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Molecular analysis of the stylar-expressed *Solanum chacoense* small asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in *Nicotiana*

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Summary

Gametophytic self-incompatibility (GSI) systems involving the expression of stylar ribonucleases have been described and extensively studied in many plant families including the Solanaceae, Rosaceae and Scrophulariaceae. Pollen recognition and rejection is governed in the style by specific ribonucleases called S-RNases, but in many self-incompatibility (SI) systems, modifier loci that can modulate the SI response have been described at the genetic level. Here, we present at the molecular level, the isolation and characterization of two *Solanum chacoense* homologues of the *Nicotiana* HT modifier that had been previously shown to be necessary for the SI reaction to occur in *N. alata* (McClure *et al.*, 1999). HT homologues from other solanaceous species have also been isolated and a phylogenetic analysis reveals that the *HT* genes fall into two groups. In *S. chacoense*, these small proteins named ScHT-A and ScHT-B are expressed in the style and are developmentally regulated during anthesis identically to the S-RNases as well as following compatible and incompatible pollination. To elucidate the precise role of each HT isoform, antisense ScHT-A and RNAi ScHT-B lines were generated. Conversion from SI to self-compatibility (SC) was only observed in RNAi ScHT-B lines with reduced levels of ScHT-B mRNA. These results confirm the role of the HT modifier in solanaceous SI and indicate that only the HT-B isoform is directly involved in SI.

Keywords: self-incompatibility, S-RNase, Solanaceae, HT-modifier gene, RNA interference.

Introduction

Self-incompatibility (SI) constitutes an important mechanism for preventing inbreeding through specific pollen recognition and rejection. In the most widespread type of gametophytic self-incompatibility (GSI), the haploid pollen is rejected when the *S*-allele it expresses, matches either of the two *S*-alleles expressed in the sporophytic tissue of the pistil. For *Solanaceae*, the GSI phenotype is specified by a highly multiallelic *S*-locus (de Nettancourt, 1977, 1997) whose only known product is a secreted ribonuclease (McClure *et al.*, 1989) expressed in the transmitting tissue of the style (Anderson *et al.*, 1986; Matton *et al.*, 1998) and called an S-RNase. Gain-of-function experiments in SI plants have shown that expression of an S-RNase transgene is sufficient to alter the SI phenotype of the pistil but not that of pollen (Lee *et al.*, 1994; Matton *et al.*, 1997; Murfett *et al.*, 1994). Furthermore, transgenic plants made to express high levels of S-RNase in pollen did not acquire the new phenotype (Dodds *et al.*, 1999), indicating that the pollen *S* gene (unknown to date) is clearly distinct from the S-RNase (Kao and McCubbin, 1996). In order to determine if expression of an active S-RNase is the sole determinant of SI in styles, transformation of closely related self-compatible (SC) species with S-RNases were attempted. Transformation of SC *Nicotiana tabacum* or *N. plumbaginifolia* with an *S*-allele from the SI species *N. alata* did not result in the acquisition of the SI phenotype (Murfett *et al.*, 1996), nor did the introgression of a chromosome fragment bearing the *S*-locus from the SI *Lycopersicon hirsutum* in SC

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L. esculentum (Bernatzky *et al.*, 1995), or the expression of an S-RNase from the SI *L. peruvianum* in the SC *L. esculentum* (Kondo *et al.*, 2002b). Conversely, when an *S*-allele from the SC *Petunia hybrida* was introduced in SI *P. inflata*, it became functional in rejecting its corresponding self-pollen, indicating that factors expressed in the *P. inflata* SI genetic background were needed for the SI reaction to occur (Ai *et al.*, 1991). These results strongly suggest that other factors are necessary for the SI reaction to occur. Some of these factors that affect the SI response have been described in numerous SI systems and often been named *S*-locus inhibitors or modifiers (de Nettancourt, 1977). In *S. chacoense*, an *S*-locus inhibitor (SIi) has been mapped to the distal end of chromosome 12, but has not been cloned yet (Hosaka and Hanneman, 1998a,b). To date, the only modifier functionally characterized at the molecular level is the *HT* gene, a stylar-expressed small asparagine-rich protein in *N. alata* (McClure *et al.*, 1999). The *NaHT* (*Nicotiana alata* HT) cDNA was isolated from a differential screen for SI stylar-specific transcripts, and antisense *Nicotiana* HT plants became SC, although they still expressed normal levels of stylar S-RNases. Recent correlative evidences from mRNA expression studies in *Lycopersicon* species also suggest the involvement of the HT modifier in SI (Kondo *et al.*, 2002a,b). Here, we describe the characterization of HT homologues that are co-ordinately expressed with the S-RNases during pistil development in the SI species *S. chacoense*, and show that only the HT-B isoform is involved in SI.

Results

Isolation of the Solanum HT homologues and sequence comparison

The *ScHT-A$_1$*, *ScHT-A$_2$* and *S$_{14}$-RNase* cDNAs were isolated from a pollinated pistil cDNA library (see Experimental procedures section). The *ScHT-A$_1$* cDNA codes for a small protein of 99 amino acid residues with a highly predicted N-terminal signal peptide as determined from the SignalP algorithm (Nielsen *et al.*, 1997). The predicted cleavage site for ScHT-A$_1$ is before Arg-25, producing a mature polypeptide of 75 amino acids (8 kDa). The *ScHT-A$_2$* cDNA is incomplete in the 5′ region, but would comprise all of the mature protein (77 residues, 8.3 kDa) as predicted from the ScHT-A$_1$-deduced cleavage site. Both ScHT-A$_1$ and ScHT-A$_2$ predicted mature proteins are acidic with pIs of 3.98 and 4.11, respectively. Amino acid sequence comparison of the predicted mature polypeptides indicate that ScHT-A$_1$ and ScHT-A$_2$ are 96% identical (93% nucleotide sequence identity) and most probably correspond to allelic variants of the same gene (see linkage analysis of the ScHT-A isoforms below). The ScHT-B$_1$ isoform was obtained by PCR amplification with an upstream primer located in the signal peptide region and a downstream primer located 3′ of the predicted stop codon from the *N. alata* HT and *S. chacoense* HT-A$_1$ isoforms. The ScHT-B$_1$ mature protein comprises 79 amino acids (MW, 8.7 kDa) with an acidic pI of 4.67, and is approximately 51% identical (57% similar) at the amino acid level to the ScHT-A isoforms. No N-glycosylation sites are found on either polypeptides, but six cysteine residues that could be involved in disulfide bonding are conserved between all HT homologues, except from the *S. pinnatisectum* B$_1$ isoform that lacks one cysteine, and are found flanking a striking C-terminal region containing 16–20 Asp (D) or Asn (N) residues. In the mature ScHT proteins, asparagine and aspartic acid residues account for roughly 30% of the total amino acids. A sequence alignment of the deduced amino acid sequences corresponding to the mature protein region of the *S. chacoense* HT isoforms as well as HT homologues from other SI solanaceous plants, including *L. peruvianum*, *N. alata*, *S. pinnatisectum*, *S. bulbocastanum* and from the SI species *S. tuberosum,* is shown in Figure 1(a). All *Solanum* and *Lycopersicon* sequences were obtained by PCR amplification with the same primer pairs as described for the amplification of *ScHT-B$_1$*. Although all the HT sequences share some specific structural features, e.g. a C-terminal Asn/Asp-rich region flanked by conserved cysteine residues, they can be easily classified in two groups when the amino-terminal half of the protein is considered. Based on the CLUSTALX alignment, a phylogenetic analysis was performed to determine if this preliminary classification would hold true. Figure 1(b) shows that all the B isoforms fell into a highly supported cluster, while more sequence data would be needed to determine if the A-type sequences form one or more group. Interspecific amino acid sequence identities between the predicted mature polypeptides ranges from 76 to 86% in the A-isoform group, and 36–92% in the B-isoform group. The ScHT-A$_1$ and ScHT-A$_2$ (94%), SbHT-B$_1$ and SbHT-B$_2$ (98%) and SpHT-B$_1$ and SpHT-B$_2$ (97%) are most probably alleles of the same genes in their respective species. When the only non-*Solanum* sequence is removed (NaHT-B), the B-isoform group sequence identity is in the range of 77–92%. One surprising feature is the very high conservation of the predicted signal peptides between species, as determined from the available complete *HT* cDNA sequences (*ScHT-A$_1$*, *NaHT-B*, *LpHT-A$_1$* and *LpHT-B$_1$*), ranging from 66 to 100% identity (82–100% similarity), when compared to the mature protein sequences (data not shown). This intriguing situation is also observed with the sporophytic SI (SSI) pollen *S* gene where the signal peptides are also far more similar to each other (mean of 77% identity and 89% similarity) than the mature protein sequences (29% identity and 38% similarity on average) when the sequences of five different SSI pollen *S* genes are compared (Schopfer *et al.*, 1999; Takayama *et al.*, 2000).

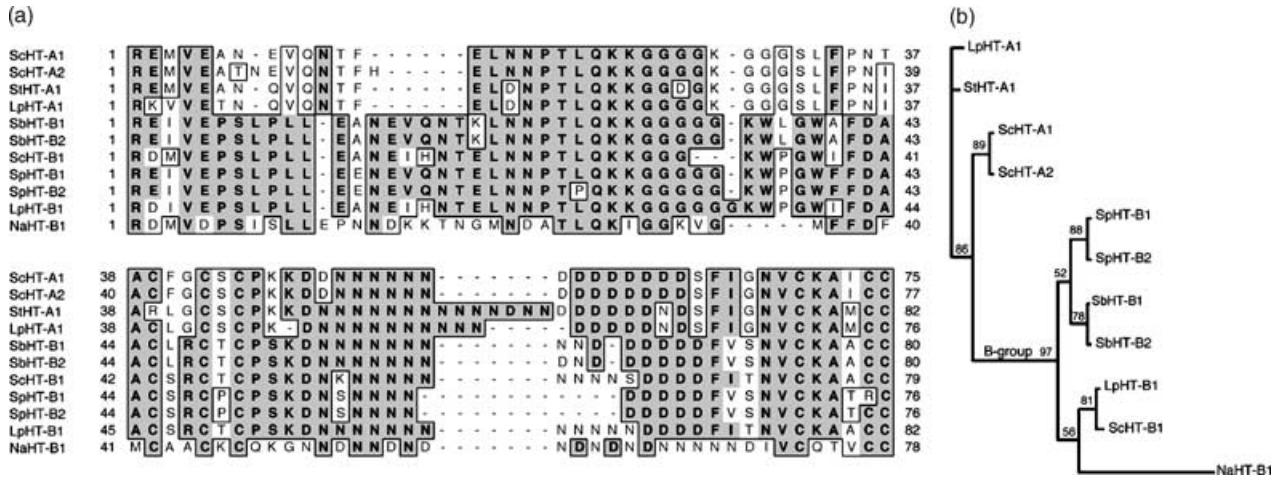

Figure 1. Sequence alignment (a) and phylogenetic analysis (b) of the deduced mature protein sequences of *ScHT-A*$_1$, *ScHT-A*$_2$ and *ScHT-B*$_1$ with related sequences from other solanaceous species.
(a) CLUSTALX alignment was used to produce a phylogenetic analysis of related HT sequences in six solanaceous species.
(b) A jacknife analysis using Paup 4.08b was used to produce the phylogram which is shown.

Tissue-specific and developmental regulation of the ScHT modifiers

Tissue-specific expression of ScHT-A and ScHT-B isoforms was determined using RNA extracted from different tissues of *S. chacoense*. Since the *ScHT-A*$_1$ and *ScHT-A*$_2$ cDNAs are 93% identical at the DNA level, the RNA-gel blot analyses most probably reflect the expression of both genes, although the probe used at all time was *ScHT-A*$_1$. Overall DNA sequence identity between the *ScHT-A* and Sc-HT-B isoforms is around 73%, and long stretches of identity might also produce cross-hybridization. In order to avoid this, an oligonucleotide specific to the B isoform and corresponding to the N-terminal sequence, PSLPLLEA, was synthesized. Both *ScHT-A* and *ScHT-B* isoforms are almost exclusively expressed in styles with very weak expression detected in ovary upon prolonged exposures (data not shown). No *ScHT-A* or *B* mRNAs could be detected in leaf, stem, root, petal, anther, pollen or pollen tube tissues (data not shown). This expression pattern is identical to the one observed for the S-RNases (Matton *et al*., 1998). Since the S-RNase genes are themselves developmentally regulated during anthesis (Anderson *et al*., 1986; Cornish *et al*., 1987), we determined the RNA expression pattern of *ScHT-A* and *ScHT-B*, and compared with the one obtained from *S*$_{14}$*-RNase* (Figure 2a,b). Both ScHT isoforms and the *S*$_{14}$*-RNase* are identically regulated during pistil development and reach a maximum level of expression around anthesis day (Figure 2a,b). Figure 2(a,b) also shows that, in unpollinated flowers, *ScHT-A*, *ScHT-B* and *S*$_{14}$*-RNase* mRNA levels decline from around 2 days after anthesis, coinciding with a reduced fertilization receptivity.

In S-RNase-mediated GSI, rejection of the pollen tubes mostly occurs in the top half of the style. To determine if there could be a correlation with pollen tube arrest and the expression levels of genes involved in SI, mRNA levels of *ScHT-A* and *S*$_{14}$*-RNase* were measured in the upper and lower halves of styles around peak expression time (Figure 2c). Both genes were more strongly expressed in the upper half of the style, consistent with the site of most pollen tube arrest as determined by aniline blue staining in *S. chacoense* styles (Matton *et al*., 1999).

Effect of compatible and incompatible pollination on ScHT *and* S-RNase *gene expression*

In many species, pollination is known to induce deterioration and death of the secretory cells in the stigmatic region and in the transmitting tissue of the style (Cheung, 1996). We have previously shown that some genes that respond to pollination, also respond to wounding stress and wound hormone treatments, mainly jasmonates (Lantin *et al*., 1999a,b). Wounding, as well as wound hormone treatment (JA, ABA, MeJA) and elicitors of defense responses (salicylic acid, arachidonic acid), had no effect on either *ScHT-A* or *S*$_{14}$*-RNase* mRNA levels (data not shown, except for wounding in Figure 2d). Expression of these genes thus seemed to be exclusively controlled by developmental cues during pistil maturation, except for a differential response toward the type of pollination. *ScHT-A* and *S-RNases* responded differentially to a compatible or an incompatible pollination. In Figure 2(d), flowers were pollinated with either compatible or incompatible pollen and tissues were harvested 48 h later. For the wounding treatment, the upper part of the style including the stigma was slightly crushed with tweezers and tissues were also harvested 48 h later. Following a compatible pollination, or wounding, both *ScHT-A* and *S*$_{14}$*-RNase* mRNA levels declined similar to

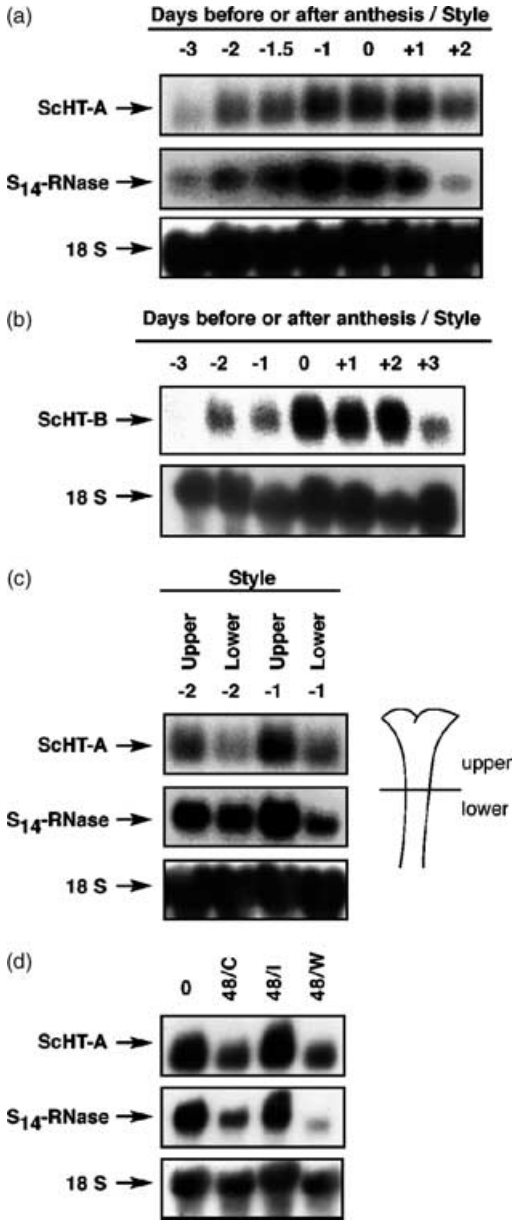

Figure 2. RNA expression analysis of *ScHT* transcript levels in styles.
(a) Developmental expression pattern of *ScHT-A* and S_{14}*-RNase* mRNA levels in unpollinated pistil tissues. ScHT-A and S_{14}-RNase transcript levels were determined by RNA-gel blot analysis of unpollinated pistil tissues, 3 days before anthesis (−3) to 2 days (+2) after anthesis. Ten micrograms of total style RNA from each developmental stage was probed with the *ScHT-*A_1 cDNA insert, stripped and re-probed with the S_{14}*-RNase* cDNA insert.
(b) Developmental expression pattern of *ScHT-B* mRNA levels in unpollinated pistil tissues. Same conditions as in (a), except that an identical RNA-gel blot was probed with the *ScHT-*B_1 specific oligonucleotide.
(c) Differential expression of *ScHT-A* and S_{14}-RNase transcript levels in upper and lower part of the style. *ScHT-A* and S_{14}*-RNase* transcript levels were determined by RNA-gel blot analysis in upper and lower halves of styles collected 2 (−2) and 1 day (−1) before anthesis. Conditions same as in (a).
(d) Effect of compatible and incompatible pollination on *ScHT-A* and S_{14}*-RNase* transcript levels. *ScHT-A* and S_{14}*-RNase* transcript levels were determined by RNA-gel blot analysis in unpollinated styles at anthesis day (0), in styles collected 48 h after a fully compatible ($S_{11}S_{12} \times S_{13}S_{14}$) pollination (48/C), in styles collected 48 h after a fully incompatible ($S_{13}S_{14} \times S_{13}S_{14}$) pollination (48/I) and in styles collected 48 h after wounding (48/W). Conditions same as in (a). To ascertain equal loading conditions, all RNA-gel blots were stripped and re-probed with an 18S ribosomal cDNA probe from *S. chacoense*.

the developmentally regulated decrease observed in unpollinated flowers (compare Figure 2a,d). An incompatible pollination had the opposite effect. The *ScHT-A* and S_{14}*-RNase* mRNA levels stayed as high as found on anthesis day, indicating that the developmentally programmed decrease in *S-RNase* and *ScHT* mRNA levels could be reversed, at least transiently, following an incompatible pollination.

Polymorphism of the HT modifiers and linkage to the S-locus

Using the *ScHT-*A_1 cDNA insert as a probe, an F_1 population from a parental cross ($S_{11}S_{12} \times S_{13}S_{14}$) was tested for polymorphism and linkage to the *S*-locus. A fraction of the F_1 progeny tested is shown in Figure 3. The S-RNase genotype of the progeny had been determined previously (Rivard *et al.*, 1994) and was confirmed by PCR analyses with allele-specific primers (data not shown). The *ScHT-A* gene is highly polymorphic as four different RFLPs could be detected in these plants. Although four different *S*-alleles also segregated in this population, the *ScHT-A* alleles were completely unlinked to the *S*-locus, as any combination of *ScHT-A* alleles could be found with all four S-RNases in this population. The same population was re-probed with the *ScHT-B* cDNA. Two new RFLPs specific to the B form were observed (data not shown). Although cross-hybridization does occur between the *ScHT-A* and *ScHT-B* cDNA probes (data not shown), no single RFLP could be linked with the *S-RNase* gene.

Two-hybrid analysis of ScHT and S-RNase protein interaction

A few putative roles have been proposed for the *N. alata* HT protein (McClure *et al.*, 1999). Recently it was shown that S-RNases, in both compatible or incompatible interactions, are taken up by pollen tubes, but the entry mechanism is still unknown (Luu *et al.*, 2000). One possibility is that other stylar factors involved in SI, such as the HT protein, could accompany or interact directly with the S-RNases as they are being transported into the growing pollen tubes. Since HT proteins from either *S. chacoense* or *N. alata* are fairly acidic proteins with pI around 4, and since S-RNases are basic proteins (S_{14}-RNase mature protein, predicted pI is 9.12), ScHT proteins could interact directly with S-RNases, albeit not in a sequence-specific manner, as determined by the linkage analysis (Figure 3). Another possibility would be that the HT proteins could interact with the pollen tubes and

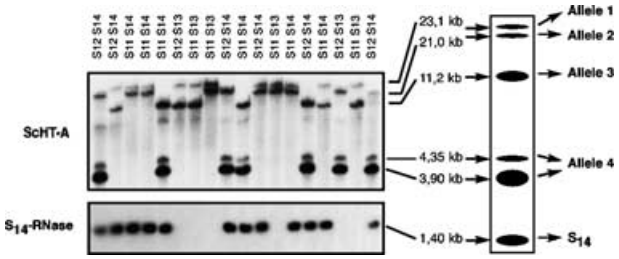

Figure 3. DNA-gel blot analysis of *ScHT-A* and linkage analysis with the *S*-locus.
Left panels: genomic DNA (10 μg) from *S. chacoense* leaves isolated from an F_1 population ($S_{11}S_{12} \times S_{13}S_{14}$) segregating for four *S*-alleles was digested with *Hind*III and probed with the *ScHT-A*$_1$ cDNA insert (upper panel). Identitical DNA-gel blots had been previously probed with the corresponding S-RNase cDNAs to determine the genotype of the plants (labeled on top of each lane) and tested by crossing. One *S*-allele hybridization is shown for the *S_{14}-RNase* (lower panel). Molecular sizes of the fragments appear on the right.
Right panel: schematic drawing of the banding pattern of the four *ScHT-A* alleles.

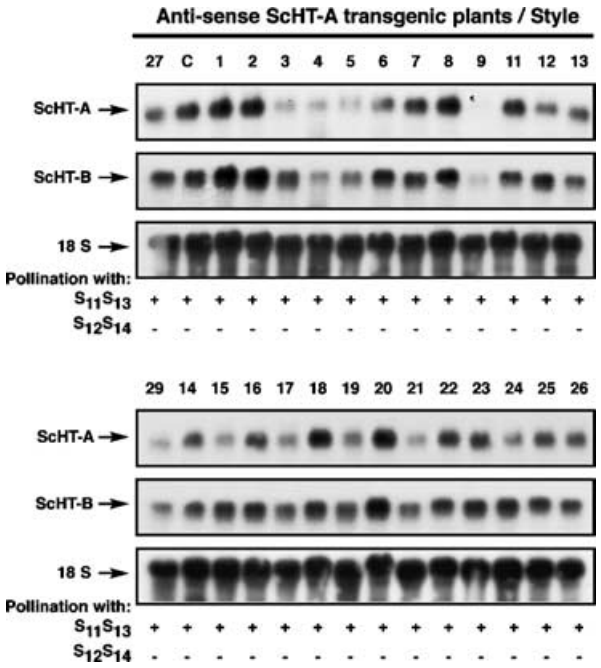

Figure 4. RNA expression analysis of *ScHT* transcript levels in styles of *ScHT-A* antisense transgenic plants and genetic cross results.
ScHT-A and *ScHT-B* transcript levels were determined by RNA-gel blot analysis of unpollinated styles at anthesis day from 27 antisense *ScHT-A*$_1$ transgenic plants. Control plant (C) is the untransformed host (genotype $S_{12}S_{14}$). All crosses were done on at least 10 flowers with pollen from a fully compatible plant (genotype $S_{11}S_{13}$) or a fully incompatible plant (genotype $S_{12}S_{14}$). Ten micrograms of total style RNA from each plant was probed with the *ScHT-A*$_1$ cDNA insert and the *ScHT-B*$_1$ specific oligonucleotide. Equal loading conditions were verified with an 18S ribosomal cDNA probe from *S. chacoense*.

facilitate S-RNase uptake. To test if the HT protein can interact directly with the S-RNase, the *ScHT–A*$_1$ and *ScHT–B*$_1$ cDNAs were PCR amplified with or without the putative signal peptide, and inserted in frame downstream of the yeast GAL4 DNA-binding domain in the pBDGAL4 vector. Since the linkage analysis (Figure 3) showed that all combinations of *ScHTs* and *S-RNases* could be found in the segregating population, this strongly suggested that no allele-specific interactions would be expected. Thus, a single *S-RNase* gene was used to test putative protein–protein interactions between the ScHT and S-RNase protein products. A modified *S_{11}-RNase* that was previously produced by site-directed mutagenesis was fused to the yeast GAL4 activating domain in the pADGAL4 vector. Because of their intrinsic ribonuclease activity, the S_{11}-RNase used in the two-hybrid analysis was mutagenized to remove one histidine residue involved in the active site of the enzyme (C3 domain) and replaced with a leucine, thus abolishing the ribonuclease activity that could have prevented proper growth in yeast cells. No direct interaction could be detected between the *ScHT-A* protein, with or without the predicted signal peptide, or the *ScHT-B* protein without the predicted signal peptide and the modified S_{11}-RNase, as no yeast growth could be observed on histidine-depleted media (data not shown).

Molecular characterization of the antisense Solanum HT-A plants

To determine if the function of the *ScHT* genes is conserved in solanaceous species other than *N. alata*, antisense *HT-A* plants were produced. The *ScHT-A*$_1$ cDNA was inserted in the antisense orientation downstream of the CaMV 35S promoter with doubled enhancer in the pBIN19 vector and flanked by the nopaline synthase terminator. *S. chacoense* plants of $S_{12}S_{14}$ genotype were transformed with *Agrobacterium tumefaciens* LBA4404 strain containing the *ScHT-A*$_1$ antisense construct. Twenty-seven primary transformants were selected. Because *ScHT-A* and *ScHT-B* share 73% nucleotide sequence identity, and since stretches of perfect identity are found between these two sequences, some antisense lines for *ScHT-A* might also be suppressed in *ScHT-B* mRNA accumulation. Figure 4 shows RNA-gel blots of these 27 transgenic plants probed with either the *ScHT-A*$_1$ complete cDNA or the *ScHT-B*$_1$ specific oligonucleotide, and the results of the genetic crosses with either compatible or incompatible pollen are shown below. In some antisense lines where *ScHT-A* mRNA accumulation had been suppressed, *ScHT-B* levels were also affected, albeit to a lesser extent (AS plant #4 and 9). Although numerous plants showed strong reduction in *ScHT-A* mRNA levels (plants #3–5, 9, 29), none set seeds upon self-pollination.

Molecular characterization of the RNAi Solanum HT-B plants

Because antisense *S. chacoense HT-A* plants did not become SC, even with an almost 15-fold reduction in *ScHT-A*

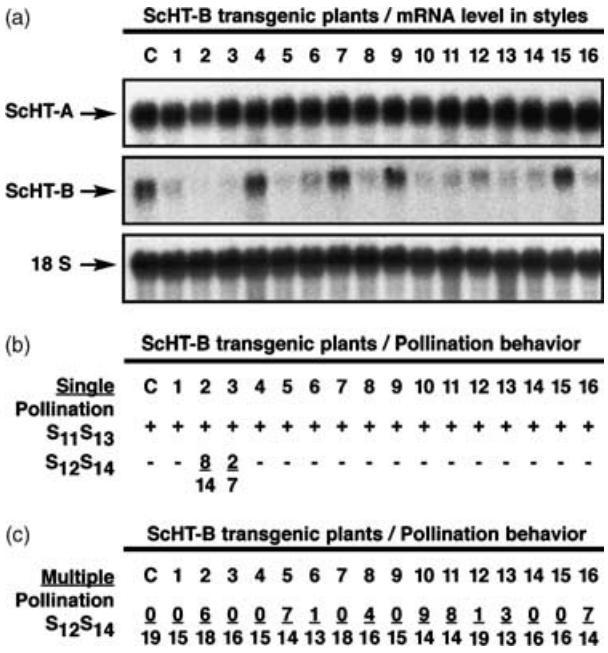

Figure 5. RNA expression analysis of *ScHT* transcript levels in styles of *ScHT-B* RNAi transgenic plants and genetic cross results.
(a) *ScHT-A* and *ScHT-B* transcript levels were determined by RNA-gel blot analysis of unpollinated styles at anthesis day from 16 RNAi *ScHT-B*$_1$ transgenic plants. Control plant (C) is the untransformed host (genotype $S_{12}S_{14}$). Ten micrograms of total style RNA from each plant was probed with the *ScHT-A*$_1$ cDNA insert and the *ScHT-B*$_1$ specific oligonucleotide. Equal loading conditions were verified with an 18S ribosomal cDNA probe from *S. chacoense*.
(b) Genetic cross results with pollen from SI or SC plants. All crosses were done on at least 10 flowers per plant on anthesis day, with pollen from a fully compatible plant (genotype $S_{11}S_{13}$) or a fully incompatible plant (genotype $S_{12}S_{14}$). The plus (+) sign indicates a fully compatible pollination: crosses were successful and seed set occurred in more that 90% of the pollinated flowers. The minus (–) sign indicates a fully incompatible pollination: no crosses were successful and no seed set occurred. For intermediate phenotypes, the number of successful pollination leading to seed set per flower pollinated is indicated.
(c) Genetic cross results with pollen from a SI plant after repeated pollination (multiple pollination). Same as in (b) except that the flowers were repeatedly pollinated with pollen from a fully SI parent (genotype $S_{12}S_{14}$). Pollination was done on anthesis day, and then after 24, 48 and 72 h.

transcripts (*ScHT-A* AS plant #9, as determined by densitometric scans), and since correlative evidences showing weak or complete loss of expression of *HT-B* homologues, but not of HT-A homologues in some SC *Lycopersicon* species, have been recently obtained (Kondo *et al.*, 2002a,b), we also decided to target the *ScHT-B* gene through an RNA interference (RNAi) strategy. The *ScHT-B*$_1$ cDNA was inserted first in the sense orientation downstream of the CaMV 35S promoter, followed by a 327-bp spacer, and by the *ScHT-B*$_1$ cDNA again, but in the antisense orientation. This RNAi construct was then inserted in the *A. tumefaciens* LBA4404 strain and used to transform *S. chacoense* plants of the $S_{12}S_{14}$ genotype. Sixteen primary transformants were initially selected. All *ScHT-B* RNAi lines were cross-pollinated with pollen from fully compatible ($S_{11}S_{13}$) or fully incompatible ($S_{12}S_{14}$) genotypes. Two plants (#2 and #3) sired seeds upon self-pollination (pollen from genotype $S_{12}S_{14}$), and could be scored as partially or semi-compatible (Figure 5b). *ScHT-A* and *ScHT-B* mRNA levels were then determined in mature flowers at anthesis. Figure 5a shows an RNA-gel blot of all the transgenic plants probed with either the *ScHT-A*$_1$ complete cDNA or the *ScHT-B*$_1$-specific oligonucleotide, and the results of the genetic crosses with either compatible or incompatible pollen (Figure 5b). Unlike the *ScHT-A* antisense experiment (Figure 4), the RNA interference strategy specifically targeted the *ScHT-B* transcript as no significant variation in the *ScHT-A* mRNA levels could be observed (Figure 5a). Only the transgenic plants with the most reduced *ScHT-B* mRNA level became partially SC (plants #2 and 3), suggesting that a threshold level of *ScHT-B* is necessary to maintain the SI phenotype, and that only the HT-B isoform is involved in GSI.

The ScHT-B *gene affects flower longevity and stylar abscission following an incompatible pollination*

One intriguing observation, following an incompatible pollination, was that flowers of *ScHT-B* RNAi plants that had lower levels of *ScHT-B* transcripts, stayed much longer on the plant than control or transgenic plants not affected in *ScHT-B* mRNA levels (plants #4, 7, 9 and 15). Under normal conditions, abscission of unpollinated flowers in *S. chacoense* occurs approximately 5 days after anthesis (Figure 6, unpollinated G4). After a compatible pollination, ovary swelling is clearly detectable 3 days after pollination and stylar abscission occurs approximately 4 days after pollination (Figure 6, V22 × G4). Following an incompatible pollination, abscission is delayed by an average of 24 h when compared to unpollinated flowers (Figure 6, G4 × G4). In *ScHT-B*-suppressed lines, flower abscission was further delayed and only occurred after an 8–9-day period following initial pollination (data not shown). This extended flower longevity phenotype caused by a lower than normal *ScHT-B* mRNA level prompted us to re-examine pollination behaviour with SI pollen under a multiple pollination scheme. In this experiment, *ScHT-B* transgenic plants and untransformed control plants were pollinated on anthesis day, and then on the following 3 days with similar pollen load. Fruit formation was then monitored from day 6 to 12 after pollination. As a control, the transformation host genotype was also repeatedly pollinated. Even after multiple pollination, the untransformed plant (control) and the transgenic plants not affected in *ScHT-B* levels (plants #4, 7, 9 and 15) never sired seeds, indicating that multiple pollination alone, even over a 72-h period, was not sufficient to bypass the SI recognition and rejection system (Figure 5c). For the remaining 12 transgenic plants with altered level of *ScHT-B* mRNA, a total of nine plants were scored as

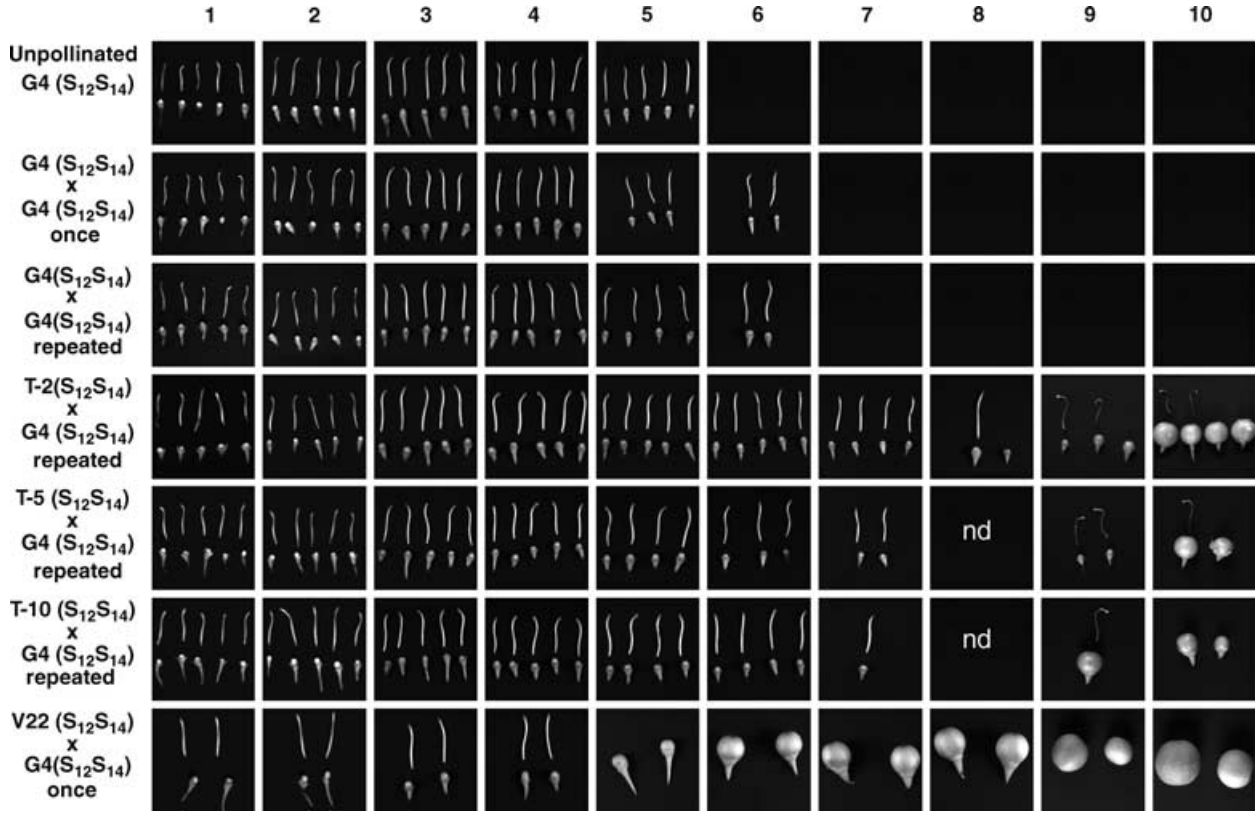

Figure 6. Pistil morphology of *ScHT-B* transgenic and control plants after SI and SC pollination.
Floral and stylar abscission was monitored, from one day after anthesis or from one day after pollination for control plants, and for three transgenic plants (T-2, T-5 and T-10) until day 10. In all cases, pollination was performed with pollen from the G4 line (genotype $S_{12}S_{14}$). Five flowers per plant and per day (except for the V22 × G4 cross) were hand pollinated on consecutive days and, for the remaining flowers, dissected at the end of the time-course. Only the remaining floral parts at the end of the time-course are displayed. ND, not determined. Once, single pollination. Repeated, consecutive pollination on day 0–2 and if possible on day 3, depending on the corolla closure.

semi-compatible (plants #2, 5, 6, 8, 10–13 and 16) upon repeated pollination with fully incompatible pollen (genotype $S_{12}S_{14}$). Floral and stylar abscission were also monitored, from one day after anthesis or from one day after pollination for control plants and three transgenic plants (T-2, T-5 and T-10) that showed a SC behaviour following self-pollination. Five flowers per plant and per day were hand pollinated on consecutive days in order to collect all samples on the same day (except for the unpollinated control plant and the fully compatible cross V22 × G4). As such, with the remaining flowers at the end of the 10-day period, the whole time-course was displayed. Pistil morphology for these plants is shown in Figure 6. Transgenic plants, T-2, T-5 and T-10, clearly showed an increased stylar longevity, with turgid styles that appeared receptive to pollination until day 7 or 8, after pollination. Furthermore, stylar abscission from the developing fruit was also delayed, with some styles still attached even after withering (Figure 6, plants T-2 and T-5 on day 9 and 10; plant T-10 on day 9). When compared to a fully compatible cross (V22 × G4), fruit formation was also delayed in self-pollinated T-2, T-5 and T-10 transgenic plants. These results confirm the involvement of the *ScHT-B* gene in SI and suggest that it might act through an increased flower receptivity period.

Discussion

Mechanisms underlying the breakdown of GSI have been recently reviewed and grouped in three broad categories (Stone, 2002). First, loss of SI occurs following the duplication of the *S*-locus and the presence of heterozygous pollen (heteroallelic for the *S*-locus) (Golz *et al.*, 2000). Secondly, mutations affecting either the expression of the S-RNase or its activity also lead to a SC phenotype (Royo *et al.*, 1994). Thirdly, mutations not affecting the enzymatic activity of the S-RNase have also been described at the genetic level and include many so-called modifier loci. Numerous experiments have demonstrated that although the S-RNase is responsible for pollen recognition and rejection in the style (Lee *et al.*, 1994; Matton *et al.*, 1997; Matton *et al.*, 1999; Murfett *et al.*, 1994), other stylar factors are also necessary for the proper expression of the SI phenotype (Ai *et al.*, 1991; Bernatzky *et al.*, 1995; Kondo *et al.*, 2002b; Murfett

et al., 1996). Such factors, often considered as modifier loci, are present in the genetic background of SI plants, unlinked to the *S*-locus, and have often been lost in SC relatives of SI species. Complementation phenomena of the genetic background have been described in *L. hirsutum* where the F_1 population from two independent SC accessions were all SC, while SI offsprings could be recovered in the F_2 generation from these F_1 plants, strengthening the multigenic nature of the gametophytic SI (Ricks and Chetelat, 1991). One such candidate for a modifier gene is the *N. alata HT* gene (McClure *et al.*, 1999). The *NaHT* gene was cloned based on a differential screen between stylar expressed mRNAs from SC *N. plumbaginifolia* and an SC accession of *N. alata* that is defective in S-RNase expression but that is competent to express SI (Murfett *et al.*, 1996). Anti-sense *NaHT* plants with reduced level of the HT protein but with normal levels of S-RNases were either fully or partially SC (McClure *et al.*, 1999). This strongly suggests that the *NaHT* gene is a good candidate for such a modifier factor necessary for the SI reaction to occur. In the present study, we have characterized *NaHT* homologues from four different *Solanum* species, and have focussed our attention on the putative function of the *S. chacoense HT* homologues (*ScHT-A* and *ScHT-B*) in GSI.

Phylogenetic analyses of the isolated NaHT homologues clearly demonstrated that two different HT isoforms exist and that isoform B is probably the most closely related to the *NaHT* gene. All the HT proteins shared some common features. Firstly, a highly conserved N-terminal region that is strongly predicted to be a signal peptide. The sequence conservation was high enough to originally derive PCR primer pairs from only the *NaHT* and *ScHT-A*$_1$ sequences, and amplify both HT isoforms from numerous *Solanum* (this study) and *Lycopersicon* species (Kondo *et al.*, 2002a,b). Secondly, all HT homologues possess a C-terminal region composed of consecutive stretches of asparagine and aspartic acid residues, flanked by conserved cysteines probably involved in disulfide bridges. Although sequence identity is quite variable, ranging from 36 to 92% in the B-isoform group (77–92% when only *Solanum* sequences are considered), the overall structure conservation combined with identical expression pattern would suggest that the *ScHT-B* isoform and the NaHT protein are probably true orthologues. Both *ScHT-A*, *ScHT-B* and *NaHT* are almost exclusively stylar-expressed as for the *S-RNases*, and all are developmentally regulated during pistil maturation (this study and McClure *et al.*, 1999). Interestingly, we found higher expression levels of both *ScHT* and *S-RNase* genes in the upper style region (Figure 2c), consistent with the pattern of pollen tube arrests that occurs in the top half of the style in *S. chacoense* (Matton *et al.*, 1999). The developmental regulation of S-RNase transcripts accumulation enables the production of selfed progeny in some GSI species when using very young flower buds (bud-pollination), but is very difficult to achieve in *S. chacoense*. One reason could be the elevated level of both S-RNase and HT transcripts, even 2 days before anthesis, and detectable 3 days before anthesis, combined with a preferential upper style accumulation. One intriguing observation was the differential expression pattern of both *S-RNase* and *HT* transcripts following an incompatible pollination compared to an unpollinated or a compatibly pollinated flower (Figure 2d). As the flower ages, *S-RNase* and *HT* transcript levels decreases markedly, but low expression levels coincide with reduced fertilization receptivity, and eventually, flower abscission. Surprisingly, *S-RNase* and *ScHT-A* transcript levels do not decrease following an incompatible pollination (for at least 2 days after pollination, Figure 2d), and this cannot be the result of only stigmatic and transmitting tissue deterioration and death, since this is also induced by a compatible pollination. Furthermore, mechanical wounding or wound hormone treatments had no effect on *S-RNase* and *ScHT-A* transcript levels. This strongly suggests that the presence of dead pollen tubes or molecules liberated from the arrested pollen tubes, either increase the transcription of these genes, or reduce their mRNA turnovers, ensuring that the S-RNases and HT proteins are still present in sufficient amount to reject newly incoming pollen from incompatible genotypes. The maintenance of high steady-state levels of *S-RNases* and *ScHT* mRNAs following an initial incompatible pollination, would also lead to a prolonged reproductive barrier, an important issue since flower senescence is retarded following an incompatible pollination (Figure 6, G4 $\times$ G4).

In order to determine the role of the *ScHT* genes in SI, functional analysis of *ScHT-A* and *ScHT-B* protein–protein interactions with an S-RNase were tested in the yeast two-hybrid system, and transgenic plants with strongly suppressed levels of both isoforms were generated. Although *ScHT-A* and *ScHT-B* deduced mature proteins have acidic pIs and the S-RNase is basic (predicted pI = 9.25 for S_{11}-RNase mature protein), no direct interactions based either on specific or electrostatic attractions could be detected in the two-hybrid system, as no yeast growth could be observed, with or without the predicted signal peptide. Such direct interaction had also not been detected with the purified HT protein from *N. alata*, although in that case, the NaHT protein appeared to be unstable in stylar extracts (McClure *et al.*, 1999). Both results suggest that HT proteins and S-RNases do not interact directly.

Recently, correlative evidences for the involvement of the NaHT homologues in *Lycopersicon* species have been obtained (Kondo *et al.*, 2002a,b). In the three *Lycopersicon* SI species tested, all expressed functional S-RNases as well as *HT-A* and *HT-B* mRNAs. In the seven *Lycopersicon* SC species tested, no or low stylar ribonuclease activity was observed. This alone would most probably be sufficient to explain their SC phenotype, since a threshold level of

S-RNase expression is necessary to confer an SI phenotype (Lee *et al.*, 1994; Matton *et al.*, 1997). Intriguingly, in the seven *Lycopersicon* SC species tested, transcription of the HT-B isoform was either weakly or not detected at all, and the HT-B isoform produced had internal stop codons, while the HT-A isoform was strongly expressed at the mRNA level, although some SC species also produced defective (frame-shifted) HT-A transcripts. Apart from the *N. alata* transgenic antisense lines, no other functional analysis had been made prior to the one presented here. In *N. alata*, plants with reduced levels of the NaHT protein were either fully or partially SC, suggesting that the amount of NaHT protein is important (McClure *et al.*, 1999). In the present study, antisense *ScHT-A* and RNAi *ScHT-B* plants were generated. Figure 4 showed that even with a 15-fold decrease in *ScHT-A* mRNA levels, *ScHT-A* AS plant #9 remained SI. *ScHT-B* mRNA levels in that transgenic line were also affected, although to a lesser extent. This could suggest that a threshold level of *ScHT-B* is sufficient to maintain an SI phenotype. In a second series of experiments, *ScHT-B* suppression was achieved through an RNAi strategy. Unlike the antisense plants, the *ScHT-B* RNAi plants were only affected in *ScHT-B* mRNA expression (Figure 5). Plants with severely reduced *ScHT-B* transcripts became SC and sired seeds upon pollination with pollen from an incompatible genotype. RNAi plant #2 had the most severely reduced *ScHT-B* mRNA levels and consistently set seeds upon self-pollination. RNAi plant #3 had a less stable phenotype, and only sired seeds occasionally. Although at first only two plants became partially SC upon selfing, all the transgenic plants with reduced *ScHT-B* mRNA levels also showed an extended, albeit slightly variable, floral longevity upon pollination with incompatible pollen. This observation led to the hypothesis that the *ScHT-B* gene could be involved in modulating the receptivity period of the flower, perhaps through a control over the abscission of the floral organs. This increase in floral longevity, and in particular in stylar turgescence and receptivity might partially explain the SC phenotype since it could increase the chances of pollen tubes to reach the ovary. To test this, repeated pollination were performed on 3–4 consecutive days, on the 16 RNAi *ScHT-B* transgenic plants and control plants. None of the *ScHT-B* transgenic plants unaffected in *ScHT-B* mRNA expression, or the untransformed control plant, had an extended floral longevity and none were fertilized upon selfing. Of the remaining 12 transgenic plants expressing reduced levels of *ScHT-B* mRNA, nine were able to sire seeds. Fruits formed on these plants were smaller than the ones obtained from a compatible cross, and after 10 days, were comparable in size with fruits produced from a compatible pollination after 6 days (Figure 6, compare the fruits from plants T-2, T-5 and T-10 with the ones from the V22 $\times$ G4 cross). These results are entirely consistent with our hypothesis that reduced level of *ScHT-B* mRNA affects the receptivity period of the flower and that the SC phenotype observed in *ScHT-B* transgenic plants does not result only from the developmentally regulated decrease in both *S-RNase* and *ScHT-B* mRNA levels (Figure 2 a,b), since repeated incompatible pollination could not induce fertilization in control or unaffected *ScHT-B* transgenic plants. Furthermore, in *N. alata HT* antisense plants, pollen tube growth in the style is observed even when the S-RNase level is high, although in that case, fertilization and production of fruits could not be observed because the recipient plant used was a sterile hybrid between *N. alata* and *N. plumbaginifolia* and only pollen tube growth in the style was used to score the SI or SC phenotype.

Our results clearly indicate that there is an increase in flower longevity and pollination receptivity, associated with a decrease in *ScHT-B* transcripts. One possibility would be that the HT-B isoform is involved in a pathway regulating floral abscission. Pollination is known to affect the physiological state of the flower. Pollinated flowers (compatible) senesce rapidly compared to unpollinated flowers or those pollinated by incompatible pollen grains in the case of an SI plant (Gilissen, 1977; Singh *et al.*, 1992). Early studies in *Petunia* ovaries showed an increase in polyribosomes activity, 6–12 h after pollination, well before the arrival of the pollen tubes in the ovary (approximately 50 h) (Deurenberg, 1976). Pollination-induced wilting of the corolla can be prevented if the style is removed early after pollination (Gilissen, 1984). These and other results (Stead, 1992) have led to the hypothesis that a pollination-induced signal is transmitted through the pistil and precedes the growing pollen tube. Ethylene has been shown to have a strong effect on flower abscission in solanaceous species (van Doorn, 2002a,b). Furthermore, pollination itself induces ethylene synthesis (Hall and Forsyth, 1967), and it has been shown that in *P. hybrida*, pollination induces two distinct phases of ethylene production in the flower (Singh *et al.*, 1992). The first phase is common to both self- and cross-pollinated flowers and is dependent on pollen-borne ACC (ethylene precursor). The second phase results from *de novo* synthesis of ethylene from the flower and occurs 18 h after a compatible pollination. Following an incompatible pollination, the production of ethylene is delayed to 3 days after pollination (Singh *et al.*, 1992). Since RNAi *ScHT-B* plants showed delayed floral abscission, we tested these plants for alteration in the expression of ethylene-related genes. No differences could be observed in the expression pattern of two genes involved in ethylene biosynthesis (ACC synthase and ACC oxidase), or in ethylene perception and signal transduction (ethylene receptor ETR1 and EIL-3) in *ScHT-B* transgenic plants (data not shown). Since the ACC synthase and the ACC oxidase genes are part of multigene families (at least eight members for the ACC synthase and four members for the ACC oxidase in *S. lycopersicon*) (Llop-Tous *et al.*, 2000), specific probes will need

to be designed for individual members in order to determine if a given isoform is affected in *ScHT-B* mutant background.

From our results, we propose that the *ScHT-B* isoform is involved in at least two phenomena. Firstly, elevated levels of both S-RNases and *ScHT-B* would be necessary for the SI reaction to occur, as determined from McClure's work (McClure *et al.*, 1999) and from the phenotype of the *ScHT-B* RNAi plant T-2. When the *ScHT-B* mRNA levels are below a threshold level, pollen tubes would be able to reach the ovary and effect fertilization. The developmentally regulated decrease in both *S-RNase* and *ScHT* mRNA levels (Figure 2a,b) would normally lead to an SC phenotype in aged flowers, but is counterbalanced by floral abscission. The increase in both *S-RNase* and *ScHT* mRNA levels following an incompatible pollination (Figure 2d) would also ensure the maintenance of a strong reproductive barrier over a longer period of time. This could be of importance since flowers pollinated with incompatible pollen last longer by an average of 1 day on the plant than unpollinated flowers (Figure 6, G4 × G4), and the receptivity period for a successful pollination is normally limited to the first 2–3 days after anthesis. Secondly, the *ScHT-B* RNAi transgenic plants display a novel phenotype that includes a longer floral longevity with delayed stylar abscission and, perhaps, more relevant for the SC phenotype of those plants, the persistence of turgid styles, even 9 days after anthesis (Figure 6). This phenotype, observed in plants with reduced levels of *ScHT-B* mRNAs, would enable pollen tube growth in older styles with reduced S-RNase level, pass their normal flower lifespan and pollination receptivity period. As suggested by McClure (McClure *et al.*, 1999), the HT protein could interact directly with pollen tubes and facilitate S-RNase uptake. Our two-hybrid results would support the fact that *ScHT-B* does not interact directly with the S-RNase. Furthermore, if the HT-B protein is involved in S-RNase uptake, a reduced level of HT-B protein would increase the number of pollen tubes not affected by the presence of the S-RNase, enabling fertilization to take place. This is entirely consistent with our results, since repeated pollination in *ScHT-B* RNAi plants increase the percentage of fertilized ovules. Although its mode of action still remains unclear, our data demonstrate a specific role for the HT-B isoform in SI and points towards a role in the control of flower senescence and abscission.

Experimental procedures

Plant material and transformation

The diploid ($2n = 2x = 24$) *S. chacoense* Bitt. SI genotypes used included line 314 ($S_{11}S_{12}$), 582 ($S_{13}S_{14}$), G4 ($S_{12}S_{14}$) and V22 ($S_{11}S_{13}$). Plants were grown in greenhouse with 14–16 h of light per day. Transformation was done as described previously and the transformation host plant was line G4 (Matton *et al.*, 1997). The *ScHT-A*$_1$ cDNA was cloned in antisense orientation downstream of a CaMV 35S promoter with doubled enhancers (Skuzeski *et al.*, 1990) and flanked by the nos terminator in the pBIN19 vector (Bevan, 1984). For RNA interference experiments, a new vector was constructed. This new vector, called pDARTH (O'Brien and Matton, unpublished), includes a CaMV 35S promoter with doubled enhancers (Skuzeski *et al.*, 1990), an extended multiple cloning site and a 327-bp intron from a histone deacetylase (*HD2*) gene from *S. chacoense* (our unpublished results). The *ScHT-B*$_1$ cDNA sequence (324 bp) was cloned in sense and antisense orientation separated by the *HD2* intron.

Isolation of the ScHT *cDNAs and PCR amplification of other solanaceous* HT *genes*

The *ScHT-A*$_1$, *ScHT-A*$_2$ and *S*$_{14}$*-RNase* cDNAs were initially isolated from a pollinated pistil cDNA library using virtual subtraction (Li and Thomas, 1998). In this procedure, genes corresponding to low-expressed mRNA species are preferentially isolated. Because the initial screen was for rare mRNA species expressed in ovary tissues, and since the library also contained cDNAs expressed in styles, genes that were highly expressed in styles but only weakly expressed in ovaries were also recovered. A second screening round with a probe derived from stylar mRNAs, uncovered all of the stylar expressed genes, including the *ScHT-A*$_1$, *ScHT-A*$_2$ and *S*$_{14}$*-RNase* cDNAs. For the isolation of the *ScHT-B*$_1$ cDNA and of related sequences in other solanaceous species, three degenerate primers were designed based on the most conserved amino acid sequence of *ScHT-A*$_1$ from *S. chacoense* and HT from *N. alata* (McClure *et al.*, 1999). The sequence of the upstream primers (HT-NS1: 5′-TTT CTT TGG TTC TT(A/T) TGA T(A/T)A TAT CAT CA-3′; HT-NS2: 5′-ATA TCA TCA GA(A/G) GTT ATT GC(A/T) AGG GA(A/T) ATG-3′) are derived from the predicted signal peptide sequence, and the sequences of the downstream primers (HT-C1 : 5′-TCC TTT ATT CAA CCA AT(C/T) TCA TAT TA-3′; HT-C2B: 5′-CAA AAA TAT TAC ATA ATA TTT TGT AGT CG-3′) are derived from the C-terminus of the HT protein. The *S. chacoense* HT-B1 isoform was obtained by PCR amplification of cDNAs from a pollinated pistil library while HT isoforms from *S. pinnatisectum*, *S. bulbocastanum* and *S. tuberosum* were obtained by PCR amplification of genomic DNA.

Isolation and gel blot analysis of RNA and DNA

Total RNA was isolated as described previously (Jones *et al.*, 1985). RNA concentration was determined by measuring its absorbance at 260 nm and verified by agarose gel electrophoresis following ethidium bromide staining. To confirm equal loading of total RNA on RNA-gel blots, a 1-kb fragment of the *S. chacoense* 18S RNA was PCR amplified and used as a probe (Lantin *et al.*, 1999a). Genomic DNA isolation was performed via a modified CTAB extraction method (Reiter *et al.*, 1992) or with the Plant DNeasy kit from Qiagen. DNA-gel blot analysis, including restriction, electrophoresis and capillary transfer to a positively charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Baie D'Urfé, Québec) were performed as described previously (Sambrook *et al.*, 1989). Hybridization of the membrane was performed under high stringency conditions at 65°C as described previously (Church and Gilbert, 1984) for 16–24 h, and following hybridization, the membrane was washed at room temperature twice with 2X SSC/0.1% SDS for 30 min, twice with 1X SSC/0.1% SDS at 50°C for 30 min and twice with 0.1X SSC/0.1% SDS at 55°C for 10 min (1X SSC is 0.15 M

NaCl, 0.015 M sodium citrate, pH 7.0). RNA-gel blot analyses were performed as described in Sambrook *et al.* (1989), following the formaldehyde denaturing protocol. RNAs were capillary transfered to Hybond N+ nylon membranes and cross-linked (120 mJ cm^{-2}) with a Hoefer UVC 500 UV Crosslinker. Hybridization of the membranes was performed under high stringency conditions at 45°C in 50% deionized formamide, 5X Denhardt solution, 0.5% SDS, 200 μg ml^{-1} denatured salmon sperm DNA and 6X SSC for 16–24 h. Following hybridization, the membranes were washed at room temperature twice with 2X SSC/0.1% SDS for 30 min, twice with 1X SSC/0.1% SDS at 50°C for 30 min and twice with 0.1X SSC/0.1% SDS at 55°C for 10 min. Probes for DNA-gel blot analysis were synthesized from random-labeled isolated DNA inserts (Roche Diagnostic, Laval, Québec) with α-^{32}P dCTP (ICN Biochemicals, Irvine, CA). For RNA-gel blot analyses, cDNA probes were made with α-^{32}P dATP with the Strip-EZ DNA labeling kit (Ambion, Austin, TX) and oligonucleotide probes were labeled with γ-^{32}P dATP (Sambrook *et al.*, 1989). The membranes were autoradiographed at −85°C with one intensifying screen on Kodak Biomax MR film (Interscience, Markham, Ontario).

Site-directed mutagenesis of the S_{11}-RNase and yeast two-hybrid analysis

A mutated S_{11}-RNase gene with the conserved His-114 residue (CAT) located in the C3 active site domain was converted to a leucine residue (CTT) by site-directed mutagenesis using the following oligonucleotide (mutated nucleotide is underlined): 5′-CTAAAGCTTGGATCCTGCTGT-3′ (Altered sites II *in vitro* mutagenesis system, Promega, WI). The original construct contained both the S_{11} intron and 3′ end of the gene, and was expressed in transgenic *S. chacoense* plants (Matton *et al.*, 1997; Matton *et al.*, 1999) under the control of the style specific chitinase promoter (Harikrishna *et al.*, 1996). The spliced His^- S_{11}-RNase cDNA was recovered from reversed transcribed style mRNAs, and the coding region corresponding to the mature protein was PCR amplified (Pwo DNA polymerase, Roche Diagnostics, Laval, Québec) and fused in frame with the DNA-binding domain of the GAL4 protein in the pBDGAL4 yeast vector (TRP1 selection marker) (Stratagene, LaJolla, CA). The *ScHT-A*$_1$ coding region was PCR amplified with or without the predicted signal peptide and inserted in frame with the GAL4 activation domain in the pADGAL4 vector (LEU2 selection marker). For the *ScHT-B* construct, only the coding region without the predicted signal peptide was inserted in frame with the GAL4 activation domain in the pADGAL4 vector. Integrity of the DNA constructs was verified by sequencing. The constructs were transformed sequentially in the yeast strain PJ69-4A (James *et al.*, 1996) and selected through their ability to grow on Trp^- and Leu^- media. Protein–protein interaction assays were performed on media lacking Trp, Leu and His and on media lacking Trp, Leu and Ade.

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