A Novel Thioredoxin h Is Secreted in Nicotiana alata and Reduces S-RNase in Vitro*

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Thioredoxins (Trxs) are small conserved proteins that play an important role in cellular redox regulation. When the Trx active site WCGPC is reduced, it is able to reduce the disulfide bonds of target proteins. Trxs are widely distributed in nature from prokaryotes to eukaryotes. In photosynthetic organisms, Trxs have been shown to be highly polymorphic and to participate in several central cellular processes (1, 2). The diversity of physiological roles in which Trxs participate depends entirely on the target proteins (3–5). The 20 genes encoding Txrs in the Arabidopsis thaliana genome (6) and the proteomic analysis (2) of their targeted proteins by Trx h3 in A. thaliana reinforce the wide range of functions in which these proteins are involved.

In plants, five different types of Trxs have been described: m, f, x, o, and h. Types m, f, and x are chloroplastic, whereas types o and h Trxs are mitochondrial and type h Trxh are assumed to be localized in the cytoplasm (5, 7–11). The mitochondrial and cytoplasmic Trxs are reduced by mitochondrial (11) or cytoplasmic (12) NADPH-dependent thioredoxin reductases (NTR).

Although Trxs have traditionally been considered to be cytoplasmic proteins, recent analyses (13) revealed that they can be further differentiated into three subgroups. Subgroup II includes Trxh with N-terminal extensions. The biochemical function of these extensions remains unclear, and the available algorithms do not predict any targeting signal. Gelhaye et al. (14) reported on a Trx h subgroup II from poplar (Populus tremula), PtTrx2, the N-terminal extension of which is necessary to target this Trx h to mitochondria. In addition, APP13-1, a Trx h present in the rice (Oryza sativa) phloem sap, mediates its own transport from cell to cell through plasmodesmata only if its N-terminal extension is present (15, 16).

Here, we report an analysis of a Trx h that belongs to the subgroup II, from Nicotiana alata, called NaTrxh. The N-terminal extension of NaTrxh was not recognizable as a secretion signal, and yet we demonstrated that NaTrxh accumulates in the extracellular matrix (ECM) of the stylar transmitting tract (TT) in N. alata. Moreover, fusion of NaTrxh to the green fluorescent protein (GFP) directed secretion of this fusion protein in two heterologous systems. We used two separate biochemical approaches, monobromobimane (mBBr) labeling and 4-aminophenylarsine oxide (PAO) chromatography, to detect potential substrates of NaTrxh. S-RNase was clearly among the proteins reduced by NaTrxh, raising the possibility that it is involved in pollination, particularly in self-incompatibility (SI).

**EXPERIMENTAL PROCEDURES**

Plant Materials—Nicotiana plumbaginifolia (inventory number TW107, accession number 43B) was obtained from Tobacco Germplasm Collection number TW107, Crops Research Laboratory, U. S. Department of Agriculture- Agricultural Research Service, Oxford, NC. Self-compatible (SC) N. alata cv Breakthrough was obtained from Thompson and Morgan, Jackson, NJ. Self-incompatible (SI) N. alata S105S105 and S110S110 have been described previously (17–19). Nicoti...
ana benthamiana and A. thaliana Columbia (Col-0) ecotype plants were grown under greenhouse conditions using a 16:8 photoperiod at 22 °C.

Recombinant NaTrxh Overexpression and Purification—The NaTrxh cDNA with BamHI and EcoRI sites was generated using the following primers: forward, 5′-CCGCGGATCCATGGATCATACTTACCA-3′; reverse, 5′-CCGCGGATCCATGGATCATACTTACCA-3′. The PCR product was cloned into the N-terminal of the GFP in the pHBT vector (20). The protein expression was observed by confocal microscopy after 3 days. A. thaliana Col-0 transformation was carried out by bombardment (25) using squashed in-platelet (grown aseptically in 0.5× Murashige and Skoog with 1% sucrose) with DNA-coated tungsten particles (Tungsten M-17). The NaTrxh-GFP fusion was cloned into pBluN19 (21) under control of the 35S cauliflower mosaic virus (CaMV35S) promoter.

After Agrobacterium tumefaciens pGV2260 (22) transformation, an agroinfiltration of N. benthamiana leaves was carried out, as described previously (23, 24). The protein expression was observed by confocal microscopy after 1 week. A. thaliana Col-0 transformation was carried out by bombardment (25) using squashed in-platelet (grown aseptically in 0.5× Murashige and Skoog with 1% sucrose) with DNA-coated tungsten particles (Tungsten M-17). The NaTrxh-GFP fusion was cloned into pBluN19 (21) under control of the 35S cauliflower mosaic virus (CaMV35S) promoter.

NaTrxh-GFP Fusion Constructs and Transient Expression—An NaTrxh cDNA with BamHI and Ncol sites was produced by PCR and fused in-frame to the N-terminal of the GFP in the pHBT vector (20). Primers used are as follows: forward, 5′-CCGCGGATCCATGGATCATACTTACCA-3′; reverse, 5′-AGCAGTGGTGATGGATG-3′. The NaTrxh-GFP fusion was cloned into pBluN19 (21) under control of the 35S cauliflower mosaic virus (CaMV35S) promoter.

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N. alata Total Protein Extracts—Total protein extracts from mature styles including stigmas, anthers, ovaries, sepals, petals, and young leaves from N. alata were obtained using 0.05 M sodium acetate, pH 5.0, 0.05 M NaCl, 1% (v/v) 2-mercaptoethanol as extraction buffer.

Sequential Style Protein Extractions—Two different salt concentration buffers for 2.5 h at 4 °C: (a) low salt buffer (100 mM Tris HCl, pH 8.0, 2 mM Na2S2O4); (b) high salt buffer (400 mM NaCl, 40 mM Tris HCl, pH 8.0, 2 mM Na2S2O4). The NaTrxh protein was purified by sequentially washing the resin twice with 1 mM 2-mercaptoethanol buffer. All the fractions were concentrated by cold acetone precipitation and were analyzed by 12.5% SDS-PAGE, blotted onto nitrocellulose, and immunostained with anti-NaTrxh antibody.

Protein Gel Blot Analysis and Immunostaining—Proteins were fractionated in a 12.5% SDS-PAGE, blotted onto nitrocellulose, and immunostained with anti-S105-RNase (1:10,000 dilution), anti-NaTrxh (1:1,000 dilution) (32), or anti-NaTrxh (1:1,000 dilution).

In Vitro Identification of NaTrxh Target Proteins—Reduction of the disulfide bonds of target proteins was determined using the two-dimensional SDS-PAGE system (33) using the low salt buffer extract without Na2S2O4. Before the first dimension, proteins are labeled with mBBr after non-reducing (control) or reducing (with dithiothreitol (DTT) or Na2S2O4) conditions. The second dimension gel was observed using UV light (Fluor-S, Fuji Corp., Saddle Brook, NJ). Low salt protein extracts from N. alata S105S105 in 50 mM Tris HCl, pH 7.9, were passed over PAO resin (Invitrogen) after reduction by NaTrxh, 20 mM 2-mercaptoethanol, or without reduction. After washing, the unbound fraction was collected. Bound proteins were eluted by sequentially washing the resin twice with 1 mM 2-mercaptoethanol containing buffer, five times with 5 mM 2-mercaptoethanol, and three times with 500 mM 2-mercaptoethanol buffer. All the fractions were concentrated by lyophilization and were analyzed by 12.5% SDS-PAGE. Gels were blotted onto nitrocellulose and immunostained with anti-S105-RNase antibody.

In Vitro Protein-Protein Interactions by Affinity Chromatography—Protein extracts (1 mg) from N. alata S105S105 were eluted with 50 mM glycine, 50 mM NaCl, pH 2.6. The samples were neutralized by the addition of 1 M Tris. BSA-Affi-Gel and Gly-Affi-Gel Columns—BSA (20 mg) in 0.1 M MOPS, pH 7.5, was immobilized to Affi-Gel-15 (Bio-Rad), as recommended by the manufacturer. Gly-Affi-Gel column was made by blocking Affi-Gel-15 with 1.0 M glycine, pH 8.0, as recommended by the manufacturer.

Phylogenetic Analysis—Amino acid sequences of plant Trxs were aligned by Clustal X (29). Based on the alignment, a phylogenetic tree was constructed by the neighbor-joining method (30). A phylogenetic test was done based on 1,000 bootstrap replicates. Phylogenetic analysis was conducted using MEGA version 2.1 (31). The GenBank™ accession numbers used for this analysis are as follows: Trx type f, A. thaliana (Q9XFH8), Pisum sativum (X63537), and Spinacia oleracea (X14959); Trx-like proteins, Hordeum bulbosum (AF159385), Lolium perenne (AF159387), Phalaris coerulescens (AF159388), Secale cereale (AF159386), A. thaliana (AAG51342); Trx type h, Ipomea batatas (AY444228), A. thaliana (AAG52566, AAD39316, S58119, S58118, S58123, S58120, and S29448), Brassica napus (U59379), O. sativa (D26547), N. alata (NaTrxh) (DQ012448), Nicotiana tabacum (Q07090 and X58527); Trx type m, A. thaliana (AAF15949, O47837, Q9SEU6, and AAF15950), P. sativum (X76269), S. oleracea (X51462), Zea mays (L40957), Trx type x, A. thaliana (AAF15952); and Trx type o, A. thaliana (AF396650).

Specific antibodies against NaTrxh were eluted with 50 mM glycine, 50 mM NaCl, pH 2.6. The samples were neutralized by the addition of 1 M Tris. BSA-Affi-Gel and Gly-Affi-Gel Columns—BSA (20 mg) in 0.1 M MOPS, pH 7.5, was immobilized to Affi-Gel-15 (Bio-Rad), as recommended by the manufacturer. Gly-Affi-Gel column was made by blocking Affi-Gel-15 with 1.0 M glycine, pH 8.0, as recommended by the manufacturer.

Microscopy and Immunolabeling—N. alata S105S105 styles were obtained using 0.05 M sodium acetate, pH 5.0, 0.05 M NaCl, 1% (v/v) 2-mercaptoethanol as extraction buffer.
**Thioredoxin Type h Secretion**

dehydrated in an ethanol series, and embedded in Paraplast Plus (Pollysciences Inc., Warrington, PA). Sections of 6–7 μm were blocked with phosphate-buffered saline plus 3% BSA, 0.01% sodium azide, 0.1% Triton X-100 for 4 h at 4 °C. Sections were simultaneously incubated with the primary rabbit anti-NaTrxhrec antibody (1:50 dilution) and the primary mouse anti-S<sub>C10</sub>-RNase antibody (1:1,000 dilution) at 4 °C overnight. Sections were then incubated with both secondary goat anti-rabbit Alexa Fluor 568-fluorochrome (magenta signal) conjugated and goat anti-mouse fluorescein isothiocyanate-fluorochrome (green signal) conjugated antibodies for 4 h at 4 °C. Sections were observed using confocal fluorescence microscopy.

**RESULTS**

**Isolation of the Trx h cDNA**—We isolated a cDNA (AFLP25F), which is differentially expressed between *N. alata* cv Breakthrough, an SC mutant plant that does not express the S-RNase, and SC *N. plumaginifolia*. The full-length cDNA sequence of this transcript (Fig. 1A) showed extensive sequence similarity with Trx genes from plants. The predicted open reading frame contains the sequence WCGPC, described as the conserved Trx active site (3, 4).

To identify the type of Trx encoded by the AFLP25F cDNA (i.e. m, f, x, or h), we performed a phylogenetic analysis using the deduced AFLP25F amino acid sequence and other plant Trxs. The phylogenetic tree in Fig. 1B displays five major groups. Four of those groups correspond to organelar thioredoxins (f, m, x, and o types). The AFLP25F protein sequence clearly clusters with Trxs h, subgroup II. The AFLP25F product was named NaTrxh.

To evaluate the disulfide reductase activity of NaTrxh, we expressed this gene in *E. coli* as a GST fusion protein. Fig. 2A shows the recombinant NaTrxh (NaTrxh<sub>rec</sub>) purified after cleavage from GST. Its ability to reduce insulin disulfide bonds using DTT as an electron donor (28) is shown in Fig. 2B. The results show that NaTrxh<sub>rec</sub> was able to reduce insulin disulfide bridges with a similar qualitative activity profile as the recombinant *E. coli* Trx (34). Furthermore, when NaTrxh<sub>rec</sub> was incubated with NADPH and recombinant *E. coli* NTR, the thiol groups of NaTrxh<sub>rec</sub> were labeled with mBBr. Fig. 3 shows NaTrxh<sub>rec</sub> and recombinant *E. coli* Trx sulfhydryl labeling in a time-dependent manner only when NADPH and NTR were present. Thus, similar to other Trxs h, oxidized NaTrxh<sub>rec</sub> can be regenerated by the addition of NADPH and NTR (12, 35).

**NaTrxh Expression Pattern in N. alata**—To analyze the expression pattern of NaTrxh in plants, we prepared specific affinity-purified anti-NaTrxh antibodies. Fig. 3A (right panel) shows that this antibody detects specifically NaTrxh<sub>rec</sub> with no cross reaction to *E. coli* Trx. This antibody was used to analyze the presence of the NaTrxh in different tissues of *N. alata*. NaTrxh protein is detected in all the tissues analyzed (Fig. 3B, right panel). This protein is particularly abundant in floral tissues including petals, ovaries, and styles, and it is present in lower levels in anthers, sepals, and leaves.

Biochemical fractionation studies suggest an extracellular location for NaTrxh. SI *N. alata* S<sub>105</sub>S<sub>105</sub> styles were hand-bisected, and the secreted proteins that accumulate in this tissue were differentially extracted using sequential washes with low salt and high salt buffers, as described (26). Using this procedure, soluble ECM proteins are eluted by a low salt buffer, whereas more tightly bound proteins are released only after the high salt buffer wash. The extraction profile obtained for the NaTrxh protein was similar to S<sub>105</sub>-RNase and to the transmitting tissue-specific protein of *N. alata* (NaTTS) (Fig. 4). It is known that these two proteins are secreted and that they accumulate in the stylar TT ECM. NaTrxh is eluted with the higher molecular mass (i.e. 55–110-kDa) NaTTS isoforms (Fig. 4C, *LS* lane) that are thought to be present in the ECM (26), suggesting that NaTrxh is also secreted into the ECM of the stylar TT.

**NaTrxh Is Secreted into the Extracellular Matrix of the Transmitting Tissue**—To further investigate NaTrxh localization and its secretion to the stylar TT ECM, we performed an immunohistochemical analysis using three-dimensional confocal microscopy. Cross sections of SI *N. alata* S<sub>C10</sub>S<sub>C10</sub> styles were simultaneously immunolabeled with a mouse anti-S<sub>C10</sub>-RNase antibody (32) and with the affinity-purified polyclonal anti-NaTrxh antibody. Fig. 5, A2, A4 and A6, show that both
NaTrxh and S_{105} R-Nase colocalized outside of the TT cells, indicated by the yellow signal generated from the mixture of both green (S_{C105} R-Nase) and magenta (NaTrxh) fluorochrome signals. To show that the anti-NaTrxh antibody is specifically reacting with NaTrxh in the TT cells, we pretreated the antibody prior to apply to the tissue with NaTrxh rec. As shown in Fig. 5, A3 and A5, after pretreatment, only the green signal of S_{C105} R-Nase was detected.

The experiments shown in Figs. 4 and 5A demonstrate that NaTrxh is secreted into the ECM. However, NaTrxh does not possess a canonical secretory signal peptide, and different secretion signal algorithms give conflicting results. For example, the Bendtsen neural network algorithm (36) does not predict any signal peptide, whereas the hidden Markov model algorithm predicts a signal peptide with a probability of 0.953 with a maximal cleavage site probability of 0.593 between amino acid residues 16 and 17 (Fig. 1A). Likewise, the Secretome 1.0 predictor (37) predicts that NaTrxh (NN-score 0.874) is a non-classical secreted protein.

NaTrxh Sequence Contains the Information for Its Secretion—To further corroborate that NaTrxh is indeed secreted in plants, an NaTrxh-GFP fusion protein expressed from the CaMV35S promoter (20) was constructed. This construct was used to analyze GFP expression pattern in transient assays in N. benthamiana and A. thaliana leaves (Fig. 5B). When the NaTrxh-GFP fusion protein is expressed, most of the GFP protein accumulates in the cell wall in either N. benthamiana (Fig. 5, B2 and B4) or A. thaliana (Fig. 5, B10 and B12). As expected, the GFP protein without fusion, used as control, was mainly localized in the cytoplasm (Fig. 5, B6 and B8). Thus, NaTrxh is sufficient to cause secretion of GFP, supporting the idea that this protein is targeted outside of the cell in N. alata.

S-RNase Is Reduced by NaTrxh in Vitro—To investigate possible substrates for NaTrxh, we performed in vitro reduction reactions with stylar proteins and NaTrxh rec plus NADPH and NTR. Reduced sulphhydryls were labeled with the fluorescent probe mBBr and visualized after separation in a two-dimensional SDS-PAGE system in which non-reducing conditions were used for the first dimension and reducing ones were used for the second dimension (33). For these experiments, we used the low salt extractable ECM stylar protein fraction from SI N. alata S_{105} R-Nase prepared without a reducing agent in the extraction buffer. As shown in Fig. 6, A and B, there is a small amount of mBBr labeling where S_{105} R-Nase is the most prominent labeled protein observed (Fig. 6, A and B). Proteins reduced after the first dimension show altered mobility and shift off the diagonal in the second dimension. If reduction is incomplete, two spots are visible, as seen for S_{105} R-Nase (Fig. 6, A and B). Proteins fully reduced with DTT prior to mBBr labeling show brighter fluorescence and appear as a single spot after two-dimensional SDS-PAGE (Fig. 6, C and D). As expected, many proteins are reduced by DTT, and mBBr labeling is visible along the entire diagonal. Treatment with purified NaTrxh rec, NADPH, and NTR caused a specific increase in mBBr labeling of S_{105} R-Nase similar to the one obtained by DTT (Fig. 6,
Thioredoxin Type h Secretion

FIGURE 5. NaTrxh is a secretion protein. A, NaTrxh colocalization with the secreted S$_{105}$-RNase in the ECM of TT in N. alata S$_{105}$S$_{105}$. Immunohistochemical analysis of styles treated as follows. Image 1, no primary antibody control to show self-fluorescence; images 2, 4, and 6, co-staining with NaTrxh (magenta) and S$_{105}$-RNase (green) antibodies; images 3 and 5, antibodies preincubated with NaTrxh$_{un}$-purified protein; image 6, higher magnification of the transmitting tissue where the colocalization is observed in the extracellular matrix. Bars correspond to 68 (images 1–3), 55 (images 4 and 5), and 13.3 (image 6). B, NaTrxh-GFP fusion protein is secreted to the cell wall in N. benthamiana and A. thaliana leaves. Images 1–4, NaTrxh-GFP fusion protein was introduced into N. benthamiana and A. thaliana (images 9–12) leaves. The localization of the GFP in N. benthamiana leaves (images 5–8) was used as a control. After transfection, the leaves were analyzed under phase contrast microscopy (images 1, 3, and 9) and under fluorescence microscope for GFP (images 2, 6, and 10) and for chlorophyll fluorescence (images 3, 7, and 11). Images 4, 8, and 12, merged images shown for each assay. Arrows, GFP fluorescence. CW, cell wall; Cyt, cytoplasm; Chl, chlorophyll fluorescence. Bars correspond to 10 μm (images 1–8) and 20 μm (images 9–12).

E and F). S$_{105}$-RNase changed its mobility after reduction with 2-mercaptoethanol for the second dimension (i.e., it appears as two spots in the second dimension), suggesting that NaTrxh only partially reduced S$_{105}$-RNase before the first dimension. The specificity of NaTrxh reduction is apparent from comparison of the large number of proteins that shift mobility and appear off the diagonal after treatment with NaTrxh but not with the nonspecific reduction by DTT (compare Fig. 6, C and E).

PAO affinity chromatography provided further evidence that S$_{105}$-RNase is a substrate for NaTrxh. PAO matrices specifically bind proteins with vicinal thiols that can reversibly form a covalent bond with the resin (38). Low salt extracts from SI N. alata S$_{105}$S$_{105}$ either were applied directly to the PAO matrix or were first treated with 20 mM 2-mercaptoethanol or NaTrxh$_{un}$ plus NADPH and E. coli NTR. Fig. 7 shows that little or no S$_{105}$-RNase bound to the PAO matrix unless it was first subjected to reducing conditions. Some S$_{105}$-RNase was weakly bound under all conditions and eluted with 1 mM or 5 mM 2-mercapto-ethanol (Fig. 7A). Large amounts of S$_{105}$-RNase, however, bound tightly to this column after reduction by 2-mercaptoethanol (Fig. 7B) or NaTrxh$_{un}$ (Fig. 7C). The tightly bound S$_{105}$-RNase eluted with 500 mM 2-mercaptoethanol (Fig. 7, B and C).

S$_{105}$-RNase Interacts with NaTrxh Independently of Disulfide Bridges—We performed affinity chromatography experiments to prove that S$_{105}$-RNase specifically interacts with NaTrxh. An NaTrxh-Affi-Gel affinity column was prepared and used to pass over a stylar protein extract from SI N. alata S$_{105}$S$_{105}$ obtained without reducing agents. Fig. 8A shows that S$_{105}$-RNase is specifically retained in the NaTrxh-Affi-Gel matrix because S$_{105}$-RNase is not released from the column when this was washed under harsh conditions (1% Tween 20 and 0.2 M NaCl), but it is eluted only with a low pH buffer (Fig. 8A, B lane). Therefore, we conclude that S$_{105}$-RNase retention in the column is due to its specific interaction with NaTrxh because it was not retained when the BSA-Affi-Gel (Fig. 8B) or the Gly-Affi-Gel columns (Fig. 8C) were used.

DISCUSSION

In the present work, we identified a 16.8-kDa Trx h (NaTrxh). Sequence analysis of this protein showed that it belongs to the Trx h subgroup II (13), and it also showed that it is a novel thioredoxin in Nicotiana since only two different Trxs h have been reported in N. tabacum (39), which are clustered in the subgroup I (Fig. 1B). Trx h subgroup II is characterized by the presence of an N-terminal extension thought to be important for targeting (16, 14). Despite the fact that this domain characterizes all the proteins of this subgroup, its function remains unclear. Scattered information has linked this domain to a particular function; the ability to move from cell to cell of a rice phloem sap Trx h has been associated with its N-terminal extension (16). Similarly, Gelhaye et al. (14) reported that poplar Ptrxh2 is associated with mitochondria, proving that its N-terminal extension functions as a mitochondrial transit peptide.

Using different approaches in this work, we demonstrated that NaTrxh is secreted from the cell in different plant species. Moreover, the biochemical data showed that NaTrxh behaves as a soluble component in the ECM and that its fusion to GFP is sufficient to cause GFP secretion. To our knowledge, accumulation of Trx h in the extracellular space has not been previously reported. However, the mechanism by which this N-terminal extension promotes secretion is still unclear. This sequence might function as a non-canonical secretion signal for processing through the endoplasmic reticulum and Golgi apparatus for eventual secretion. It is also possible that NaTrxh secretion occurs through an alternative pathway. In mammalian cells, it has been reported that a Trx is secreted through a pathway that does not involve the Golgi apparatus, the redox status of the cell, or its own Trx redox state (40).

Trxs modify the structure and activity of target proteins by reducing disulfide bonds. They have been implicated in diverse physiological processes. For example, cytoplasmic Trxs are involved in the Brassica sporophytic SI system. Bower et al. (41) identified two Trxs h, THL-1 and THL-2, that specifically interact with the protein kinase domain of the S-locus receptor (SRK), the female determinant of S-specificity in Brassica (42). Cabrillac et al. (43) suggested that THL-1 binds to SRK and inhibits its auto-phosphorylation. When SRK binds SCR (S-locus cysteine-rich protein), the male determinant (44), it appears to release THL-1 concomitant with activation of kinase activity.
Our results show that, similarly to other plant Trxs (THL-1, THL-2) (5, 41), NaTrxh is ubiquitously expressed in *N. alata*. This result suggests that NaTrxh may function in the regulation of the redox state of extracellular proteins. In the particular case of the style, the ECM in the TT is especially rich in secreted proteins including S-RNases and arabinogalactan proteins such as NaTTS (26). As NaTrxh appears to be able to regulate these proteins, it might explain its higher abundance in pistils when compared with other tissues. We have also observed that NaTrxh is expressed at approximately a 6-fold higher level in *N. alata* than in *N. plumbaginifolia* (data not shown), and this is consistent with its higher level of expression of secreted proteins in *N. alata*. The abundance of secreted proteins in the TT ECM afforded an opportunity to directly test whether they are substrates for NaTrxh reduction. If NaTrxh functions to reduce ECM proteins, then the maintenance of its reduced active state would require NTR in the ECM as well. There is not yet evidence for a secreted NTR in plants. In animal systems, however, an NTR is secreted together with a Trx in monocytes and also in malignant leukemia or melanoma cell lines (45). Another possibility is that NaTrxh functions after uptake into the pollen tube cytoplasm, where NADPH and NTR are more likely to be present. In *N. alata*, NaTrxh could be taken up by the pollen tubes along with other stylar proteins, such as the S-RNase (46) and 120K (47). 3

Using mBBr labeling and PAO chromatography, we showed that S-RNase is clearly a substrate for NaTrxh. S-RNase was preferentially labeled by mBBr even in the presence of other potential substrates such as NaTTS, 120K, and the class III pistil-specific extensin-like proteins (48).

We detected a stable interaction between S-RNase and NaTrxh, which is specific and independent of any disulfide bridge. It is known that the interactions based on disulfide bridges between a Trx and its target protein are ephemeral since it only depends on the intermolecular disulfide bridge that is formed when the first cysteine residue of the target protein is reduced by the N-terminal cysteine, and it is quickly attacked by the second cysteine of the Trx, releasing the reduced target protein (49, 50). Therefore, it seems that $S_{105}$-RNase reduction by

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3 B. A. McClure, unpublished data.
NaTrxh is specific and depends on previous substrate recognition by NaTrxh. These observations may have significance for SI. The specific interaction of S-RNase with NaTrxh and its consequent reduction could favor its interaction with SLF (S-locus F-box protein), the pollen determinant of S-specificity (S1). Alternatively, reduction of S-RNase may alter its conformation and facilitate its uptake. Oxley and Bacic (52) have observed changes in S-RNase conformation after reduction. Thus, because of its remarkable secretion into the ECM, NaTrxh may play a role in pollen rejection. Further studies of this possibility are warranted.

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