATPase Subunit VHA-a isoform 2
Sopen11g021110.1	1.90E-04	129.8	3.1	Protein of unknown function, DUF593
Sopen06g009470.1	2.13E-04	0.8	3.1	Hypothetical protein

A linear discriminant function was trained on the expression values of LA1777 and LA0407, and then used to classify other accessions as UI or non-UI.

601
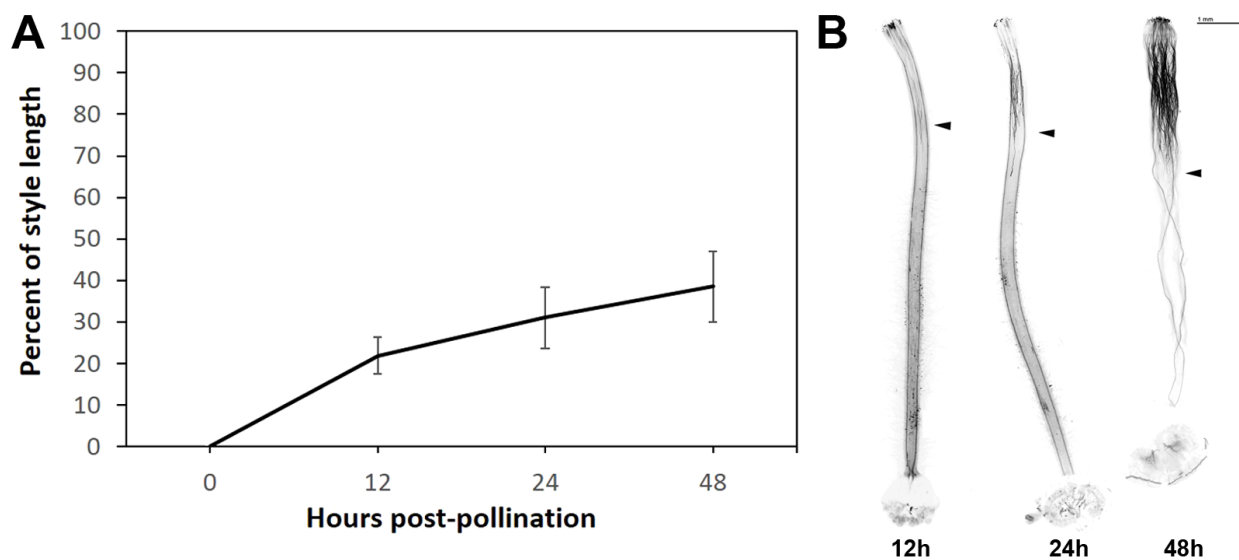
Figure 1. Interpopulation pollen tube growth over time. Pistils of *Solanum habrochaites* LA1777 were pollinated with LA0407 pollen and harvested at 12, 24 and 48 h after pollination. A, Time course of LA0407 pollen tube growth in styles of LA1777 with standard deviation for each time point. B, Representative image of LA0407 pollen tube growth in LA1777 pistils at each time point; arrowhead points to the location where the majority of pollen tubes are rejected.

Tissue	Treatment	Female	Male	Compatibility
Pollen	NA	NA	LA0407	NA
Style	Unpollinated	LA0407	NA	NA
Style	Self	LA0407	Self	C
Style	Intrapopulation	LA0407	LA0407	C
Style	Interpopulation	LA0407	LA1777	C
Pollen	NA	NA	LA1777	NA
Style	Unpollinated	LA1777	NA	NA
Style	Self	LA1777	Self	I
Style	Intrapopulation	LA1777	LA1777	C
Style	Interpopulation	LA1777	LA0407	I

Figure 2. Experimental design for *Solanum habrochaites* RNA-seq experiment. Top panel: compatibilities within and between *S. habrochaites* populations LA0407 and LA1777. SC, self-compatible; SI self-incompatible. Bottom panel: Tissues (pollen, style) and stylar treatment types (intrapopulation, interpopulation, self- pollinations and unpollinated) were collected for three biological replicates of LA0407 and LA1777. C, compatible pollination; I, incompatible pollination; NA, not applicable.

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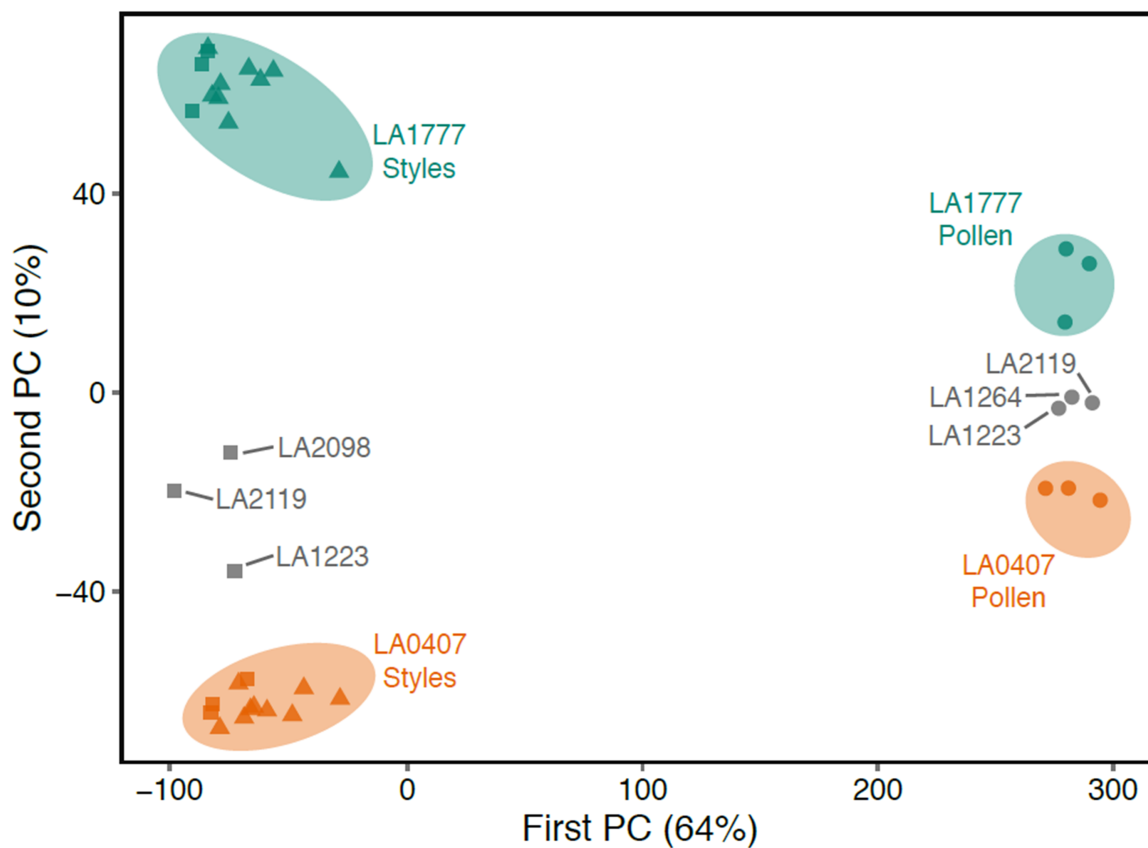
Figure 3. Genome-wide variation in gene expression across five populations of *Solanum habrochaites*, summarized by their first two principal components. The largest source of variation results from differences in expression between pollen (circles) and styles (unpollinated, squares; pollinated, triangles), on the first PC (horizontal axis). Variation across populations, on the other hand, accumulates on the second PC (vertical axis).
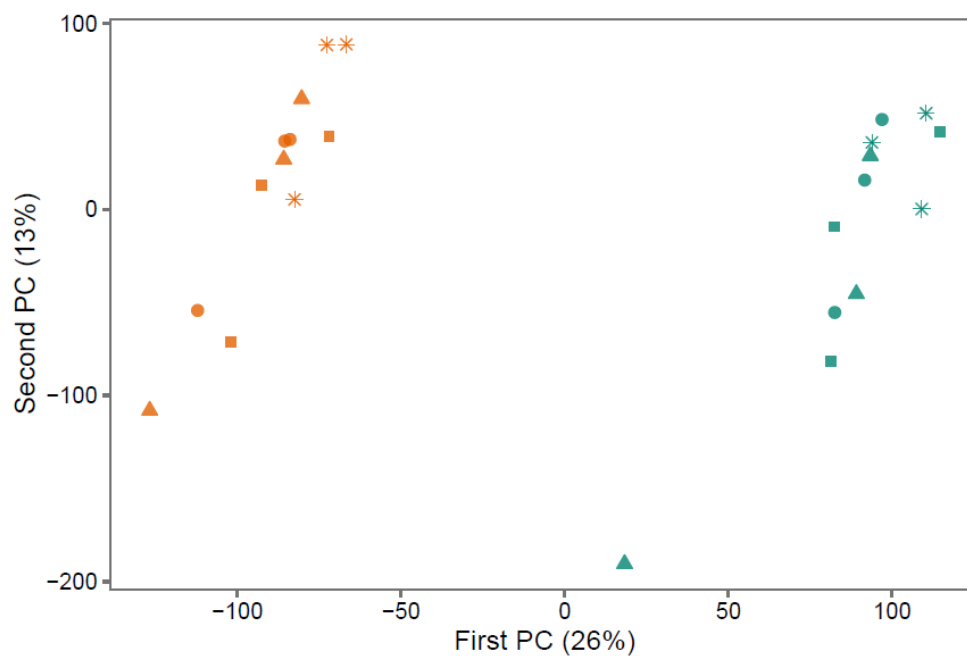
Figure S1. The genome-wide patterns of expression in styles from two populations of *Solanum habrochaites* (LA1777 in orange and LA0407 in green), summarized by a principal components analysis. Most variance among samples is due to differences between populations (horizontal axis), and we found no large differences among experimental treatments at this scale (unpollinated styles, stars; self-pollinated, circles; intrapopulation-pollinated, triangles; interpopulation pollinated, squares).

A

```
Penn    MALVSWAKKELSKLKLQNKPKRLTLPQTSTKCLALPLIQEVILDADLRCTHCQNRVSSVI
Lyco    ---VSWAKKELSKLKLQNKPKRLTLPQTSTKCLALPLIQEVILDADLRCTHCQNRVSSVI
        *****************************************************
```

```
Penn    SNVEDVESIVVHVLEKKVTLIRKSTSK
Lyco    SNIEDVESIVVHVLEKKVTLIRKSTSK
        **:***********************
```

B

```
Penn    MAGRVMLGVCVIFFVVASVASITPAPSPNVAESPVDNNVIGTLDGGVGGAAPVGGPVPEG
Lyco    MAGHVMLGVCVIFFVVASVASITPAPSPNVAESPVDNNVIGTLDGGVGGAAPVGGPVPEG
        ***:********************************************************
```

```
Penn    VFSNISPESESSAATINAHLSTIAIISSIVATSFLLS
Lyco    VFSNISPESQSSAATINAHLSTIAIISSIVATSFLLS
        *********:***************************
```

Figure S2. Sequence alignments of hypothetical proteins showing the highest fold-change in UI-competent vs. UI-compromised tissues. **A,** The deduced amino acid sequence of *Solanum pennellii* Sopen12g017530 (Penn), the gene showing the highest fold change in UI-competent versus UI-compromised styles, is aligned to a *Solanum lycopersicum* (Lyco) EST (GenBank # AW092656.1) identified in an elicitor screen of tomato leaf. A BLAST search of *S. lycopersicum* gene models (SOLv2.4, solgenomics.net) did not return any results. Putative heavy metal binding domain (P-Fam 00403) is shown in blue; putative prenylation site (CaaX, where 'a' represents an aliphatic amino acid) is shown in bold "CLAL". Cysteine residues involved in both domains are underlined. Stars represent residues conserved between both sequences. **B,** The deduced amino acid sequence of *Solanum pennellii* Sopen12g014190 (Penn), the gene showing the highest fold change in UI-competent versus UI-compromised pollen, is aligned with *Solanum lycopersicum* Solyc12g033100 (Lyco). Putative signal peptide is shown in bold; distinguishing dipeptide motifs of arabinogalactan proteins (AGPs) are underlined. Hydrophobic residues are highlighted in yellow. Stars represent residues conserved between both sequences.

Method S1. Full command and parameters used for SHEAR in bioinformatics analysis of *S. habrochaites* transcriptomes.

```bash
#!/bin/bash

# These script snippets were used for the differential gene expression
analysis presented in
# Broz et al. "Transcriptomic characterization of a pollen-pistil unilateral
reproductive barrier"

# These will not run adequately without helper files (as indicated below)
# Software needed:
# scythe (github.com/vsbuffalo/scythe)
# shear (github.com/jbpease/shear)
# subread (subread.sourceforge.net/)
# STAR (github.com/alexdobin/STAR/)
# samtools (www.htslib.org/)

# Contact: Rafael F Guerrero, rafguerr@indiana.edu


# (1) Shear reads
#raw_R1_fullpaths.txt must contain all the names of the R1 fastq files to be
analyzed

while read line
do
a=${line:0:${#1}-9}

python shear.py \
--fq1 $a"_R1.fastq" \
--fq2 $a"_R2.fastq" \
--out1 $a"_sheared_R1.fastq" \
--out2 $a"_sheared_R2.fastq" \
--execscythe /N/soft/rhel6/scythe/0.992beta/scythe \
--tempdir $(pwd)/tempfiles_shear \
--trimfixed 0:0 --trimqual 20:20 --trimqualpad 0:0 --filterlength 50 --
trimpattern3 AGATC --trimpolyat 12 --trimambig --filterlowinfo 0.5 --
filterunpaired --filterqual 10 --filterambig 8

done < raw_R1_fullpaths.txt


# (2) STAR mapping
#shear_R1_fullpaths.txt must contain all the names of the R1 fastq files
preprocessed by shear
# The variable PATH_TO_GENOME must be set to the absolute path to the (STAR
indexed) reference genome

while read line
do
prefix=${line:0:${#line}-17}
```

```
dirname=${prefix}_penn
if [ ! -d "$dirname" ]; then
mkdir $dirname
cd $dirname
STAR \
--genomeDir $PATH_TO_GENOME \
--readFilesIn ${prefix}_sheared_R1.fastq /${prefix}_sheared_R2.fastq \
--runThreadN 8 \
--outReadsUnmapped  Fastx \
--genomeLoad LoadAndKeep
fi
done < shear_R1_fullpaths.txt

# (3) Sorting, filtering and indexing alignment files
#dirnames.txt must have the absolute paths to the STAR output directories
(one per library)

while read line
do
cd $line
prefix="Aligned.out"
if [ ! -f ${prefix}".sorted.bam" ]; then
samtools view -uS ${prefix}".sam" > ${prefix}".bam"
samtools sort $prefix".bam" $prefix".sorted"
samtools index $prefix".sorted.bam"
rm $prefix".bam"
fi
done < dirnames.txt

# (4) Counting reads in gene models with featureCounts from subread-1.4.6

featureCounts -T 15 -B -p -t exon -g Parent -a spenn_v2.0_exons.gff -o
all_counts_to_PENN.txt $(cat all_penn_bams.txt)
```