

## RESEARCH PAPER

# Identification and functional analysis of pistil self-incompatibility factor *HT-B* of *Petunia*

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## Abstract

Gametophytic self-incompatibility (GSI) in Solanaceae, Rosaceae, and Plantaginaceae is controlled by a multiallelic *S*-locus. The specificities of pistil and pollen are controlled by separate *S*-locus genes, *S-RNase* and *SLF/SFB*, respectively. Although the *S*-specificity is determined by the *S*-locus genes, factors located outside the *S*-locus are also required for expression of GSI. *HT-B* is one of the pistil non-*S*-factors identified in *Nicotiana* and *Solanum*, and encodes a small asparagine/aspartate-rich extracellular protein with unknown biochemical function. Here, *HT-B* was cloned from *Petunia* and characterized. The structural features and expression pattern of *Petunia HT-B* were very similar to those of *Nicotiana* and *Solanum*. Unlike other solanaceous species, expression of *HT-B* was also observed in self-compatible *Petunia* species. RNA interference (RNAi)-mediated suppression of *Petunia HT-B* resulted in partial breakdown of GSI. Quantitative analysis of the *HT-B* mRNA accumulation in the transgenics showed that a 100-fold reduction is not sufficient and a >1000-fold reduction is required to achieve partial breakdown of GSI.

**Key words:** HT-B, *Petunia inflata*, pistil, RNAi, self-incompatibility.

## Introduction

Gametophytic self-incompatibility (GSI) is a genetic system that enables the pistil to reject pollen from genetically related plants, and thus contributes to promotion of outcrossing. In the families Solanaceae, Rosaceae, and Plantaginaceae, this system is controlled by a single polymorphic *S*-locus. When the *S*-haplotype of pollen matches one of the two *S*-haplotypes of a diploid pistil, the pollen is recognized as self and rejected by the pistil (de Nettancourt, 2001). The *S*-specificities of pistil and pollen of these families are determined by different *S*-locus genes, *S-RNase* and *SLF/SFB*, respectively (Kao and Tsukamoto, 2004; McClure, 2006; McClure and Franklin-Tong, 2006). Despite its apparent simplicity, the *S*-locus alone is not sufficient to elicit the *S-RNase*-based GSI reaction. Genetic analyses have suggested that factors located outside the *S*-locus are required for expression of the GSI mechanism (Ai *et al.*, 1991; Bernatzky *et al.*, 1995; Hosaka and Hanneman, 1998a, b). Furthermore, expression of high

enough levels of *S-RNases* in transgenic self-compatible (SC) *Nicotiana tabacum*, *N. plumbaginifolia*, or cultivated tomato (*Solanum lycopersicum*) did not confer the *S*-specific pollen rejection function (Murfett *et al.*, 1996; Kondo *et al.*, 2002b). These findings indicate that non-*S*-factors are required for GSI to occur. However, very limited numbers of non-*S*-factors have been cloned and characterized so far.

The stylar 120 kDa glycoprotein (120K) is a non-*S*-factor identified in *Nicotiana*. 120K is an extracellular arabinogalactan protein capable of binding to the *S-RNase* (Cruz-Garcia *et al.*, 2005). RNA interference (RNAi)-mediated suppression of 120K resulted in the breakdown of the capability of the pistil to reject self-pollen, suggesting that it is required for GSI function (Hancock *et al.*, 2005). Another non-*S*-factor gene cloned to date is *HT-B* of *Nicotiana* and *Solanum*. *HT-B* is a pistil-expressed, extracellular, small asparagine/aspartate (N/D)-rich protein and

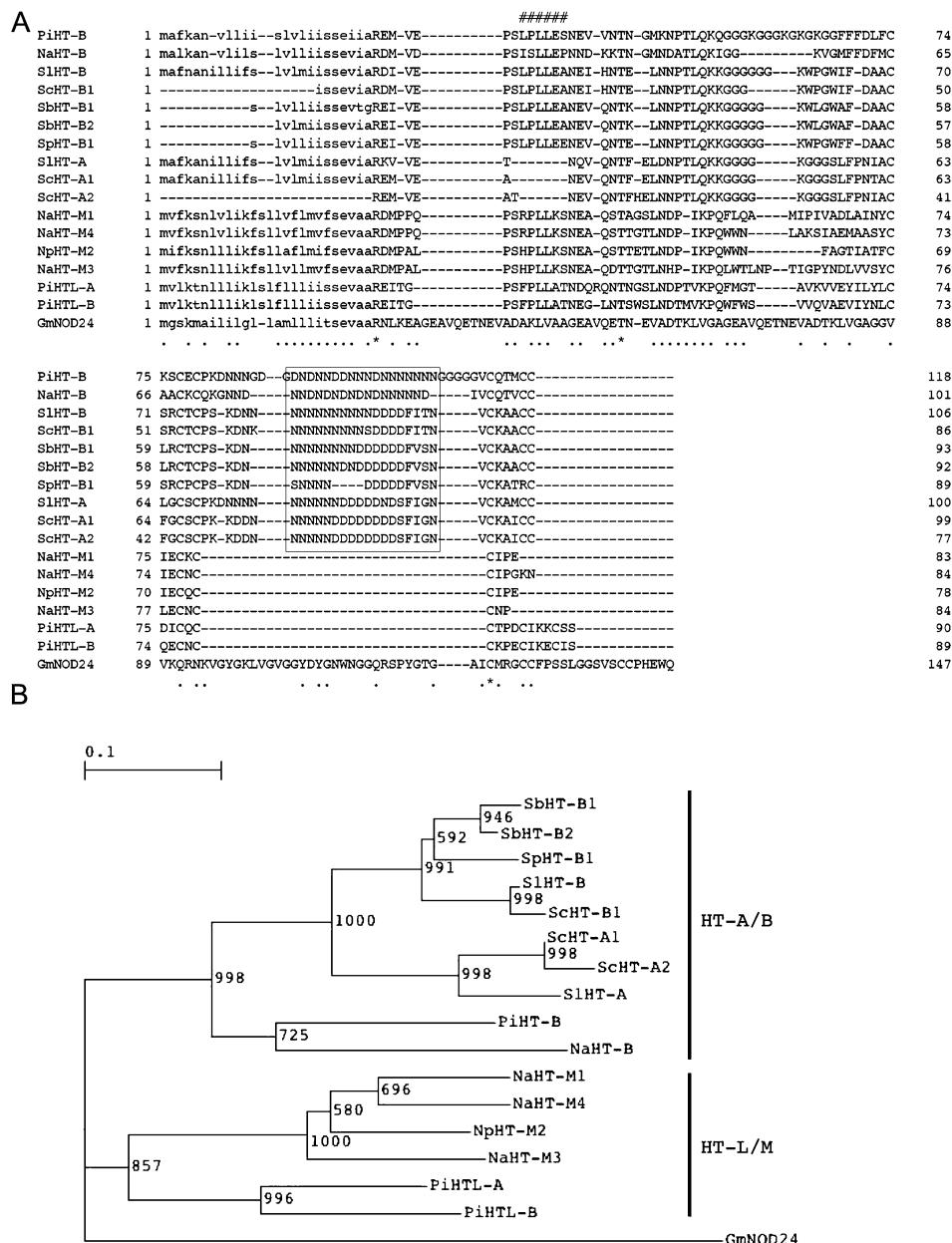
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was first identified in *Nicotiana*. Antisense suppression of *HT-B* caused loss of the pistil part function of GSI, although the transgenic plants retained normal levels of S-RNases (McClure *et al.*, 1999). In wild relatives of tomato and potato, the species of *Solanum*, *HT-B* genes have also been isolated and characterized. Kondo *et al.* (2002b) isolated two *HT*-like genes, *HT-B* and *HT-A*, and found that SC cultivated tomato (*S. lycopersicum*) has defects in both genes. They further extended the analysis of the *HT* genes to other SC and self-incompatible (SI) wild relatives of tomato, and suggested that the mutation affecting the

expression level of *HT-B* may have been primarily responsible for the evolution of SC in cultivated tomato and its wild relatives (Kondo *et al.*, 2002a). Functions of *HT-A* and *HT-B* were examined experimentally in *S. chacoense*, a wild potato. Antisense suppression of *HT-A* did not affect the SI phenotype, while RNAi-mediated silencing of *HT-B* changed the phenotype, suggesting that *HT-B* but not *HT-A* is involved in SI (O'Brien *et al.*, 2002).

Although the biochemical function of *HT-B* is not known at present, immunolocalization has shown that *HT-B* protein is taken up by the pollen tube, and then localized in



**Fig. 1.** Amino acid sequence alignment and phylogenetic tree of HT-like proteins. (A) The amino acid sequences of HT-like proteins were aligned by CLUSTAL W (Thompson *et al.*, 1994). Putative signal peptides are shown in lower case. A characteristic deletion for *HT-B* is denoted with #. The N/D-rich domain was boxed. (B) A Neighbor-Joining tree (Saitou and Nei, 1987) of HT-like proteins. NOD24 protein of soybean (GmNOD24; M10595) was chosen as an outgroup for rooting, because it is a member of the recently proposed expanded HT family, the HT/NOD-24 family, and is distantly related to solanaceous HT-like proteins (Kondo and McClure, 2008). Numbers denote bootstrap values with 1000 replicates.

the membrane of vacuoles in which S-RNases, which have been taken up, are stored (Goldraij *et al.*, 2006). Based on the finding that degradation of HT-B protein is more prominent in compatible pollination than in incompatible pollination, it was hypothesized that HT-B is involved in destabilization of vacuoles, and interaction between the S-RNase and its cognate SLF specifically inhibits the degradation of HT-B, which results in breakdown of the vacuole and release of the S-RNase into the cytoplasm of the self pollen (Goldraij *et al.*, 2006; McClure, 2006).

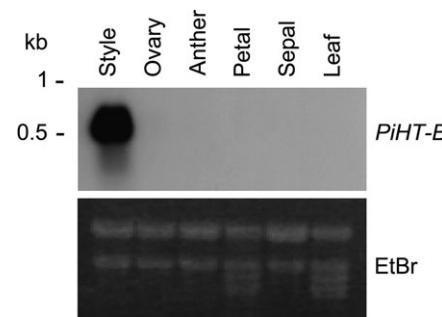
In *Petunia*, a solanaceous species, *HT-B* has not been identified yet. An attempt to clone *HT-B* from *Petunia* resulted in the isolation of a new class of *HT*-like gene, *HTL* (Sassa and Hirano, 2006). *HTL* shared several characteristics with *HT* genes of *Nicotiana* and *Solanum*, i.e. the deduced amino acid sequence including a signal peptide region and conserved cysteine residues near the C-terminus, and style-specific expression. However, the *Petunia* *HTL* protein lacked the N/D-rich domain. Furthermore, suppression by RNAi did not affect the SI phenotype of transgenic *Petunia*, suggesting that the *Petunia* *HTL* is not the orthologue of the non-S-factor *HT-B* of *Nicotiana* and *Solanum*, and is a new member of the *HT*-like gene family (Sassa and Hirano, 2006). Recently, similar *HT*-like proteins that lack the N/D-rich domain were also identified in *Nicotiana* and designated as *HT-M*. RNAi-mediated suppression of *HT-M* did not affect the SI phenotype of the transgenic *Nicotiana* (Kondo and McClure, 2008).

In this study, the isolation of the *Petunia* *HT-B* gene which encodes a protein with the N/D-rich domain is described. RNAi experiments with this gene caused partial breakdown of S-specific pollen rejection. Quantitative analysis of the *HT-B* mRNA accumulation in the transgenics showed that a 100-fold reduction is not sufficient and a >1000-fold reduction is required to achieve partial breakdown of GSI.

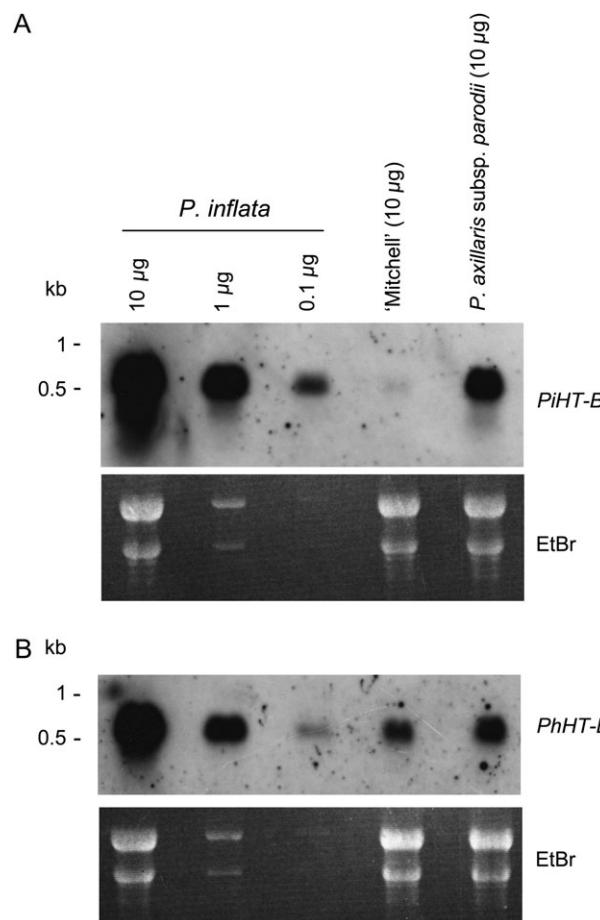
## Materials and methods

### Plant materials

SI *P. inflata* lines ( $S^{3L}S^{3L}$  and  $S^{k1}S^{k1}$ ) were described previously (Sassa and Hirano, 2006). Taking advantage of its high transformation efficiency, SC *P. hybrida* cv. Mitchell was used for transformation (Ausubel *et al.*, 1980). The SI near-isogenic line 'Mitchell' [NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^i$ / $HT\text{-}B^i$ )] was bred by introduction of  $S^{3L}$  and  $HT\text{-}B^i$  of *P. inflata* into 'Mitchell' by backcrossing using 'Mitchell' as a recurrent parent. S genotypes of segregants were analysed by multiplex PCR. DNA was extracted from the plants as described (Sassa, 2007) and used for PCR with a primer pair FSSR1 and RSSR1 for  $S^{3L}$ -RNase (~400 bp, Sassa and Hirano, 2006) and a pair of primers FS $\alpha$ 1 and RS $\alpha$ PT15 (5'-CAGATGTCTACAGTCATCAG-3') and RS $\alpha$ PT15 (5'-CGCGGATCCTCACGGTCGAAACATAATCCC-3') for  $S^m$ -RNase of 'Mitchell' (~320 bp, HS and ARP, unpublished data). *HT-B* genotypes of backcrossed progeny were

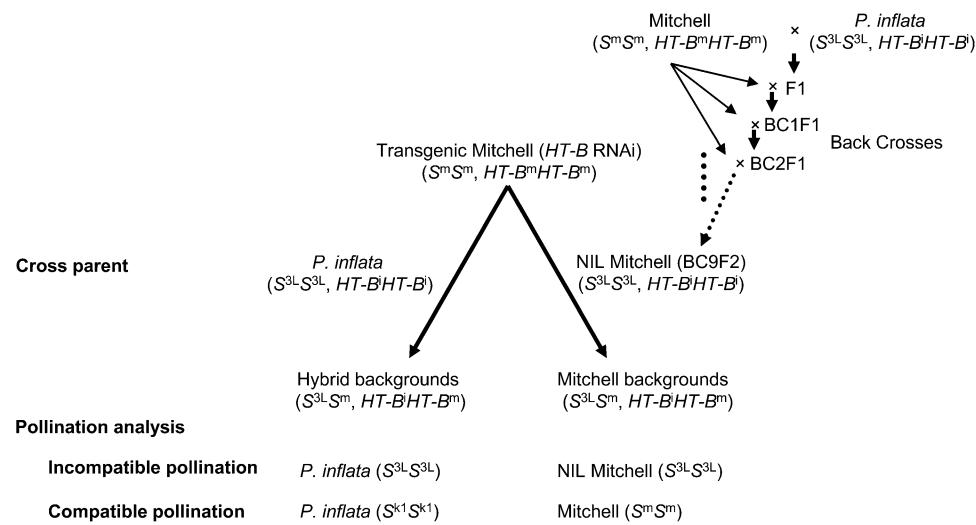


**Fig. 2.** RNA blot analysis of organ-specific expression of *PiHT-B* in *P. inflata*. A 10 µg aliquot of total RNA was loaded in each lane, blotted, and hybridized with a *PiHT-B* probe. The ethidium bromide-stained gel is shown to ascertain equal loading conditions.



**Fig. 3.** RNA blot analysis of *PiHT-B* and *PhHT-B* in styles of *P. inflata*, 'Mitchell' and *P. axillaris* subsp. *parodii*. A 10 µg aliquot of total RNA was loaded in each lane, blotted, and hybridized with *P. inflata* (A, *PiHT-B*) and 'Mitchell' (B, *PhHT-B*) probes. Aliquots of 1 µg and 0.1 µg of *P. inflata* RNA were also loaded in separate lanes to allow for comparison of the level of expression. The ethidium bromide-stained gels are shown to ascertain equal loading conditions.

analysed by PCR with FHTC $\alpha$ r1 (5'-ACGCTTCAAAAA-CAAGGAGG-3') and RHTB $\alpha$ T15 (5'-CGCGGATCCTAACACACATGGTTGGC-3') that generate fragments of 213 bp (*inflata* allele,  $HT\text{-}B^i$ =*PiHT-B*) and 190 bp (Mitchell



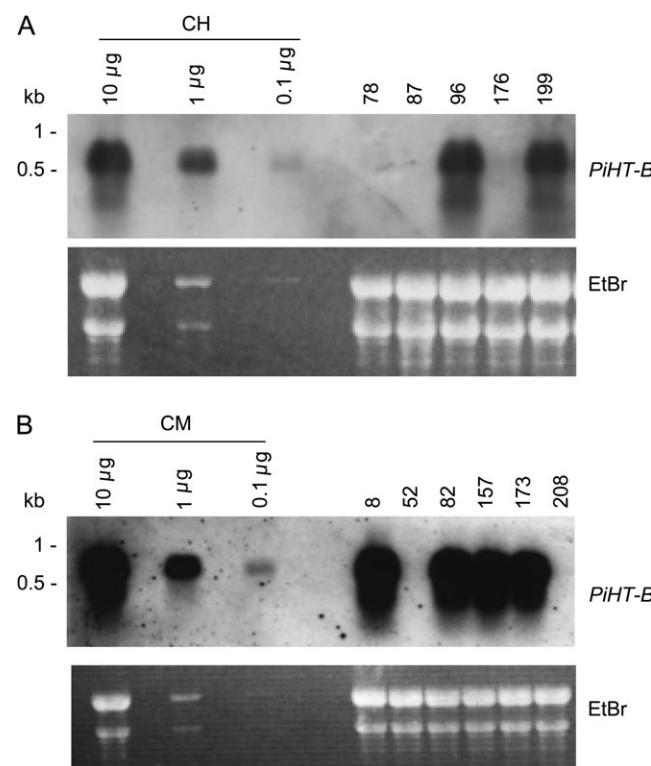
**Fig. 4.** Transgenic plant experiments testing the role of *PiHT-B* in the S-specific pollen rejection. The RNAi construct for *PiHT-B* was first introduced into the SC line 'Mitchell'. The transgenics were crossed with SI lines *P. inflata* ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ ) or NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ ) to produce 'hybrid background' or 'Mitchell background' transgenics, respectively, and used for analysis of the S-specific pollen rejection.

allele,  $HT\text{-}B^m$ =*PhHT-B*). A BC9F1 plant ( $S^{3L}S^m$ ,  $HT\text{-}B^iHT\text{-}B^m$ ) was bud pollinated with self-pollen, and a BC9F2 plant of  $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$  was selected [NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ )]. SI of NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ ) was confirmed by self-pollination.

#### Cloning of cDNA and the genomic fragment

Total RNA was extracted from the pistils of *P. inflata* ( $S^{3L}S^{3L}$ ), and reverse transcribed with oligo d(T) RACE-N primer to generate first-strand cDNA as described by Ushijima *et al.* (2003). The cDNA fragment of *PiHT-B* was first amplified by FHT-3 (5'-RWTGAAYGAYSCAACACTCC-3') and HT-C1 [5'-TCCTTTATTCAACCAAT(C/T)TCATATTA-3', Kondo *et al.* (2002b)], and the product was then used as the template for nested PCR with FHT-4 (5'-STGTCASSTTGCAMWTGCC-3') and RHTB1 (5'-CTAACACAACARCGGYTTKAC-3'). 5' RACE (rapid amplification of cDNA ends) was conducted by using a specific reverse primer RHTb1 (5'-GTCGTTGTTATCATTATCACCC-3') which was designed based on the RT-PCR fragment for *PiHT-B*. Based on the 5' RACE clone sequence, the forward primer FPH5E (5'-ATTCACAAACTAAATATCAACAAAC-3') was designed and used to amplify the full-length cDNA of *PiHT-B* by 3' RACE using a high fidelity DNA polymerase *Pyrobest* (Takara, Ohtsu, Japan). The PCR products were cloned into a pZER-O-2 vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The DDBJ/GenBank/EMBL accession number of *PiHT-B* is AB191255. The *HT-B* allele of 'Mitchell' was also cloned by RT-PCR (*PhHT-B*, AB468968).

A bacterial artificial chromosome (BAC) library of *P. inflata* ( $S^{3L}S^{k1}$ ) was constructed by using pECBAC1 (Amplicon Express, Pullman, WA, USA). The library consisting of 64 436 clones with an average insert size of 129 kb (~7.2-fold genome coverage) was screened by the



**Fig. 5.** RNA blot analysis of *PiHT-B* in styles of RNAi transgenic plants. (A) Hybrid background transgenic lines 78, 87, 96, 176, and 199 [transgenic 'Mitchell'  $\times$  *P. inflata* ( $S^{3L}S^{3L}$ )]. (B) Mitchell background transgenic lines 8, 52, 157, 173, and 208 [transgenic 'Mitchell'  $\times$  NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ )]. A 10  $\mu$ g aliquot of pistil total RNA was loaded in each lane. Aliquots of 1  $\mu$ g and 0.1  $\mu$ g of RNA of the untransformed plants were also loaded in separate lanes to assess the level of suppression. CH and CM are untransformed controls of hybrid and Mitchell backgrounds, respectively. The ethidium bromide-stained gel is shown to ascertain equal loading conditions.

*HT-B* cDNA clone of *S. peruvianum* (Kondo *et al.*, 2002b) or by *PiHT-B* as probe under low and high stringency, respectively.

#### Isolation and gel blot analysis of DNA and RNA

Isolation of nucleic acids, electrophoresis, blotting, and hybridization with digoxigenin (DIG)-labelled probes were performed as described in Sassa and Hirano (2006).

#### RNA silencing

The construct for RNA silencing was prepared by using pHANNIBAL (Wesley *et al.*, 2001). The *HT-B* coding region was amplified from cDNA by *Pyrobest* polymerase with iFPHT1 (5'-GCTCTAGACTCGAGTTAACCGTC-CAAAATATG-3') and iRPHT1 (5'-GCATCGATGGTACCAAGATAATCATGCCATTAC-3'), and was cloned into pHANNIBAL in sense and antisense orientation separated by the *Pdk* intron (Wesley *et al.*, 2001). The resultant *HT-B* silencing cassette was excised by *SacI* and *SpeI*, and inserted into the *SacI-XbaI* sites of pBINPLUS (van Engelen *et al.*, 1995) to obtain pBINiPiHT-B. The silencing construct was introduced into *Agrobacterium tumefaciens* LBA4404 to transform 'Mitchell' by the leaf disk method (Horsch *et al.*, 1985). For the analysis of the effect of *HT-B* suppression on the incompatibility phenotype, transgenic 'Mitchell' lines were crossed with SI *P. inflata* ( $S^{3L}S^{3L}$ ) or NIL Mitchell ( $S^{3L}S^{3L}$ , *HT-B<sup>i</sup>*/*HT-B<sup>i</sup>*) to introduce a functional  $S^{3L}$  allele. The heterozygous hybrid transgenics ( $S^{3L}S^{m}$ ) were pollinated with pollen from incompatible ( $S^{3L}S^{3L}$ ) or compatible genotypes ( $S^{k1}S^{k1}$  or  $S^{m}S^{m}$ ) to analyse the effect of the transgene on the  $S$ -specific pollen rejection.

#### Quantitative RT-PCR

Total RNA preparations were treated with DNase I twice, and the absence of genomic DNA in the RNA was confirmed by PCR. First-strand cDNA was synthesized from 1 µg of the DNase-treated RNA with ReveTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primer. Quantitative RT-PCR (qRT-PCR) was performed with SYBR Premix Ex Taq II (TaKaRa, Ohtsu, Japan) on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instruction. Each reaction was performed with 4 µl of a 1:20 (v/v) dilution of the synthesized cDNA with 0.4 µM of each primer in a 20 µl reaction volume. The cycling conditions were as follows: 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 10 s, and extension at 72 °C for 15 s. The specificity of the PCR amplification was verified by a dissociation curve analysis. Ser/Thr protein phosphatase 2A (PP2A), which was found to be very stably expressed in *Arabidopsis* (Czechowski *et al.*, 2005) and tomato (K Ushijima *et al.*, unpublished data), was used as an internal control to calculate the efficiency of the cDNA synthesis. The primers used are as follows; PP2A (PhPP2AQtF, 5'-AGCTTGGTGCTATGCATGC-3' and

PhPP2AQtR, 5'-TTCCTCAGCAAGGCCTAAC-3'), PiHT-B (PiHTBLQtF, 5'-CTAAATATCAACAACTTCT-ATAAG-3' and PiHTBLQtR, 5'-CTTGTGTTTGAAGCG-TAG-3'), and PiHTL (PiHTLsQtF, 5'-GGGATCTACAAA TATCAACATC-3' and PiHTLsQtR, 5'-CCTAGCAGCA-ACCTCTGA-3'). The transcript levels of PiHT-B and PiHTL were normalized to that of PP2A.

#### Pollination phenotypes

Emasculated flowers were pollinated at anthesis. After 48 h they were harvested and their pistils were fixed in acetic acid:70% ethanol (1:3) for 1 d. Pistils were then treated with 1 N NaOH for 3 h and stained with aniline blue (0.1%) for 24 h. Pollen tube growth was observed by fluorescence microscopy. For fruit set analysis, pollination was performed as described above and fruits were detached from the plants and photographed 2 weeks after pollination. All plants were crossed at least three times with each pollen donor for each type of experiment.

## Results

#### Identification of Petunia HT-B

*PiHT-B* was isolated from pistils of *P. inflata* by RT-PCR using degenerate primers designed according to *HT* sequences of *Nicotiana* and *Solanum*. Figure 1A shows the deduced amino acid sequence of PiHT-B and other related proteins.

**Table 1.** Fruit sets of RNAi transgenics for *HT-B*

Hybrid background transgenics [transgenic 'Mitchell' × *P. inflata* ( $S^{3L}S^{3L}$ )] were pollinated with pollen from *P. inflata*  $S^{k1}S^{k1}$  or *P. inflata*  $S^{3L}S^{3L}$ , while Mitchell background transgenics [transgenic 'Mitchell' × NIL Mitchell ( $S^{3L}S^{3L}$ , *HT-B<sup>i</sup>*/*HT-B<sup>i</sup>*)] were pollinated with 'Mitchell' or NIL Mitchell  $S^{3L}S^{3L}$ . Data are presented as the number of fruits over pollinations. CH and CM are untransformed controls of hybrid background and Mitchell background, respectively. DT denotes a double transformant.

Pistil	Pollen	
Hybrid background ( $S^{3L}S^{m}$ )	<i>P. inflata</i> ( $S^{k1}S^{k1}$ )	<i>P. inflata</i> ( $S^{3L}S^{3L}$ )
CH	3/3	0/3
78	3/3	0/3
87	3/3	0/3
96	3/3	0/3
176	3/3	0/3
199	3/3	0/3
94 (DT)	3/3	0/3
Mitchell background ( $S^{3L}S^{m}$ )	'Mitchell' ( $S^{m}S^{m}$ )	NIL Mitchell ( $S^{3L}S^{3L}$ )
CM	3/3	0/3
8	3/3	0/3
52	3/3	4/11*
82	3/3	0/3
157	3/3	0/3
173	3/3	0/3
208	3/3	4/17*

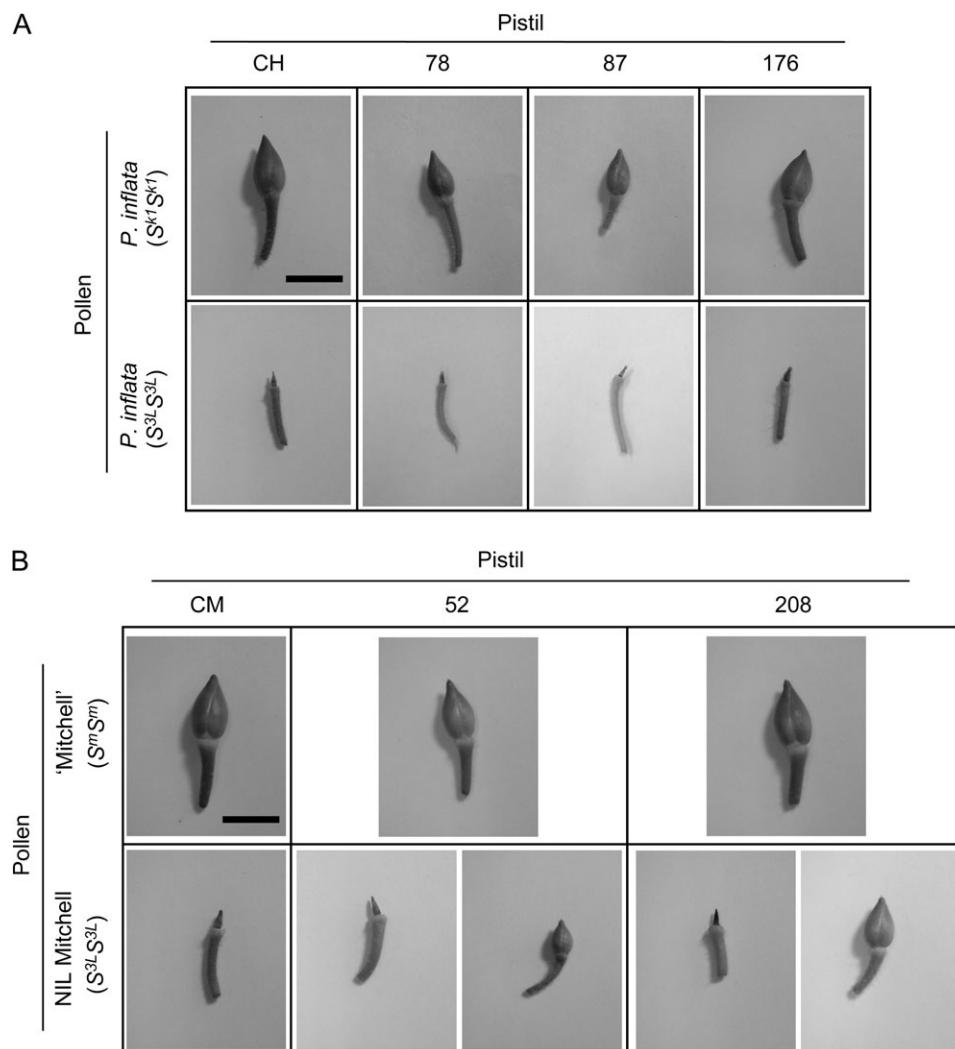
\* Fruit sizes were smaller than those obtained by compatible pollination. See text and Fig. 6B.

*PiHT-B* encodes a 119 residue amino acid protein with a predicted cleavage site before Arg24 (SignalP, Nielsen *et al.*, 1997). The pI of the mature protein is calculated to be 4.42. *PiHT-B*, unlike the previously described *PiHTL* (Sassa and Hirano, 2006) and like other HT-B proteins, contains a C-terminal N/D-rich domain (23 residues) with three cysteine residues at each side. HT-A proteins have a characteristic deletion of 5–7 residues near the N-terminal region of mature proteins (Kondo *et al.*, 2002b; O'Brien *et al.*, 2002). The corresponding region of *PiHT-B* was much closer to that of solanaceous HT-Bs and *Petunia* HTL than to HT-As (Fig. 1A). Phylogenetic analysis showed that the HT-like proteins were first classified into two groups, the HT-A/B group and the HT-L/M group (Fig. 1B). *PiHT-B* was categorized in the HT-A/B group, and was closely related to HT-B of *Nicotiana*. Identities between *PiHT-B* and the other solanaceous proteins ranged between 34.9% and 58.0%.

A *P. inflata* BAC library was screened with the *S. peruvianum* *HT-B* probe (Kondo *et al.*, 2002b) and the *PiHT-B* cDNA under low and high stringency conditions, respectively. Two positive clones, 22C21 and 21I9, were obtained. Southern blotting analysis of these two clones revealed that they exhibited very similar restriction patterns, suggesting that they are overlapping with each other (data not shown). No other positive clones were recovered from the library that is ~7.2-fold genome coverage, suggesting that there are no *HT-B*-like sequences in the genome of *P. inflata* other than *PiHT-B*. This is consistent with the result of genomic Southern blot analysis (data not shown).

#### Style-specific expression of *PiHT-B*

RNA blot analysis was performed with RNA from style-stigma (hereafter style), ovary, anther, sepal, petal, and leaf of *P. inflata*, and the results are shown in Fig. 2. *PiHT-B*



**Fig. 6.** Fruit development in RNAi lines for *HT-B*. (A) Hybrid background transgenics [transgenic 'Mitchell' × *P. inflata* ( $S^{3L}S^{3L}$ )]. (B) Mitchell background transgenics [transgenic 'Mitchell' × NIL Mitchell ( $S^{3L}S^{3L}$ ), *HT-B*/*HT-B*]. Pistils were pollinated, detached from the plants after 2 weeks, and photographed. CH and CM are untransformed controls of hybrid and Mitchell backgrounds, respectively. For plants 52 and 208, two types of responses after incompatible pollination [NIL Mitchell ( $S^{3L}S^{3L}$ )] are presented; aborted (left) and developed fruits (right). Bar=1 cm.

expression was restricted to the style as is that of other solanaceous *HT-B* genes.

*HT-B* expression was also compared between *P. inflata* (SI), *P. axillaris* subsp. *parodii* (SC), and 'Mitchell' (SC). The *HT-B* allele of 'Mitchell', *PhHT-B*, was cloned by RT-PCR and also used as a probe. Nucleotide and deduced amino acid identities between the two *Petunia* genes/proteins were 91.6% and 90.7%, respectively. As shown in Fig. 3, *P. inflata* presented a very strong signal when the blots were hybridized with either *PiHT-B* or *PhHT-B*. In contrast, 'Mitchell' showed almost no signal when the blot was hybridized with *PiHT-B* and a moderate one when hybridized with *PhHT-B*. *Petunia axillaris* subsp. *parodii* had a moderate signal with both probes, although a slightly stronger intensity is observed with *PiHT-B*.

#### RNAi-mediated suppression of *PiHT-B*

Taking advantage of its high transformation efficiency, SC line 'Mitchell' was used to introduce the RNAi construct for *PiHT-B*. The transgenics were crossed with SI lines *P. inflata* ( $S^{3L}S^{3L}$ ) or NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ ) to produce 'hybrid background' and 'Mitchell background' transgenics, respectively (Fig. 4). Independent 'Mitchell' transgenics were used to produce 'hybrid backgrounds' and 'Mitchell backgrounds'. The two types of heterozygous transgenics ( $S^{3L}S^m$ ,  $HT\text{-}B^iHT\text{-}B^m$ ), 'hybrid backgrounds' and 'Mitchell backgrounds', were used to analyse the effect of *PiHT-B* suppression on the S-specific pollen rejection function. Similarly, an *HTL*-suppressed *P. inflata* plant (Sassa and Hirano, 2006) was crossed with 'Mitchell' *HT-B* RNAi transgenic plants to produce double transformants. Transgenics were analysed by Southern blotting to confirm the presence of transgenes (data not shown). Five independent 'Mitchell'  $\times$  *P. inflata* ( $S^{3L}S^{3L}$ ) transformants (hybrid backgrounds;  $S^{3L}S^m$ ,  $HT\text{-}B^iHT\text{-}B^m$ ) were subjected to RNA blot analysis (Fig. 5A). Suppression levels varied from almost no suppression to approximately a >100-fold reduction in comparison with the untransformed control. Likewise, six independent 'Mitchell'  $\times$  NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ ) transgenics (Mitchell backgrounds;  $S^{3L}S^m$ ,  $HT\text{-}B^iHT\text{-}B^m$ ) showed different levels of suppression (Fig. 5B). Those S-heterozygous transgenics were subjected to pollination with S-homozygous lines for phenotype analyses.

Fruit set analysis revealed that all hybrid background transformants (including the double transformant) presented the same phenotype as that of the control plant regardless of the level of suppression (Table 1). The use of compatible pollen from *P. inflata* ( $S^{k1}S^{k1}$ ) resulted in the development of large fruits (Fig. 6A). Conversely, the use of incompatible pollen from *P. inflata* ( $S^{3L}S^{3L}$ ) led to no fruit formation.

The Mitchell background transgenic plants behaved in a similar manner except for those which presented a severe *HT-B* suppression, lines 52 and 208. These plants produced fruits upon pollination with  $S^{3L}$  pollen in some of the crosses (Table 1). However, as shown in Fig. 6B, these fruits

were smaller than those produced in crosses between control Mitchell background (CM;  $S^{3L}S^m$ ,  $HT\text{-}B^iHT\text{-}B^m$ ) and 'Mitchell'. The average fruit length ( $\pm SD$ ) of the CM  $\times$  'Mitchell' crosses was  $1.2 \pm 0.06$  cm, while for plants 52 and 208, when crossed with NIL Mitchell ( $S^{3L}S^{3L}$ ), the average fruit length was  $0.6 \pm 0.05$  cm and  $0.8 \pm 0.14$  cm, respectively.

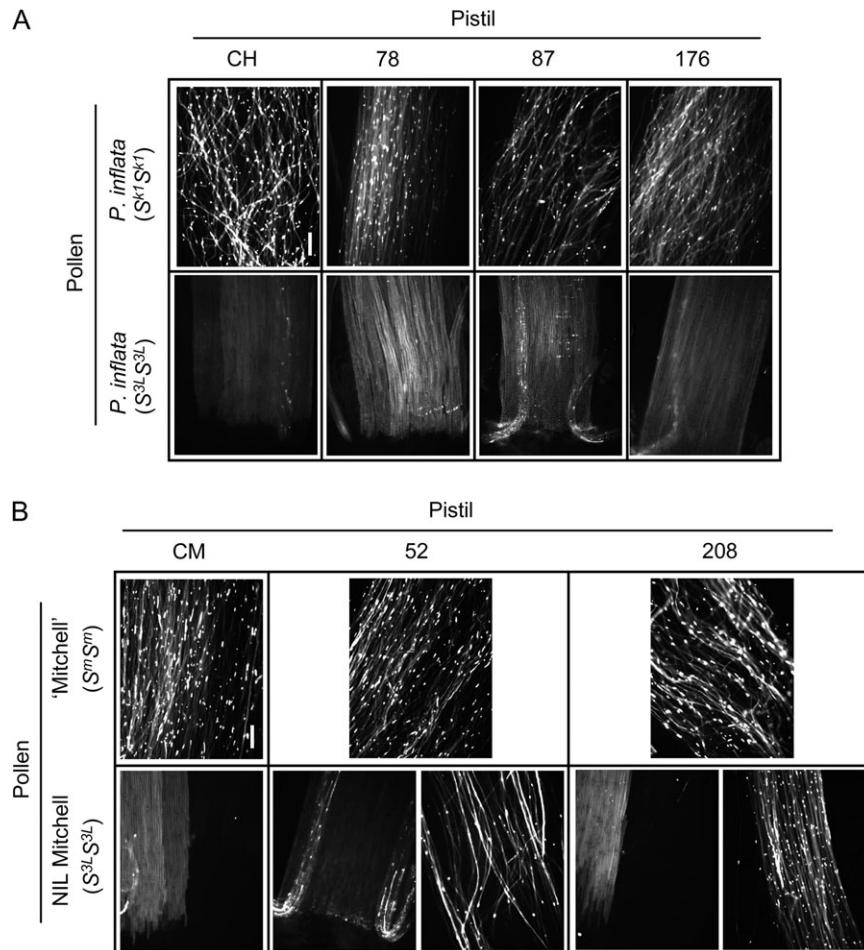
Pollen tube observation confirmed the phenotypes assessed by fruit set (Table 2; Fig 7). All hybrid background transgenics, including the double transformant, presented a characteristic incompatible phenotype when pollinated with  $S^{3L}$  pollen, i.e. strong deposition of callose in the pollen tube walls, uneven distribution of callose plugs, irregular direction and a marked decrease in the number of pollen tubes in the middle of the style, with none of them reaching the lower style. Conversely, crosses using  $S^{k1}$  pollen produced the typical compatible phenotype, in which an overwhelming number of pollen tubes grow harmoniously, with callose plugs regularly spaced, through the style to be visible in great quantities towards the ovary. Mitchell background transgenic plants showed the same phenotype when crossed with 'Mitchell' pollen (Table 2; Fig. 7B). Rejection of NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT\text{-}B^iHT\text{-}B^i$ ) pollen occurred, as expected, with the control plant (CM) and with transformants which were slightly or not *HT-B* suppressed (8, 82, 157, and 173). However, severely suppressed plants (52 and 208) showed a partial SC response upon pollination with  $S^{3L}$  pollen. In some crosses, the typical incompatible rejection phenotype was observed. However, other crosses showed partially or fully compatible phenotypes (Fig. 7B).

Real-time PCR was performed in selected plants to quantitate the level of reduction of *HT-B* gene expression.

**Table 2.** Pollen tube growth in pistils of RNAi transgenics for *HT-B*

Data are presented as the number of compatible pollinations over the total pollinations attempted. Crosses were considered as compatible when one or more pollen tubes were observed at the lower part of the style. CH and CM are untransformed controls of hybrid background and Mitchell background, respectively. DT denotes a double transformant.

Pistil	Pollen
Hybrid background ( $S^{3L}S^m$ )	<i>P. inflata</i> ( $S^{k1}S^{k1}$ )
CH	4/4
78	4/4
87	3/3
96	3/3
176	3/3
199	3/3
94 (DT)	3/3
Mitchell background ( $S^{3L}S^m$ )	'Mitchell' ( $S^mS^m$ )
CM	3/3
8	3/3
52	5/5
82	3/3
157	3/3
173	3/3
208	3/3
	<i>NIL Mitchell</i> ( $S^{3L}S^{3L}$ )



**Fig. 7.** Pollen tube phenotypes in RNAi lines for *HT-B*. (A) Hybrid background transgenics [transgenic 'Mitchell'  $\times$  *P. inflata* ( $S^{3L}S^{3L}$ )]. (B) Mitchell background transgenics [transgenic 'Mitchell'  $\times$  NIL Mitchell ( $S^{3L}S^{3L}$ ,  $HT-B^iHT-B^i$ )]. CH and CM are untransformed controls of hybrid and Mitchell backgrounds, respectively. For plants 52 and 208, two types of phenotypes in incompatible pollination [NIL Mitchell ( $S^{3L}S^{3L}$ )] are presented; incompatibility (left) and partial compatibility (right). Styles were collected 48 h after pollination, stained with aniline blue, and observed under an epifluorescence microscope. All photographs correspond to the lower part of the style. Bar=100  $\mu$ m.

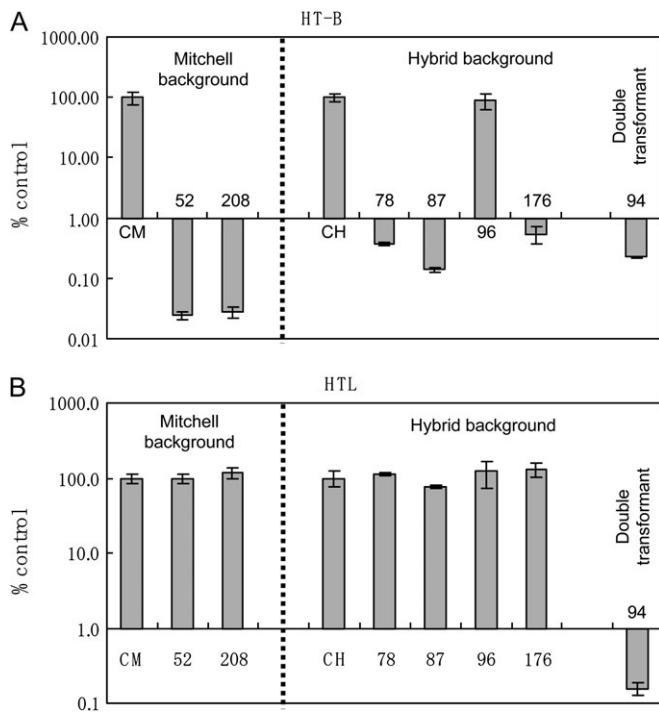
As shown in Fig. 8A, *PiHT-B* expression in the partial SC 'Mitchell' background plants 52 and 208 was reduced by  $>1000$  times in comparison with the control (CM). Suppression in hybrid background plants 78, 87, and 176 was  $>100$  times. The double transformant (plant 94) presented a suppression of  $>100$  times for both genes (Fig. 8A, B). Unaffected expression of  $S^{3L}$ -RNase in the partial SC plants 52 and 208 was confirmed through RNA blot analysis including an untransformed plant (CM) and 'Mitchell' as positive and negative controls, respectively (Fig. 9).

## Discussion

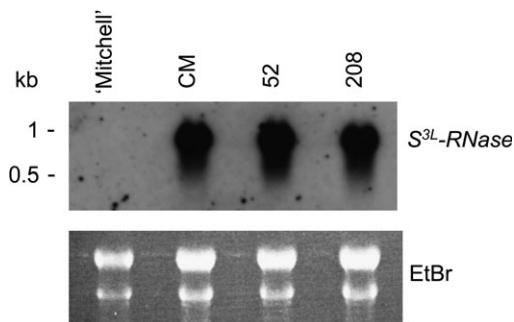
A previous attempt to clone *HT-B* of *Petunia* resulted in isolation of *PiHTL*, which was similar to *HT-B* but lacked the N/D-rich domain at the C-terminus, representing a new class of *HT*-like gene. The finding that silencing of *PiHTL* did not affect GSI suggested that *Petunia* has other genuine *HT-B* genes (Sassa and Hirano, 2006). In this study, a new

*HT*-like gene, *PiHT-B*, was isolated from *Petunia* and characterized. *PiHT-B* has characteristics which are typical of *HT-B* genes, i.e. structures including an N/D-rich domain and style-specific expression. Furthermore, silencing of *PiHT-B* changed the GSI phenotype, indicating that it is the *Petunia* orthologue of *HT-B*. In addition to *HT-B*, another isoform, *HT-A*, has been identified in *Solanum* species (Kondo *et al.*, 2002a, b; O'Brien *et al.*, 2002), while *HT-A* is not known in *Nicotiana* (McClure *et al.*, 1999). In *Petunia*, only *HT-B* was isolated, and *HT-A*-like sequences were not identified by the BAC library screening. This may reflect phylogenetic relationships between those species; subfamily Petunoideae, that includes *Petunia*, is more closely related to Nicotianoideae (*Nicotiana*) than it is to Solanoideae (*Solanum*) (Olmstead *et al.*, 1999).

*HT-B* is the first non-S-factor characterized at the molecular level, and was discovered in a differential screen as being highly expressed in *N. alata* style but not in the SC species *N. plumbaginifolia* (McClure *et al.*, 1999). Kondo *et al.* (2002a, b) isolated *HT-B* genes from SI and SC



**Fig. 8.** Quantitative analysis of the transcripts of *HT-B* and *HTL* in RNAi transgenic plants. Levels of transcripts for *HT-B* (A) and *HTL* (B) were determined by quantitative real-time PCR. Values from three independent experiments are expressed as a percentage of controls ( $\pm$ SD). CH and CM are untransformed controls of hybrid and Mitchell backgrounds, respectively.



**Fig. 9.** RNA blot analysis of *S<sup>3L</sup>-RNase* in styles of RNAi lines for *HT-B*. Severely *HT-B*-suppressed Mitchell background plants 52 and 208 were analysed. 'Mitchell' and untransformed Mitchell background (CM) are negative and positive controls, respectively. A 10  $\mu$ g aliquot of style total RNA was loaded in each lane, blotted, and hybridized with the *P. inflata* *S<sup>3L</sup>-RNase* probe. The ethidium bromide-stained gel is shown to ascertain equal loading conditions.

relatives of tomato, and showed that high *HT-B* expression levels were exclusively observed in SI species. Based on the *HT* data, it was hypothesized that a mutation causing reduced transcription of *HT-B* in an ancestral species was central to the loss of GSI in tomato relatives (Kondo *et al.*, 2002a). In *Petunia*, in contrast, *HT-B* expression was also

observed in SC lines, *P. axillaris* subsp. *parodii* and *P. hybrida* 'Mitchell', though the levels were lower than that in SI *P. inflata*. Considering that a >1000-fold reduction of *HT-B* transcript is required to achieve partial breakdown of GSI, the low but detectable levels of *HT-B* transcript are unlikely to be related to SC of those *Petunia* lines. This may suggest that different courses are possible for the evolution of SC in different taxa of Solanaceae.

RNAi-mediated silencing was conducted to analyse the function of *PiHT-B*. Among the transgenic lines with different levels of suppression of the *PiHT-B* gene, the most severely affected plants only showed partial breakdown of *S*-specific pollen rejection function. qPCR analysis showed that the accumulation of *PiHT-B* mRNA in the partial SC transgenics was reduced by >1000-fold. RNAi lines with >100-fold reduction of *PiHT-B* showed no change of GSI phenotype, suggesting that a threshold level of *PiHT-B* expression is required for GSI, and the threshold is very low at <1% of the wild-type level. In the functional analysis of *HT-B* of *S. chacoense*, RNAi-mediated silencing also achieved partial breakdown of GSI, although the level of suppression was not quantitatively assessed (O'Brien *et al.*, 2002). The requirement for threshold level expression is also known for another highly abundant pistil GSI factor, the *S-RNase*. In contrast to *HT-B*, the threshold level of the *S-RNase* is high, and strong expression is required to confer *S*-specific pollen rejection function to the pistil (Lee *et al.*, 1994; Murfett *et al.*, 1996; Qin *et al.*, 2006). The different threshold levels of these pistil factors may reflect the difference in biochemical function between them. Although the biochemical function of *HT-B* has not been clarified yet, based on the findings of the probable association of *HT-B* with the vacuole membrane of pollen tubes and its differential stability between compatible and incompatible pollination, it is hypothesized that *HT-B* is involved in destabilization of the vacuole of the pollen tube (Goldraij *et al.*, 2006; McClure, 2006). A small amount of *HT-B* protein may be sufficient for its function on the vacuole membrane. Another possibility is that *HT-B* plays an indirect role that influences the strength of GSI expression. Analysis of deletion-type or insertional disruption-type mutations of *HT-B* would clarify if complete abolishment of the protein results in complete breakdown of GSI, and if the small amount of *HT-B* protein is essential. Identification of interaction partner(s) of *HT-B* protein would also be necessary to understand the biochemical role of the protein and the mechanism of the *S-RNase*-based GSI system.

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