

**TESTING THE SI × SC RULE: POLLEN–PISTIL
INTERACTIONS IN INTERSPECIFIC CROSSES BETWEEN
MEMBERS OF THE TOMATO CLADE (*SOLANUM* SECTION
LYCOPERSICON, SOLANACEAE)¹**

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- *Premise of the study:* Interspecific reproductive barriers (IRBs) act to ensure species integrity by preventing hybridization. Previous studies on interspecific crosses in the tomato clade have focused on the success of fruit and seed set. The SI × SC rule (SI species × SC species crosses are incompatible, but the reciprocal crosses are compatible) often applies to interspecific crosses. Because SI systems in the Solanaceae affect pollen tube growth, we focused on this process in a comprehensive study of interspecific crosses in the tomato clade to test whether the SI × SC rule was always followed.
- *Methods:* Pollen tube growth was assessed in reciprocal crosses between all 13 species of the tomato clade using fluorescence microscopy.
- *Key results:* In crosses between SC and SI species, pollen tube growth follows the SI × SC rule: interspecific pollen tube rejection occurs when SI species are pollinated by SC species, but in the reciprocal crosses (SC × SI), pollen tubes reach ovaries. However, pollen tube rejection occurred in some crosses between pairs of SC species, demonstrating that a fully functional SI system is not necessary for pollen tube rejection in interspecific crosses. Further, gradations in the strength of both pistil and pollen IRBs were revealed in interspecific crosses using SC populations of generally SI species.
- *Conclusion:* The SI × SC rule explains many of the compatibility relations in the tomato clade, but exceptions occur with more recently evolved SC species and accessions, revealing differences in strength of both pistil and pollen IRBs.

Key words: interspecific reproductive barriers; pollen–pistil interactions; self-incompatibility; SI × SC rule; Solanaceae; *Solanum*; tomato clade; unilateral incompatibility; unilateral incongruity; wide hybridization; wild tomato species.

One premise of the biological species concept (BSC) is that reproductive barriers act to prevent interbreeding between species. While the BSC is not universally applicable, interspecific reproductive barriers (IRBs) between species can be detected in many cases. For example, in the tomato clade (*Solanum* sect. *Lycopersicon*) prezygotic IRBs can prevent hybridization between certain species (Rick, 1956, 1979; Martin, 1961a, b, 1964; Hardon, 1967; Rick et al., 1976; Liedl et al., 1996; Bedinger et al.,

2011). In compatible crosses, a pollen grain on a stigma germinates, forming a pollen tube that grows through the style and into the ovary to fertilize the ovum (Cheung, 1996; Dresselhaus and Franklin-Tong, 2013). However, in incompatible crosses, pollen tubes can be prevented from reaching the ovary by active rejection processes. In a number of wild tomato species, there are two types of pollen–pistil incompatibility systems involving rejection of pollen tubes in pistils. First, self-incompatibility (SI) can prevent inbreeding through the rejection of self-pollen tubes. In the Solanaceae, SI depends on the interaction of *S*-locus pistil-expressed S-RNases and pollen-expressed F-box proteins, as well as non-*S*-locus factors, such as pistil HT proteins and pollen SCF ubiquitin ligase components including Cullin1 (CUL1) (Kao and Tsukamoto, 2004; McClure, 2004; McClure and Franklin-Tong, 2006; Kubo et al., 2010; Li and Chetelat, 2014). Second, in some crosses between species, unilateral incompatibility (UI) occurs, such that pollinations are compatible in one direction and incompatible in the other direction (Levin, 1971; Grant, 1981; Hogenboom, 1984; McClure et al., 2000, 2011; Hancock et al., 2003). UI barriers, thus, contribute to the reproductive isolation of species. The directionality of UI often

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follows the SI × SC rule (Lewis and Crowe, 1958; Martin, 1967; Hogenboom, 1973): SI species reject pollen tubes from SC species, while the reciprocal SC × SI species cross is compatible. The generality of the SI × SC rule suggests that UI and SI are related, and genetic studies provide further support for this relationship. For example, both pollen and pistil UI QTL map to known SI loci in wild tomato species (Chetelat and Deverna, 1991; Bernacchi and Tanksley, 1997; Covey et al., 2010). Recent experiments provide direct evidence that SI and UI use at least three different common factors. Expression of two pistil SI factors, S-RNase and HT, introduced a UI barrier in *S. lycopersicum* (Tovar-Méndez et al., 2014). Further, when the pollen UI factor CUL1 was downregulated in *S. arcanum*, SI was suppressed (Li and Chetelat, 2014). It is important to note, however, that there are redundant UI pollen rejection mechanisms, some of which are independent of S-RNase (Murfett et al., 1996; Tovar-Méndez et al., 2014).

The tomato clade, *Solanum* section *Lycopersicon*, comprises 13 closely related species possessing diverse mating systems, making it an excellent system in which to investigate the relationship of IRBs to mating systems (Rick, 1979; Mutschler and Liedl, 1994; Peralta et al., 2008; Rodriguez et al., 2009; Bedinger et al., 2011). Four species are self-compatible (SC; autogamous) and produce red, orange, or greenish-yellow fruits and are hereafter referred to as “red-fruited” species: *S. lycopersicum* (the domesticated species), *S. pimpinellifolium*, and two species endemic to the Galápagos Islands, *S. galapagense* and *S. cheesmaniae*. The remaining nine species, with green to purple fruits, include two entirely SC taxa, *S. chmielewskii* and *S. neorickii*, and seven mostly SI species, *S. arcanum*, *S. huaylasense*, *S. peruvianum*, *S. corneliomulleri*, *S. chilense*, *S. habrochaites*, and *S. pennellii*.

Previous studies of cross compatibility between species in the tomato clade have measured the success of fruit set and seed production (Mutschler and Liedl, 1994). Since the SI × SC rule is robust in this group and SI systems act during pollen tube growth, it is of interest to examine pollen tube growth more directly in interspecific crosses. The relatively few studies that have analyzed pollen tube growth in interspecific crosses are limited in scope because generally only the domesticated species, *S. lycopersicum*, was used in reciprocal crosses with wild species (Martin, 1961a; Hardon, 1967; Liedl et al., 1996; Covey et al., 2010). These previous studies reveal a UI relationship: pistils of cultivated tomato accept pollen tubes from the wild species, but in the reciprocal crosses, pollen tubes of cultivated tomato were rejected by pistils of wild species. Lewis and Crowe (1958) found a similar result in interspecific crosses with SC *S. pimpinellifolium* as male with two SI species. Covey et al. (2010) discovered two modes of pollen tube rejection in UI crosses with *S. lycopersicum*; in most cases, rapid rejection was manifested after 1–2 mm of pollen tube growth, but pistils of *S. chmielewskii* and an SC population (LA0407) of *S. habrochaites* showed slower rejection, manifested after 6–7 mm of pollen tube growth.

In this paper, we assess whether the SI × SC rule applies consistently in the tomato clade by examining pollen tube growth in reciprocal crosses between all of the species in this group. We found that in general the SI × SC rule is followed in crosses between pairs of SI and SC species. However, we found that some SC species, and SC populations of otherwise SI species, exhibited an array of pollen–pistil behaviors in interspecific crosses, suggesting that incomplete loss of SI or other IRB factors can modulate interspecific compatibility.

MATERIALS AND METHODS

Plant material—Seeds of *S. lycopersicum* cultivars VF36, M82, and VFNT Cherry (LA1221), and accessions of the wild tomato species (Appendix S1, see Supplemental Data with the online version of this article) were obtained from the Charles M. Rick Tomato Genetics Resource Center at the University of California Davis (<http://tgrc.ucdavis.edu/>) and grown in greenhouses in Pro-Mix-BX soil with 16 h of light at 26°C and 8 h dark at 18°C, or in fields at Colorado State University or UC Davis.

Pollinations and pollen tube analysis—Flower buds were emasculated 1 d before anthesis (Bruckhin et al., 2003) and pollinated. At least three different female plants of each species were tested in each interspecific cross. Self-pollinations were performed to confirm mating system in each species. Pollinated pistils were collected after 48 h, unless otherwise noted, fixed, cleared, and stained with aniline blue fluorochrome as previously described (Covey et al., 2010). Images were composited in either Adobe Photoshop (<http://www.photoshop.com/>) or Image Composite Editor (ICE; <http://research.microsoft.com/en-us/um/redmond/groups/ivm/ice/>) after capture at 5× magnification using a DAPI emission filter. For all figures, fluorescence images of pistils were inverted, contrast adjusted to optimize appearance of pollen tubes, and placed on a white background. Lengths of pollen tubes and styles (from top of stigmas to the bottom of styles) were measured using the program ImageJ 1.33u (National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>). Images from crosses with at least seven pollen tubes in pistils were used to measure the length of the majority of pollen tubes (the point at which no more than three pollen tubes passed) and the longest pollen tube. Averages and standard deviations were calculated using Microsoft Excel software 2011 (Redmond, Washington, USA).

RESULTS

To assess the presence and strength of IRBs acting during pollen–pistil interactions, we performed reciprocal crosses between the 13 species of the tomato clade (*Solanum* section *Lycopersicon*). We examined pollen tube growth in each cross by staining pollen tube cell walls in pistils, as shown in Fig. 1. In crosses in which interspecific pollen tube rejection occurred, the points where pollen tubes ceased growth were measured from the stigma surface to the point where the majority of pollen tubes tips were observed (Fig. 1 insert, dashed line). Usually, only 1–3 pollen tubes could be observed beyond this point, and these rarely grew more than 1 mm past the majority of pollen tubes (Fig. 1 insert, solid line, Appendix S2, see online Supplemental Data). Table 1 summarizes the results of reciprocal interspecific crosses across the entire clade. Shown are SI and SC status in self-crosses and, where appropriate, seed set results, or the points where the majority of pollen tubes were observed (i.e., ovary or the distance from the stigma surface).

SC × SC crosses among the SC red-fruited species—Crosses among the four SC red-fruited tomato species (the domesticated species *S. lycopersicum* and three wild species, *S. pimpinellifolium*, *S. galapagense*, and *S. cheesmaniae*) did not result in pollen tube rejection, and fruit and seeds were produced, consistent with previously reported results (Rick, 1956, 1967, 1979; Rick et al., 1976). Pollen tube growth to ovaries in reciprocal crosses between *S. pimpinellifolium* and the other SC red-fruited species is shown in Appendix S3 (A–C; see online Supplemental Data) as representative of these types of crosses.

SC × SC crosses between SC green-fruited species—Two green-fruited SC species, *S. chmielewskii* and *S. neorickii*, are reported to be interfertile. Appendix S3 (D and E) shows that no pollen–pistil prezygotic IRBs exist between these species; pollen tubes grew to the ovaries in reciprocal crosses. However, a

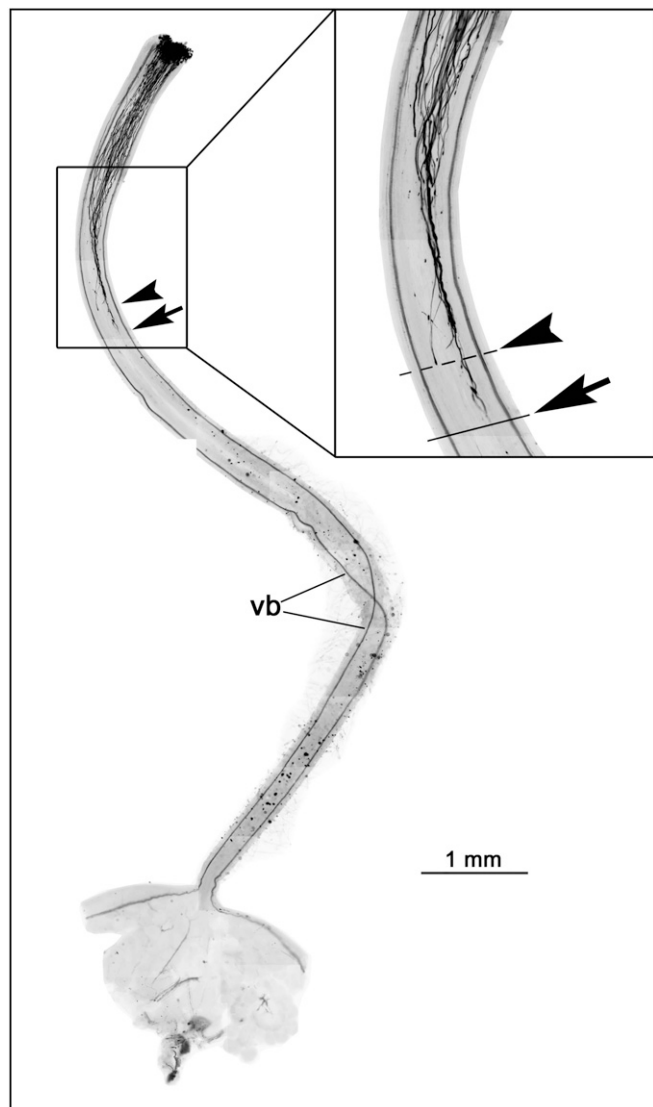


Fig. 1. Visualization and measurement of pollen tube growth in interspecific crosses. After pollinated pistils were fixed and stained as described in Materials and Methods, pollen tubes were measured from the top of the stigma to the point at which the majority (arrowhead, dashed line in insert) and the longest pollen tubes (arrow, solid line in insert) stopped growing. The image shown is from a cross between SI *Solanum corneliomulleri* (female) and SC *S. neorickii* (male), and demonstrates rejection of pollen tubes in the upper third of the style. vb = vascular bundles.

postzygotic barrier reducing the fertility of these crosses has been reported. Hybrid breakdown results in F_2 generation seeds that germinate poorly relative to seeds from self-pollination of the two parent species (Rick et al., 1976).

SC \times SC crosses between SC red- and green-fruited species—Crosses between red- and green-fruited SC species can display UI and, therefore, deviate from the SI \times SC rule in the strictest sense. Pistils of all four of the SC red-fruited species accept pollen tubes of the two SC green-fruited species (Rick et al., 1976; Rick, 1979). Appendix S3 (F and G) shows *S. pimpinellifolium* \times *S. neorickii* and *S. pimpinellifolium* \times *S. chmielewskii* as representative examples. In the reciprocal crosses, when the two SC green-fruited species *S. chmielewskii* and *S. neorickii*

are used as females in crosses with SC red-fruited species, interspecific pollen tubes can fail to reach the ovaries (Figs. 2, 3).

When *S. chmielewskii* is used as female in crosses with red-fruited species, pollen tubes grew to 7.3 mm in styles in 48 h; whether interspecific pollen tubes reached the ovaries may depend on the style length (Fig. 2). For example, pollen tubes from the four red-fruited species grew 6.5–7.3 mm but did not reach the ovaries even after 72 h in accessions with long styles (8–9 mm), such as *S. chmielewskii* accessions LA1316 or LA1317. In contrast, pollen tubes from the red-fruited species reached the ovaries in 24 h in crosses with accessions of *S. chmielewskii* with shorter (i.e., 6–7 mm) styles, such as LA1325, LA3656, and LA3653.

Solanum neorickii is an SC species with a large geographical range that extends from near Paute in central Ecuador to the Cusco area in southern Peru. When *S. neorickii* accession LA4023 from the northern limit of the species range in Paute, Ecuador was used as the female in crosses with red-fruited species as the male, pollen tubes of all four red-fruited species were rejected after about 2.5 mm of growth into styles (at about the midpoint of the style). However, pollen tubes of the red-fruited species grew into the ovaries of some accessions of *S. neorickii*, including LA2403 and LA0247, which were collected near Huanuco, Peru in the center of the species range. This parallels the results of Chmielewski (1962) and Rick et al. (1976), who reported compatibility of *S. lycopersicum* with another accession of *S. neorickii* (LA0735) from the Huanuco region. Thus, it is likely that genetic variability in *S. neorickii* influences the strength or timing of interspecific pollen tube rejection on the female side. Figure 3 shows the variation in pollen tube growth in crosses with *S. neorickii* as the female and red-fruited species as the male, with pollen tube rejection at 2.0–2.6 mm in styles of the Paute accession and pollen tubes reaching the ovaries in pistils of the Huanuco accession.

SI \times SI crosses among SI species—The seven SI species in the tomato clade (*S. arcanum*, *S. huaylasense*, *S. corneliomulleri*, *S. peruvianum*, *S. chilense*, *S. habrochaites*, and *S. pennellii*) were intercrossed, and pollen tube growth was assessed. *Solanum corneliomulleri* and *S. peruvianum* are not well resolved taxonomically, and since no differences were observed in crosses (data not shown), data were combined for these two species. Online Appendix S4 shows images of pollen tubes in representative SI \times SI crosses. In general, pollen tubes of all SI species reached the ovaries of other SI species within 48 h. However, Fig. 4 shows that when pollen from either SI or SC *S. arcanum* is used in crosses with *S. habrochaites* and *S. pennellii*, *S. arcanum* pollen tubes grew more slowly than did conspecific pollen tubes. The majority of *S. habrochaites* or *S. pennellii* pollen tubes reached the ovaries in 24 h in sibling crosses (as do sibling pollen tubes in *S. arcanum* pistils). In contrast, at 24 h post-pollination, pollen tubes of *S. arcanum* rarely reached ovaries in pistils of *S. habrochaites* and *S. pennellii*; in most crosses, the majority of pollen tubes of *S. arcanum* are about midway between the stigma and the ovary. At 48 h, pollen tubes of *S. arcanum* reached the ovary in approximately a third of the crosses, but in most crosses the majority of pollen tubes only traversed about 80% of the style. The majority of pollen tubes of *S. arcanum* reached the ovaries in crosses with *S. habrochaites* and *S. pennellii* by 72 h post-pollination.

SI \times SC and SC \times SI interspecific crosses—Reciprocal crosses were performed using the six SC and seven SI species

TABLE 1. Pollen tube growth in interspecific crosses in the tomato clade.

Female	Male												
	Self-compatible					Self-incompatible							
	<i>S. lyc</i>	<i>S. pim</i>	<i>S. gal</i>	<i>S. che</i>	<i>S. chm</i>	<i>S. neo</i>	<i>S. arc</i>	<i>S. hua</i>	<i>S. cor/S. per</i>	<i>S. chi</i>	<i>S. hab</i>	<i>S. pen</i>	
Self-compatible	SC Seed Seed Seed Seed	Seed SC Seed Seed	Seed Seed SC Seed	Seed Seed Seed SC	Seed Seed Seed Seed	Seed Seed Seed Seed	Ovary Ovary Ovary Ovary	Ovary Ovary Ovary Ovary	Ovary Ovary Ovary Ovary	Ovary Ovary Ovary Ovary	Ovary Ovary Ovary Ovary	Seed Seed Seed Seed	Seed Seed Seed Seed
Self-incompatible	7.3 ± 0.1 ^a 2.3 ± 0.26 ^b	7.0 ± 0.59 ^a 2.6 ± 0.2 ^b	7.0 ± 0.8 ^a 2.3 ± 0.15 ^b	6.5 ± 1.2 ^a 2.0 ± 0.31 ^b	Seed SC	SC	Ovary SI	Ovary SI	Ovary SI	Ovary SI	Ovary SI	Ovary SI	Ovary SI
	1.0 ± 0.13 0.8 ± 0.24	1.4 ± 0.1 1.1 ± 0.05	1.3 ± 0.12 1.2 ± 0.16	1.5 ± 0.1 0.9 ± 0.13	2.2 ± 0.19 1.9 ± 0.3	2.4 ± 0.57 1.5 ± 0.6	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary Ovary
	1.7 ± 0.18 1.5 ± 0.37	1.6 ± 0.07 1.7 ± 0.11	1.5 ± 0.15 1.9 ± 0.32	1.3 ± 0.04 1.6 ± 0.32	2.0 ± 0.22 2.8 ± 0.35	2.5 ± 0.3 2.2 ± 0.58	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary SI	Ovary Ovary	Ovary Ovary	Ovary Ovary
	1.3 ± 0.47 0.9 ± 0.07	1.1 ± 0.13 1.3 ± 0.13	1.5 ± 0.02 1.0 ± 0.23	1.2 ± 0.1 1.2 ± 0.72	2.5 ± 0.93 1.1 ± 0.12	2.5 ± 0.15 1.0 ± 0.16	Ovary ^c Ovary ^c	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary Ovary	Ovary SI

Notes: “Seed” means that seed was produced in interspecific crosses as previously reported (Rick et al., 1976; Rick, 1979, 1986). “Ovary” means that pollen tubes reached ovaries in pistils within 48 h unless otherwise noted; fertilization (fruit/seed set) tests were not attempted in this study. Numbers refer to the length in millimeters of the majority of pollen tubes ± SD in cases of pollen tube rejection. *S. lyc* = *S. lycopersicum*, *S. pim* = *S. pimpinellifolium*, *S. gal* = *S. galapagense*, *S. che* = *S. cheesmaniae*, *S. chm* = *S. chmielewskii*, *S. neo* = *S. neorickii*, *S. arc* = *S. arcanum*, *S. hua* = *S. huaylasense*, *S. cor* = *S. corneliumulleri*, *S. per* = *S. peruvianum*, *S. chi* = *S. chilense*, *S. hab* = *S. habrochaites*, *S. pen* = *S. pennellii*.

^a Pollen tubes do not reach ovaries in some accessions of *S. chmielewskii*.

^b Pollen tube rejection occurs in some accessions of *S. neorickii*.

^c The majority of pollen tubes reach ovaries in 72 h.

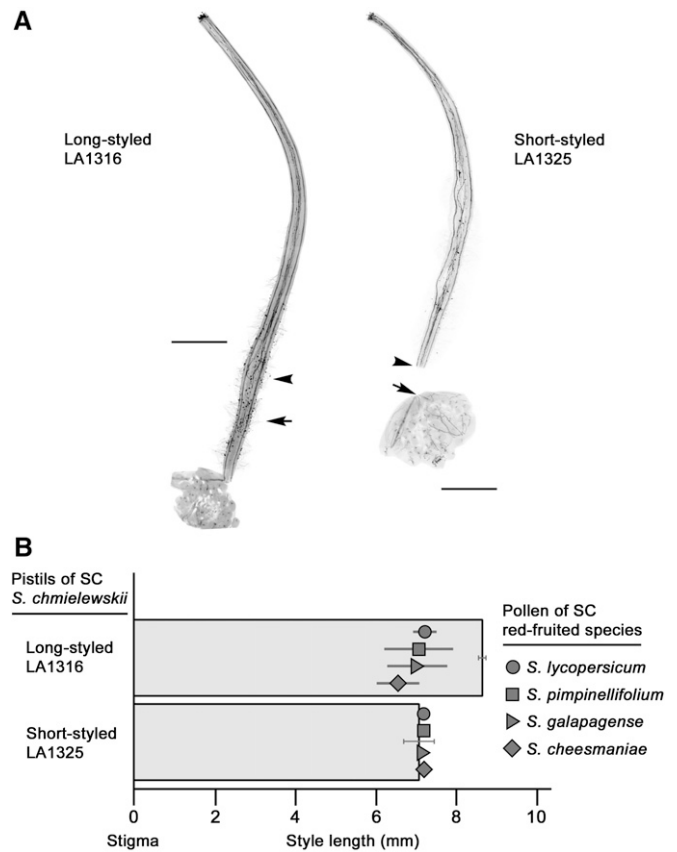


Fig. 2. Growth of pollen tubes of red-fruited SC species in pistils of different accessions of *Solanum chmielewskii*. (A) Representative images of crosses with SC *Solanum chmielewskii* LA1316 (left) and SC *S. chmielewskii* LA1325 (right) as female, pollinated with SC red-fruited *S. galapagense* LA1408. Arrowhead indicates where the majority of pollen tubes stop; arrow indicates the end of the longest pollen tube. Bars = 1 mm. (B) Lengths of pollen tubes from red-fruited species after 48 h growth in pistils of SC *S. chmielewskii* LA1316 with long styles (upper shaded rectangle) and after 24 h in LA1325 with shorter styles (lower shaded rectangle). Pollen tube lengths are shown in millimeters, with the averages of the majority of pollen tube lengths (symbols) and standard deviations (bars).

in the tomato clade described above. Consistently, pollen tubes from all SC species were rejected in pistils of all SI species, while in the reciprocal crosses SI species’ pollen tubes reached the ovaries (Table 1). Therefore, the SI × SC rule held across this set of interspecific crosses. SI species rejected pollen tubes from red-fruited SC species after 0.8–1.9 mm of growth into the styles, while pollen tubes of green-fruited SC species were rejected somewhat later, after 1.5–2.8 mm of growth (Table 1, Fig. 5 white rectangles, online Appendix S5). An exception was seen in pistils of SI *S. pennellii*, wherein pollen tubes of all SC species, including green-fruited ones, were very rapidly rejected, after 0.9–1.3 mm of growth. In all cases, interspecific pollen tubes of SC species were rejected in the upper third of the styles of SI species.

SC × SC and SI × SC crosses involving SC populations of SI species—In the tomato clade, there are several examples of SC populations of predominantly SI species, including populations of *S. pennellii*, *S. arcanum*, and *S. habrochaites*. Because some SI system components may persist in more recently evolved SC populations that could function in interspecific

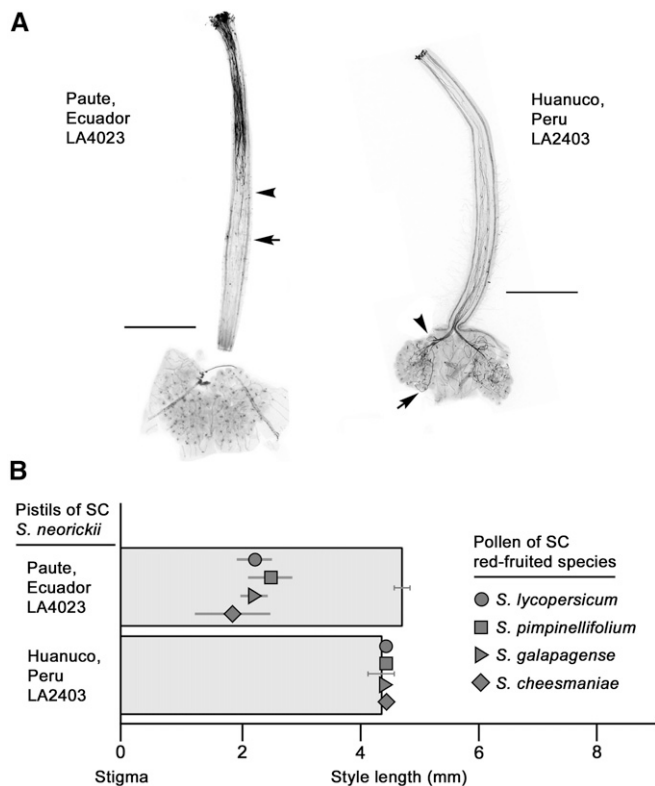


Fig. 3. Growth of pollen tubes of red-fruited SC species in pistils of different accessions of SC *Solanum neorickii*. (A) Representative images of crosses with SC *S. neorickii* accession LA4023 from Paute, Ecuador (left) and SC *S. neorickii* accession LA2403 from Huanuco, Peru (right) as female, pollinated with red-fruited SC *S. cheesmaniae* LA0522. Arrowhead indicates where the majority of pollen tubes stop; arrow indicates the end of the longest pollen tube. Bars = 1 mm. (B) Lengths of pollen tubes from red-fruited species in pistils of SC *S. neorickii* LA4023 from Paute, Ecuador (upper shaded rectangle) and LA2403 from Huanuco, Peru (lower shaded rectangle). Pollen tube lengths are shown in millimeters, with the averages of the majority of pollen tube lengths (symbols) and standard deviations (bars).

pollen tube rejection, we tested pollen tube growth in interspecific crosses using several SC populations of normally SI species.

Pistil-side differences between the SC populations and SI populations of *S. pennellii*, *S. arcanum*, and *S. habrochaites* in interspecific crosses are shown in Fig. 5. Styles of SC populations were shorter on average than those from SI populations as expected: the “selfing syndrome” found in selfing populations of numerous plant species includes smaller flowers compared with those in outcrossing populations (Ornduff, 1969; Goodwillie et al., 2010; Sicard and Lenhard, 2011). In addition, pistils of all SC populations exhibited weakened IRBs compared with SI populations of the same species. In the case of *S. pennellii* LA0716, a well-known SC accession from southern Peru, pollen tubes of all SC species were rejected, but they generally grew longer than in SI *S. pennellii* accessions—on average, 2.1 mm longer for SC red-fruited species and 4.0 mm longer for SC green-fruited species. A more dramatic difference was seen when SC *S. habrochaites* accession LA0407 was used as the male in crosses; pollen tubes reach ovaries of LA0716, but they penetrated only an average of 2 mm into styles of SI accessions of *S. pennellii* (Fig. 5).

There are distinct SC populations of *S. habrochaites* at the northern and southern margins of the species range (Rick et al.,

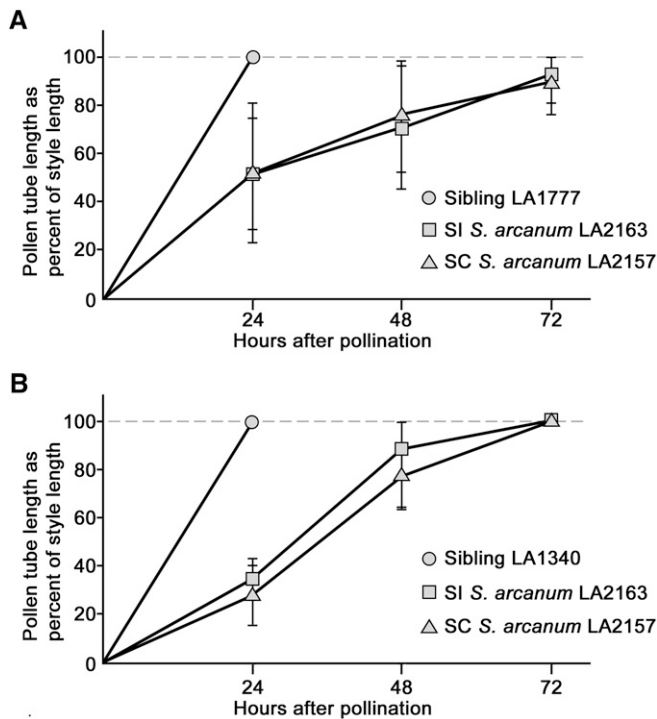


Fig. 4. Time course of *S. arcanum* pollen tube growth in pistils of (A) SI *Solanum habrochaites* LA1777 and (B) SI *S. pennellii* LA1340. Pollen tubes and styles were measured at 24, 48, and 72 h after pollination. The majority of pollen tubes reach ovaries within 24 h in intraspecific sibling crosses (circles), but the majority of pollen tubes of both SI (squares) and SC (triangles) *S. arcanum* do not consistently reach ovaries until 72 h postpollination. Pollen tube growth is shown as a percentage of style length, with the averages of the majority of pollen tube growth (symbols) and standard deviations (bars).

1979). In this study, the northern SC accession LA0407 of *S. habrochaites* was used in interspecific crosses. A number of differences in interspecific pollen tube rejection were observed in pistils of SI *S. habrochaites* accessions compared with LA0407 (Fig. 5). Pistils of SI *S. habrochaites* rejected pollen tubes of the all of red-fruited species rapidly, after 1.3 mm of growth, while pistils of LA0407 rejected *S. lycopersicum* pollen tubes at 6.7 mm on average, a finding consistent with that of Covey et al. (2010), and rejected pollen tubes of *S. pimpinellifolium* at 4.4 mm on average. Pollen tubes of the two Galápagos Island species grew even longer. Pollen tubes of *S. galapagensis* reached the ovary in 6 of 15 crosses, and those of *S. cheesmaniae* consistently reached the ovaries of SC accession LA0407 of *S. habrochaites* within 48 h. Pollen tubes of the green-fruited SC species *S. chmielewskii* and *S. neorickii* also always reached the ovaries of LA0407, but were rejected in the pistils of SI accessions of *S. habrochaites*. Therefore, our results showed that pistil IRBs in SC *S. habrochaites* LA0407 were significantly weaker than those in SI *S. habrochaites*. It should be noted that other, more northerly, SC accessions of *S. habrochaites* have been reported to produce fruit in crosses with SC red-fruited species as male and thus have even weaker pistil IRBs (Chmielewski, 1966).

Solanum arcanum LA2157 is the only known SC accession of this species. As in the other SI species, we find that IRBs in pistils of LA2157 were substantially weaker than those in pistils of SI *S. arcanum*. Pollen tubes from *S. lycopersicum* were rejected after 3.9 mm of growth in the styles of LA2157, compared with only 1 mm in styles of SI accessions of *S. arcanum*.

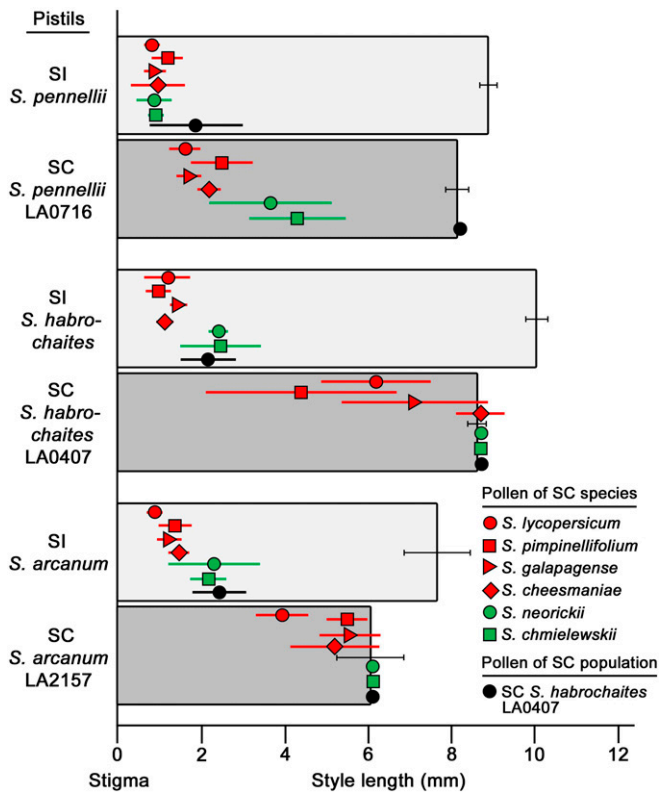


Fig. 5. Comparison of pollen tube growth in pistils of SI and SC accessions of *S. pennellii*, *S. arcanum* and *S. habrochaites*. Pollen tube lengths in pistils pollinated with pollen from SC red-fruited (red symbols) and SC green-fruited (green symbols) species and SC *S. habrochaites* accession LA0407 (black circles). Pistils of SI accessions are shown with light gray rectangles and pistils of SC accessions are shown with darker gray rectangles. Pollen tube lengths are shown in millimeters, with the averages of the majority of pollen tube lengths (symbols) and the standard deviations (bars).

Pollen tubes from the other red-fruited species grew even longer (on average 5.4 mm) in styles of LA2157; pollen tubes of *S. pimpinellifolium* and pollen tubes of the two Galápagos Island species reached the ovaries in about half of the crosses. Pollen tubes of green-fruited SC species *S. neorickii* and *S. chmielewskii*, which were rejected on SI *S. arcanum* (Table 1, Fig. 5), consistently reached ovaries of LA2157. Finally, when SC accession LA0407 of *S. habrochaites* was used as male in crosses with *S. arcanum* LA2157, pollen tubes reached the ovaries but were rejected at 2.5 mm in styles of SI accessions of *S. arcanum* in some crosses (pollen-side variation in LA0407 is discussed below).

We also tested the pollen side behavior of SC accessions of otherwise SI species in interspecific crosses. No pollen-side differences were observed for two of the three SC populations compared with SI populations. For example, when pollen from the SC accession LA0716 of *S. pennellii* was used in interspecific crosses, no differences in pollen tube growth were detected compared with crosses using pollen of SI accessions of *S. pennellii* (LA0751, LA1340, or LA2560); pistils of all SI species in the tomato clade accepted pollen tubes of both SI and SC *S. pennellii* (Table 1; online Appendix S6). Similarly, pollen tubes of SC accession LA2157 of *S. arcanum* reached the ovaries in pistils of SI species, although they grew more slowly in pistils of SI *S. habrochaites* and *S. pennellii* (Fig. 4, Appendix S6).

Since Martin (1961a, 1964) previously showed that pollen from northern SC populations of *S. habrochaites* was rejected by central SI populations of *S. habrochaites*, we hypothesized that pollen from SC accession LA0407 of *S. habrochaites* also may not behave like SI *S. habrochaites* pollen in interspecific crosses. We found that pollen tubes of SC *S. habrochaites* LA0407 reached ovaries in pistils of all SC species and SC populations of SI species (Fig. 5). However, pistils of SI *S. corneliomulleri/peruvianum*, *S. habrochaites*, and *S. pennellii* rejected pollen tubes from LA0407, while pollen tubes of SI accessions of *S. habrochaites* always reached the ovaries of the same species in 48 h (Fig. 6). There is some variability in the behavior of pollen tubes of SC accession LA0407 of *S. habrochaites*; pollen tubes of 9 of 14 LA0407 individuals tested were rejected in pistils of SI *S. arcanum*, and pollen tubes of 10 of 14 individuals tested were rejected in pistils of SI *S. chilense*. These results show that SC *S. habrochaites* LA0407 is polymorphic in this regard and suggest that pollen-side factors are segregating in this accession.

DISCUSSION

It has long been thought that SI and UI may be related, because the success of interspecific crosses often follows the SI × SC rule across many plant families, including Solanaceae (Harrison and Darby, 1955; Lewis and Crowe, 1958; Mutschler and Liedl, 1994; Liedl et al., 1996; Murfett et al., 1996; Onus and Pickersgill, 2004), Brassicaceae (Hiscock and Dickinson, 1993), Liliaceae (Harder, 1993), and Plantaginaceae (Harrison and Darby, 1955). Studies in the tomato clade provide direct support for an SI–UI relationship because some quantitative trait loci (QTLs) for pollen and pistil UI map to the *S*-locus in *S. habrochaites* and *S. pennellii* (Chetelat and Deverna, 1991; Bernacchi and Tanksley, 1997). An additional QTL for pistil UI maps to the location of the gene encoding the pistil SI factor HT (Covey et al., 2010). Moreover, specific SI and UI genes have recently been directly tested for function in both SI and UI. Expression of two known pistil SI factors (*S*-RNase and HT) in transgenic cultivated tomato creates IRBs, leading to the UI rejection of pollen tubes of red-fruited tomato species

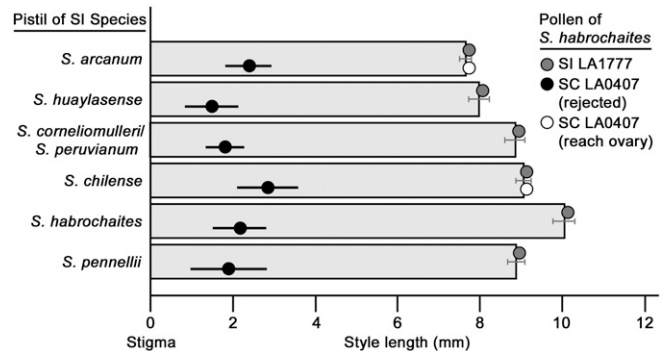


Fig. 6. Pollen tube growth of SI and SC *S. habrochaites* pollen in pistils of SI species. Pollen tube lengths of SI *S. habrochaites* accession LA1777 (gray circles) and SC *S. habrochaites* accession LA0407 in pistils (shaded rectangles) of SI species. Pollen tubes of LA0407 are generally rejected in the upper third of styles in SI species (black circles). However, in some crosses with SI *S. arcanum* and SI *S. chilense*, LA0407 pollen tubes reach ovaries (open circles). Pollen tube lengths are shown in millimeters, with the averages of the majority of pollen tube lengths (symbols) and the standard deviations (bars).

(Tovar-Méndez et al., 2014). On the pollen side, the pollen UI factor *CUL1* has been shown to be required for pollen tube growth in intraspecific pistils expressing a functional S-RNase (Li and Chetelat, 2010, 2014). These results clearly show that SI and UI use common pollen- and pistil-side factors, and, thus, mechanistic overlap is also expected.

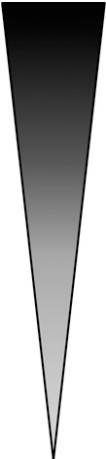
While SI and UI mechanisms overlap, there are also significant differences between these two incompatibility systems. In SI, the recognition and destruction of self-pollen tubes by S-RNases is exquisitely allele-specific: a single S-RNase causes rejection of only one specific pollen S-haplotype. However, UI does not show this level of specificity. For example, we find that pistils of all individuals tested in all SI species, which presumably express a wide array of S-RNases, reject pollen tubes from all SC species. This result, as well as the finding that the *S₆* S-RNase from *S. arcanum* LA2163 can recapitulate an IRB in transgenic cultivated tomato (Tovar-Méndez et al., 2014), is consistent with the lack of allele specificity in UI. There can be some degree of specificity in pistil-side UI, however, since occasional S-RNases show different behavior. For example, unlike all other S-RNases tested, *S₉₈₁₁* S-RNase fails to cause rejection of pollen of *Nicotiana plumbaginifolia* in interspecific crosses (Beecher et al., 2001). UI is similarly allele-nonspecific on the pollen side, since pollen tubes of all individuals of SC species are rejected in crosses with all SI species. The allele specificity of pollen-side SI resides in combinations of pollen *S*-locus F-Box proteins (Kubo et al., 2010), which are also components of SCF ubiquitin ligases. In the case of red-fruited SC species, our results make sense because these species exhibit a loss-of-function mutation in the *CUL1* gene (Li and Chetelat, 2010), an essential component of SCF ubiquitin ligases (Hua and Kao, 2006; Sims et al., 2010). Therefore, all SCF ubiquitin ligase complexes without *CUL1* would be rendered nonfunctional, eliminating the possibility of *S*-allele-specific pollen rejection.

Redundancy is another major difference between SI and UI. Table 2 provides clear evidence for redundant UI mechanisms

in crosses involving SC populations of SI species. For example, it is known that pollen from the SC red-fruited species can be rejected by an S-RNase-dependent mechanism (Tovar-Méndez et al., 2014), yet SC *S. pennellii* LA0716, *S. habrochaites* LA0407 and *S. arcanum* LA2157, which all lack functional S-RNase (Kowyama et al., 1994; Royo et al., 1994; Covey et al., 2010; Chalivendra et al., 2013), consistently reject pollen from these species. Transgenic plant studies also provide clear evidence for S-RNase-independent IRBs (Murfett et al., 1996; Tovar-Méndez et al., 2014).

These results clarify the conditions under which the SI × SC rule applies and what conditions allow exceptions to the rule. In our study, reciprocal crosses between pairs of SC and SI species follow the SI × SC rule (Table 1). However, interspecific crosses with SC populations of otherwise SI species show revealing deviations from the SI × SC rule. Lewis and Crowe (1958) made a distinction between interspecific crossing behavior of long-standing and recently evolved SC species and populations. Our finding that pollen tubes of SC accessions of *S. pennellii* and *S. arcanum* behave like those of functional SI accessions in interspecific crosses (Appendix S6), along with previous findings that pollen of these SC biotypes is fully compatible on pistils of SI accessions of the corresponding species (Hardon, 1967; Rick, 1986), is consistent with the notion that functional pollen SI/IRB factors have been retained in these SC populations even after the loss of SI due to pistil-side mutations. We also observed exceptions to a corollary of the SI × SC rule that posits that SC × SC crosses should be compatible because SC pistils should lack the capacity to reject SC pollen. Our results do not always meet this expectation, at either the species or population level. For example, pistils of SC *S. neorickii* and SC populations of *S. pennellii*, *S. arcanum*, and *S. habrochaites* can actively reject pollen tubes of the SC red-fruited species. These observations agree with prior reports of pollen rejection or lack of seed set in some SC × SC crosses (Martin, 1961a, 1967; Hardon, 1967; Rick, 1986). These results are best understood as reflecting redundant interspecific pollen rejection systems or

TABLE 2. Relative strength of pistil rejection systems in interspecific crosses in the tomato clade.

Rejection	Source of pistil	Effect	Source of pollen	S-RNase
	SI <i>S. pennellii</i>	Reject	All SC species and SC <i>S. habrochaites</i> LA0407	Functional ^a
	Other SI species	Reject	All SC species but reject those of SC green-fruited species more slowly than pistils of SI <i>S. pennellii</i>	Functional ^{a, b}
	SC <i>S. pennellii</i> LA0716	Reject	All SC species but more slowly than pistils of SI species	Absent ^a
	SC <i>S. habrochaites</i> LA0407	Reject	SC <i>S. lycopersicum</i> and <i>S. pimpinellifolium</i> and variably those of <i>S. galapagense</i>	Absent ^a
	SC <i>S. arcanum</i> LA2157	Reject	SC <i>S. lycopersicum</i> and variably those of <i>S. pimpinellifolium</i> , <i>S. galapagense</i> and <i>S. cheesmaniae</i>	Present but nonfunctional ^c
	SC <i>S. neorickii</i> (some accessions)	Reject	SC red-fruited species	Varies; absent or low activity ^b
	SC <i>S. chmielewskii</i> (some accessions)	Impede	SC red-fruited species from reaching ovaries in long styles	Absent ^{a, b}
	SC red-fruited species	Accept	Any species	Absent ^b
	Weakest			

Notes: SC = self-compatible; SI = self-incompatible.

^a Covey et al. (2010).

^b Kondo et al. (2002a, b).

^c Kowyama et al. (1994) and Royo et al. (1994).

persistence of partial interspecific pollen rejection systems, even after the loss of SI (Murfett et al., 1996; Tovar-Méndez et al., 2014). We propose that the variation in whether pollen tubes reach ovaries in interspecific crosses is due to different constellations of pollen and pistil IRB components.

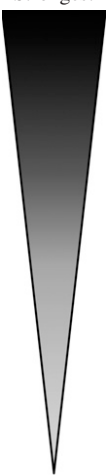
Pistil IRB rejection systems range from very strong in the SI species (especially in *S. pennellii*) to virtually absent in the SC red-fruited species (Tables 1, 2). Pistils of SC species and populations, which lack functional S-RNase, display a range of weaker pollen tube rejection strength. The SC populations of SI species (Fig. 5) range from rejecting pollen tubes of all SC species (SC *S. pennellii* LA0716) to consistently rejecting only pollen tubes of *S. lycopersicum* (SC *S. arcanum* LA2157). Active rejection of pollen tubes from red-fruited species occurs only in some accessions of SC *S. neorickii*, indicating that there is genetic variation in pistil IRBs in this species (Fig. 3). Pollen tubes of the red-fruited species grow quite long in styles of SC green-fruited *S. chmielewskii*, reaching ovaries in some cases; a possible mechanical reproductive barrier (style length) may impede pollen tubes from reaching the ovaries (Fig. 2). It should be noted, however, that self-pollen tubes can reach ovaries in accessions of SC *S. pimpinellifolium* with styles that are longer than 8 mm (Bedinger et al., 2011); thus, there may be additional systems that limit interspecific pollen tube growth in *S. chmielewskii*. Pistils of the red-fruited species, which express neither S-RNase nor HT protein (Kondo et al., 2002b; Covey et al., 2010), do not reject pollen tubes of any tomato clade species.

There is a gradation in strength of pollen resistance systems in interspecific crosses as well (Table 3). Pollen tubes of all SI species possess IRB resistance systems that allow growth to the ovaries in all the other species (Table 1; Appendix S4), with the caveat that pollen tubes of *S. arcanum* grow somewhat more slowly in pistils of some SI species (Fig. 4). Two SC populations of generally SI species, *S. pennellii* LA0716 and *S. arcanum* LA2157, retain robust pollen-side IRB resistance as well, even after the loss of SI (Appendix S6), explaining their deviation from the SI × SC rule. In the case of SC *S. habrochaites* LA0407, pollen IRB resistance is attenuated, as its pollen tubes are rejected by pistils of all SI species (Fig. 6). Resistance has

not been completely lost, however; SC *S. habrochaites* LA0407 pollen tubes reach ovaries in pistils of all SC species and SC *S. pennellii* and SC *S. arcanum* accessions (Fig. 5). Pollen tubes of the SC green-fruited species *S. chmielewskii* and *S. neorickii* are rejected in pistils of SI species and in pistils of SC *S. pennellii*, yet grow longer than pollen tubes of the SC red-fruited species in most SI pistils (Table 1, Fig. 5; Appendix S5) and reach ovaries in pistils of SC accessions of *S. arcanum* and *S. habrochaites*. Pollen tubes of the red-fruited SC species are rejected by all SI species and by SC *S. pennellii* but vary in their ability to reach ovaries in SC populations of *S. arcanum* and *S. habrochaites* (Fig. 5). The weakest degree of pollen IRB resistance is seen in *S. lycopersicum*, since pollen tubes reach ovaries only in crosses with other SC red-fruited species. The red-fruited species are missing at least one important pollen factor: CUL1, a component of SCF ubiquitin ligase that is required for resistance to S-RNases in both SI and UI (Li and Chetelat, 2010, 2014).

The IRBs observed in this study are relevant to natural populations of wild tomato species, given the numerous sites in South America with two or more sympatric wild tomato species (<http://tgrc.ucdavis.edu>). For example, sympatric populations of *S. pimpinellifolium* have been independently documented growing in association with six of the seven wild tomato SI species. Active rejection of interspecific pollen tubes would be expected to prevent hybridization at these sites if pollen from *S. pimpinellifolium* was transferred by pollinators to stigmas of SI species. There is also at least one example of sympatric SI *S. arcanum* and SI *S. habrochaites* (<http://tgrc.ucdavis.edu>, accessions LA1351 and LA1352, respectively) in northern Peru, and comparatively slow growth of *S. arcanum* pollen tubes in pistils of *S. habrochaites* could contribute to reproductive isolation at this site. Our results are also relevant to understanding early stages of speciation in natural populations: since pollen tubes of SC accession LA0407 of *S. habrochaites* are rejected by ancestral SI populations of *S. habrochaites* (Martin, 1961a, 1964), the partial loss of pollen IRBs in LA0407 could represent a step in the establishment of reproductive isolation, as a population differentiates into a separate lineage.

TABLE 3. Relative strength of pollen resistance systems in interspecific crosses in the tomato clade.

Resistance	Source of pollen	Pollen tubes reach ovary in
 Strongest	SI species (other than <i>S. arcanum</i>) and SC <i>S. pennellii</i> LA0716	All species
	SI <i>S. arcanum</i> and SC <i>S. arcanum</i> LA2157	All species but more slowly than those of other SI species in pistils of <i>S. habrochaites</i> and SI <i>S. pennellii</i>
	SC <i>S. habrochaites</i> LA0407	All SC species and populations; grow longer than those of red-fruited species in styles of SI species
	SC <i>S. neorickii</i> and SC <i>S. chmielewskii</i>	All SC species and SC <i>S. habrochaites</i> LA0407 and SC <i>S. arcanum</i> LA2157; grow longer than those of red-fruited species in styles of SI species
	SC <i>S. cheesmaniae</i>	SC red-fruited species and SC <i>S. habrochaites</i> LA0407; variably reach ovaries in SC <i>S. arcanum</i> LA2157
	SC <i>S. galapagensis</i>	SC red-fruited species; variably reach ovaries in SC <i>S. habrochaites</i> LA0407 and SC <i>S. arcanum</i> LA2157
	SC <i>S. pimpinellifolium</i>	SC red-fruited species; variably reach ovaries in SC <i>S. arcanum</i> LA2157
	SC <i>S. lycopersicum</i>	Only SC red-fruited species
Weakest		

Notes: SC = self-compatible; SI = self-incompatible.

The spectrum of pistil and pollen IRB phenotypes revealed in this study represents a rich genetic resource that illustrates the value of preserving and utilizing the natural genetic diversity in wild crop relatives. Our results will inform the design of further studies aimed at uncovering mechanisms controlling pollen–pistil interactions in interspecific crosses. For example, comparative transcriptomic analysis of genotypes that vary in pistil rejection or pollen resistance behaviors, in conjunction with newly available genomic sequences (Tomato Genome Consortium, 2012; Aflitos et al., 2014; Lin et al., 2014), should identify candidate IRB genes. The function of candidate genes can then be verified by generating transgenic plants for use in test crosses with the species and populations of varying IRB strength that we have characterized. In addition to aiding in mechanistic studies, the results of this study advance our understanding of pollen–pistil interactions in crosses between species, such that we are better positioned to transfer valuable agronomic traits from wild germplasm into crop species, either directly or through the development of bridging lines for wide crosses.

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