the plant journal

SEB Society for Experimental Biology

The Plant Journal (2017) **89,** 718–729 doi: 10.1111/tpj.13416

HT proteins contribute to S-RNase-independent pollen rejection in *Solanum*

Alejandro Tovar-Méndez, Lu Lu[†] and Bruce McClure*

Division of Biochemistry, University of Missouri, 117 Schweitzer Hall, Columbia, MO 65211, USA

Received 1 August 2016; revised 26 October 2016; accepted 28 October 2016; published online 8 November 2016.
*For correspondence (e-mail mcclureb@missouri.edu).
[†]Present address: College of Life Sciences, Northwest Normal University, 967 Anning East Road, Lanzhou, Gansu, 730070, China.

SUMMARY

Plants have mechanisms to recognize and reject pollen from other species. Although widespread, these mechanisms are less well understood than the self-incompatibility (SI) mechanisms plants use to reject pollen from close relatives. Previous studies have shown that some interspecific reproductive barriers (IRBs) are related to SI in the Solanaceae. For example, the pistil SI proteins S-RNase and HT protein function in a pistil-side IRB that causes rejection of pollen from self-compatible (SC) red/orange-fruited species in the tomato clade. However, S-RNase-independent IRBs also clearly contribute to rejecting pollen from these species. We investigated S-RNase-independent rejection of *Solanum lycopersicum* pollen by SC *Solanum pennellii* LA0716, SC. *Solanum habrochaites* LA0407, and SC *Solanum arcanum* LA2157, which lack functional S-RNase expression. We found that all three accessions express HT proteins, which previously had been known to function only in conjunction with S-RNase, and then used RNAi to test whether they also function in S-RNase-independent pollen rejection. Suppressing *HT* expression in SC *S. pennellii* LA0716 allows *S. lycopersicum* pollen tubes to penetrate farther into the pistil in *HT* suppressed plants, but not to reach the ovary. In contrast, suppressing *HT* expression in SC. *Solanum habrochaites* LA0407 and in SC *S. arcanum* LA2157 allows *S. lycopersicum* pollen tubes to penetrate to the ovary and produce hybrids that, otherwise, would be difficult to obtain. Thus, HT proteins are implicated in both S-RNase-dependent and S-RNase-independent pollen rejection. The results support the view that overall compatibility results from multiple pollen–pistil interactions with additive effects.

Keywords: interspecific reproductive barrier, tomato, *Solanum lycopersicum, Solanum habrochaites, Solanum pennellii, Solanum arcanum*.

INTRODUCTION

Interspecific reproductive barriers (IRBs) are important for understanding plant evolution as well as for crop improvement. IRBs acting in the post-pollination, prezygotic phase of reproduction rely on a particular aspect of the angiosperm life cycle – that is, interactions between the growing pollen tube and the sporophytic pistil. Barriers can be active or passive. In an active barrier, the pistil expresses specific proteins that inhibit pollen that does not possess appropriate resistance. An effective passive barrier can arise from divergence between pollen and pistil. This effect is also known as incongruity (de Nettancourt, 2001). IRBs acting during the prezygotic stage can contribute to reproductive isolation in communities of co-flowering plants where, for example, interspecific pollen can account for greater than 50% of the pollen on the stigma (Ashman and Arceo-Gomez, 2013). In a practical context, these IRBs complicate using wild species' germplasm for crop improvement (Zamir, 2001; Jansky *et al.*, 2013). Although only a few post-pollination/prezygotic IRB mechanisms have been characterized, it is clear they are more complex and diverse than the better understood self-incompatibility (SI) mechanisms that promote outcrossing within species. A particularly confounding example of this complexity is that multiple, redundant IRB mechanisms can contribute to interspecific incompatibility, even between a single pair of species (Murfett *et al.*, 1996; McClure *et al.*, 2000). Progress can be made, nevertheless, by choosing an experimental system with reduced complexity. Species in the tomato clade (*Solanum* Section *Lycopersicum*) offer such an advantage because the crossing relationships are well characterized (Bedinger *et al.*, 2011; Baek *et al.*, 2015) and

because one pistil-side IRB based on S-RNase and on HT proteins has been defined (Tovar-Méndez *et al.*, 2014).

Baek *et al.* (2015) performed a comprehensive analysis of tomato clade interspecific compatibility. The clade consists of 13 species, including four self-compatible (SC) red/orange-fruited species and nine green-fruited species (i.e., seven predominantly SI species and two SC species) (Peralta *et al.*, 2008). Most interspecific crosses conform to the SI × SC rule: pollen from the SC species is rejected on pistils of related SI species, but the reciprocal pollinations are compatible (Lewis and Crowe, 1958; Bedinger *et al.*, 2011). For example, SI *S. pennellii* × SC *S. lycopersicum* is incompatible, but SC *S. lycopersicum* × SI *S. pennellii* is compatible. This very common IRB pattern is referred to as unilateral incompatibility (UI) because crosses are compatible in only one direction (Lewis and Crowe, 1958). The SI × SC rule predicts that SC × SC crosses will be compatible, and this is usually the case in the tomato clade. For example, as predicted, SC *S. lycopersicum* and SC *S. pimpinellifolium* are cross compatible in both directions. However, there are noteworthy exceptions where unexpected SC × SC incompatibilities occur. Consequently, while the generality of the SI × SC rule suggests mechanistic linkage between IRBs and SI, the exceptions point toward mechanistically distinct IRBs.

The SI × SC rule applies widely, but there is only direct evidence for linkage between IRBs and S-RNase-based SI. In Solanaceae and some other families, pistil-expressed S-RNase proteins cause rejection of self-pollen in SI, and *S*-locus-F-box proteins (SLFs) act as pollen-side recognition proteins (Takayama and Isogai, 2005; Iwano and Takayama, 2012). S-RNases are abundant proteins expressed in the pistil at maturity (Anderson *et al.*, 1986). They are highly polymorphic, acting as the determinants of *S* specificity on the pistil side, and their ribonuclease activity is thought to inhibit incompatible self-pollen (Anderson *et al.*, 1989; McClure *et al.*, 1990; Huang *et al.*, 1994; Murfett *et al.*, 1994). In SI Solanaceae, pollen expresses an array of SLF proteins that are thought to collectively define pollen side *S* specificity (Kubo *et al.*, 2010, 2015; Williams *et al.*, 2014). Additional factors required for SI function include pistil-side proteins, such as the asparagine-rich HT proteins (McClure *et al.*, 1999; O'Brien *et al.*, 2002; Puerta *et al.*, 2009) and a 120 kDa glycoprotein (120K, Hancock *et al.*, 2005), and pollen-side proteins, such as Cullins that are components of SCF complexes (Hua and Kao, 2006; Zhao *et al.*, 2010; Li and Chetelat, 2014). For example, HT protein expression coincides closely with *S*-specific pollen rejection in *Nicotiana*, and suppressing *HT* expression prevents self-pollen rejection (McClure *et al.*, 1999; O'Brien *et al.*, 2002; Puerta *et al.*, 2009).

Genetic and molecular studies have shown that some of the same SI factors also function in IRBs. However, SI and IRBs differ in terms of specificity and the precise factor requirements. In SI, pollen rejection is highly specific: a single S-RNase causes rejection of a single *S* haplotype (Takayama and Isogai, 2005; Iwano and Takayama, 2012). IRBs, in contrast, show broad specificity, and a single S-RNase can cause rejection of pollen from species or groups of species (Murfett *et al.*, 1996; Tovar-Méndez *et al.*, 2014). For example, introducing functional *S-RNase* and *HT* genes into *S. lycopersicum* creates a pistil-side IRB with all four SC red/orange-fruited species in the tomato clade (Tovar-Méndez *et al.*, 2014). Similarly, S-RNase causes rejection of pollen from both *N. plumbaginifolia* and *N. tabacum*, however the HT protein is required for rejection of *N. plumbaginifolia* pollen but not *N. tabacum* pollen (Murfett *et al.*, 1996; Hancock *et al.*, 2005). On the pollen side, three pollen-side QTLs from *S. pennellii* are needed to overcome an S-RNase-based barrier in the pistil (Chetelat and De Verna, 1991); one corresponds to a Cullin gene, *CUL1*, and a second corresponds to an *SLF* gene, *SLF-23*. Both genes function in SI and in IRBs (Li and Chetelat, 2010, 2014, 2015). This is strong evidence that some IRBs are mechanistically linked to S-RNase-based SI in Solanaceae.

Nevertheless, not all IRBs conform to the SI × SC rule. Although the rule correctly predicts that crosses between SC species will be compatible, there are clear examples of SC × SC incompatibilities. These non-conforming IRBs are likely to be novel and independent of S-RNase and, therefore, offer the potential to elucidate new pollen–pistil interactions. For example, using an innovative bioassay and fractionation of *N. tabacum* transmitting tract extracts, Eberle *et al.* (2013) implicated Pistil Extensin-Like Protein III (PELPIII, Goldman *et al.*, 1992; de Graaf *et al.*, 2003) in rejection of pollen from SC *N. repanda* and SC *N. obtusifolia* by SC *N. tabacum*, IRBs that are clearly not related to SI. Previous studies have shown that PELPIII binds to *N. tabacum* pollen tube walls (de Graaf *et al.*, 2003), but a role in interspecific pollen rejection was not known.

We selected three SC × SC IRBs in the tomato clade to investigate S-RNase-independent IRBs. The tomato clade is evolving rapidly (Pease *et al.*, 2016a,b), and SC accessions have been identified in three of the six predominantly SI green-fruited species: *S. pennellii*, *S. arcanum*, and *S. habrochaites*. These three SC accessions have been shown to reject pollen from cultivated tomato, *S. lycopersicum* and the other red-fruited SC species. Recent studies show that pollen tubes fail to reach the ovary (Covey *et al.*, 2010; Baek *et al.*, 2015) and classic studies show that fruit set does not occur (Hardon, 1967; Rick *et al.*, 1976; Rick, 1982). We therefore investigated these three accessions as independent examples of SC × SC IRBs that do not conform to the SI × SC rule. The three chosen accessions represent recently evolved SC biotypes and provide opportunities to investigate redundant IRBs. In particular, they lack active S-RNase, thus reducing the complexity of

their IRBs and providing materials to elucidate S-RNase-independent IRBs. The selected SC accessions also display a full range of IRB strength since they reject pollen from red-fruited species at very different positions in the pistil. Although HT proteins have been implicated only in S-RNase-dependent SI and IRBs, we found HT proteins in each of these SC accessions and, consequently, hypothesized an involvement in S-RNase-independent pollen rejection. RNAi experiments found this situation to be true and, thus, support a broader role for HT proteins in pollen rejection than heretofore recognized.

RESULTS

HT proteins accumulate in three SC accessions that display S-RNase-independent interspecific pollen rejection

We selected SC *S. pennellii* LA0716, SC *S. arcanum* LA2157, and SC *S. habrochaites* LA0407 to investigate S-RNase-independent interspecific pollen rejection. Each accession lacks active S-RNase, but the defect is different in each case. SC *S. pennellii* LA0716 does not express S-RNase protein, and the gene is absent in the genome sequence (Chalivendra *et al.*, 2013; Li and Chetelat, 2014). SC *S. arcanum* LA2157 expresses an inactive S-RNase with a H33N mutation in conserved region C2, which is implicated in ribonuclease activity (McClure *et al.*, 1989; Kawata *et al.*, 1990; Ioerger *et al.*, 1991; Royo *et al.*, 1994). The *S-RNase* gene in SC *S. habrochaites* LA0407 contains an insertion in its promoter region, and it is not expressed (Covey *et al.*, 2010). Figure 1(a) confirms that S-RNase protein is not detectable in pistil extracts from *S. habrochaites* LA0407 or *S. pennellii* LA0716 and that the inactive H33N *S. arcanum* LA2157 mutant protein is detectable, as expected. Figure 1(b–d) shows that each accession, nevertheless, rejects SC *S. lycopersicum* cv. VF36 pollen and, therefore, represents an example of an S-RNase-independent interspecific pollen rejection. The pattern in SC *S. pennellii* LA0716 is referred to as early rejection since *S. lycopersicum* pollen tubes penetrate only 1–2 mm into the pistil after 24 h. *Solanum arcanum* LA2157 and *S. habrochaites* LA0407, in contrast, display a late rejection response since *S. lycopersicum* pollen tubes traverse >75% of the pistil length after 24 h (Covey *et al.*, 2010).

Chalivendra *et al.* (2013) showed that S-RNase-independent rejection of *S. lycopersicum* pollen in *S. pennellii* LA0716 is developmentally controlled and that pollen rejection coincides with *HT* gene expression. Figure 1(a) shows that HT proteins also accumulate in *S. arcanum* LA2157 and in *S. habrochaites* LA0407. Crucially, control pollinations show that HT protein has no effect on *S. lycopersicum* cv. VF36 pollen tubes. Transformed *S. lycopersicum* plants expressing *HT-A* from *S. pennellii* LA2560 remain fully compatible and pollen tubes penetrate to the ovary, similar to untransformed *S.lycopersicum* (Figure 1e; Tovar-Méndez *et al.*, 2014). Thus, we hypothesized that HT proteins, in conjunction with other pistil-side factors, are required for S-RNase-independent interspecific pollen rejection as well as S-RNase-dependent SI and UI (McClure *et al.*, 1999; O'Brien *et al.*, 2002; Hancock *et al.*, 2005; Tovar-Méndez *et al.*, 2014).

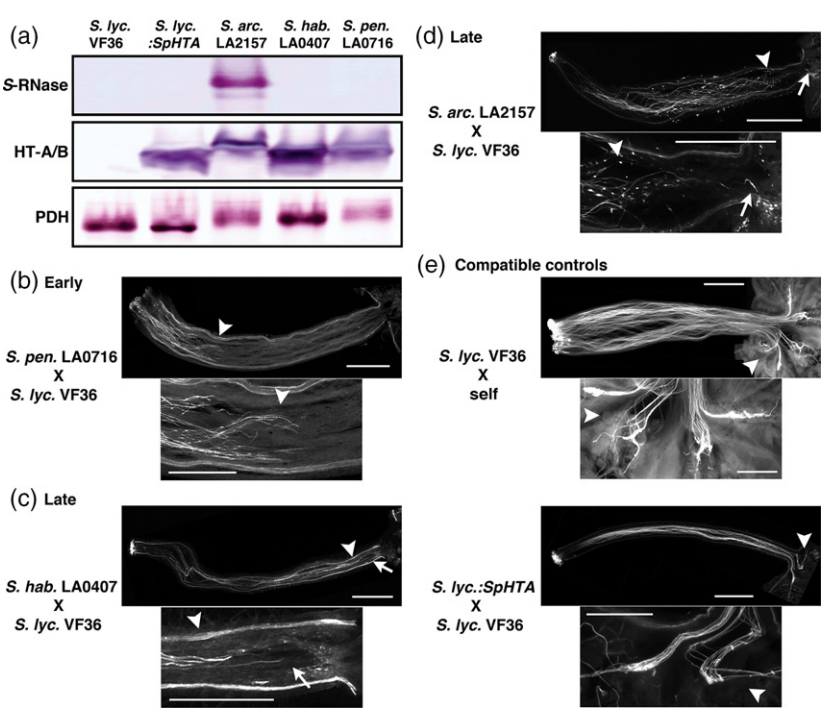

Figure 1. *HT* protein accumulation in species that display S-RNase-independent pollen rejection.
(a) Pistil extracts immunostained with antibodies to the conserved C2 region of S-RNase, HT-A and HT-B proteins, and pyruvate dehydrogenase (loading control).
(b–e) Pistils stained with aniline blue fluorochrome 24 h (*Solanum habrochaites* LA0407 and *Solanum arcanum* LA2157) or 72 h (*Solanum pennellii* LA0716) after pollination with *Solanum lycopersicum* cv. VF36 pollen. Arrowhead, position most pollen tubes stop. Arrow, longest pollen tube. Bar = 1 mm; 0.5 mm, insert. [Colour figure can be viewed at wileyonlinelibrary.com].

HT suppression delays interspecific pollen rejection in SC *S. pennellii* LA0716

We performed RNAi experiments to test the role of *HT* genes in S-RNase-independent interspecific pollen rejection. SI *Solanum* species usually express two *HT* genes, *HT-A* and *HT-B* (Kondo *et al.*, 2002; O'Brien *et al.*, 2002); although, *S. habrochaites* expresses only *HT-A* (Covey *et al.*, 2010). An RNAi construct containing both *HT-A* and *HT-B* sequences from *S. pennellii* LA0716 allows a single construct to suppress expression in all three accessions under investigation (Figure S1). The construct was transformed into *S. pennellii* LA0716 (*Spen-iHT*), *S. arcanum* LA2157 (*Sarc-iHT*), and *S. habrochaites* LA0407 (*Shab0407-iHT*) and then crossed into other accessions as needed to determine the effects in T_0 and T_1 plants.

Figure 2 shows results for eight *HT* suppressed *Spen-iHT* plants and three unsuppressed controls. We detected HT proteins using an antibody that reacts with both HT-A and HT-B proteins since both appear to function in interspecific pollen rejection (Tovar-Méndez *et al.*, 2014). Figure 2(a) shows that pistil extracts from unsuppressed controls (*Spen-iHT*-1–3) contained similar amounts of HT protein as the untransformed *S. pennellii* LA0716, and that three of the suppressed plants showed very low levels of HT protein (*Spen-iHT*-4–6), and that the five remaining had no detectable protein (*Spen-iHT*-7–11). Subsequent qRT-PCR experiments confirmed that plants *Spen-iHT*-4–6 and *Spen-iHT*-7–11 express *HT* transcripts (i.e., *HT-A* plus *HT-B*) at levels about 5% and less than 1%, respectively, of the levels observed in untransformed pistils (Figure S2).

HT suppressed T_0 plants rejected *S. lycopersicum* pollen, but the rejection response was delayed when compared with controls. Figure 2(b,c) shows that unsuppressed controls (i.e., *Spen-iHT*-1–3) behaved like untransformed *S. pennellii* LA0716, with *S. lycopersicum* pollen tubes rarely penetrating more than 2 mm into the pistil after 72 h (1.5 ± 0.4 mm unsuppressed and 1.4 ± 0.8 mm untransformed). However, *S. lycopersicum* pollen tubes penetrated significantly farther into the style in *HT* suppressed plants (*Spen-iHT*-4–11, Figure 2b; $P < 0.01$, Table S1). *Solanum lycopersicum* pollen tubes grew about 2–6.5 mm after 72 h (mean, 3.5 ± 1.3 mm) in various *Spen-iHT* T_0 plants but never penetrated the ovary.

We obtained similar results in T_1 *Spen-iHT* plants. We selfed one unsuppressed control and three *HT* suppressed *Spen-iHT* T_0 plants and then analyzed the T_1 progeny for HT protein and interspecific pollen rejection. Figure 3(a) shows protein blot results for three control *Spen-iHT*-2 T_1 progeny expressing HT proteins and four T_1 progeny from T_0 plants *Spen-iHT*-6, -7, and -11 (i.e., one expressing HT protein and three with undetectable HT proteins for each T_0). Figure 3(b) shows that the effect on *S. lycopersicum* pollen tubes segregates with *HT* suppression. The progeny

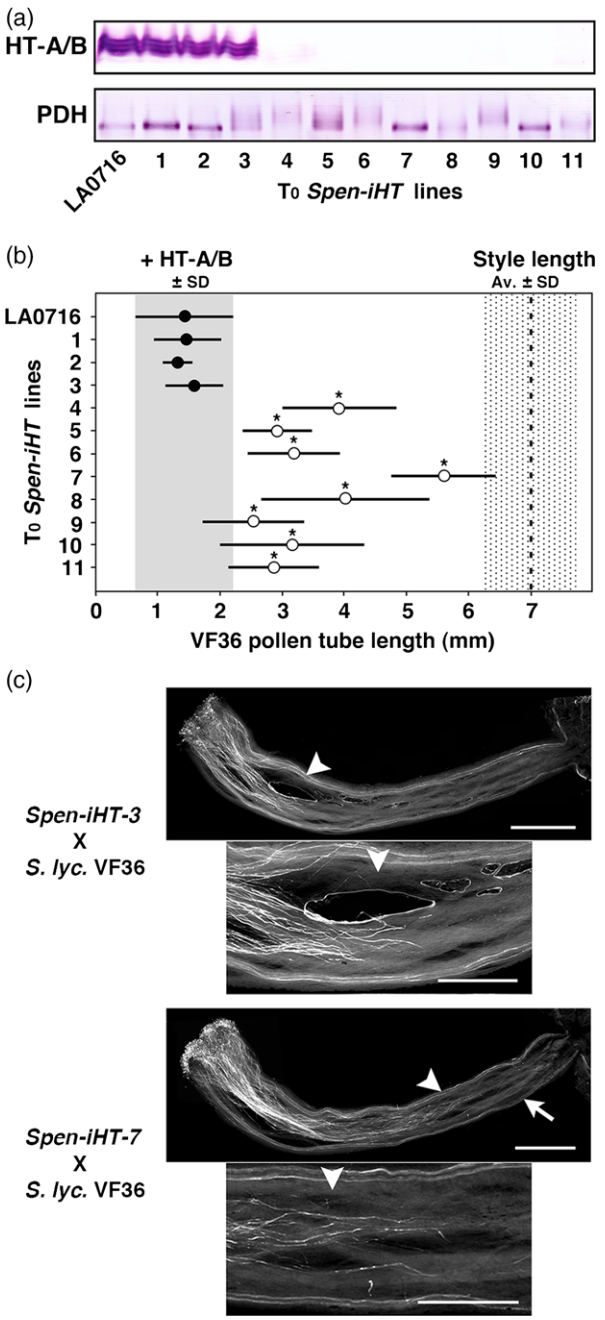

Figure 2. *Solanum lycopersicum* pollen tubes in *HT* suppressed *Solanum pennellii* LA0716 T_0.

(a) HT proteins in untransformed *S. pennellii* LA0716 and *Spen-iHT* T_0 plants. *Spen-iHT*-1–3, unsuppressed controls *Spen-iHT*-4–11, *HT* suppressed. PDH, loading control.

(b) *Solanum lycopersicum* cv. VF36 pollen tube lengths. Shown are mean and standard deviation (SD) of the positions reached by most pollen tubes at 72 h (filled, controls; open, *HT* suppressed). Grey, mean and SD pollen tube penetration in untransformed and unsuppressed controls. Stippling, mean and SD style length. Asterisk, value significantly different from untransformed and unsuppressed controls ($P < 0.01$, one-tailed *T*-test).

(c) Sample images showing the position reached by most pollen tubes at 72 h (arrowhead) and the longest visible pollen tube (arrow) in an unsuppressed control and *HT* suppressed T_0 plant. Bar = 1 mm; 0.5 mm, insert. [Colour figure can be viewed at wileyonlinelibrary.com].

of unsuppressed *Spen-iHT*-2 and unsuppressed T_1 progeny from suppressed *Spen-iHT*-6, -7, and -11 all behaved similarly and *S. lycopersicum* pollen tubes traversed only 1–2 mm into the style after 72 h (mean, 1.7 ± 0.4 mm, Figure 3b). However, the *HT* suppressed progeny of *Spen-iHT*-6, -7, and -11 permitted *S. lycopersicum* pollen tubes to penetrate significantly farther (Figure 3b; 1.5–4.3 mm, mean, 3.3 ± 0.9 mm; $P < 0.01$, Table S2). Together, the results from the T_0 and T_1 *S. pennellii* LA0716 plants show that suppressing *HT* expression has a quantitative effect that partially mitigates *S. lycopersicum* pollen tube rejection. However, *S. lycopersicum* pollen tubes did not penetrate to the ovary, even in *Spen-iHT* plants that had no detectable HT protein.

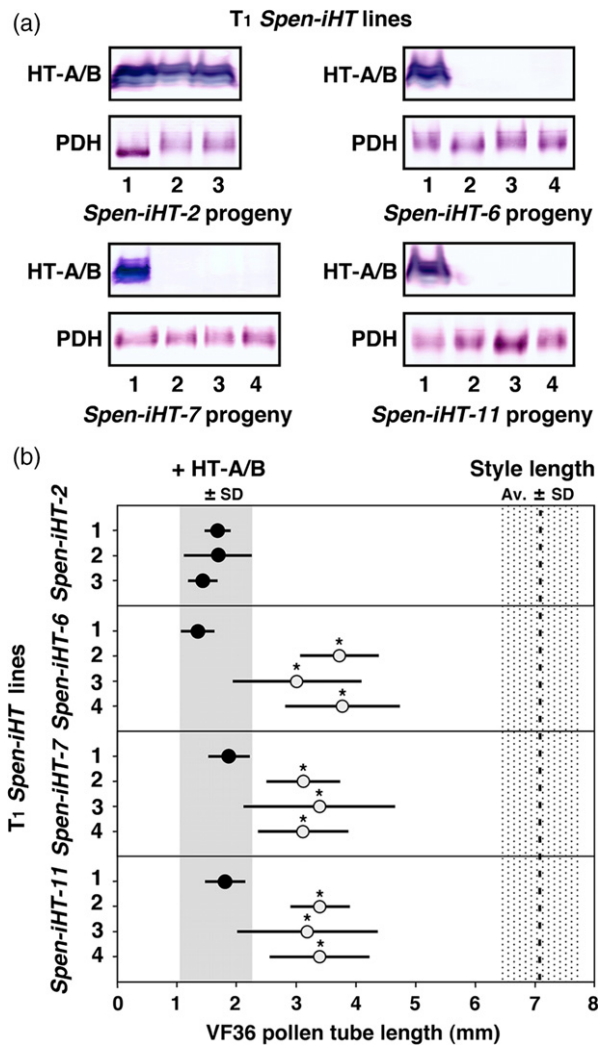

Figure 3. *Solanum lycopersicum* pollen tube penetration segregates with *HT* suppression in *Solanum pennellii* LA0716 T_1.
(a) HT proteins. *Spen-iHT*-2 T_1 progeny, unsuppressed controls; *Spen-iHT*-6, -7, and -11, one unsuppressed and three *HT* suppressed T_1 progeny are shown. PDH, loading control.
(b) *Solanum lycopersicum* cv. VF36 pollen tube lengths. Shown are mean and standard deviation (SD) of the positions reached by most pollen tubes at 72 h (filled, unsuppressed control; open, *HT* suppressed). Grey, mean and SD pollen tube penetration in unsuppressed plants. Stippling, mean and SD style length. Asterisk, value significantly different from unsuppressed controls ($P < 0.01$, one-tailed *T*-test). [Colour figure can be viewed at wileyonlinelibrary.com].

HT suppression permits production of SC *S. habrochaites* × *S. lycopersicum* hybrids

The RNAi construct effectively suppressed *HT* expression in *S. habrochaites* LA0407. However, this accession is recalcitrant to transformation, and only a single T_0 event was recovered after several attempts. Figure 4(a) shows protein blot and interspecific pollination results for the *Shab0407-iHT-1* T_0 plant and seven T_1 progeny. The T_0 plant showed low levels of HT protein. We selfed it as well as the seven T_1 progeny and selected three unsuppressed and four *HT* suppressed individuals for analysis. *HT* suppressed T_1 plants showed similarly low levels of HT protein compared to the T_0 plant, and the unsuppressed T_1 plants had comparable HT level as the untransformed controls (Figure 4a). *Solanum habrochaites* LA0407 shows a late interspecific pollen rejection response, and *S. lycopersicum* pollen tubes penetrate almost to the base of the style after 24 h (Covey *et al.*, 2010). Thus, pollen tube penetration, *per se*, is not a good test of whether *HT* suppression mitigates interspecific pollen rejection in this accession (Figure S3). Therefore, we assessed fruit development to allow time for differential pollen tube growth. Developing fruits were weighed 7 days after pollination (i.e., the time when flowers abscise after incompatible pollinations), and their mass compared to the mass of self fruits (Figure 4b, bars, *HT* suppressed; stippling, self fruit). Untransformed controls and T_1 plants expressing HT protein never set fruit after *S. lycopersicum* pollination and their flowers abscised. In contrast, the T_0 plant and its *HT* suppressed T_1 progeny consistently set interspecific fruits (Figure 4b,c), albeit usually at about half the size of conspecific self fruit. However, self fruit size was highly variable and control and *HT* suppressed distributions were not significantly different (Figure 4c and Table S3). When we germinated the seeds from the interspecific fruits, the resulting plants showed intermediate phenotypes expected of *S. habrochaites* × *S. lycopersicum* hybrids (Figure 4d) and both parental alleles of a *Cullin1* gene (Figure S4).

We further investigated the effects of *HT* suppression in *S. habrochaites* by crossing the RNAi construct into another SC accession, *S. habrochaites* LA2860. Like *S. habrochaites* LA0407, LA2860 does not express S-RNase protein (Figure S5), but rejection of *S. lycopersicum* pollen tubes occurs somewhat earlier than in LA0407 (Figures S5b versus S3). We crossed *Shab0407-iHT*-1 with *S. habrochaites* LA2860 and then tested the resulting *Sxhab2860-iHT* T_1 progeny for *HT* expression and *S. lycopersicum* pollen rejection. Figure 5 shows protein blot and pollination results for two unsuppressed controls and four

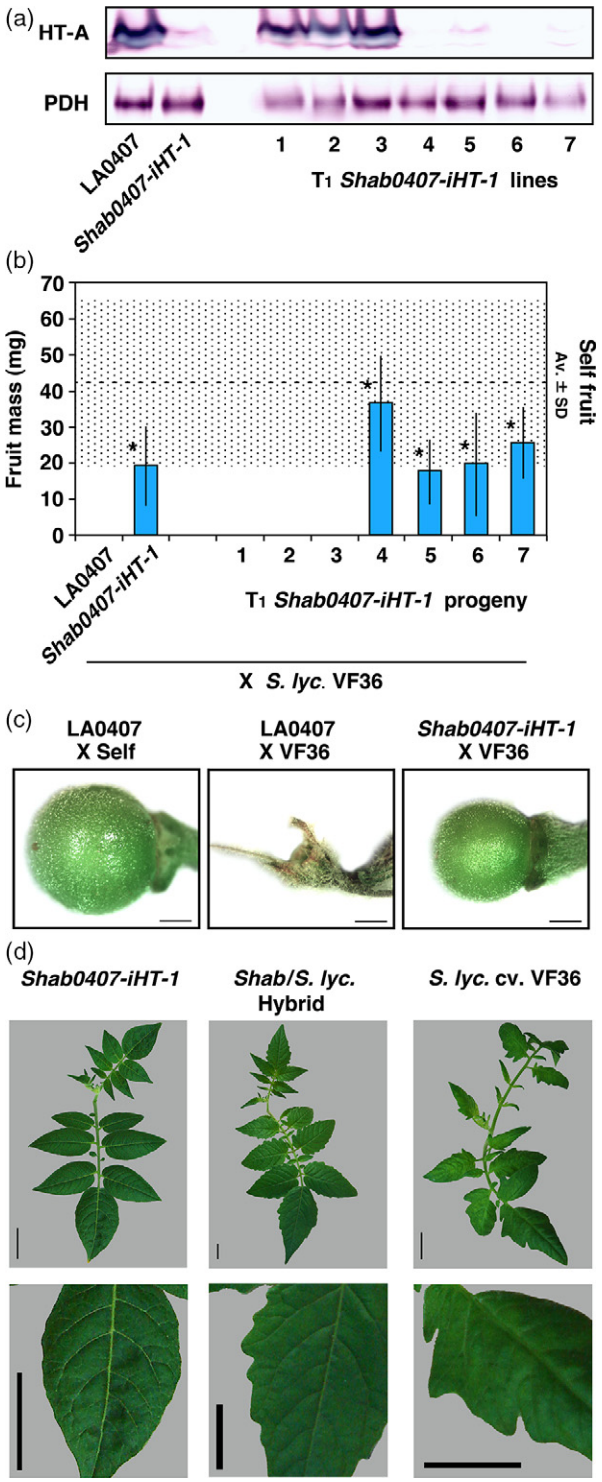

Figure 4. *Solanum lycopersicum* compatibility on *HT* suppressed *Solanum habrochaites* LA0407.
(a) HT-A protein in untransformed *S. habrochaites* LA0407, *Shab0407-iHT-1* T_0, and T_1 progeny. PDH, loading control.
(b) Fruit set 7 days after *Solanum lycopersicum* cv. VF36 pollination. Mean mass (mg) and SD of developing fruits are shown. No fruits were recovered after pollinating untransformed controls or unsuppressed T_1 plants. Stippling, mean mass and SD of self fruits (i.e., conspecific control). Asterisk, value significantly different from untransformed and unsuppressed controls ($P < 0.01$, one-tailed *T*-test).
(c) Sample images 7 days after pollination showing a developing control self fruit (left) and results from pollinating untransformed *S. habrochaites* LA0407 (center, abscised flower with no fruit) or *HT* suppressed *Shab0407-iHT*-1 (right) with pollen from *S. lycopersicum* var. VF36. Bar = 1 mm.
(d) Leaf morphology in *Shab0407-iHT*-1 (left), *S. lycopersicum* cv. VF36 (right), and hybrid (center). Bar = 1 cm.

HT suppressed *Sxhab2860-iHT* T_1 progeny. Unsuppressed *Sxhab2860-iHT* T_1 control progeny showed high levels of HT proteins and, on average, *S. lycopersicum* pollen tubes penetrated 40% of the style length. The four *HT* suppressed T_1 progeny showed low HT protein levels that were comparable to the *HT* suppressed *Shab0407-iHT*-1 self-progeny (Figures 5a versus 4a), and *S. lycopersicum* pollen tubes traversed about 90% of these styles after 24 h (Figure 5b and Table S4). Like *Shab0407-iHT*-1 HT suppressed progeny, these four *Sxhab2860-iHT* T_1 plants routinely set fruit after *S. lycopersicum* pollination, and the fruits contained both viable seeds and partially developed seed-like structures. Fully developed seeds germinated, and the resulting plants showed intermediate phenotypes expected of *S. habrochaites* × *S. lycopersicum* hybrids (Figure 5c) and both *S. habrochaites* and *S. lycopersicum* alleles of a *Cullin1* gene (Figure S4).

HT suppression permits production of SC *S. arcanum* × *S. lycopersicum* hybrids

We tested the effects of *HT* suppression in T_0 and T_1 SC *S. arcanum* LA2157, an accession that expresses a catalytically inactive S-RNase protein (Royo *et al.*, 1994). Figure 6(a) shows similar levels of HT proteins in untransformed and unsuppressed *Sarc-iHT* T_0 and little or no HT proteins in six *HT* suppressed transformants. *Solanum arcanum* LA2157 displays late *S. lycopersicum* pollen tube rejection (Baek *et al.*, 2015). As expected, *S. lycopersicum* pollen tubes penetrate to near the base of the style in *HT* suppressed *Sarc-iHT* T_0 plants (Figure S6) after 24 h. However, Figure 6(b) shows that *HT* suppressed plants routinely set fruit after *S. lycopersicum* pollination, while unsuppressed and untransformed plants did not. On average, these fruits were smaller than self fruits (Figure 6b, bars versus stippling; Figure 6c and Table S5) but contained viable seed as well as poorly developed seed-like structures.

We obtained similar results when one unsuppressed and three *HT* suppressed *Sarc-iHT* T_0 plants were selfed. Four progeny of unsuppressed *Sarc-iHT*-1 accumulated HT protein and failed to set fruit after *S. lycopersicum* pollination (Figure 6d,e and Table S6). The T_1 progeny of three *HT* suppressed plants (i.e., *Sarc-iHT*-3, -4, and -5) segregate for *HT* expression and, again, the *HT* suppressed plants set fruit after *S. lycopersicum* pollination (Figure 6d,e and Table S6). About half the seeds in the resulting fruit

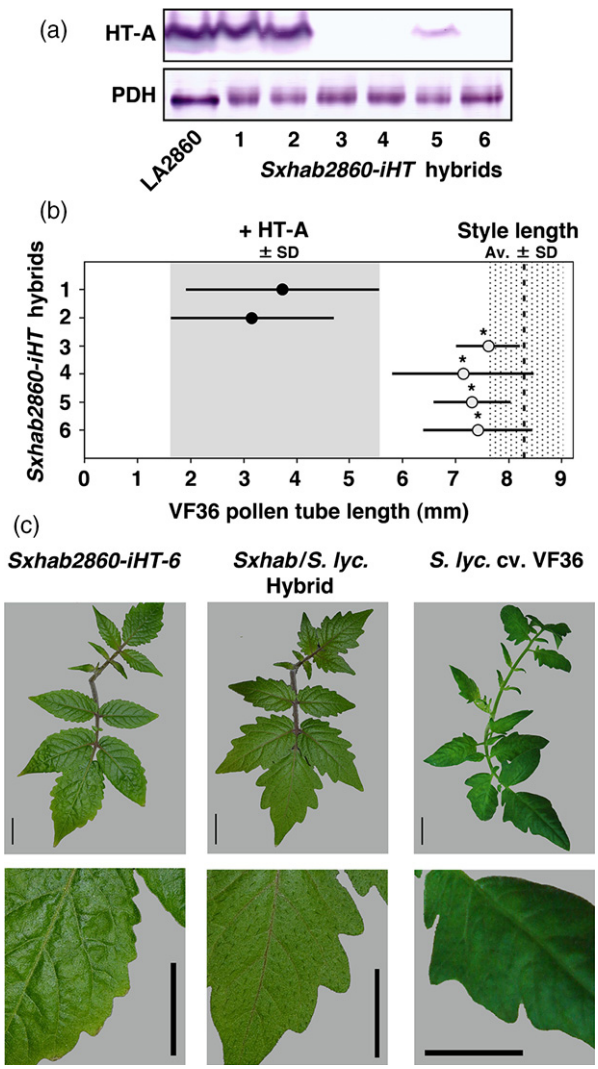

Figure 5. *Solanum lycopersicum* compatibility segregates with *HT* suppression in *S. habrochaites* LA0407/LA2860 hybrid.
(a) HT-A protein in untransformed *S. habrochaites* LA2860 *Sxhab2860-iHT* T_1 progeny. PDH, loading control.
(b) *Solanum lycopersicum* cv. VF36 pollen tube lengths. Shown are mean and SD pollen tube positions at 24 h (filled, control; open, *HT* suppressed). Grey, mean and SD pollen tube positions in untransformed and unsuppressed controls. Stippling, mean and SD style length. Asterisk, value significantly different from unsuppressed controls ($P < 0.01$, one-tailed *T*-test).
(c) Leaf morphology in normal (left, right) and *Sxhab2860* × *S. lycopersicum* cv. VF36 hybrid plants (center). Bar = 1 cm.

germinated and gave rise to *S. arcanum* × *S. lycopersicum* hybrids (Figures 6f and S4).

DISCUSSION

We tested whether HT proteins have a role in S-RNase-independent interspecific pollen rejection. HT proteins are expressed in SC species and in accessions that lack S-RNase expression but that nevertheless display interspecific UI. The three accessions examined – *S. pennellii* LA0716, *S. habrochaites* LA0407, and *S. arcanum* LA2157 – represent independent losses of S-RNase, yet each retains *HT* expression and interspecific UI with *S. lycopersicum* (Figure 1; Baek *et al.*, 2015). Moreover, previous results show that *HT* expression in *S. pennellii* LA0716 pistils coincides with developmental onset of *S. lycopersicum* pollen rejection (Chalivendra *et al.*, 2013). Importantly, pollen rejection is not due to the HT protein alone, since *S. lycopersicum* expressing *HT-A* from *S. pennellii* has no effect on compatibility (Figure 1e; Tovar-Méndez *et al.*, 2014). There is no reason to assume that *HT* expression in SC accessions is rare. For example, the *HT* gene was first cloned from SC *N. alata* cv. Breakthrough (McClure *et al.*, 1999), an accession that lacks S-RNase and displays S-RNase-independent rejection of pollen from *N. tabacum* and *N. glutinosa* (Murfett *et al.*, 1996). However, all these SC accessions are also examples of recent SI to SC transitions, and *HT* expression could be a relic SI pistil function. Since S-RNase-independent interspecific pollen rejection is poorly characterized, we chose a loss-of-function RNAi approach to test whether suppressing *HT* expression affects interspecific compatibility.

Suppressing *HT* expression in *S. pennellii* LA0716 had a quantitative effect on interspecific pollen tube growth, making the pistil more permissive for *S. lycopersicum* pollen tubes. On average, *S. lycopersicum* pollen tubes grew approximately two and a half times as far in *HT* suppressed T_1 plants as compared with unsuppressed siblings (Figure 2). However, *HT* suppression never permitted outright compatibility of *S. lycopersicum* pollen tubes in *S. pennellii* LA0716 (Figure 2); rather, it converted an early response to one that could be described as an intermediate response (i.e., intermediate between early and late) (Covey *et al.*, 2010). This quantitative effect is different from the effect on SI in *Nicotiana*, where suppressing HT protein below the limit of detection has a qualitative effect and results in compatibility, or failure of *S*-specific pollen rejection (McClure *et al.*, 1999). In *S. chacoense* and *Petunia*, however, SI effects are only observed when *HT* expression is suppressed at very low levels (O'Brien *et al.*, 2002; Puerta *et al.*, 2009), and the effects are sometimes partial. In our experiments, effects on *S. lycopersicum* pollen tube penetration were always partial and did not markedly differ among *S. pennellii* LA0716 T_0 plants where total *HT* transcript levels varied between about 5% to <0.5% of control (Figures 2, S2). We interpret this quantitative effect as suggesting that a further (i.e., an *HT*-independent) barrier to *S. lycopersicum* pollen tube growth exists in *S. pennellii* LA0716, but other interpretations are possible. For example, it is possible that suppressing HT merely attenuates a barrier rather than eliminating it entirely, resulting in a quantitative effect.

HT suppression in *S. habrochaites* LA0407 and *S. arcanum* LA2157, which display late rejection of

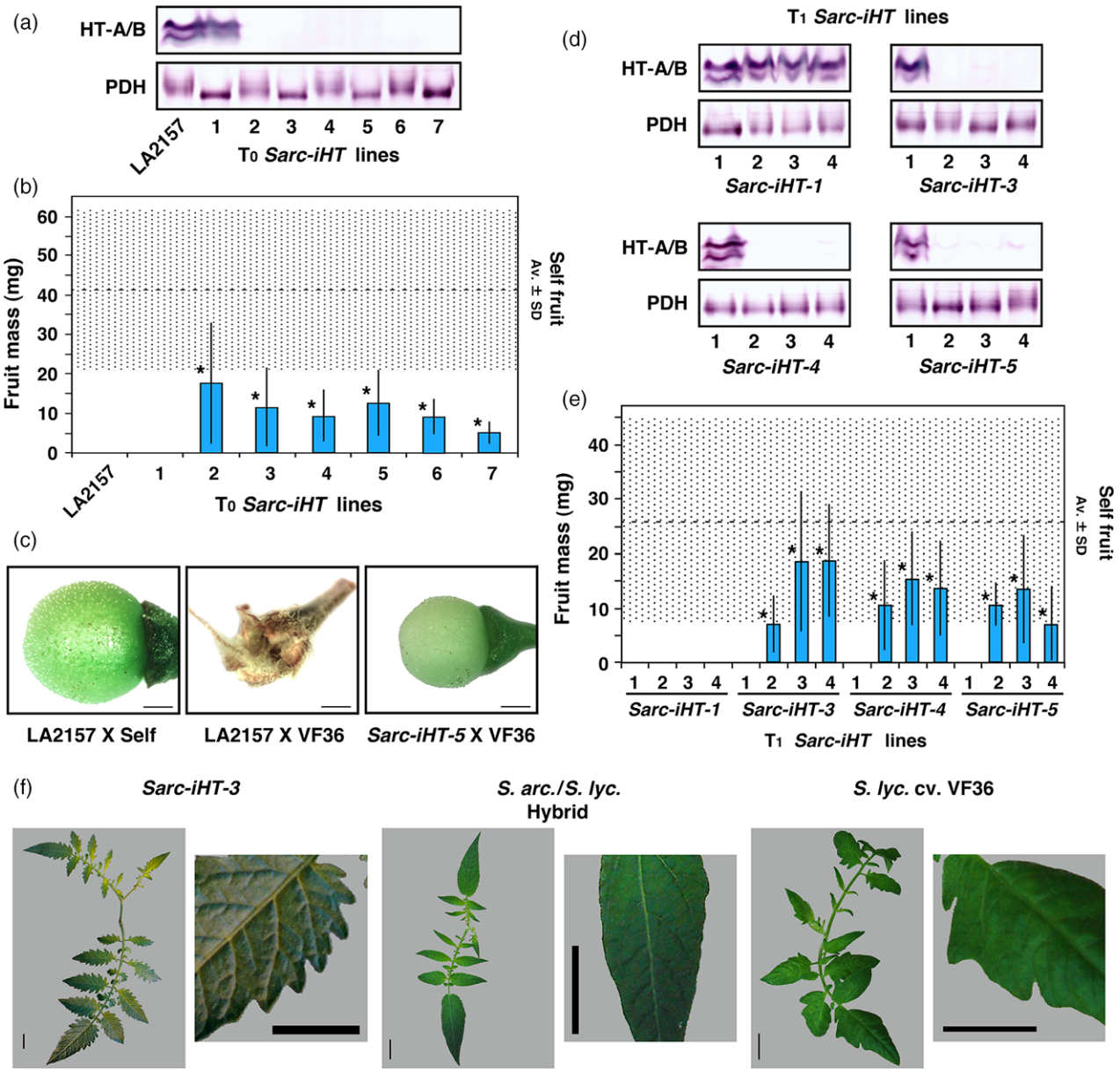

Figure 6. *Solanum lycopersicum* compatibility on *HT* suppressed *Solanum arcanum* LA2157.
(a, d) HT proteins in untransformed *S. arcanum* LA2157, *Sarc-iHT* T_0 and T_1 progeny plants. PDH, loading control.
(b, e) Fruit set 7 days after *S. lycopersicum* cv. VF36 pollination. Shown are mean mass (mg) and SD of developing fruits. No fruits were recovered after pollinating untransformed controls or unsuppressed T_0 and T_1 plants. Stippling, mean mass (mg) and SD of self fruits (i.e., conspecific control). Asterisk, value significantly different from untransformed and unsuppressed controls ($P < 0.01$, one-tailed *T*-test).
(c) Sample images showing a developing control self fruit (left) and results from pollinating untransformed *S. arcanum* LA2157 (center, abscised flower with no fruit) or *HT* suppressed *Sarc-iHT*-5 (right) with pollen from *S. lycopersicum* var. VF36. All images taken 7 days after pollination. Bar = 1 mm.
(f) Leaf morphology in normal *S. arcanum* LA2157 (left) and *S. lycopersicum* cv. VF36 (right) and *Sarc-iHT-3* × *S. lycopersicum* cv. VF36 hybrid plants (center). Bar = 1 cm.

S. lycopersicum pollen tubes, resulted in compatibility and formation of interspecific hybrids. While we obtained only a single *HT* suppressed *S. habrochaites* LA0407 transformant, fruit set segregated with *HT* suppression in self-progeny and when the RNAi construct was crossed into SC *S. habrochaites* LA2860, which also lacks S-RNase expression (Figures 4, 5 and S5). This qualitative effect on *S. lycopersicum* compatibility occurred even though low levels of HT protein were observed in some transformants (Figures 4 and 5). Fruits that formed after pollination of *HT* suppressed *Shab0407* or *Sxhab2860* plants by *S. lycopersicum* contained viable seeds and the resulting plants showed the intermediate leaf morphology expected of hybrids (Figures 4 and 5). *HT* suppressed *S. arcanum*

LA2157 behaved similarly. We observed fruit set in six independent *HT* suppressed T_0 lines and nine T_1 progeny plants (Figure 6). These fruits contained viable seeds as well as inviable seed-like structures. Germinated seeds gave rise to plants with intermediate leaf morphology, as expected (Figure 6).

Together, the results clearly implicate HT proteins in S-RNase-independent interspecific pollen rejection and point to HT-dependent mechanisms in three *Solanum* species. Although the most parsimonious explanation is that the same S-RNase-independent/HT-dependent mechanism operates in all three species, this hypothesis has not been explicitly tested. Recognition of S-RNase-independent/HT-dependent pollen rejection is significant as it provides an entry point for further analysis. As HT proteins by themselves have no known effect on pollen tube growth, we believe that they are necessary, but not sufficient, for this newly recognized pollen rejection mechanism (or mechanisms). We speculate that one or more additional factors are required (i.e., perhaps with functions analogous to S-RNase and 120K). This implies that species such as *S. lycopersicum*, which lack this S-RNase-independent/HT-dependent pollen rejection mechanism, also lack functional versions of these additional factors. Genetic and high-throughput sequencing experiments are underway to identify these factors (Pease *et al.*, 2016a,b). The action of HT proteins in both S-RNase-dependent and S-RNase-independent mechanisms has implications also for understanding compatibility, how it changes, and how it might be manipulated.

We suggest that overall compatibility in *Solanum* results from active pistil-pollen interactions that have additive effects and may be redundant with respect to a specific species pair. Incongruity also has been invoked to explain interspecific incompatibility (Hogenboom, 1973, 1975). This concept describes situations where pollen and pistil are poorly matched, and it surely explains failure of crosses between highly diverged species. Our experiments, however, are better explained by active pollen rejection. For example, developmental studies show that pollen rejection mechanisms are functional late in pistil development and that immature pistils are compatible (Chalivendra *et al.*, 2013). Moreover, as we show, and in contrast to the expectation under the incongruity model, loss of barriers result in compatibility (e.g., in *S. habrochaites* and *S. arcanum*, Figures 4–6), not incompatibility. The evidence in *Solanum* suggests that pistil-side mechanisms present pollination barriers and that pollen-side mechanisms provide resistance. S-RNase-based SI is the best-known example of a pistil-barrier/pollen-resistance system (Takayama and Isogai, 2005), but S-RNase-dependent and S-RNase-independent interspecific pollen rejection also conform to this pattern (Chetelat and De Verna, 1991; Li and Chetelat, 2010; Chalivendra *et al.*, 2013; Li and Chetelat, 2014; Tovar-Méndez *et al.*, 2014; Li and Chetelat, 2015). We infer additivity and redundancy of S-RNase-dependent and S-RNase-independent pistil-side mechanisms from considering results of plant transformation studies in the context of crossing studies of SI and SC accessions. For instance, expressing *S-RNase* and *HT* genes in *S. lycopersicum* results in plants that reject pollen from *S. lycopersicum* and other red-fruited *Solanum* species (Tovar-Méndez *et al.*, 2014), yet SC accessions that lack S-RNase nevertheless reject the same types of pollen. Thus, both RNase-dependent and S-RNase independent pistil-side mechanisms are inferred in the SI progenitors.

The existence of multiple pollen rejection barriers may be both surprising and potentially confusing. Clearly, pistils of wild SI species, such as *S. habrochaites*, *S. arcanum*, and *S. pennellii*, are rarely, if ever, challenged with pollen from *S. lycopersicum.* Nevertheless, this pollen is useful experimentally because, as a derived SC species, it appears to have lost its resistance mechanisms. What in our experimental system appear to be redundant pollen rejection mechanisms may function between different species in a natural context. Redundancy also may complicate experiments because defects in one rejection mechanism do not necessarily result in compatibility. However, individual mechanisms can be identified in a step-wise fashion using experimental materials with reduced complexity (i.e., known to be defective in specific factors, as in the accessions lacking functional S-RNase expression used here).

Our interpretation is that the S-RNase-independent/HT-dependent mechanism indicated by our RNAi experiments is redundant with S-RNase-dependent/HT-dependent pollen rejection in SI progenitors. The results in *S. pennellii* LA0716 suggest additional complexity. We speculate that SI *S. pennellii* pistils express at least three barriers active against *S. lycopersicum* pollen: an S-RNase + HT barrier, an S-RNase-independent/HT-dependent barrier, and a distinct barrier that requires neither S-RNase nor HT protein. The latter barrier is sufficient for *S. lycopersicum* pollen rejection even when the former two barriers are removed. This example of additivity and redundancy gives additional insight into the complexity of interspecific pollen rejection. Moreover, our results suggest that *HT* suppressed *S. pennellii* LA0716 is an appropriate experimental background to investigate a novel pistil-side barrier that requires neither S-RNase nor HT protein.

Our results show that the S-RNase-independent/HT-dependent mechanism is the only mechanism causing rejection of *S. lycopersicum* pollen in SC *S. habrochaites* LA0407 and in *S. arcanum* LA2157. Still, we do not conclude that the corresponding SI progenitors necessarily express only this barrier and the S-RNase-independent/HT-dependent mechanism. For example, SC *S. habrochaites* LA0407 is a more derived SC accession (i.e., compared to other SC accessions) collected about 200 km north of the nearest SI population of *S. habrochaites*, and it appears to

have undergone several additional changes after loss of SI (Broz *et al.*, 2016).

The evident complexity and redundancy of interspecific pollen rejection affects how compatibility changes as mating systems evolve. The transition from SI to SC is common; SC is favored by reproductive assurance and transmission advantage, but SI lineages persist, nevertheless, presumably because genetic diversity allows greater resiliency. Since *S-RNase* is a single gene, loss of S-RNase function is a common route to SC. Moreover, since it has roles in both SI and interspecific pollen rejection, the effects are pleiotropic. Our results show that *HT* genes also have pleiotropic effects in both interspecific and intraspecific pollen rejection, suggesting further linkages between these two processes. Most *Solanum* species express two functionally redundant *HT* genes (i.e., *HT-A* and *HT-B*, Tovar-Méndez *et al.*, 2014), so loss of *HT* function is not expected to be a common route to SC. Consequently, the S-RNase-independent/HT-dependent interspecific pollination barrier should be commonly preserved in recently evolved SC accessions. However, *S. habrochaites* is exceptional, as only *HT-A* is functional in this species (Covey *et al.*, 2010). Broz *et al.* (2016) recently described an accession from the extreme northern part of the *S. habrochaites* range – SC *S. habrochaites* LA1223 – that has mutations in *S-RNase*, *HT-A*, and *HT-B* genes. As expected, this accession accepts pollen from *S. lycopersicum* and other tomato clade species (Broz *et al.*, 2016) due to the progressive loss of redundant pollen rejection mechanisms.

On a practical level, the wider role of HT proteins in interspecific pollen rejection has implications for prebreeding programs aimed at utilizing traits from crop wild relatives. Wild species have great potential for crop improvement, but crosses with crop species are sometimes difficult (Zamir, 2001; Jansky *et al.*, 2013). For example, Villamon *et al.* (2005) described potato relatives in the *Piurana* clade with strong late blight resistance; however, because of strong IRBs, extensive efforts to develop prebreeding lines resulted in few interspecific hybrids with cultivated potato. Our results with *S. habrochaites* LA0407 and *S. arcanum* LA2157 show that *HT* suppression routinely permits production of hybrids with *S. lycopersicum* as the pollen parent. This finding suggests HT protein may be a possible route for pre-breeding interspecific hybrids, either by suppressing HT expression or by identifying natural variants, such as *S. habrochaites* LA1223, that fail to express HT protein.

EXPERIMENTAL PROCEDURES

Plant materials

Solanum arcanum LA2157, *S. habrochaites* LA0407 and LA2860, *S. pennellii* LA0716, and *S. lycopersicum* cultivar VF36 (accession no. LA0490) were obtained from the C. M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu). *Solanum lycopersicum* cv. M82 expressing the *HT-A* gene from *S. pennellii* LA2560 has been described (Tovar-Méndez *et al.*, 2014).

RNAi construct and plant transformation

The CDS sequences of *HT-A* (XM_015204546.1, Sopen12g029190) and *HT-B* (XM_015205372.1, Sopen12g029200) *S. pennellii* LA0716 genes were synthesized as a single 639-bp DNA sequence (www.genscript.com), cloned into pHANNIBAL (Wesley *et al.*, 2001), and transferred to *Agrobacterium tumefaciens* LBA4404. *Solanum arcanum* LA2157, *S. habrochaites* LA0407, and *S. pennellii* LA0716 hypocotyls were transformed using a standard protocol (McCormick, 1991) with minor modifications. Fresh cut hypocotyls from 2-week-old seedlings were co-cultivated for 10 min in a medium supplemented with 40 mg acetosyringone L^{-1}. Selective media contained 100 mg L^{-1} of each kanamycin (plant selection), carbenicillin, cefotaxime, and timentin. Co-cultivation and selective shoot elongation media were supplemented with 2 mg L^{-1} zeatin–riboside, selective shoot elongation medium was supplemented with 0.1 mg L^{-1} zeatin–riboside, and selective root inducing medium was supplemented with 2 mg L^{-1} indole-3-butyric acid.

qRT-PCR analysis of *HT-A/B* gene expression

Total RNA was extracted from *S. pennellii* LA0716 T_0 pistils using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from 2 μg DNase-treated total RNA with (dT)16 and Moloney murine leukemia virus reverse transcriptase (Promega). Protocols for qPCR analysis followed the Real-Time PCR Application Guide (Bulletin 5279; Bio-Rad, Hercules, CA, USA). qRT-PCR analysis was performed using a CFX96 real-time system and SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and the following gene-specific primers (forward and reverse): *HT-A* (5′-GCAAGGGAAATGGTTGAGGCAA-3′ and 5′-TTTTGGGCAACTGCAACCCA-3′, 92% efficiency) and *HT-B* (5′-TGCAAAGGATATAGTTGAGCCTTCACT-3′ and 5′- TGGGCAAGGGCAACGTG-3′, 94% efficiency). Gene expression levels were normalized with actin (XM_015217969.1, Sopen04g005680, 5′-ATGGTCAAGGCTGGGTTCG-3′ and 5′-CAGGGGCAACACGAAGCT-3′, 94% efficiency). qPCR cycling conditions were as recommended for the Supermix reagent. Absence of genomic DNA contamination was confirmed by PCR from total RNA. PCR and dissociation curve analyses confirmed primer specificity in each assay.

Immunoblot analysis of HT proteins

Proteins were extracted from the stigma and style of freshly opened flowers and extract from 1.5 mg fresh weight was separated, blotted, and immunostained as described previously (Tovar-Méndez *et al.*, 2014). HT-A and HT-B proteins were simultaneously detected by an affinity-purified antibody prepared against the peptide LEANEIHNTELNNPTLQKKGGC-amide (21st Century Biochemicals, http://www.21stcenturybio.com/), which was used at a 1:5000 dilution. The anti α-subunit of the mitochondrial pyruvate dehydrogenase (PDH) antibody was a gift from Professor Douglas D Randall, and it was used at 1:50 dilution for protein loading control.

Pollination phenotypes

Pollination phenotypes were determined after analysis of three to 13 crosses. Flowers were emasculated 1 day before opening, and pollinations were performed the next day by covering the stigma with pollen (Chalivendra *et al.*, 2013). Two assays were used. For

the pollen tube length assays, pistils were collected, stained with aniline blue fluorochrome (Biosupplies, Melbourne, Vic., Australia), and imaged as described previously (Covey *et al.*, 2010; Tovar-Méndez *et al.*, 2014). Measurements were made from the stigma to the pollen tube front as well as the point where the longest pollen tubes stopped. Mean style lengths (shown in Figures 2, 3, 5, S3, S5, and S6) were measured at the same time and refer to the distance from the stigma to the base of the style. In *S. arcanum* LA2157 and *S. habrochaites* LA0407 that showed late rejection of *S. lycopersicum* pollen, compatibility was assessed by measuring the ovary (i.e., developing fruit) mass 7 days after pollination. When comparing suppressed plants and controls *F*-tests were used to determine whether variances were equal or unequal and the appropriate one-tailed *T*-test was then used to assess differences, a *P*-value less than 0.01 is reported as significant.

Cullin1 alleles in hybrid plants

A Cullin-1 gene was used to test whether *S. arcanum* and *S. habrochaites* × *S. lycopersicum* hybrids were obtained (Figure S4). Genomic DNA was isolated from control and hybrid plants using the Qiagen DNeasy Plant Mini kit. *Cullin1* alleles were amplified as described (Li and Chetelat, 2010) using primers 5′-CAGGAACGTGAGGGTGAGA-3′ and 5′-ACTCCACAAAAGTAAC CCCTTCA-3′. The amplicon includes the site of a 426-bp deletion present in the *S. lycopersicum* allele compared with *S. arcanum* and *S. habrochaites*.

ACKNOWLEDGEMENTS

We thank the C. M. Rick Tomato Genetics Resource Center staff for supplying seed stocks; Gahyun Park and Brendan Lahm for help with pollinations; Dr. Douglas D. Randall, University of Missouri, for supplying the anti-PDH antibody; Drs Pat Bedinger, Amanda Broz, Roger Chetelat, and Leonie Moyle for helpful comments; and Melody Kroll for proofreading the manuscript. The project was supported by NSF grant MCB 1127059.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *HT-A* and *HT-B* RNAi construct. *HT-A* (Sopen12g029190) and *HT-B* (Sopen12g029200) CDS sequences from *Solanum pennellii* LA0716 were used to simultaneously suppress expression of both genes.

Figure S2. Relative *HT* transcript levels in *Solanum pennellii* LA0716 T_0 HT-RNAi plants.

Figure S3. *Solanum lycopersicum* var. VF36 pollen tube length in pistils of untransformed *S. habrochaites* LA0407 control (filled) and *HT* suppressed *Shab0407-iHT*-1 T_0 (open).

Figure S4. *Cullin1* alleles in hybrid plants. *Cullin1* sequences were amplified from genomic DNA as described (Li and Chetelat, 2010).

Figure S5. S-RNase, pollen tube growth, and leaf morphology in *Solanum habrochaites* LA2860.

Figure S6. *Solanum lycopersicum* var. VF36 pollen tubes in pistils of untransformed *S. arcanum* LA2157 and *HT* suppressed *Sarc-iHT*-3 after 24 h (filled, control; open, *Sarc-iHT*-3).

Table S1. *Solanum lycopersicum* cv. VF36 pollen tube lengths in control and T0 HT-RNAi *S. pennellii* LA0716 styles (Figure 2b).

Table S2. *Solanum lycopersicum* cv. VF36 pollen tube lengths in control and T1 HT-RNAi *S. pennellii* LA0716 styles (Figure 3b).

Table S3. Control and HT-RNAi *Solanum habrochaites* LA0407 fruit mass 7 days after pollination with *S. lycopersicum* cv. VF36 pollen (Figure 4b).

Table S4. *Solanum lycopersicum* cv. VF36 pollen tube lengths in T1 HT-RNAi *S. habrochaites* hybrid (LA0407 × LA2860) styles (Figure 5b).

Table S5. Control and T_0 HT-RNAi *Solanum arcanum* LA2157 fruit mass 7 days after pollination with *S. lycopersicum* cv. VF36 pollen (Figure 6b).

Table S6. T_1 HT-RNAi *Solanum arcanum* LA2157 fruit mass 7 days after pollination with *S. lycopersicum* cv. VF36 pollen (Figure 6e).

REFERENCES

Anderson, M.A., Cornish, E.C., Mau, S.L. *et al.* (1986) Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. *Nature*, **321**, 38–44.

Anderson, M., McFadden, G., Bernatzky, R., Atkinson, A., Orpin, T., Dedman, H., Tregear, G., Fernley, R. and Clark, A.E. (1989) Sequence variability of three alleles of the self-incompatibility gene of *Nicotiana alata*. *Plant Cell*, **1**, 483–491.

Ashman, T.L. and Arceo-Gomez, G. (2013) Toward a predictive understanding of the fitness costs of heterospecific pollen receipt and its importance in co-flowering communities. *Am. J. Bot.* **100**, 1061–1070.

Baek, Y.S., Covey, P.A., Petersen, J.J., Chetelat, R.T., McClure, B. and Bedinger, P.A. (2015) Testing the SI × SC rule: pollen–pistil interactions in interspecific crosses between members of the tomato clade (*Solanum* section *Lycopersicon*, Solanacea). *Am. J. Bot.* **102**, 302–311.

Bedinger, P.A., Chetelat, R.T., McClure, B. *et al.* (2011) Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation. *Sex. Plant Reprod.* **24**, 171–187.

Broz, A., Randle, A., Sianta, S., Tovar-Méndez, A., McClure, B.A. and Bedinger, P. (2016) Mating system transitions in *Solanum habrochaites* impact interactions between populations and species. *New Phytol.* **213**, 440–454.

Chalivendra, S.C., Lopéz-Casado, G., Kumar, A. *et al.* (2013) Developmental onset of reproductive barriers and associated proteome changes in stigma/styles of *Solanum pennellii*. *J. Exp. Bot.* **64**, 265–279.

Chetelat, R.T. and De Verna, J.W. (1991) Expression of unilateral incompatibility in pollen of *Lycopersicon pennellii* is determined by major loci on chromosomes 1, 6, and 10. *Theor. Appl. Genet.* **82**, 704–712.

Covey, P.A., Kondo, K., Welch, L. *et al.* (2010) Multiple features that distinguish unilateral incongruity and self-incompatibility in the tomato clade. *Plant J.* **64**, 367–378.

Eberle, C.A., Anderson, N.O., Clasen, B.M., Hegeman, A.D. and Smith, A.G. (2013) PELPIII: the class III pistil-specific extensin-like *Nicotiana tabacum* proteins are essential for interspecific incompatibility. *Plant J.* **74**, 805–814.

Goldman, M.H., Pezzotti, M., Seurinck, J. and Mariani, C. (1992) Developmental expression of tobacco pistil-specific genes encoding novel extensin-like proteins. *Plant Cell*, **4**, 1041–1051.

de Graaf, B.H.J., Knuiman, B.A., Derksen, J. and Mariani, C. (2003) Characterization and localization of the transmitting tissue-specific PELPIII proteins of *Nicotiana tabacum*. *J. Exp. Bot.* **54**, 55–63.

Hancock, C.N., Kent, L. and McClure, B.A. (2005) The stylar 120 kDa glycoprotein is required for *S*-specific pollen rejection in *Nicotiana*. *Plant J.* **43**, 716–723.

Hardon, J.J. (1967) Unilateral incompatibility between *Solanum pennellii* and *Solanum esculentum*. *Genetics*, **57**, 795–808.

Hogenboom, N.G. (1973) A model for incongruity in intimate partner relationships. *Euphytica*, **22**, 219–233.

Hogenboom, N.G. (1975) Incompatibility and incongruity: two different mechanisms for the non-functioning of intimate partner relationships. *Proc. R. Soc. Lond. B*, **188**, 361–375.

Hua, Z.H. and Kao, T.H. (2006) Identification and characterization of components of a putative *Petunia S*-locus F-box-containing E3 ligase complex involved in *S*-RNase-based self-incompatibility. *Plant Cell*, **18**, 2531–2553.

Huang, S., Lee, H.S., Karunanandaa, B. and Kao, T.H. (1994) Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. *Plant Cell*, **6**, 1021–1028.

Ioerger, T.R., Gohlke, J.R., Xu, B. and Kao, T.H. (1991) Primary structural features of the self-incompatibility protein in Solanaceae. *Sex. Plant Reprod.* **4**, 81–87.

Iwano, M. and Takayama, S. (2012) Self/non-self discrimination in angiosperm self-incompatibility. *Curr. Opin. Plant Biol.* **15**, 78–83.

Jansky, S.H., Dempewolf, H., Camadro, E.L., Simon, R., Zimnoch-Guzowska, E., Bisognin, D.A. and Bonierbale, M. (2013) A case for crop wild relative preservation and use in potato. *Crop Sci.* **53**, 746–754.

Kawata, Y., Sakiyama, F., Hayashi, F. and Kyogoku, Y. (1990) Identification of two essential histidine residues of ribonuclease T2 from *Aspergillus oryzae*. *Eur. J. Biochem.* **187**, 255–262.

Kondo, K., Yamamoto, M., Itahashi, R., Sato, T., Egashira, H., Hattori, T. and Kowyama, Y. (2002) Insights into the evolution of self-compatibility in *Lycopersicon* from a study of stylar factors. *Plant J.* **30**, 143–153.

Kubo, K., Entani, T., Takara, A. *et al.* (2010) Collaborative non-self recognition system in S-RNase-based self-incompatibility. *Science*, **330**, 796–799.

Kubo, K., Paape, T., Hatakeyama, M., Entani, T., Takara, A., Kajihara, K., Tsukahara, M., Shimizu-Inatsugi, R., Shimizu, K.K. and Takayama, S. (2015) Gene duplication and genetic exchange drive the evolution of *S*-RNase-based self-incompatibility in *Petunia*. *Nat. Plants*, **1**, 1–9.

Lewis, D. and Crowe, L.K. (1958) Unilateral interspecific incompatibility in flowering plants. *Heredity*, **12**, 233–256.

Li, W. and Chetelat, R.T. (2010) A pollen factor linking inter- and intraspecific pollen rejection in tomato. *Science*, **330**, 1827–1830.

Li, W. and Chetelat, R.T. (2014) The role of a pollen-expressed *Cullin1* protein in gametophytic self-incompatibility in *Solanum*. *Genetics*, **196**, 439–442.

Li, W. and Chetelat, R.T. (2015) Unilateral incompatibility gene *ui1.1* encodes an *S*-locus F-box protein expressed in pollen of *Solanum* species. *Proc. Natl Acad. Sci. USA*, **112**, 4417–4422.

McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F. and Clarke, A. (1989) Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature*, **342**, 955–957.

McClure, B.A., Gray, J.E., Anderson, M.A. and Clarke, A.E. (1990) Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. *Nature*, **347**, 757–760.

McClure, B.A., Mou, B., Canevascini, S. and Bernatzky, R. (1999) A small asparagine-rich protein required for S-allele-specific pollen rejection in *Nicotiana*. *Proc. Natl Acad. Sci. USA*, **96**, 13548–13553.

McClure, B.A., Cruz-Garcia, F., Beecher, B. and Sulaman, W. (2000) Factors affecting inter- and intraspecific pollen rejection in *Nicotiana*. *Ann. Bot.* **85**, 113–123.

McCormick, S. (1991) Transformation of tomato with *Agrobacterium tumefaciens*. In *Plant Tissue Culture Manual: Fundamentals and Applications* (Lindsey, K., ed.). Dordrecht: Kluwer Academic Publishers, pp. B6/1–B6/9.

Murfett, J., Atherton, T.L., Mou, B., Gasser, C.S. and McClure, B.A. (1994) *S*-RNase expressed in transgenic *Nicotiana* causes *S*-allele-specific pollen rejection. *Nature*, **367**, 563–566.

Murfett, J., Strabala, T.J., Zurek, D.M., Mou, B., Beecher, B. and McClure, B.A. (1996) *S* RNase and interspecific pollen rejection in the genus *Nicotiana*: multiple pollen-rejection pathways contribute to unilateral incompatibility between self-incompatible and self-compatible species. *Plant Cell*, **8**, 943–958.

de Nettancourt, D. (2001) *Incompatibility and Incongruity in Wild and Cultivated Plants*, 2nd edn. Berlin: Springer-Verlag.

O'Brien, M., Kapfer, C., Major, G., Laurin, M., Bertrand, C., Kondo, K., Kowyama, Y. and Matton, D.P. (2002) Molecular analysis of the stylar-expressed *Solanum chacoense* asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in *Nicotiana*. *Plant J.* **32**, 1–12.

Pease, J.B., Guerrero, R.F., Sherman, N.A., Hahn, M.W. and Moyle, L.C. (2016a) Molecular mechanisms of postmating prezygotic reproductive isolation uncovered by transcriptome analysis. *Mol. Ecol.* **25**, 2592–2608.

Pease, J.B., Haak, D.C., Hahn, M.W. and Moyle, L.C. (2016b) Phylogenomics reveals three sources of adaptive variation during a rapid radiation. *PLoS Biol.* **14**, e1002379.

Peralta, I.E., Spooner, D.M. and Knapp, S. (2008) Taxonomy of wild tomatoes and their relatives (*Solanum* sect. *Lycopersicoides*, sect. *Juglandifolia*, sect. *Lycopersicon*; Solanaceae). *Syst. Bot. Monogr.* **84** 1–186.

Puerta, A.R., Ushijima, K., Koba, T. and Sassa, H. (2009) Identification and functional analysis of pistil self-incompatibility factor HT-B of *Petunia*. *J. Exp. Bot.* **60**, 1309–1318.

Rick, C.M. (1982) A new self-compatible wild population of *L. peruvianum*. *Rep. Tomato Genet. Coop.* **32**, 43–44.

Rick, C.M., Kesicki, E., Fobes, J.F. and Holle, M. (1976) Genetic and biosystematic studies on two new sibling species of *Lycopersicon* from interandean Perú. *Theor. Appl. Genet.* **47**, 55–68.

Royo, J., Kunz, C., Kowyama, Y., Anderson, M., Clarke, A.E. and Newbigin, E. (1994) Loss of a histidine residue at the active site of *S*-locus ribonuclease is associated with self-compatibility in *Lycopersicon peruvianum*. *Proc. Natl Acad. Sci. USA*, **91**, 6511–6514.

Takayama, S. and Isogai, A. (2005) Self-incompatibility in plants. *Annu. Rev. Plant Biol.* **56**, 467–489.

Tovar-Méndez, A., Kumar, A., Kondo, K., Ashford, A., Baek, Y.S., Welch, L., Bedinger, P.A. and McClure, B.A. (2014) Restoring pistil-side self-incompatibility factors recapitulates an interspecific reproductive barrier between tomato species. *Plant J.* **77**, 727–736.

Villamon, F.G., Spooner, D.M., Orrillo, M., Mihovilovich, E., Perez, W. and Bonierbale, M. (2005) Late blight resistance linkages in a novel cross of the wild potato species *Solanum paucissectum* (series *Piurana*). *Theor. Appl. Genet.* **111**, 1201–1214.

Wesley, S.V., Helliwell, C.A., Smith, N.A. *et al.* (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581–590.

Williams, J.S., Natale, C.A., Wang, N., Li, S., Brubaker, T.R., Sun, P.L. and Kao, T.H. (2014) Four previously identified *Petunia inflata S*-Locus F-Box genes are involved in pollen specificity in self-incompatibility. *Mol. Plant*, **7**, 567–569.

Zamir, D. (2001) Improving plant breeding with exotic genetic libraries. *Nat. Rev. Genet.* **2**, 983–989.

Zhao, L., Huang, J., Zhao, Z., Li, Q., Sims, T.L. and Xue, Y. (2010) The Skp1-like protein SSK1 is required for cross-pollen compatibility in *S*-RNase-based self-incompatibility. *Plant J.* **62**, 52–63.

