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Restoring pistil-side self-incompatibility factors recapitulates an interspecific reproductive barrier between tomato species

Alejandro Tovar-Méndez[1], Aruna Kumar[1,†], Katsuhiko Kondo[1,‡], Amy Ashford[2], You S. Baek[2], Lillian Welch[2,§], Patricia A. Bedinger[2] and Bruce A. McClure[1,*]

[1]*Division of Biochemistry, University of Missouri-Columbia, 117 Schweitzer Hall, Columbia, MO 65211 USA, and*
[2]*Department of Biology, Colorado State University, Fort Collins, CO 80523-1878 USA*

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*For correspondence (e-mail mcclureb@missouri.edu).
†Present address: Amity Institute of Biotechnology, Amity University, Sector 125, Noida, Uttar Pradesh 201303, India.
‡Present address: Group of Plant Stress Physiology, Institute of Plant Science and Resources, Okayama University, 2-20-1Tyuou, Kurashiki, Okayama 710-0046, Japan.
§Present address: Department of Hematology/Oncology, Oregon Health Sciences Center, R&D 19 Building 103, Room E223, 3710 SW US Veterans Hospital Road, Portland, OR 97239, USA.

SUMMARY

Interspecific reproductive barriers are poorly understood, but are central to the biological species concept. The pre-zygotic barriers between red- and green-fruited species in the tomato clade of the genus *Solanum* provide a model to better understand these barriers in plants. Compatibility usually follows the SI x SC rule: pollen from self-compatible (SC) red-fruited species is rejected on pistils of the predominantly self-incompatible (SI) green-fruited species, but the reciprocal crosses are compatible. This suggests that the interspecific reproductive barrier may be linked to the intraspecific SI mechanism. However, pollen from the SC red-fruited species is also rejected by SC accessions of green-fruited species that lack S-RNase, a key protein expressed in pistils of SI *Solanum* species. Thus, multiple mechanisms may contribute to the barrier between red- and green-fruited species. We tested whether an S-RNase-dependent barrier is sufficient for rejection of pollen from red-fruited species by introducing functional *S-RNase*, *HT-A* and *HT-B* genes from SI species into *Solanum lycopersicum* (cultivated tomato). We found that expressing *S-RNase* in combination with either *HT-A* or *HT-B* in the pistil is sufficient to cause rejection of pollen from all four red-fruited species. Thus, redundant mechanisms must operate side by side to prevent crosses between red- and green-fruited species in the clade, underlining the complexity of interspecific pollination barriers. Our results also have implications for mating system transitions. We suggest that these transitions must occur in a specific sequence, and that the transition from SI to SC also affects interspecific compatibility.

Keywords: interspecific compatibility, unilateral incompatibility, gametophytic self-incompatibility, tomato, mating system, *Solanum*.

INTRODUCTION

Studies of interspecific reproductive barriers are important for crop improvement and for understanding how species diverge and maintain their identity. Wild relatives of crop species often possess adaptations to a wide range of environments; these adaptations may include useful agronomic traits such as resistance to abiotic and biotic stresses. In order to effectively incorporate these valuable traits into crop species, it is important to understand the basis of interspecific reproductive barriers between plant species, as these may limit the use of wild germplasm. The tomato clade is a useful model for studies of interspecific reproductive barriers because a variety of barriers exist between the 12 species, and the compatibility relationships are generally known. Moreover, the clade is probably undergoing active speciation in the highly diverse and fragmented environment of the western slopes of the Andes where the species are endemic (Nakazato *et al.*, 2010).

Failure of interspecific pollination may be attributed to incongruity when pollen and pistil are poorly matched due to evolutionary divergence, as may occur between distantly related species, or incompatibility when the pistil expresses active rejection factors that are not balanced by corresponding pollen resistance factors (McClure *et al.*, 2000). Failure of an interspecific cross may be attributed to either process to a greater or lesser extent, but there is evidence that active processes operate in the tomato clade.

For example, Chalivendra *et al.* (2013) recently showed that pollen from cultivated tomato, *Solanum lycopersicum*, is compatible on immature *S. pennellii* pistils but is rejected by mature pistils. This response is consistent with active pollen rejection, as the immature *S. pennellii* pistil clearly expresses factors needed for pollen germination, pollen tube growth and guidance, while factors required for recognition and rejection of *S. lycopersicum* pollen are added later. However, the identity of these factors and their mechanisms of action are not well known.

Self-incompatibility (SI) systems that prevent inbreeding within a species are the best understood mechanisms for pollen recognition and rejection. As SI species reject self-pollen, out-crossing dominates their mating behavior, favoring genetic diversity. The specificity of pollen rejection in SI is usually controlled by a single locus, called the *S* locus. SI *Solanum* species display S-RNase-based systems in which pistil-expressed S-RNase proteins control the specificity of pollen rejection, and pollen-expressed *S*-locus F-box (SLF) proteins provide for specificity (i.e. compatibility or incompatibility) on the pollen side (Iwano and Takayama, 2012). S-RNases, together with other pistil factors, act as cytotoxins specifically directed against incompatible pollen (McClure *et al.*, 1990). Compatible pollen clearly overcomes rejection, and SLF and other pollen proteins must function as resistance factors although various mechanisms have been proposed to account for this (Goldraij *et al.*, 2006; Kubo *et al.*, 2010). S-RNase-based SI is thus an example of an active pollen rejection mechanism, and is developmentally controlled; immature pistils are competent to support pollination, and SI functions are expressed when the pistil is mature. Pollen recognition and rejection are exquisitely specific in SI: pollen is rejected only when the pollen *S*-haplotype is identical to either of the two *S*-haplotypes in the diploid pistil. Modifier genes are also required for both pistil and pollen SI functions but do not contribute to *S*-specificity *per se* (McClure *et al.*, 1999; Tsukamoto *et al.*, 2003; Hancock *et al.*, 2005; Hua and Kao, 2006; Puerta *et al.*, 2009; Zhao *et al.*, 2010; Jiménez-Durán *et al.*, 2013). On the pistil side, modifier genes, including HT proteins, the 120 kDa glycoprotein and the protease inhibitor NaStep, have been directly implicated in *S*-haplotype-specific pollen rejection using RNAi or antisense experiments in *Nicotiana* (McClure *et al.*, 1999; O'Brien *et al.*, 2002; Hancock *et al.*, 2005; Jiménez-Durán *et al.*, 2013). As pollen-expressed SLF genes are thought to function in an SCF ubiquitin ligase complex, pollen modifier genes include at least those genes encoding a Skp1 homolog (Zhao *et al.*, 2010), a Cullin1 (Hua and Kao, 2006) and/or S-RNase binding proteins (Hua and Kao, 2006).

Most *Solanum* species are SI, but SI has been lost multiple times in the tomato clade alone (Rick and Chetelat, 1991; Igic *et al.*, 2008; Bedinger *et al.*, 2011). Kondo *et al.* (2002a) suggested that loss of SI in the four red- or orange-fruited SC species (*S. lycopersicum*, *S. pimpinellifolium*, *S. galapagense* and *S. cheesmaniae*; hereafter referred to as 'red-fruited' species) is associated with mutations in *HT-A*, *HT-B* and/or *S-RNase* genes. Two green-fruited SC species, *S. chmielewskii* and *S. neorickii*, represent independent losses of SI. SC populations of otherwise SI species are also known. For example, the SC *S. arcanum* accession LA2157 expresses catalytically inactive S-RNase (Kowyama *et al.*, 1994; Royo *et al.*, 1994), while SC *S. pennellii* accession LA0716 and SC accessions of *S. habrochaites* do not express S-RNase (Covey *et al.*, 2010; Chalivendra *et al.*, 2013). It is noteworthy that these examples represent defects in pistil-side SI factors that contribute to pollen rejection. As the balance between pistil rejection functions and pollen resistance determines overall compatibility, such changes may have consequences beyond the shift from SI to SC.

Interspecific pollination barriers act at the species level, and thus recognition and rejection are inherently less specific than in SI. Moreover, there is evidence of considerable complexity, because multiple mechanisms contribute to interspecific reproductive barriers, even between a single pair of species. However, there is also evidence that some interspecific pollination barriers in Solanaceae are related to SI. Unilateral incompatibility (UI) is an interspecific relationship on which pollinations are only compatible in one direction. It often occurs between SI species and their SC relatives, and often follows the SI x SC rule (i.e. SI plants reject pollen from SC species, but the reciprocal cross is compatible), suggesting that SI factors participate in this type of UI (Lewis and Crowe, 1958). This type of UI is common in the tomato clade. Pollen from red-fruited SC *S. lycopersicum* is rejected by green-fruited SI relatives, such as *S. habrochaites* and *S. pennellii*. Genetic studies of these systems show major-effect QTLs on both the pistil and pollen sides that coincide with the *S* locus (Chetelat and Deverna, 1991; Bernacchi and Tanksley, 1997). The pollen-side UI factor encoded by *ui6.1* is a Cullin1 protein that is similar to a protein from *Petunia* that has been implicated in SI (Hua and Kao, 2006; Li and Chetelat, 2010). Furthermore, the product of *ui6.1* only functions when the gene is expressed in conjunction with *ui1.1*, a distinct pollen UI QTL located at the *S* locus (Li and Chetelat, 2010; Li *et al.*, 2010). An additional QTL that contributes to pistil-side UI (Bernacchi and Tanksley, 1997) includes the *HT* gene locus (Covey *et al.*, 2010).

In another solanaceous genus, *Nicotiana*, SI *N. alata* rejects pollen from SC relatives, including *N. plumbaginifolia*, *N. longiflora*, *N. tabacum* and *N. glutinosa*. Direct manipulation of *S-RNase* and *HT* expression in *Nicotiana* causes gain or loss of specific UI responses (Murfett *et al.*, 1996; Hancock *et al.*, 2005), providing evidence that redundant rejection mechanisms may contribute to UI between a single pair of species. For example, both

S-RNase-dependent and S-RNase-independent mechanisms in *N. alata* contribute to rejection of pollen from *N. tabacum* (Murfett *et al.*, 1996). S-RNase-dependent and S-RNase-independent mechanisms also exist side by side in *S. pennellii* (Covey *et al.*, 2010; Chalivendra *et al.*, 2013). Redundant mechanisms may confound efforts to elucidate interspecific pollination barriers, because loss of pistil barriers for one mechanism does not necessarily alter compatibility if a redundant mechanism is sufficient for rejection.

The tomato clade is ideal for elucidating connections between SI and UI as well as identifying unrelated interspecific reproductive barriers. Figure 1 shows compatibility and incompatibility relationships between SC *S. lycopersicum* and some of its wild relatives. Crosses between *S. lycopersicum* and the other red-fruited SC species are fully compatible (Figure 1, right). All four red-fruited species lack functional *S-RNase* and *HT* genes (Kondo *et al.*, 2002a). For example, the *HT-A* and *HT-B* genes in these species display nonsense mutations and either no transcript is accumulated (*HT-B*) or a transcript is expressed that results in a severely truncated peptide (HT-A) (Kondo *et al.*, 2002a). By contrast, the green-fruited species show UI: *S. lycopersicum* accepts pollen from green-fruited species, but the reciprocal pollinations are incompatible (Figure 1, left). Figure 1 also shows some complexities of interspecific compatibility and departures from the SI x SC rule. Kondo *et al.* (2002a) reported defects in *S-RNase* and *HT* gene expression in the closely related green-fruited SC species *S. neorickii* and *S. chmielewskii*, indicating loss of pistil-side SI function, but UI between *S. lycopersicum* and these species is still observed. Thus, UI between *S. neorickii* and *S. chmielewskii* and the red-fruited species is S-RNase-independent. Likewise, Figure 1 shows UI between *S. lycopersicum* and two SC accessions of the largely SI species *S. pennellii* (accession LA0716) and *S. habrochaites* (accession LA0407). These SC accessions do not express *S-RNase* (Covey *et al.*, 2010; Chalivendra *et al.*, 2013), and therefore also reject pollen from red-fruited species by S-RNase-independent mechanisms. A simplistic interpretation of the SI x SC rule is that an intact SI system is necessary and sufficient for UI. For instance, if SI and UI were mechanistically identical, loss of SI would result in concomitant loss of UI; that is why, SI x SC rule exceptions have been used as evidence to suggest that SI and UI are unrelated (Hogenboom, 1972; de Nettancourt, 1997). However, such examples may be equally explained by the alternative hypothesis that pistils of the SI green-fruited species express redundant pollen rejection mechanisms (i.e. S-RNase-dependent and S-RNase-independent mechanisms, either of which is sufficient for pollen rejection), and that pollen from the red-fruited species does not express resistance factors for either mechanism.

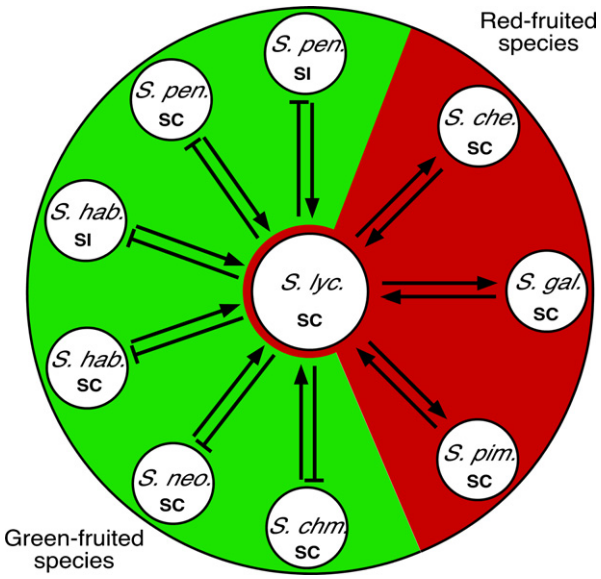

Figure 1. *S. lycopersicum* interspecific compatibilities.
Interspecific compatibilities between *S. lycopersicum* and wild tomato species used in this study (Bedinger *et al.*, 2011). Arrows, compatible pollinations; barred lines, incompatible pollinations. *S. lycopersicum* is compatible with all SC red-fruited species, but shows UI with green-fruited species. Abbreviations: *S. che.*, *S. cheesemaniae*; *S. gal.*, *S. galapagense*; *S. pim.*, *S. pimpinellifolium*; *S. chm.*, *S. chmielewskii*; *S. neo.*, *S. neorickii*; *S. hab.*, *S. habrochaites*; *S. pen.*, *S. pennellii*.

We tested the hypothesis that S-RNase-dependent UI is sufficient to form an interspecific reproductive barrier between red- and green-fruited species by re-introducing the functional pistil-side SI genes *S-RNase*, *HT-A* and *HT-B* into *S. lycopersicum*, and testing the effects on pollen from tomato relatives. We found that restoration of these factors was sufficient to recapitulate a pistil-side interspecific UI barrier, and that both *S-RNase* and *HT* genes are required. As restoring a pistil barrier in a species whose pollen cannot overcome it results in self-sterility, our results further show that mating system transitions must progress with loss of pistil-side function occurring first. This, together with the linkage between SI and UI, suggests that mating system transitions may have effects on compatibility between species as well as within species.

RESULTS

The experimental model

The gain-of-function experiment tested whether an S-RNase-dependent mechanism is sufficient for unilateral incompatible pollen rejection in the pistil. Figure 2 shows the experimental strategy. The hypothesis was that pistil-side SI factors (i.e. S-RNase and HT proteins) create a specific UI barrier similar to the natural barrier between red- and green-fruited tomato species because the former lack appropriate pollen resistance to UI barriers.

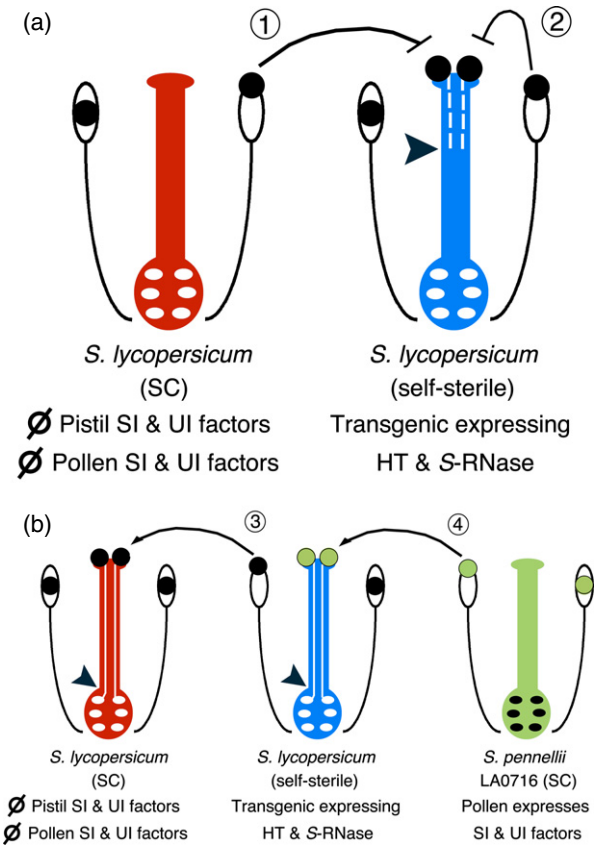

Figure 2. Experimental approach.
Pistils and anthers of transgenic plants (blue) and untransformed testers *S. lycopersicum* (red) and *S. pennellii* accession LA0716 (green) are shown. The hypothesized extents of incompatible pollen tube growth (dashed lines) and compatible pollen tube growth (solid lines) are indicated (arrow heads).
(a) Functional *S-RNase* and *HT* genes from SI tomato relatives were introduced into *S. lycopersicum* (red). Transgenic pistils expressing S-RNase and HT proteins (blue) were hypothesized to reject pollen from untransformed *S. lycopersicum* (1) and to be self-sterile (2), as *S. lycopersicum* pollen does not express appropriate S-RNase resistance factors.
(b) Specificity tests. A specific UI response is indicated by the ability to reject one viable type of pollen while accepting other types. Arrow (3) indicates the viability of pollen from plants expressing S-RNase and HT proteins on untransformed *S. lycopersicum* pistils. Arrow (4) indicates the competence of pistils to support pollen tube growth tested with pollen from *S. pennellii* accession LA0716, which is resistant to S-RNase.

S. lycopersicum and other red-fruited species have defects in both *S-RNase* and *HT* genes, so functional genes were cloned from SI species. Previous experiments tested the effects of S_6*-RNase* from SI *S. arcanum* accession LA2163 when expressed in *S. lycopersicum* cv. Ailsa Craig (Kondo *et al.*, 2002b), and it was convenient to add functional *HT-A* and *HT-B* genes from *S. pennellii* accession LA2560 to cv. M82 to test the effects of pyramiding the transgenes. We hypothesized that recapitulating pistil-side rejection in the absence of corresponding pollen resistance causes self-sterility as well as rejection of pollen from untransformed *S. lycopersicum* (Figure 2a). Control crosses to untransformed *S. lycopersicum* tested whether self-sterility resulted from lack of pollen viability (Figure 2b, left). Self-sterility in these experiments should not be confused with SI. SI is characterized by *S*-haplotype-specific pollen rejection, and pollen from SI species is resistant to any S-RNase apart from self S-RNase (McClure, 2009; Iwano and Takayama, 2012). Species-level pollen rejection in UI is not *S*-haplotype-specific, probably as a result of a complete lack of pollen resistance to all S-RNases (Murfett *et al.*, 1996; Beecher *et al.*, 2001). Thus, the species-level specificity test for the recapitulated UI barrier utilized pollen that shows functional S-RNase resistance, i.e. pollen from *S. pennellii* accession LA0716 (Figure 2b, right).

S-RNase *or* HT *genes alone are insufficient to recapitulate a pistil UI barrier*

S_6-RNase from SI *S. arcanum* accession LA2163 was previously cloned, and its expression in *S. lycopersicum* has no effect on pistil compatibility (Kondo *et al.*, 2002b); numerous self-pollen tubes reach the base of the style in plants expressing S_6-RNase (Figure S1). In most *Solanum* species, there are two *HT* genes, *HT-A* and *HT-B.* We expressed the *HT-A* and *HT-B* genes from SI *S. pennellii* accession LA2560 separately in *S. lycopersicum*, and also found no effect on compatibility (Figure S1). *S. lycopersicum* cv. VF36 pollen was used as a tester throughout this study as it flowered more dependably than other cultivars.

Pyramiding HT-A *or* HT-B *genes with* S-RNase *recapitulates a specific pistil UI barrier*

To test the effects of expressing *HT* transgenes in combination with S_6*-RNase*, we crossed appropriate transgenic plants and analyzed T_1 and T_2 progeny. We found that HT protein expression is lower in progeny plants than in T_0 plants (Figure S2); however, in combination with S_6*-RNase*, the levels of HT-A protein are sufficient to change the pollination phenotype. Figure 3 shows that plants expressing both HT-A and S_6-RNase display a specific pistil UI barrier. For example, T_1 plants HTA/S6-2, -3 and -4 reject self or *S. lycopersicum* cv. VF36 pollen and accept pollen from *S. pennellii* accession LA0716 (Figure 3a–c). A specific UI response is indicated because pollen from the two species behaves differently: *S. lycopersicum* pollen is rejected, but pollen from *S. pennellii* accession LA0716 is not. T_1 plants HTA/S6-1 and -5 did not inherit the *HT-A* transgene and do not display UI (Figure 3a–c). As the transgene promoter is expressed late in pistil development, it is possible to generate T_2 plants by pollinating immature T_1 buds. Results are shown for six T_2 progeny of plant HTA/S6-3. T_2 plants HTA/S6-6 and -7 inherited no transgenes or a single transgene and accept *S. lycopersicum* pollen. In contrast, plants HTA/S6-8–11 inherited both transgenes and show the recapitulated UI barrier (Figure 3a–c and Table S1). The incompatible self or *S. lycopersicum* cv. VF36 pollen tubes

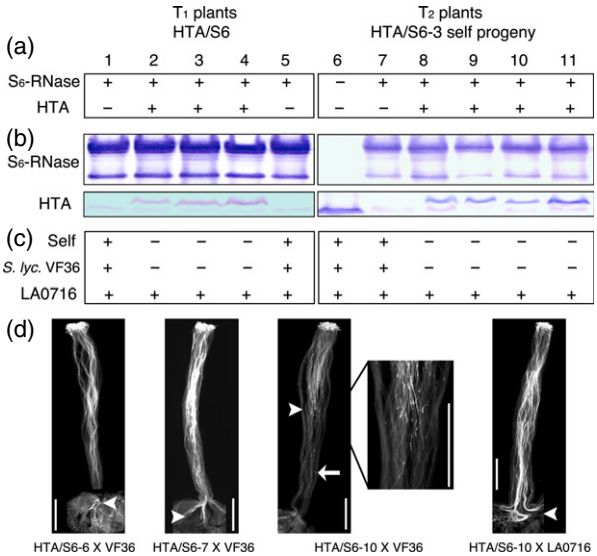

Figure 3. Recapitulating UI using HT-A and S_6-RNase (HTA/S6).
Plants HTA/S6-1–5, T_1 progeny from HTA-3 × S6 crosses; plants HTA/S6-6–11, T_2 progeny after forced selfing of plant HTA/S6-3.
(a) The transgene presence (+) or absence (−) was determined by PCR.
(b) Immunostained HT-A and S_6-RNase proteins in pistil extracts.
(c) Pollination summary scored after 24 h. Incompatible (−), few or no pollen tubes at style base. Compatible (+), > 20 pollen tubes at style base.
(d) Typical pollen tube results. Scale bars = 1 mm. Inset, enlargement showing incompatible pollen tube tips. The arrowheads indicate where most pollen tubes stop. The arrow indicates the longest pollen tube. Full pollination data are shown in Table S1.

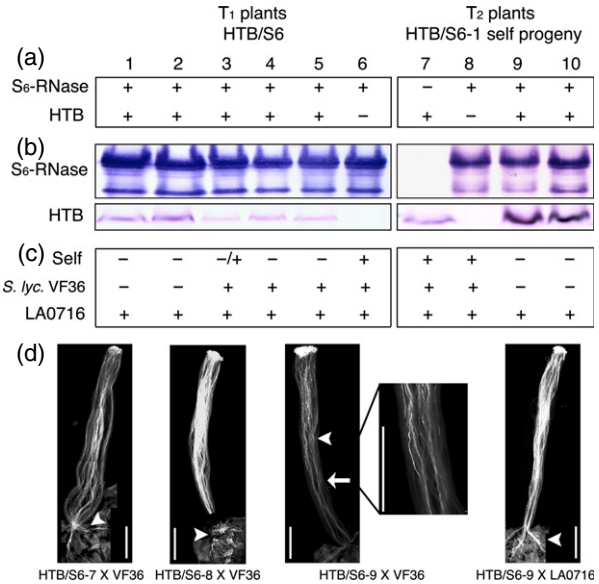

Figure 4. Recapitulating UI using HT-B and S_6-RNase (HTB/S6).
Plants HTB/S6, T_1 progeny from HTB-3 × S6 (HTB/6-1–3) or HTB-4 × S6 (HTB/S6-4–6) crosses. Plants HTB/S6-7–10, T_2 progeny after forced selfing of T_1 plant HTB/S6-1.
(a) PCR genotyping showing the presence (+) or absence (−) of transgenes.
(b) Immunostained HT-B and S_6-RNase proteins in pistil extracts.
(c) Pollination summary as in Figure 3. A plant with intermediate levels of HT-B was scored as + in some pollinations and - in others. Mixed results are shown as −/+.
(d) Typical pollen tube staining results on recapitulated UI pistils as in Figure 3. Scale bars = 1 mm. Inset, enlargement showing incompatible pollen tube tips. Full pollination data are shown in Table S2.

typically penetrate only approximately 2.5 mm, and display intense callose staining near the tip, which is characteristic of rejection (e.g. plant HTA/S6-10; Figure 3d).

Plants expressing both *HT-B* and *S_6-RNase* also show a specific UI barrier (Figure 4 and Table S2). We again observed lower HT protein levels in progeny plants than in T_0 plants (Figure S2). T_1 HTB/S6 plants expressing high levels of both HT-B and S_6-RNase protein show a strong and specific UI barrier by rejecting both *S. lycopersicum* cv. VF36 pollen and self pollen while remaining fully compatible with pollen from SC *S. pennellii* accession LA0716 (plants HTB/S6-1 and -2; Figure 4 and Table S2). Plants expressing lower levels of HT-B protein show intermediate UI, consistent with a threshold effect (plants HTB/S6-3, -4 and -5; Figure 4), and a plant that did not inherit the *HT-B* transgene expressed no HT-B and showed no UI barrier (plant HTB/S6-6, Figure 4). Plants expressing low levels of HT-B usually show more than 20 *S. lycopersicum* cv. VF36 pollen tubes at the base of the style, but usually show fewer than 20 self-pollen tubes at the base of the style (scored as + or −, respectively, in Figure 4c). Both sources of *S. lycopersicum* pollen (i.e. self-pollen and pollen from untransformed *S. lycopersicum* cv. VF36) show dramatically reduced numbers of pollen tubes at the base of the style compared to pollen from SC *S. pennellii* accession LA0716. This is indicative of a weak UI barrier that allows poor but nevertheless significant penetration by *S. lycopersicum* pollen (Table S2 and Figure S3). Plant HTB/S6-3, with very low HT-B expression, shows the weakest UI response. One of the highly expressing HTB/S6 T_1 plants was self-pollinated at the immature stage, and yielded T_2 plants segregating for the transgenes. Again, only plants expressing both transgenes show a specific UI barrier (plants HTB/S6-7 and -8 versus plants HTB/S6-9 and -10; Figure 4).

Together, the results in Figures 3 and 4 show that pyramiding transgenes from various SI species in order to express both HT proteins and S-RNase is sufficient to recapitulate a pistil-side UI barrier and cause rejection of pollen from SC *S. lycopersicum.* As predicted (Figure 2), plants that are otherwise SC but expressing rejection factors in the pistil display self-sterility and reject pollen from untransformed *S. lycopersicum*. Moreover, both HT-A and HT-B are functional in this UI system as either protein causes specific rejection of *S. lycopersicum* pollen.

The HT/S6 UI barrier distinguishes pollen from red-fruited and green-fruited tomato species

We used pollen from the three other red-fruited SC species (*S. cheesmaniae* accession LA0522, *S. galapagense*

accession LA0438 and *S. pimpinellifolium* accession LA3798) and four green-fruited SC or SI species (SC *S. chmielewskii* accession LA1316, SC *S. neorickii* accession LA4023, SI *S. habrochaites* accession LA1777 and SI *S. pennellii* accession LA2560) as a further test of species-level specificity. Plants expressing only HT-A, HT-B or S_6-RNase accept pollen from all seven sources (Table 1 and Figures S4–S6). However, plants expressing either HT protein plus S_6-RNase (HT/S6 plants) specifically reject pollen from the red-fruited species but accept pollen from both SC and SI green-fruited species (Table 1, solid box). Figure 5 shows that, after 24 h, pollen tubes from red-fruited species typically penetrate approximately 2–3 mm, and that compatible pollen tubes from any of the green-fruited species reach the ovary (approximately 5 mm; Figure 5 and Tables S3 and S4). This pollen tube length is similar but longer than the *S. lycopersicum* pollen tubes penetrating SI *S. pennellii* accession LA2560 or SI *S. habrochaites* accession LA1777, but shorter than expected in a 'late UI' response that is observed in some SC green-fruited species, in which pollen tubes transverse 60–70% of the style (Covey *et al.*, 2010).

DISCUSSION

S-RNase-dependent UI is sufficient as an interspecific reproductive barrier between wild tomato species

Molecular studies of intraspecific SI systems have advanced more than studies of UI, partly because the *S*-haplotype-specificity of pollen rejection simplifies phenotypic analysis and because the genetics of the well-studied SI systems are known. Within a species, compatibility is the default, and *S*-haplotype-specific pollen rejection may be regarded as a mechanism superimposed on this compatibility to enhance outcrossing. Interspecific pollen rejection is less tractable because compatibility is not necessarily the default and pollen may fail to reach the ovary for many reasons. Thus, gain-of-function experiments that transfer candidate interspecific barrier genes into otherwise SC species are especially helpful for identifying active interspecific pollen rejection mechanisms.

Our results show that introducing functional *S-RNase* and *HT* genes from green-fruited SI species into *S. lycopersicum* causes rejection of pollen from all four red-fruited tomato species (Figures 3–5 and Table 1). The common ancestor of the entire tomato clade was probably SI (Spooner *et al.*, 2005; Igic *et al.*, 2008), and therefore expressed a full complement of functional SI factors. Our gain-of-function experiment restored pistil-side functions lost in the transition to SC in the red-fruited group (Kondo *et al.*, 2002a). Previous studies showed that S-RNase alone is not sufficient for self-pollen rejection in *S. lycopersicum* (Kondo *et al.*, 2002b) and this is now understood on the basis of the requirement for both S-RNase and HT proteins. Similar results were obtained in *Nicotiana*, in which where expression of S-RNase alone in *N. plumbaginifolia*, a species that does not express HT-A- or HT-B-like proteins, does not cause rejection of *N. plumbaginifolia* pollen, but pollen rejection does occur in *N. plumbaginifolia* x SC *N. alata* hybrids (Murfett *et al.*, 1996). In these *Nicotiana* experiments, the identity of the non-S-RNase factors supplied from the SC *N. alata* background was not deter-

Table 1 The recapitulated HT/S6 barrier distinguishes red-fruited and green-fruited tomato relatives

	Red-fruited			Green-fruited				
	SC pollen						SI pollen	
	S. che. LA0522	*S. gal.* LA0438	*S. pim.* LA3798	*S. chm.* LA1316	*S. neo.* LA4023	*S. pen.* LA0716	*S. hab.* LA1777	*S. pen.* LA2560
Pistil								
S. lyc. VF36	+	+	+	+	+	+	+	+
S6	+	+	+	+	+	+	+	+
HTA-3	+	+	+	+	+	+	+	+
HTB-3	+	+	+	+	+	+	+	+
HTA/S6	–	–	–	+	+	+	+	+
HTB/S6	–	–	–	+	+	+	+	+
S. hab. LA1777	–	–	–	–	–	+	SI	+
S. pen. LA2560	–	–	–	–	–	+	+	SI

Plants expressing single *HT-A*, *HT-B* or *S_6-RNase* transgenes and HTA/S6 and HTB/S6 plants were pollinated as shown. *S. lycopersicum* cv. VF36 and SI accessions of *S. habrochaites* and *S. pennellii* were used as positive and negative controls. Pollination phenotypes: –, incompatible; +, compatible. Abbreviations: *S. che.*, *S. cheesemaniae*; *S. gal.*, *S. galapagense*; *S. pim.*, *S. pimpinellifolium*; *S. chm.*, *S. chmielewskii*; *S. neo.*, *S. neorickii*; *S. hab.*, *S. habrochaites*; *S. pen.*, *S. pennellii*.
Full results are shown in Table S3.

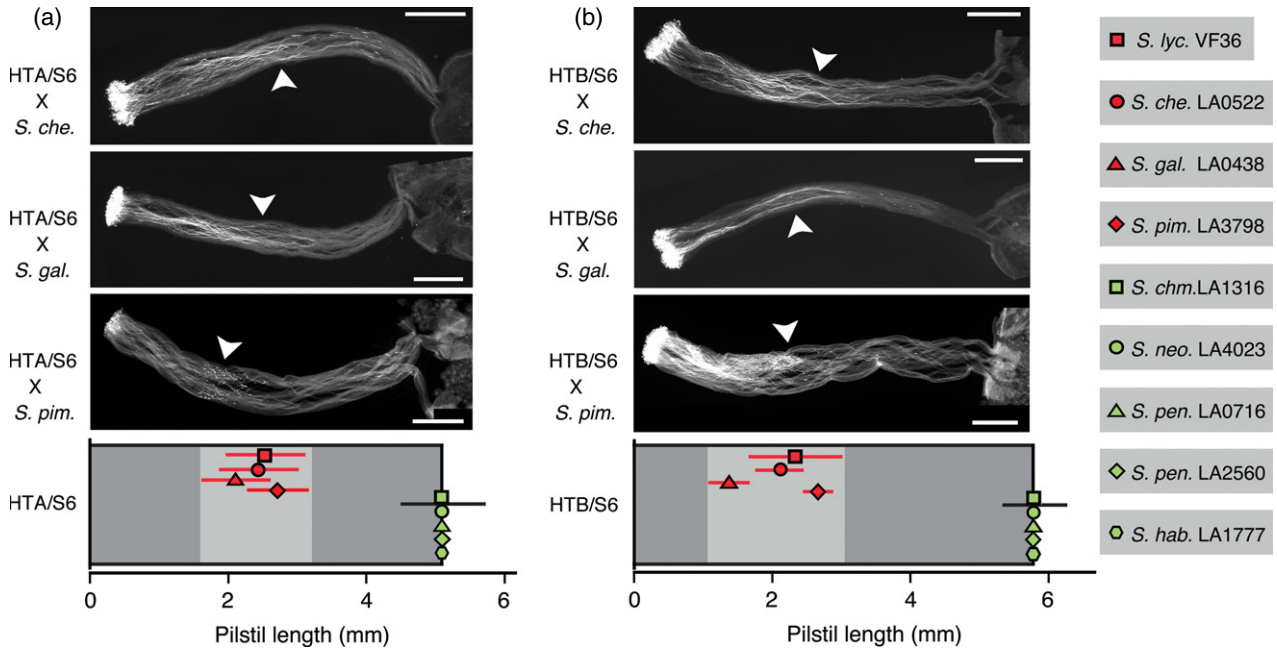

Figure 5. The recapitulated UI barrier distinguishes red-fruited and green-fruited tomato relatives.
(a) HTA/S6 plants.
(b) HTB/S6 plants.
Top, typical images showing rejected pollen tubes from red-fruited species. Scale bar = 1 mm. Bottom, results from all pollinations showing the mean position (± SD) at which growth of most pollen tubes from red-fruited species (red symbols) stopped compared to green-fruited species (green symbols). The symbols indicate the mean pistil length, the horizontal lines show variability. Full results are shown in Table S3. The lengths of the longest pollen tubes are shown in Table S4. Further pollen tube images for red-fruited species are shown in Figures S4–S6.

mined, although HT-B was shown to be required (Hancock *et al.*, 2005).

The requirement for both S-RNase and HT proteins suggests mechanistic similarity between S-RNase-dependent SI and UI, at least in terms of pistil-side function. However, it should be stressed that the two mechanisms are not identical. Crucially, SI is highly specific, and a given S-RNase only causes rejection of one *S*-haplotype, while the HT/S6 UI barrier operates at the species level and is inherently less specific. Because of this difference in specificity, we were able to recapitulate a UI barrier using genes from two SI species and testing for rejection of pollen from four red-fruited species. This difference in specificity has also been observed in *Nicotiana* (Murfett *et al.*, 1996; Beecher *et al.*, 2001), and there may also be other differences between S-RNase-dependent SI and UI (Hancock *et al.*, 2005).

It is noteworthy that Figures 3 and 4 show that HT-A and HT-B function redundantly in the reconstructed HT/S6 UI barrier. O'Brien *et al.* (2002) used RNAi to suppress expression of *HT-A* and *HT-B* in the SI potato relative *S. chacoense*, and concluded that only HT-B is required for SI. However, we recently discovered that the SI tomato species *S. habrochaites* expresses only *HT-A* (Covey *et al.*, 2010). Although this apparent conflict remains unresolved, the transformation system used here may be used in future studies addressing the function of either HT-A or HT-B.

The recapitulated HT/S6 UI barrier mirrors the natural barrier that separates the red-fruited tomato species from most of the green-fruited tomato species (Figure 5 and Table 1). Pollen from red-fruited species appear to entirely lack resistance to S-RNase-dependent UI, i.e. pollen from all red-fruited species is rejected by pistils of any SI green-fruited species, regardless of *S*-haplotype, and is also rejected by HT/S6 plants. A similar rejection mechanism for all red-fruited species is indicated because, in each case, rejection requires both HT proteins and S-RNase (Table 1). The growth of compatible SI *S. pennellii* accession LA2560 and SI *S. habrochaites* accession LA1777 pollen tubes to the ovary in HT/S6 plants is also as expected, as SI pollen rejection is *S*-haplotype-specific (i.e. pollen is resistant to rejection by non-self S-RNases). The growth of SC *S. pennellii* accession LA0716 pollen tubes is probably due to intact pollen resistance to S-RNase-based pollen rejection, as this accession retains the compatibility with SI *S. pennellii* accessions.

Table 1 illustrates the complexities of interspecific reproductive barriers that arise from redundancy and species-level specificity. Clearly, expressing the HT/S6 barrier in *S. lycopersicum* is sufficient to cause rejection of pollen from red-fruited species (Table 1, HT/S6, solid box), and this is clear evidence that SI and UI share common factors. Nevertheless, it is equally clear that pollen from red-fruited

species is also rejected by SC *S. pennellii* accession LA0716, an accession that does not express S-RNase (Covey *et al.*, 2010; Chalivendra *et al.*, 2013). Thus, the S-RNase-dependent and S-RNase-independent rejection mechanisms active in SI accessions (e.g. SI *S. pennellii* accession LA2560) are redundant for pollen from red-fruited species. In contrast, pollen from *S. chmielewskii* and *S. neorickii* is resistant to the recapitulated HT/S6 barrier. However, SI pistils reject pollen from these species (Table 1, dashed box), probably due to one or more S-RNase-independent mechanisms. At present, it is not known whether the same S-RNase-independent rejection mechanism acts on pollen from red-fruited species as well as *S. chmielewskii* and *S. neorickii*. In any case, both S-RNase-dependent and S-RNase-independent rejection mechanisms entail pistil-side pollen rejection and pollen-side resistance. Moreover, pollen-side resistance may be direct or indirect. For example, in S-RNase-based SI in Solanaceae, both the collaborative S-RNase degradation model (Kubo *et al.*, 2010) and the compartmentalization model (Goldraij *et al.*, 2006) postulate the involvement of factors that confer pollen resistance. In the former model, the S-RNase/SLF protein interaction leads directly to resistance by S-RNase degradation, while resistance is indirect and arises from S-RNase sequestration in the latter model.

Changes in intraspecific and interspecific compatibility evolve in stages, with loss of pistil function as a necessary first step

The SC red-fruited tomato species possess mutations in multiple genes expressed in both pistil and pollen that affect S-RNase-based pollen rejection. It is not clear from these data alone how these species, which are interfertile, became reproductively isolated from the rest of the clade. Li and Chetelat (2010) recently reported that most accessions of the red-fruited tomato species display mutations in the *ui6.1* gene encoding CUL1, and proposed that CUL1 forms part of a pollen resistance mechanism for S-RNase-based UI between red-fruited SC species and green-fruited SI species. Mutations in the *ui6.1* gene, leading to loss of pollen resistance, may have resulted in UI between the ancestor of the red-fruited clade and the bulk of the green-fruited tomato clade. Li and Chetelat (2010) hypothesized that a *ui6.1* gene mutation may become fixed only in a background where the corresponding pistil factors are not functional, as lack of pollen resistance would otherwise cause sterility. Here, we show that restoring functional pistil-side SI factors (i.e. *HT* and *S-RNase* genes) in red-fruited SC *S. lycopersicum* results in self-sterility (Figures 3 and 4). Thus, if a loss-of-function mutation occurred on the pollen side while pistil-side function was intact, the mutation would not persist because it would not be transmissible. We infer that loss-of-function shifts from SI to SC in Solanaceae are mechanistically constrained to occur in sequence, with loss of pistil-side function occurring first. This inference is supported by the absence of known SC tomato species or populations that lack pollen SI function but retain pistil function. In contrast, SC mutations affecting only the pistil side are known; for example, *S. pennellii* accession LA0716 and *S. arcanum* accession LA2157 display S-RNase mutations but produce pollen that functions on pistils of conspecific SI accessions. Loss of individual *SLF* genes may be an exception to this rule if they result in pollen rejection on some but not all pistil S-RNase haplotypes (Kubo *et al.*, 2010).

In summary, our results show that it is possible to reconstruct UI barriers by bringing together SI genes from multiple species, and provide insight into the necessary stages that plants must pass through as their compatibility evolves. Clearly, pollination barriers acting at very different levels share common factors. Defining pollination barriers in this way also highlights differences between barriers. Although SI and UI are clearly connected in some cases, as we have shown here, this will probably not be true for all interspecific reproductive barriers. It is intriguing that *HT* genes are associated with many of these UI systems, raising the possibility that they are implicated in both S-RNase-dependent and S-RNase-independent UI pollen rejection mechanisms (Covey *et al.*, 2010; Chalivendra *et al.*, 2013). Why SC species accumulate defects in intraspecific and interspecific reproductive barriers remains unexplained, but, in principle, loss of SI may allow accumulation of loss-of-function mutations in non-essential factors associated with these mechanisms. Reproductive assurance and transmission advantage are thought to drive the initial shift from SI to SC (Goodwillie *et al.*, 2005; Goldberg and Igic, 2012). Coupling of this shift to further losses of crossing barriers, as has occurred in red-fruited tomato species and several *Nicotiana* species, suggests multiple connections between intraspecific and interspecific pollen rejection mechanisms.

EXPERIMENTAL PROCEDURES

PLANT MATERIALS

S. pennellii accessions LA0716 and LA2560, *S. chmielewskii* accession LA1316, *S. neorickii* accession LA4023, *S. pimpinellifolium* accession LA3798 and *S. lycopersicum* cultivar VF36 (accession LA0490) were obtained from the C.M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu). The *S. lycopersicum* (cv. Ailsa Craig) line ACS6-39 expressing S_6-RNase has been described previously (Kondo *et al.*, 2002b), and was obtained from Yasuo Kowyama (Graduate School of Bioresources, Mie University, Tsu, Japan).

HT *GENE EXPRESSION CONSTRUCTS AND PLANT TRANSFORMATION*

HT-A and *HT-B* genes were amplified from genomic DNA of *S. pennellii* SI accession LA2560 (Covey *et al.*, 2010), and

expressed under the control of the tomato chitinase *Chi2;1* gene promoter as described previously (Murfett *et al.*, 1996). The construct was transferred to pCAMBIA 3300 (www.cambia.org), and transformed into *S. lycopersicum* cv. M82 at the Ralph M. Parsons Plant Transformation Facility (University of California, Davis, CA).

ANALYSES OF TRANSGENIC PLANTS EXPRESSING HT PROTEIN AND/OR S_6-RNASE

Transformants (T_0) showing the highest expression of HT-A or HT-B proteins in mature pistils were selected from among 10 independent events (HT-A) or 11 independent events (HT-B). For protein blots, HT-A and HT-B proteins in pistil extracts (1.5 mg fresh weight/lane, samples included the stigma and style but not the ovary) were detected using specific antisera as described previously (Covey *et al.*, 2010). Plants expressing HT proteins and S_6-RNase were generated by crossing selected HT-expressing T_0 plants with ACS6-39 plants expressing S_6-RNase. The presence of *HT* and *S_6-RNase* transgenes was verified by PCR using forward primer 5′-TTGAAAATTCTTCACCTCTTCG-3′ from the *Chi2;1* promoter sequence and a reverse primer specific for *HT-A* or *HT-B* (5′-TAGGAAACAATGATCCCCCA-3′ and 5′-ACAGGCTGCATCAAAA ATCC-3′, respectively). The primers for *S_6-RNase* were 5′-AAA TGCGCTAGACAAACGCT-3′ and 5′-CATTTCCAGGTGGTTTTC GT-3′. Primers 5′-ACCTGAGGAAATTGGCTGTG-3′ and 5′-ATGTTG CTCTCGGCTTCAGT-3′ for tubulin were used as a control. HT-A or HT-B proteins were detected as described previously (Covey *et al.*, 2010). S_6-RNase was detected using a specific guinea pig anti-S_6-RNase antibody raised against the peptide Acetyl-CDVPPEVDYVQ-IEDHKILNA-$CONH_2$. Compatibility tests were based on 3–10 crosses. Flowers were emasculated 1 day before opening, and pollinated the following day (Chalivendra *et al.*, 2013). Pollinated pistils were collected after 24 h, stained with aniline blue fluorochrome as described previously(Covey *et al.*, 2010), and photographed using an Olympus IX-170 microscope (www.olympusamerica.com). Pollen tube lengths in incompatible crosses were measured from the top of the stigma to the point where most of the pollen tubes stopped.

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SUPPORTING INFORMATION

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

Figure S1. Expression of S_6-RNase and HT proteins in *S. lycopersicum*.

Figure S2. HT protein levels in HTA-3, HTB-3 and HT/S6 T2 pistil extracts.

Figure S3. Pollen tube growth in a transgenic *S. lycopersicum* HTB/S6-4 T_1 plant.

Figure S4. Growth of the *S. cheesmaniae* accession LA0522 pollen tube in transgenic *S. lycopersicum* plants.

Figure S5. Growth of the *S. galapagense* accession LA0438 pollen tube in transgenic *S. lycopersicum* plants.

Figure S6. Growth of the *S. pimpinellifolium* accession LA3798 pollen tube in transgenic *S. lycopersicum* plants.

Table S1. Pollen tubes at the base of the style 24 h after pollination in HT-A x S6 progeny.

Table S2. Pollen tubes at the base of the style 24 h after pollination in HT-B x S6 progeny.

Table S3. Pollen tubes from wild species at the base of the style 24 h after pollination in HT/S6 plants.

Table S4. Lengths of longest pollen tubes from red-fruited species in HTA/S6 and HTB/S6 pistils.

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