NaStEP: A Proteinase Inhibitor Essential to Self-Incompatibility and a Positive Regulator of HT-B Stability in Nicotiana alata Pollen Tubes1[W][OA]

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In Solanaceae, the self-incompatibility S-RNase and S-locus F-box interactions define self-pollen recognition and rejection in an S-specific manner. This interaction triggers a cascade of events involving other gene products unlinked to the S-locus that are crucial to the self-incompatibility response. To date, two essential pistil-modifier genes, 120K and High Top-Band (HT-B), have been identified in Nicotiana species. However, biochemistry and genetics indicate that additional modifier genes are required. We recently reported a Kunitz-type proteinase inhibitor, named NaStEP (for Nicotiana alata Stigma-Expressed Protein), that is highly expressed in the stigmas of self-incompatible Nicotiana species. Here, we report the proteinase inhibitor activity of NaStEP. NaStEP is taken up by both compatible and incompatible pollen tubes, but its suppression in Nicotiana spp. transgenic plants disrupts S-specific pollen rejection; therefore, NaStEP is a novel pistil-modifier gene. Furthermore, HT-B levels within the pollen tubes are reduced when NaStEP-suppressed pistils are pollinated with either compatible or incompatible pollen. In wild-type self-incompatible N. alata, in contrast, HT-B degradation occurs preferentially in compatible pollinations. Taken together, these data show that the presence of NaStEP is required for the stability of HT-B inside pollen tubes during the rejection response, but the underlying mechanism is currently unknown.

To avoid low-fertility progeny, many plants have developed a cell-cell interaction mechanism to promote outcrossing, through the recognition and discrimination of both self and nonself pollen. This recognition system is controlled by the highly polymorphic self-incompatibility S-locus, which determines pollination specificity in both the pollen and pistil. Pollen is rejected when male and female S-haplotypes coincide (de Nettancourt, 1977, 2001; Franklin et al., 1995).

In Solanaceae, Plantaginaceae, and Rosaceae, the S-locus product in the pistil is an extracellular glycoprotein named S-RNase (Anderson et al., 1986; McClure et al., 1989). During pollination, S-RNase is taken up by both compatible and incompatible pollen tubes (Luu et al., 2000) and targeted to a vacuole (Goldraij et al., 2006). In the later stages of an incompatible cross, the S-RNase-containing vacuole is disrupted and the S-RNases are released to the pollen tube cytoplasm, where RNA degradation can occur (McClure et al., 2011).

The S-pollen gene encodes an SLF or SFB (SLF/SFB; for S-locus F-box) protein, which is a member of the F-box protein family (Entani et al., 2003; Sijacic et al., 2004). In vitro binding assays show that PiSLF in Petunia inflata physically interacts with S-RNases, although this interaction is stronger with nonself S-RNases than with self S-RNases (Hua and Kao, 2006). Additional protein-protein interaction assays suggest that SLFs/SFB may be a component of an SCF (for Skp1-Cullin-f-box) or SCF-like complex (Qiao et al., 2004; Hua and Kao, 2006). Notably, data from Zhao et al. (2010) in Petunia hybrida show that reduction of PhSSK1 (for P. hybrida SLF-interacting Skp-like) and its Antirrhinum hispanicum ortholog, AhSSK1, is also required for cross-pollen compatibility.

Although S-RNase and SLF/SFB define pollen rejection S-specificity, modifier genes unlinked to the S-locus are required for self-incompatibility (St; Martin, 1968; Ai et al., 1991; Murfett et al., 1996; Tsukamoto et al., 1999). To date, only two pistil-modifier genes have been identified: High Top-Band (HT-B) and 120K. In Nicotiana spp., HT-B is an 8.6-kD acidic protein with a domain consisting of 20 Asn and Asp residues toward its C terminus (McClure et al., 1999; Kondo and McClure, 2000). In Nicotiana alata, 120K (2010) has a C-terminal domain containing a C2 domain (Tsukamoto et al., 1999). 120K has been shown to interact with S-RNase, and this interaction is stronger with nonself S-RNases than with self S-RNases (Hua and Kao, 2006).

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2008). Loss-of-function assays prove HT-B to be essential for pollen rejection in *Nicotiana* spp., *Solanum* spp., and *Petunia* spp. (McClure et al., 1999; Kondo et al., 2002; O’Brien et al., 2002; Sassa and Hirano, 2006; Puerta et al., 2009), although it is not expressed in SI *Solana num habrochaites*, prompting the speculation that in this species a related gene, HT-A, may function as a substitute (Covey et al., 2010). Immunolocalization shows that HT-B is readily taken up by pollen tubes during pollination. Its steady-state levels decrease slightly in pollen tubes from incompatible pollinations. However, in compatible crosses, HT-B levels decrease 75% to 97%, probably as a result of protein degradation (Goldraij et al., 2006).

120K is a style-specific 120-kD arabinogalactan protein (Schultz et al., 1999) that is taken up by pollen tubes (Lind et al., 1996) and appears to be associated with S-RNase-containing vacuoles (Goldraij et al., 2006). 120K forms complexes with S-RNases and other proteins (Cruz-García et al., 2005) in vitro, and suppression of 120K expression prevents specific pollen rejection (Hancock et al., 2006). Protein-protein interaction assays demonstrate that 120K interacts with the pollen-specific protein NaPCCP (a pollen C2 domain-containing protein), a protein that binds phosphatidylinositol 3-phosphate and is associated with the pollen tube endomembrane system (Lee et al., 2008, 2009).

Two models have been proposed to explain pollen rejection in Solanaceae. (1) The S-RNase degradation model (Hua and Kao, 2006; Hua et al., 2007, 2008; Kubo et al., 2010) focuses on S-RNase-SLF interactions that bring about preferential nonself S-RNase degradation. In this model, strong nonself S-RNase-SLF interactions lead to the degradation of nonself S-RNases by the ubiquitin-26S proteasome system, allowing pollen tubes to escape from its cytotoxic effect. Weak self S-RNase-SLF interactions, in contrast, permit the persistence of sufficient free S-RNase that pollen tube RNA is degraded, resulting in self-pollen rejection. Notably, by functional and protein-protein interaction assays in *Petunia* spp., Kubo et al. (2010) found at least three types of divergent SLF proteins encoded at the S-locus, each recognizing a subgroup of nonself S-RNases. The authors proposed the collaborative nonself recognition model, where multiple SLF proteins interact with nonself S-RNases to protect nonself pollen from degradation (Kubo et al., 2010). (2) The compartmentalization model incorporates the observations that pollen tubes internalize both self and nonself S-RNases and targets them to vacuoles and that HT-B is degraded in compatible crosses but is stable in incompatible crosses (Goldraij et al., 2006). In incompatible crosses, the S-RNase-containing vacuoles are ultimately disrupted and S-RNases are released to the cytoplasm, where they degrade RNA, leading to rejection of self-pollen. In compatible crosses, the integrity of the S-RNase-containing vacuoles is preserved, allowing pollen tube growth to continue. Thus, in this model, self or nonself S-RNase-SLF interactions determine the specificity of pollen rejection indirectly.

Biochemical and genetic data show that pistil-modifier genes apart from HT-B and 120K are required for SI. We recently described NaStEP (for *N. alata* Stigma-Expressed Protein), an abundant, pistil-specific stigma protein found in SI *Nicotiana* spp. (Busot et al., 2008). Its abundance in SI species made NaStEP a strong modifier gene candidate. Here, we demonstrate that NaStEP is taken up by pollen tubes, has subtilisin inhibitory activity, and that suppressing its expression in transgenic hybrids disrupts pollen rejection. Moreover, when NaStEP-suppressed hybrids are pollinated, HT-B protein is degraded in both compatible and incompatible pollen tubes, while in wild-type SI *N. alata*, HT-B is preferentially stabilized in incompatible pollen tubes.

**RESULTS**

**NaStEP Suppression**

To test whether NaStEP is required for S-specific pollen rejection, we introduced an RNA interference (RNAi):*NaStEP* construct into self-compatible (SC) *Nicotiana plumbaginifolia* (i.e., *S*0*S*0) and crossed T0 transformants with SI *N. alata S*105*S*0 to generate *S*105*S*0 T1 progeny family K08. Figure 1A shows an analysis of pistil extracts from two hybrids with no detectable NaStEP, K08-2 and K08-3; a partially suppressed hybrid, K08-9; and an unsuppressed hybrid, K08-16. Population J08 was obtained by crossing hybrid K08-2 to SI *N. alata S*105*C10. This second population enabled testing the effects of NaStEP suppression on a second S-haplotype in a further generation. The entire J08 population consisted of 25 plants (13 *S*105*C10 and 12 *S*105*S*0). Ten J08 plants showed suppression and 15 expressed normal levels of NaStEP (Fig. 1B; Supplemental Fig. S1A). Five representatives are shown in Figure 1B. Plants J08-3, J08-6, and J08-8 showed no detectable NaStEP, while plants J08-2 and -12, with nearly normal expression levels, were used as controls (Fig. 1B). The RNAi effect was specific to NaStEP in both the K08 and J08 populations, as little or no change in S-RNase, HT-B, or 120K levels was observed (Fig. 1).

**NaStEP Is Required for S-Specific Pollen Rejection**

Pollination tests showed that NaStEP expression is essential for S-specific pollen rejection in *Nicotiana* spp. The effect on pollination phenotype was assessed by observing pollen tubes at the base of the style (Fig. 2C) 72 h post pollination after challenging with *S*105*S*0 or *S*105*C10 pollen (Fig. 2, A and B; Supplemental Tables S1 and S2). As expected, untransformed control *S*105*S*0 and *S*105*C10 hybrids (i.e. *N. plumbaginifolia × SI N. alata S*105*S*0 or *N. plumbaginifolia × SI N. alata S*105*C10) hybrids expressing *S*105-RNase or *S*105-RNase, respectively) displayed S-specific pollen rejection by only rejecting pollen with a matching S-haplotype (Fig. 2, A and B, top panels). In contrast, fully suppressed hybrids (K08-2, K08-3, J08-3, J08-6, and J08-8) showed many (more than 50) pollen tubes at the base of the
style, regardless of the pollen S-haplotype, and thus did not display S-specific pollen rejection (Fig. 2, A and B; Supplemental Tables S1 and S2). Moreover, the S105S0 K08-9 hybrid that displayed partially suppressed NaStEP allowed a few pollen tubes to reach the base of the style (Fig. 2A).

**NaStEP in Interspecific Pollen Rejection**

To test the role of NaStEP in interspecific pollen rejection, fully suppressed S105S0 hybrids were pollinated with SI Rastroensis (Nicotiana rastroensis), SC N. longiflora, SC N. plumbaginifolia, SC N. tabacum, SC N. benthamiana, or SC N. glauca pollen. Table I shows that untransformed S105S0 hybrids and SI N. alata S105S105 accepted pollen from SI Rastroensis and SC N. longiflora but rejected pollen from all other SC species. NaStEP-suppressed hybrids behaved the same with one notable exception, N. plumbaginifolia. Fully suppressed hybrids accepted N. plumbaginifolia pollen, and the partially suppressed hybrid showed partial compatibility (Table I). Thus, NaStEP is required

**Figure 1.** NaStEP suppression in Nicotiana spp. A, Ten-milligram total protein extracts from pistil in transformed SC N. plumbaginifolia × SI N. alata (S105S0) hybrids. NaStEP is not detectable in plants K08-2 and K08-3 and is partially detectable in K08-9. S-RNase, HT-B, and 120K protein levels were not greatly affected. B, Ten-milligram total protein extracts from pistil in K08-2 × SI N. alata (S105S105) progeny. NaStEP is not detectable in plants J08-3, J08-6, and J08-8. S-RNase, HT-B, and 120K protein levels were not greatly affected. The untransformed control is SC N. plumbaginifolia × SI N. alata (S105S105 or S0C10S10).

**Figure 2.** NaStEP suppression disrupts S-specific pollen rejection. A, NaStEP RNAi N. plumbaginifolia × SI N. alata (S0C10S10) hybrids were pollinated with S0 or S10 pollen and prepared for imaging after 72 h. Images were taken at or near the base of the style (arrow in C). The plants K08-2 (S0S10) and K08-3 (S0C10) show both S0 and S10 pollen tubes reaching the base of the style. B, Plants from the J08 population were pollinated with S10 or S10S0 pollen. The plants J08-3 (S0C10S0), J08-6 (S0C10S0), and J08-8 (S0C10S0) show both S0 and S10 pollen tubes reaching the base of the style. Untransformed (UT) hybrid (N. plumbaginifolia × SI N. alata S105S105 or S0C10S10) pollinated with S10 or S10S0 pollen was used as a control. Bars = 50 μm. Results represent a replicate of three pollination assays. C, Pistil diagram showing the observation area for pollen tube growth.
for interspecific rejection of *N. plumbaginifolia* pollen but not for the rejection of pollen from the other SC species that were tested.

**NaStEP Is Taken Up by Pollen Tubes**

We recently demonstrated that NaStEP is sorted to a vacuole in mature papillary stigmatic cells. Upon pollination, the papillary cell wall becomes perforated and NaStEP is relocalized onto the stigma apoplast (Busot et al., 2008). Since NaStEP is essential for pollen rejection (Fig. 2), we hypothesized that it might be taken up and function inside the pollen tube. We performed double-label experiments using antibodies for callose and NaStEP in compatible (*N. alata* S\textsubscript{105} × *N. alata* S\textsubscript{C10}) and incompatible (*N. alata* S\textsubscript{105} × *N. alata* S\textsubscript{105} S\textsubscript{105}) crosses 6 and 9 h after pollination (Fig. 3, green signal). At these times, pollen grains had germinated and pollen tubes had entered the upper style. Figure 3 shows that NaStEP accumulates abundantly in the stigmatic cells. Figure 3 also reveals that NaStEP enters both compatible and incompatible pollen tubes, and once there, its appearance looks like small dots. Relocalization of NaStEP from the stigma surface to the pollen tube would position it to function in internal pollen tube processes.

**NaStEP Positively Regulates HT-B Stability in Pollen Tubes**

NaStEP is a Kunitz-type proteinase inhibitor family member (Busot et al., 2008) and could modulate the stability of other proteins in the pollen tubes. HT-B is of special interest since it shows differential stability in compatible versus incompatible pollen tubes (Goldraij et al., 2006). To test this possibility, we carried out immunolocalization experiments to evaluate HT-B levels in compatible and incompatible pollen tubes. HT-B levels were classified by staining intensity as low or not stained, medium, or high (sample images are shown in Supplemental Fig. S2). In suppressed *S\textsubscript{105}S\textsubscript{0} hybrid challenged with *S\textsubscript{105} pollen, most pollen tubes contained high levels of HT-B 16 h post pollination, but by 36 h, a striking shift occurred and HT-B was almost undetectable in most pollen tubes (RNAi *S\textsubscript{105}S\textsubscript{0} × S\textsubscript{105} pollen; Fig. 4A). As reported previously (Goldraij et al., 2006), no such shift occurred in control crosses where *S\textsubscript{105}S\textsubscript{0} hybrid or SI *N. alata* S\textsubscript{105} was challenged with *S\textsubscript{105} pollen, as HT-B is stabilized in incompatible crosses (SI *N. alata* S\textsubscript{105} S\textsubscript{105} × S\textsubscript{105} pollen; Fig. 4B). Compatible crosses also behaved as expected and displayed HT-B degradation. When suppressed *S\textsubscript{0}S\textsubscript{0} hybrid or SI *N. alata* S\textsubscript{105} S\textsubscript{105} plants were challenged with *S\textsubscript{C10} pollen, most pollen tubes showed little or no detectable HT-B by 36 h.

### Table 1. NaStEP does not account for interspecific pollen rejection

Transformed K08-2 (S\textsubscript{105}S\textsubscript{0}), K08-3 (S\textsubscript{105}S\textsubscript{0}), and K08-16 (S\textsubscript{105}S\textsubscript{0}) hybrids were pollinated with SI Rastroensis (R), SC *N. longiflora* (NL), SC *N. plumbaginifolia* (Np), SC *N. tabacum* (Nt), SC *N. benthamiana* (Nb), and SC *N. glauca* (Ng) pollen. After 72 h of pollination, styles were prepared for imaging. Images were taken at or near the base of the style. Compatible (+), incompatible (−), and semicompatible (+/−) pollinations are indicated. Each symbol (+ or −) represents a replicate of each pollination assay.

| Pistil       | SI R | SC NL | SC Np | SC Nt | SC Nb | SC Ng |
|--------------|------|-------|-------|-------|-------|-------|
| S\textsubscript{105}S\textsubscript{105} | +,+,+ | +,+,+ | −,−,− | −,−,− | −,−,− | −,−,− |
| Untransformed hybrid | +,+,+ | +,+,+ | −,−,− | −,−,− | −,−,− | −,−,− |
| Np × NaS\textsubscript{105}S\textsubscript{105} | +,+,+ | +,+,+ | +,+,+ | −,−,− | −,−,− | −,−,− |
| K08-2 S\textsubscript{105}S\textsubscript{0} | +,+,+ | +,+,+ | +,+,+ | −,−,− | −,−,− | −,−,− |
| K08-3 S\textsubscript{105}S\textsubscript{0} | +,+,+ | +,+,+ | −,−,− | −,−,− | −,−,− | −,−,− |
| K08-16 S\textsubscript{105}S\textsubscript{0} | +,+,+ | +,+,+ | −+/−,− | −,−,− | −,−,− | −,−,− |

**Figure 3.** NaStEP is taken up by pollen tubes. A and B, Six-hours-postpollination pistils. A, SI *N. alata* S\textsubscript{0}S\textsubscript{105} pistils pollinated with *S\textsubscript{C10} pollen (compatible). B, SI *N. alata* S\textsubscript{105}S\textsubscript{105} pistils pollinated with *S\textsubscript{105} pollen (incompatible). C and D, Nine-hours-postpollination pistils. C, SI *N. alata* S\textsubscript{105}S\textsubscript{0}S\textsubscript{105} pistils pollinated with *S\textsubscript{C10} pollen (compatible). D, SI *N. alata* S\textsubscript{105}S\textsubscript{0}S\textsubscript{105} pistils pollinated with *S\textsubscript{0} pollen (incompatible). Sections from pollinated pistils were simultaneously probed with anti-NaStEP (green) or anti-callose (white) antibodies. PT, Pollen tube; STC, stigmatic cell. Bars = 5 mm.
Thus, NaStEP suppression interferes with S-specific stabilization of HT-B in otherwise incompatible pollen tubes; HT-B degradation occurred regardless of S-haplotype. These results are consistent with a role for NaStEP in HT-B stability.

Experiments on pistil extracts also showed HT-B degradation regardless of pollen S-genotype in suppressed hybrids. Figure 5 shows immunoblot analyses of extracts prepared 16, 36, and 72 h post pollination. SI N. alata S<sub>105</sub>-S<sub>105</sub> controls showed preferential degradation of HT-B after compatible pollination, as reported previously (Fig. 5B; Goldraij et al., 2006). Compatible pollination of NaStEP-suppressed hybrid (S<sub>C10</sub>S<sub>C10</sub>) showed very similar results (Fig. 5A; S<sub>05</sub> pollen). Incompatible S<sub>C10</sub> pollen gave similar results as controls at 36 h, but HT-B was undetectable after 72 h. Thus, degradation of HT-B occurred regardless of pollen S-genotype in the NaStEP-suppressed hybrid, although the effect at the whole-organ level was delayed compared with observations of HT-B inside pollen tubes (Fig. 4 versus Fig. 5).

Model of the NaStEP Three-Dimensional Structure

A model for the NaStEP three-dimensional structure was produced and used for structure-based comparisons with other Kunitz-type inhibitors to infer its possible function. Per-residue GROMOS energies revealed a well-refined final three-dimensional model (Fig. 6). The Rd.HMM score (Martínez-Castilla and Rodríguez-Sotres, 2010) for the NaStEP amino acid sequence was nearly 0.3 times the sequence length before relaxation (E value of $1.8 \times 10^{-8}$) and 0.92 after relaxation (E value of $6.4 \times 10^{-31}$). The Rd.HMM structurally aware alignment (Martínez-Castilla and Rodríguez-Sotres, 2010) of the model was in frame with the NaStEP amino acid sequence, and no other sequence in the National Center for Biotechnology Information nonredundant database had a higher score. Using a cutoff E value of 10, only orthologs from N. alata (GenBank accession no. ABX76298.1) and N. glutinosa (GenBank accession no. AAF15901.1) were identified as possible sequence fits with the proposed three-dimensional model. However, the scores for these sequences were only one-half of the score obtained with the NaStEP sequence. Molecular dynamics (MD) simulations (data not shown) also indicate that the proposed structure is of high quality.

Rd.HMM hits with poor scores suggested structural similarity with the barley (Hordeum vulgare) subtilisin/amyrase inhibitor. Superimposition of the three-dimensional structure of the barley subtilisin/amyrase inhibitor and the NaStEP three-dimensional model using STAMP (Russell and Barton, 1992) confirmed the close structural relationship. The barley subtilisin/amyrase inhibitor structure was used to model the disulfide bridging pattern and to locate a putative subtilisin reactive site in NaStEP (F130–K136). This site is shown in purple in Figure 6, A and B, and is boxed in Figure 6C. Comparison of the reactive sites reveals important sequence differences that could affect specificity. Both sequences have a basic residue (Fig. 6C), although its position differs. Importantly, STAMP does not align the TTKL1 sequence in NaStEP with the TTCL sequence in the
NaStEP Is a Subtilisin Inhibitor

NaStEP was purified by ammonium sulfate precipitation and anion-exchange chromatography and tested for its ability to inhibit subtilisin. Fractions were analyzed by SDS-PAGE, and those enriched in NaStEP (Supplemental Fig. S3, A and B) were tested for activity. The results showed that the major Coomassie blue-stainable bands corresponded to anti-NaStEP reactive bands. Active bands were also excised, fragmented, and sequenced by mass spectrometry. The results showed greater than 60% coverage and perfect agreement with the predicted NaStEP sequence (Supplemental Fig. S3C, red letters).

A reverse zymogram assay was used to test NaStEP for subtilisin inhibition activity. In this assay, proteins migrate through the gel and subtilisin inhibition is reflected by preventing the degradation of gelatin. Figure 7 shows two subtilisin inhibition bands with approximate molecular masses between 20 and 35 kD in the enriched NaStEP fractions. The aprotinin control ran near 15 kD and also showed strong inhibitory activity. An immunoblot of this gel stained with anti-NaStEP detected both inhibition bands, confirming the subtilisin inhibitory activity of this protein (Fig. 7, A and B versus C). It is not clear why NaStEP runs as multiple bands in these assays, but it may be related to protein oxidation or oligomerization.

DISCUSSION

Pollen rejection in Nicotiana spp. depends on S-specific interactions between S-RNase and SLF/SFB as well as other modifier genes. Until now, HT-B and 120K were the only identified modifier genes functionally tested in pollen rejection in Solanum spp., Nicotiana spp., and Petunia spp. (McClure et al., 1999; Kondo et al., 2002; O’Brien et al., 2002; Hancock et al., 2005; Sassa and Hirano, 2006; Puerta et al., 2009). Here, loss-of-function assays implicate NaStEP as a third modifier gene essential for the SI pollen rejection response in Nicotiana spp., since the suppressed hybrid (SC N. plumbaginifolia × SI N. alata) loses the ability to reject pollen in an S-specific manner. Likewise, we present data supporting NaStEP involvement in interspecific pollen rejection. Previous studies had shown undetectable transcript and protein levels of NaStEP in the SC species N. plumbaginifolia and N. tabacum but abundant expression in the mature stigmas of SI Nicotiana spp., where it is targeted to a vacuole in the papillar cells and released into the exudate upon pollination (Busot et al., 2008). The accumulation of NaStEP in stigmas suggests a function for this protein during the early stages of pollen germination or pollen tube growth. The roles of stigma-specific proteins in pollen are diverse and may or may not include a function in SI. As an example, the Brassica S-receptor kinase (SRK) protein functions at early pollination times and is essential to the pollen-rejection response (Stein et al., 1991; Takasaki et al., 2002; Brien et al., 2002; Hancock et al., 2005; Sassa and Hirano, 2006; Puerta et al., 2009).
tube growth may be excluded. Therefore, NaStEP suppression disrupts pollen rejection in an S-specific manner, and at least one main function of this gene is required for pollen rejection.

NaStEP function appears to be restricted to S-RNase-based self as well as interspecific pollen rejection, because its suppression in Nicotiana spp. did not result in any other evident phenotype changes. Interspecific pollen rejection occurs through multiple and complex mechanisms, which in Nicotiana spp. happen in both an S-RNase-dependent and an S-RNase-independent manner (Pandey, 1981; Murfett et al., 1996). Interspecific pollen rejection mediated by S-RNases has been shown to depend strictly on modifier genes common to SI, such as HT-B and 120K (McClure et al., 1999; Hancock et al., 2005). Suppressed HT-B or 120K transgenic hybrids of Nicotiana spp. (N. plumbaginifolia × SI N. alata) accept pollen from the SC N. plumbaginifolia, which are otherwise rejected by the untransformed control hybrids (SC N. plumbaginifolia × SI N. alata; Hancock et al., 2005). Our results using the NaStEP RNAi-silenced hybrid and pollen from different Nicotiana species (Table I) highlight the inability of NaStEP-suppressed hybrids to reject N. plumbaginifolia or N. alata pollen, in spite of normal S-RNase levels. These results show that NaStEP is required for S-specific pollen rejection and for rejecting pollen from N. plumbaginifolia or N. alata pollen, in spite of normal S-RNase levels. Taken together, our results are congruent with a role for NaStEP as a
pistil-modifier gene in SI and point to its involvement in an S-RNase interspecific pollen-rejection pathway dependent on modifier genes, as shown for HT-B and 120K.

Upon pollination, NaStEP is taken up by pollen tubes regardless of their S-haplotype. Although the subcellular destination of NaStEP within the pollen tube is currently unknown, in the S-RNase-based pollen-rejection systems, pistil proteins essential to SI and exerting their function within the pollen tube, such as S-RNases, HT-B, and 120K, have been shown to associate with the pollen tube endomembrane system (Goldraij et al., 2006). Although the molecular mechanism leading to pollen rejection is completely different between gametophytic and sporophytic systems, the events that occur in association with the endomembrane system seem to be relevant to the pollen-rejection response in both systems. Samuel et al. (2009) showed in Brassica spp. that Exo70 A1, a subunit of an exocyst complex involved in polarized secretion in yeast (Saccharomyces cerevisiae) and animals (Novick et al., 2006; Zársky et al., 2009), functions in the polarized transport of vesicles in the stigma and is necessary for the hydration, germination, and growth of compatible pollen tubes. During the SI response, the S-specificity determinants SRK-SCR negatively regulate Exo70 A1, through the activation of ARC1 (for ARM repeat-containing1 protein), an E3 ubiquitin ligase. ARC1 targets Exo70 A1 for degradation, provoking a deregulation of the exocyst function, thereby promoting pollen rejection.

In N. alata, following both compatible and incompatible pollinations, S-RNases, HT-B, and 120K are taken up by the pollen tube and become associated with the endomembrane system. S-RNases are sequestered into a vacuole, where they remain contained until its destabilization releases them into the cytoplasm (Goldraij et al., 2006; McClure et al., 2011). Similarly, biochemical fractionation experiments give evidence of the HT-B association with the microsomal fraction (Kondo and McClure, 2008). Furthermore, immunolocalization studies show the colocalization of 120K with the S-RNases in the pollen tube vacuole (Goldraij et al., 2006). The incorporation of NaStEP into the pollen tube occurs early in pollination (6 h), and its function might be related, directly or indirectly, with the pollen tube endomembrane system. This is supported by the preservation of HT-B from degradation, once inside the pollen tube, when NaStEP is present, disclosing a link between NaStEP and HT-B stability. In wild-type SI N. alata, the HT-B remains stabilized in incompatible pollen tubes, even at times when pollen rejection has been effective, whereas it disappears from compatible pollen tubes (Goldraij et al., 2006; Kondo and McClure, 2008). By contrast, in the absence of NaStEP, HT-B signal loss was similar in both compatible and incompatible pollen tubes, which, according to the S-RNase compartmentalization model (Goldraij et al., 2006), would maintain the S-RNase vacuole stable, allowing pollen tube growth.

However, the mechanism by which NaStEP stabilizes HT-B remains to be elucidated. NaStEP is homologous to Kunitz-type protease inhibitors (Busot et al., 2008) and exhibits a subtilisin inhibition activity. A reasonable model could contemplate the inhibitory role of NaStEP on a subtilisin-like component of a proteolytic cascade targeting HT-B during compatible pollen tube growth. However, the putative subtilisin-like component has yet to be identified, and a control mechanism for the NaStEP action remains unknown, since this protein is also present during SI pollination events, where rejection does take place. Besides their possible involvement in SI, plant proteases and protease inhibitors have been increasingly associated with several developmental processes such as plant-pathogen-insect interactions (Li et al., 2008; Hartl et al., 2010) and more recently in program cell death (Chichkova et al., 2010), which makes it a very intense field of study.

Our results have led us to consider the timing of the events during the pollen-rejection response. We propose that NaStEP accumulates in the stigmatic exudate during pollination (Busot et al., 2008) and is taken in to the pollen tubes soon after germination in order to protect HT-B (directly or indirectly) in pollen tubes from degradation through its protease inhibitor activity. The HT-B protein is incorporated at later times, once the pollen tube has reached the transmitting tissue in the style. This temporal offset in the incorporation of stylar proteins would allow HT-B to avoid premature degradation and prevent the breakdown of the SI response, since this protein mediates in the rupture of the S-RNase-containing vacuole in the incompatible pollen tubes. This hypothesis is consistent with our observations in the NaStEP-suppressed

Figure 7. NaStEP is a subtilisin inhibitor. A, Coomassie blue staining of a NaStEP-purified fraction. B, Reverse zymography revealing NaStEP inhibition of subtilisin proteolytic activity. C, Immunoblotting against NaStEP after zymography.
hybrids that, in the majority of the pollen tubes from incompatible crosses, HT-B is not detected after 36 h. Likewise, our data show that HT-B might exert its function in pollen rejection around 16 h post-pollination, which is when it reaches high levels of accumulation in both compatible and incompatible pollen tubes, and after which its levels decline until they become undetectable. Although the exact time in which the S-RNase-SLF/SFB interaction occurs within the pollen tube and the destabilization of the S-RNase-containing vacuole are not known, we propose that these occur before HT-B degradation is set.

Finally, the development of a reliable and biologically appropriate three-dimensional model was particularly useful to interpret three pieces of experimental data: (1) the increased efficiency of the transit signal in the full protein, compared with the β-region alone, is consistent with a role for the NaStEP C terminus in the stability of the N terminus found in the three-dimensional model; (2) the role of the β-segment as a secretory signal sequence and (3) the activity of NaStEP as a subtilisin inhibitor are both in agreement with the intrinsically disordered nature of these regions and their high solvent accessibility, as deduced from the model. In addition, the model suggests a divergence between the barley subtilisin inhibitor and NaStEP, which suggests a process of subfunctionalization of an ancestral protein during the evolution of the SI mechanisms in Nicotiana spp. Accordingly, a similar process of divergence might have taken place on its target protease, and this information could be useful in the search for this putative component.

MATERIALS AND METHODS

Plant Materials

SI N. alata (S105S105 S120S120), SC N. glauca, and SC N. tabacum ‘Praeco’ have been described previously (Murrett et al., 1994, 1996; Beecher and McClure, 2001). SC N. plumbaginifolia (inventory no. TW107, accession 43B) and SC N. longiflora (inventory no. TW79, accession 30A) were obtained from the U.S. Tobacco Germplasm Collection (Crop Research Laboratory), SI Rastroensis (Nicotiana rustroensis) and SC N. benthamiana have been described previously (Juárez-Díaz et al., 2006; Lee et al., 2009). All plant materials were grown under greenhouse conditions.

RNAi Construct, Plant Transformation, and Transgenic Hybrid Generation

The complementary DNAs for the sense and antisense NaSTEP were amplified as follows: sense oligonucleotide forward primer, 5′-CGCCCTCGAGATGAAATCCTTTATTTTCAGCTTCCTC-3′; reverse, 5′-GCCGGTAGTCCACGACATTTTCACTTCCTGG-3′; antisense oligonucleotide forward primer, 5′-GCCGGATCCATGAAATCCTTTATTTTCAGCTTCCTGG-3′; reverse, 5′-GCCGCTCGAGATGAAATCCTTTATTTTCAGCTTCCTGG-3′. The PCR products were cloned into a pHANNIBAL vector (Commonwealth Scientific and Industrial Research Organization Plant Industry; Wesley et al., 2001). N. plumbaginifolia was transformed as described (Murrett et al., 1992). Thirty independent lines were analyzed as primary transformants. We recovered one transformed N. plumbaginifolia line, which was crossed with SI N. alata (S105S105). Four transgenic hybrids (N. plumbaginifolia × SI N. alata (S105S105)) were obtained and designated as K08-2 (S105S105), K08-3 (S105S105), K08-9 (S105S105), and K08-16 (S105S105). To generate an advanced hybrid transgenic population (J08), we crossed the SC fertile hybrid K08-2 (presumably tetraploid) N. plumbaginifolia × SI N. alata (S105S105) with SI N. alata (S105S105).
was centrifuged (20,000g, 30 min, 4°C), the supernatant was discarded, and the pellet was resuspended in column buffer (50 μl Tris-HCl, pH 8.5, 100 μl NaCl). Dialysis was performed overnight using the column buffer. The extract was loaded onto an HPLC system (1 ml min⁻¹) using a 15-ml Source-Q column (GE Healthcare) preequilibrated with column buffer, and a linear NaCl elution gradient (200–600 μl) was used. Most of NaStEP eluted gradually before the NaCl gradient was initiated.

Protein Gel-Blot Analysis

Equal amounts of protein were separated using 12% or 15% SDS-PAGE and blotted onto nitrocellulose. Immunoblotting was performed essentially as described (Cruz-Garcia et al., 2005; Goldraij et al., 2006; Busot et al., 2008). Primary antibody dilutions were as follows: anti-120K (1:10,000), anti-HT-B (1:5,000), anti-NaStEP (1:15,000), anti-Suc-RNase (1:10,000), and anti-SG-RNase (1:10,000; Supplemental Fig. S1) as described (Cruz-Garcia et al., 2005; Goldraij et al., 2006; Busot et al., 2008).

Zymogram Analysis

Reverse zymograms were performed as suggested by Lantz and Ciborowski (1994). Two sets of gels were used, one without and one including gelatin (0.1% w/v) copolymerized with the polyacrylamide. After electrophoresis, the gels without gelatin were stained with Coomassie Brilliant Blue R-250 and blotted onto nitrocellulose to be immunostained with anti-NaStEP antibodies as described above. The gelatin gel was rinsed twice with 2.5% (v/v) Triton X-100 solution, twice with 2.5% (v/v) Triton X-100 + 50 mM Tris-HCl, pH 7.4, solution, and twice with 50 mM Tris-HCl, pH 7.4, each for 10 min, in order to remove SDS. The gelatin gel was incubated for 2 h at 37°C in a buffer solution (50 mM Tris-HCl, 200 μM NaCl) to which 2 μg ml⁻¹ glutathione was added. Gels were fixed (10% methanol, 10% acetic acid solution) and stained with Coomassie Brilliant Blue R-250. Destaining of the gels revealed the inhibition bands at sites where gelatin was not digested by the protease. Confirmation of NaStEP presence at these sites was obtained by immunostaining with anti-NaStEP antibodies as described above. Aprotinin was used as a positive control, because it is a Kunitz proteinase inhibitor homologue. J Exp Bot 59: 3187–3201 Chavelas-Adame EA, Hernández-Dominguez EE, Gaytán-Mondragón S, Rosales-Lein L, Valencia-Turcotte L, Rodriguez-Soto R (2011) A hitchhiker’s guide to the modeling of the three-dimensional structure of proteins. Int Biotechnol Color J 1: 26–35 Chichkova NV, Shaw J, Galiliuna RA, Drury GE, Tuzhikov AJ, Kim SH, Kalkum M, Hong TB, Gorshkova EN, Torrance L, et al (2010) Phytoasparagine, a retrofuscolysin cell death promoting plant proteinase with caspase specificity. EMBO J 29: 1149–1161 Covey PA, Kondo K, Welch L, Frank E, Sianta S, Kuma A, Nuzhe E, Lopez-Casado G, van der Knaap E, Rose JK, et al (2010) Multiple features that distinguish unilateral incongruity and self-incompatibility in the tomato cloade. Plant J 64: 367–378 Cruz-Garcia F, Hancock CN, Kim D, McClure B (2005) Stylar glycoproteins bind to S-RNase in vitro. Plant J 42: 295–304 de Nettancourt D (1977) Incompatibility in Angiosperms: Monographs on Theoretical and Applied Genetics 3. Springer-Verlag, New York de Nettancourt D (2001) Incompatibility and Incongruity in Wild and Cultivated Plants. Springer-Verlag, New York Deutscher MP (1990) Maintaining protein stability. Methods Enzymol 182: 83–89 Entani T, Iwano M, Shiba H, Che FS, Isogai A, Takayama S (2003) Comparative analysis of the self-incompatibility (S-) locus region of Prunus mume: identification of a pollen-expressed F-box gene with allelic diversity. Genes Cells 8: 203–213 Franklin FCH, Lawrence MJ, Franklin-Tong VE (1995) Cell and molecular biology of self-incompatibility in flowering plants. Int Rev Cytol 158: 1–64 Goldraij A, Kondo K, Lee CB, Hancock CN, Sivaguru M, Vazquez-Santana S, Kim S, Phillips TE, Cruz-Garcia F, McClure BA (2006) Compartmentalization of S-RNase and HT-B degradation in self-incompatible Nicotiana. Nature 439: 805–810 Hancock CN, Kent L, McClure BA (2005) The stylar 120 kDa glycoprotein is required for S-specific pollen rejection in Nicotiana. Plant J 43: 716–723 Harty M, Giri AP, Kaur H, Baldwin IT (2010) Serine protease inhibitors specifically defend Solanum nigrum against generalist herbivores but do not influence plant growth and development. Plant Cell 22: 4158–4175 Hess B, Kutzner C, van der Spoel D, Lindahl E (2008) GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. J Chem Theory Comput 4: 435–447

Supplemental Table S2. Pollen tubes at the base of the style 72 h post pollination in a NaStEP-suppressed hybrid from the K08 population.

ACKNOWLEDGMENTS

We are grateful to Anabel Bieler-Antolin for her assistance with fluorescence microscopy, to Gabriel Orozco-Hoyuela for confocal microscopy help, to María Teresa Oliviera-Flores and Laurel Fabila-Ibarra for plant transformation and greenhouse support, and to Guillermo Mendelza for mass spectrometry analysis. We particularly thank the anonymous reviewers for comments to a previous version of the manuscript.

Received April 9, 2012; accepted November 12, 2012; published November 13, 2012.
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