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.
**Results**

Isolation of the Solanum HT homologues and sequence comparison

The ScHT-A1, ScHT-A2 and S14-RNase cDNAs were isolated from a pollinated pistil cDNA library (see Experimental procedures section). The ScHT-A1 cDNA codes for a small protein of 99 amino acids with a highly predicted N-terminal signal peptide as determined from the SignalP algorithm (Nielsen et al., 1997). The predicted cleavage site for ScHT-A1 is before Arg-25, producing a mature polypeptide of 75 amino acids (8 kDa). The ScHT-A2 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-A1-deduced cleavage site. Both ScHT-A1 and ScHT-A2 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-A1 and ScHT-A2 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-B1 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-A1 isoforms. The ScHT-B1 mature protein comprises 79 amino acids (MW, 8.7 kDa) with an acidic pl 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 B1 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-B1. 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-A1 and ScHT-A2 (94%), SbHT-B1 and SbHT-B2 (98%) and SpHT-B1 and SpHT-B2 (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-A1, NaHT-B, LpHT-A1 and LpHT-B1), 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).
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-A1 and ScHT-A2 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-A1. 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 S14-RNase (Figure 2a, b). Both ScHT isoforms and the S14-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 S14-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 S14-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 S14-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 S14-RNase mRNA levels declined similar to

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**Figure 1.** Sequence alignment (a) and phylogenetic analysis (b) of the deduced mature protein sequences of ScHT-A1, ScHT-A2 and ScHT-B1 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.
the developmentally regulated decrease observed in unpollinated flowers (compare Figure 2a,d). An incompatible pollination had the opposite effect. The ScHT-A and S14-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 cDNA insert as a probe, an F1 population from a parental cross was tested for polymorphism and linkage to the S-locus. A fraction of the F1 progeny tested is shown in Figure 3. The S-RNase genotype of the progeny had been determined previously 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 pl around 4, and since S-RNases are basic proteins (S14-RNase mature protein, predicted pl is 9.12), ScHT proteins could interact directly with the pollen tubes and...
facilitate S-RNase uptake. To test if the HT protein can interact directly with the S-RNase, the ScHT–A1 and ScHT–B1 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 S11-RNase that was previously produced by site-directed mutagenesis was fused with or without the predicted signal peptide, and inserted in frame downstream of the yeast GAL4 activating domain in the pADGAL4 vector. Because of their intrinsic ribonuclease activity, the S11-RNase used in the two-hybrid analysis was mutated 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 S11-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-A1 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 S12S14 genotype were transformed with Agrobacterium tumefaciens LBA4404 strain containing the ScHT-A1 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-A1 complete cDNA or the ScHT-B, 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 mRNA levels, a modified version of the antisense construct was made. This construct contained a specific oligonucleotide from the ScHT-B protein, and the results of the crosses are shown below.

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 F1 population (S11S13 × S12S14) segregating for four S-alleles was digested with HindIII and probed with the ScHT-A cDNA insert (upper panel). Identical 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 S11-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 transgenic plants. Control plant (C) is the untransformed host (genotype S12S14). All crosses were done on at least 10 flowers with pollen from a fully compatible plant (genotype S11S13) or a fully incompatible plant (genotype S13S14). Ten micrograms of total style RNA from each plant was probed with the ScHT-A1 cDNA insert and the ScHT-B1 specific oligonucleotide. Equal loading conditions were verified with an 18S ribosomal cDNA probe from S. chacoense.
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 cDNA was inserted first in the sense orientation downstream of the CaMV 35S promoter, followed by a 327-bp spacer, and by the ScHT-B1 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 S12S14 genotype. Sixteen primary transformants were initially selected. All ScHT-B RNAi lines were cross-pollinated with pollen from fully compatible (S11S13) or fully incompatible (S12S14) genotypes. Two plants (#2 and #3) sired seeds upon self-pollination (pollen from genotype S12S14), 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-A1 complete cDNA or the ScHT-B1-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
semi-compatible (plants #2, 5, 6, 8, 10–13 and 16) upon repeated pollination with fully incompatible pollen (geno-
type S_{12}S_{14}). Floral and stylar abscission were also mon-
tored, 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) 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/C2).

As such, with the remaining flowers at the end of the 10-day
period, the whole time-course was displayed. Pistil mor-
phology 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-polli-
nated 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 duplica-
tion 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

Such factors, often considered as modifier loci, are present in the genetic background of SI plants, unlked 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 F₁ population from two independent SC accessions were all SC, while SI offspring could be recovered in the F₂ generation from these F₁ 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 share 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 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-isofom 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 arrest 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 (budding 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 unpolinated 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 × 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 pl and the S-RNase is basic (predicted pl = 9.25 for S₁, 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., 1997). Intriguingly, 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-BmRNA 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 abortion of the floral organs. This increase in floral longevity, and in particular in styril 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 × 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 2a,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 longer transgenic plants display a novel phenotype that includes a ground. 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 longer transgenic plants display a novel phenotype that includes a ground.

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

The ScHT-A1, ScHT-A2 and S. lycopersicum RNAi cDNAs were isolated from a pollinated pistil cDNA library using virtual subtraction (Li and Thomas, 1999). In this procedure, genes corresponding to low-expressed mRNA species are preferentially isolated. Because the initial screen was for mRNA species expressed in ovary tissues, and since the library also contained cDNAs expressed in styles, genes that were highly expressed in ovary tissues were recovered by a second screening round with a probe derived from stylar mRNAs, uncovered all of the stylar expressed genes, including the ScHT-A1 and ScHT-B1 cDNAs. For the isolation of the ScHT-B1 cDNA and of related sequences in other solanaceous species, three degenerate primers were designed based on the most conserved amino acid sequence of ScHT-A1 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 TTT A/TG TGA T(A/T)A TAT CAT CA-3'; HT-NS2: 5'-ATA TCA TCA GAA(A/G) GTT ATT GCA(A/T) AGG GAA(A/T) ATG-3') are derived from the predicted signal peptide sequence, and the sequences of the downstream primers (HT-C1: 5'-TTC TTT ATT CCA CAA AT(C/I)T TCA TAT TA-3'; HT-C2B: 5'-CAA AAA TAT TAC ATA AT A TTT GTC AGT CG-3') are derived from the C-terminus of the HT protein. The ScHT-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 and digested with agarase gel electrophoresis following ethidium bromide staining. To confirm equal loading of total RNA onto RNA gel blots, a 1-kb fragment of the ScHT-B1 cDNA was PCR amplified and used as a probe (Lantin et al., 1999). 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 transferred to Hybond N+ nylon membranes and cross-linked (120 mJ cm⁻²) with a Hafer UV CVC 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, LaJolla, CA). For RNA-gel blot analyses, cDNA probes were made with a-32P dCTP (ICN Biochemicals, Irvine, CA). For RNA-gel blot analyses, cDNA probes were made with a-32P dATP with the Strip-EZ DNA labeling kit (Ambion, Austin, TX) and oligonucleotide probes were labeled with γ-32P 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 S1₁-RNase and yeast two-hybrid analysis

A mutated S1₁-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' - CTAAAAGCTTGGATCCTGCTT3' (Altered sites in italics). The original construct contained both the S₁₁ 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-S1₁-RNase cDNA was recovered from reverse transcribed style mRNAs, and the coding region corresponding to the mature protein was PCR amplified (Pwo DNA polymerase, Roche Diagnostics, LaJolla, Quebec) 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-A1 coding region was PCR amplified with or without the predicted signal peptide and inserted in frame with the GAL4 activation domain in the pBDGAL4 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 pBDGAL4 vector. Integrity of the DNA constructs was verified by sequencing. The constructs were transfected 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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References


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