In Planta Assessment of the Role of Thioredoxin h Proteins in the Regulation of S-Locus Receptor Kinase Signaling in Transgenic Arabidopsis

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The self-incompatibility (SI) response of the Brassicaceae is mediated by allele-specific interaction between the stigma-localized S-locus receptor kinase (SRK) and its ligand, the pollen coat-localized S-locus cysteine-rich protein (SCR). Based on work in Brassica spp., the thioredoxin h-like proteins THL1 and THL2, which interact with SRK, have been proposed to function as oxidoreductases that negatively regulate SRK catalytic activity. By preventing the spontaneous activation of SRK in the absence of SCR ligand, these thioredoxins are thought to be essential for the success of cross pollinations in self-incompatible plants. However, the in planta role of thioredoxins in the regulation of SI signaling has not been conclusively demonstrated. Here, we addressed this issue using Arabidopsis thaliana plants transformed with the SRKb-SCRb gene pair isolated from self-incompatible Arabidopsis lyrata. These plants express an intense SI response, allowing us to exploit the extensive tools and resources available in A. thaliana for analysis of SI signaling. To test the hypothesis that SRK is redox regulated by thioredoxin h, we expressed a mutant form of SRKb lacking a transmembrane-localized cysteine residue thought to be essential for the SRK-thioredoxin h interaction. We also analyzed transfer DNA insertion mutants in the mutant form of SRKb lacking a transmembrane-localized cysteine residue thought to be essential for the success of cross pollinations in self-incompatible plants.

Many flowering plants possess self-incompatibility (SI), a genetic system that promotes outcrossing by preventing self-fertilization. In the Brassicaceae family, the SI response is controlled by haplotypes of the S locus, each of which contains two genes that encode highly polymorphic proteins, the S-locus receptor kinase (SRK), which is a plasma membrane resident single-pass transmembrane Ser/Thr receptor kinase displayed at the surface of stigma epidermal cells (Stein et al., 1991; Takasaki et al., 2000), and the S-locus Cysteine-rich protein (SCR), which is the pollen coat-localized ligand for SRK (Schopfer et al., 1999; Kachroo et al., 2001; Takayama et al., 2001). SRK and SCR exhibit allele-specific interactions, whereby only SRK and SCR encoded by the same S-locus haplotype interact. In a self-pollination, the binding of this "self" pollen-borne SCR to the extracellular domain of SRK activates the SRK kinase, thereby triggering a cellular response in stigma epidermal cells that causes inhibition of pollen germination and tube penetration into the stigma epidermal cell wall (for review, see Tantikanjana et al., 2010).

Tight regulation of SRK kinase activity and its signaling cascade is critical for productive pollen-stigma interactions because constitutive (i.e. SCR-independent) activity of the receptor is expected to result in sterile stigmas that reject both compatible and incompatible pollen. In the classical view of ligand-activated receptor kinases, the receptor occurs as catalytically inactive monomers in the absence of ligand and only becomes activated upon ligand-induced dimerization (for review, see Lemmon and Schlessinger, 2010). However, some receptor kinases in both animals (Chan et al., 2000; Ehrlich et al., 2011) and plants (Giranton et al., 2000; Wang et al., 2005, 2008; Shimizu et al., 2010; Bücher et al., 2013) form catalytically inactive dimers or oligomers in the absence of ligand, with receptor activation presumably resulting from ligand-induced higher order oligomerization or conformational changes (Lemmon and Schlessinger, 2010). Similar to the latter receptors, SRK forms oligomers in unpollinated stigmas, i.e. in the absence of SCR (Giranton et al., 2000), at least partly via ligand-independent dimerization domains located within the SRK extracellular domain (Naithani et al., 2007). It has been proposed that these ligand-independent SRK oligomers are maintained in an inactive state by thioredoxins, the ubiquitous small oxidoreductases that reduce disulfide bridges in proteins (Buchanan and Balmer, 2005). This hypothesis is supported by the following observations: (1) two Brassica napus thioredoxins, the Thioredoxin H-Like proteins THL1 and THL2, were identified as SRK interactors in a yeast (Saccharomyces cerevisiae) two-hybrid screen that used...
the B. napus SRK910 kinase domain as bait (Bower et al., 1996); (2) when purified from pistils or insect cells, the *Brassica oleracea* SRK3 variant was found to exhibit constitutive autophosphorylation activity in vitro, and this activity was inhibited by *Escherichia coli*-expressed THL1 proteins and was restored by addition of pollen coat proteins containing self but not of pollen coat proteins containing nonself SCR (Cabrillac et al., 2001); (3) the catalytic activity of THL1 was required for its inhibition of SRK3 autophosphorylation activity in vitro (Cabrillac et al., 2001); and (4) antisense suppression of THL1/THL2 gene expression in the stigmas of a self-compatible *B. napus* strain reportedly produced a low-level constitutive incompatibility (Haffani et al., 2004), as might be expected if the THL1/THL2 proteins prevent the spontaneous activation of SRK-mediated signaling in stigmas.

These observations notwithstanding, the in planta role of thioredoxin *h* proteins as negative regulators of SRK activity has not been conclusively demonstrated. To date, this proposed function has only been evaluated in a self-compatible strain of *B. napus* (Haffani et al., 2004). Consequently, it is not known if the proposed inhibitory effect of these thioredoxins on SRK catalytic activity is manifested in self-incompatible stigmas and if it applies to all SRK variants, be they derived from *Brassica* spp. or other self-incompatible species of the Brassicaceae such as *Arabidopsis lyrata*.

In this study, we tested the in planta role of thioredoxin *h* proteins in the regulation of SI signaling using a transgenic self-incompatible *Arabidopsis thaliana* model that we generated by transforming *A. thaliana* with the SRKb-SCRb gene pair isolated from the *Sb* haplotype of self-incompatible *A. lyrata* (Kusaba et al., 2001; Nasrallah et al., 2002, 2004). We had previously shown that the stigmas of *A. thaliana* SRKb-SCRb transfectants can exhibit an SI response that is as robust as the SI response observed in naturally self-incompatible *A. lyrata*, demonstrating that *A. thaliana*, which harbors nonfunctional S-locus haplotypes (Kusaba et al., 2001; Sherman-Broyles et al., 2007; Shimizu et al., 2008; Boggs et al., 2009c), has nevertheless retained all other factors required for SI. In view of the availability in *A. thaliana* of a highly efficient transformation method and numerous genetic resources, the *A. thaliana* SRK-SCR transgenic model has enabled the use of experimental approaches that are difficult or impossible to implement in *Brassica* species and has thus proven to be an invaluable platform for in planta analysis of SRK and SI signaling (Liu et al., 2007; Boggs et al., 2009a, 2009b; Tantikanjana et al., 2009; Tantikanjana and Nasrallah, 2012).

We therefore used this transgenic *A. thaliana* self-incompatible model to determine if abolishing the proposed SRK-thioredoxin *h* interaction or eliminating expression of the major thioredoxin *h* proteins expressed in stigmas would affect the outcome of self- or cross pollination. To this end, we expressed a mutant form of SRKb that lacked the Cys residue previously shown to be required for the interaction of SRK with THLs (Mazzurco et al., 2001), and we analyzed plants carrying knockout insertional mutations in thioredoxin *h* genes. Our results are inconsistent with the proposed role of thioredoxin *h* proteins as negative regulators of SRK catalytic activity and SI signaling.

**RESULTS**

**THL1 and THL2 Orthologs in the *A. thaliana* Stigma**

The *A. thaliana* thioredoxin *h* family of proteins consists of eight members (Reichheld et al., 2002). Phylogenetic analysis shows that three of these, the Thioredoxin H3 (AtTRX3), H4 (AtTRX4), and H5 (AtTRX5) genes, are the most closely related to *Brassica* spp. THL1 and THL2 proteins (Fig. 1A). Furthermore, AtTRX3, AtTRX4, and AtTRX5 are unique among all *A. thaliana* thioredoxin proteins in having the same reduction-oxidation (redox)-active site as *Brassica* spp. THL1 and THL2 proteins, which consists of the Trp-Cys-Pro-Pro-Cys sequence instead of the canonical sequence Trp-Cys-Gly-Pro-Cys found in other *A. thaliana* thioredoxin proteins (Fig. 1B; Gelhaye et al., 2005). Because the amino acid residue immediately after the first Cys within the active site of thioredoxin is thought to play a major role in the protein’s activity and specificity (Bréhelin et al., 2000), the substrate specificities of *Brassica* spp. THL1 and THL2 and of AtTRX3, AtTRX4, and AtTRX5 are likely to be different from those of other *A. thaliana* thioredoxin *h* proteins. This conclusion is supported by the observation that AtTRX3 and AtTRX4 interact with *Brassica* spp. SRKs, while AtTRX1 and AtTRX2, two thioredoxin *h* proteins that contain the Trp-Cys-Gly-Pro-Cys active site sequence (Fig. 1B), do not (Mazzurco et al., 2001). Thus, we focused on the AtTRX3, AtTRX4, and AtTRX5 proteins as possible regulators of SRK catalytic activity in the *A. thaliana* stigma.

Using absolute quantitative real-time PCR (Wong and Medrano 2005), we found that AtTRX3, AtTRX4, and AtTRX5 are all expressed in *A. thaliana* stigmas, albeit at various levels (Fig. 1C). AtTRX3 was expressed at the highest levels, followed by AtTRX4 and AtTRX5, which were expressed at approximately 11- and 19-fold lower levels, respectively. This result is consistent with microarray data available in GENEVSTIGATOR (Supplemental Fig. S1; Zimmermann et al., 2004) and with the results of our previous genome-wide transcriptional analysis of genes expressed specifically in stigma epidermal cells (Tung et al., 2005), both of which show *ATRX* expression levels to be 6-fold higher than those of other thioredoxin *h* genes in *A. thaliana* stigmas.

Two lines of evidence indicate that AtTRX3 and AtTRX4, and not AtTRX5, are the orthologs of *Brassica* spp. THL1 and THL2, respectively (Supplemental Table S1). Amino acid sequence comparisons show that AtTRX3 is 78% identical to THL1 and AtTRX4 is 85% identical to THL2, while AtTRX5 is only 69% and 60% identical to THL1 and THL2, respectively.
Furthermore, the *A. thaliana* chromosomal regions that encompass the *AtTRX3* and *AtTRX4* genes exhibit a high degree of synteny with the *Brassica rapa* chromosomal regions that contain *Bra027469* and *Bra025762* (Fig. 1D; Supplemental Table S1), two thioredoxin *h* genes whose products exhibit, respectively, 98% and 99% amino acid sequence identity to *B. napus* THL1 and THL2. By contrast, the *A. thaliana* chromosomal region containing *AtTRX5* is syntenous with a *B. rapa* chromosomal region that contains another thioredoxin *h* gene, *Bra014037*, whose protein product is 84% identical to *AtTRX5* (Fig. 1D; Supplemental Table S1),
and is thus the likely ortholog of AtTRX5. This observation, together with the very low expression level of AtTRX5 in stigmas, led us to focus on AtTRX3 and AtTRX4 for assessing the potential role of thioredoxin h proteins in the pollination responses of the *A. thaliana* stigma.

**A TM-Localized Cys Essential for the SRK-THL Interaction Is Not Required for the Inhibition of SRK-Mediated Signaling in the Absence of SCR Ligand**

In their analysis of the interaction of *Brassica* spp. SRKs with THL1 and THL2, Mazzurco et al. (2001) concluded that the SRK-THL1/THL2 interaction requires a Cys residue located near the C-terminal end of the predicted transmembrane (TM) domain of SRK (Fig. 2A). This conclusion was predicated on the results of yeast two-hybrid interaction assays showing that the SRK-THL1/THL2 interaction was abolished when the TM-localized Cys was deleted or replaced by Ser, while the ability to interact with THL1/THL2 was acquired when a Cys was inserted at the appropriate position in the TM domain of an SRK-related receptor kinase that lacked this Cys and did not interact with THL1/THL2 (Mazzurco et al., 2001). Furthermore, the fact that this TM-localized Cys residue was conserved in all SRK sequences available at the time but was missing from SRK-related proteins that do not function in SI was interpreted as indicating that interaction with THL was a distinctive feature of SRK and the SI response it determines (Mazzurco et al., 2001).

Public databases now contain 44 SRK sequences containing the TM domain. A survey of these sequences, which are derived from self-incompatible strains of various species, showed that the majority of SRK variants contained a Cys residue at the appropriate location within the region that corresponds to the SRK TM domain as predicted by the TMpred program (Hofmann and Stoffel, 1993). By contrast, this TM-localized Cys residue is replaced by Trp, Phe, Ser, or Arg in five SRK variants from *B. oleracea* (BoSRK68), *A. lyrata* (AlSRKb and AlSRK3), *Arabidopsis halleri* (AhSRK9), and *Capsella grandiflora* (CgSRK7; Fig. 2A). Moreover, the TM-localized Cys is not required for the negative regulation of SRK in planta. The SRKs that lack this Cys have not been reported to produce a constitutive SI response in their native species or when introduced into *A. thaliana*. For example, *A. thaliana* stigmas expressing the CgSRK7 variant (Fig. 2A) exhibit a robust SI response toward self SCR-expressing pollen but do not exhibit constitutive rejection of nonself pollen (Boggs et al., 2009b). These observations raise doubt about the notion that THLs, by virtue of their interaction with SRK through this TM-localized Cys residue, function as negative regulators of SRK in the absence of SR ligand.

The yeast interaction studies of Mazzurco et al. (2001) had also shown that the interaction of *Brassica* spp. SRKs with the *A. thaliana* AtTRX3 and AtTRX4 proteins, like the SRK-THL1/THL2 interaction, requires the TM-localized Cys residue of SRK. Therefore, we reasoned that in transgenic *A. thaliana* stigmas, *A. lyrata*-derived SRKb, which contains this Cys residue, might interact with AtTRX3 and AtTRX4 and that mutating the TM-localized Cys in SRKb would abolish this interaction. In this manner, we would be able to assess in planta the importance of this Cys residue and of the SRK-thioredoxin h interaction for the regulation of SRK-mediated signaling. It should be noted that it is not feasible to test the *Brassica* spp. SRK variants used in the yeast interaction studies, such as the *B. napus* SRK910 variant (Bower et al., 1996; Mazzurco et al., 2001). Despite repeated attempts, it has not been possible to transfer the SI trait to *A. thaliana* by expressing *Brassica* spp. SRKs, including SRK910 (Bi et al., 2000) or several *B. oleracea* and *B. rapa* receptors belonging to highly diverged classes of SRKs (our unpublished results). This result indicates that *Brassica* spp. SRKs do not function in *A. thaliana*, possibly because the approximately 15 million years of evolution that separate
the Brassica spp. and A. thaliana lineages may have produced extensive divergence between the SRK substrate(s) in the two taxa.

To assay for the role of the TM-localized Cys residue, we used the AtS1pr::SRKb chimeric gene in which the SRKb transcriptional unit is placed downstream of the stigma epidermal cell-specific AIT1 promoter (Boggs et al., 2009a). We replaced the TM-localized Cys (C463) of SRKb with a Trp codon to recapitulate the substitution that occurs in two SRK variants (Fig. 2A), and the resulting AtS1pr::SRKb(C463W) gene was introduced into A. thaliana plants of the C24 accession. As controls for this experiment, we used C24 plants transformed with the wild-type AtS1pr::SRKb transgene, which expresses a robust and SI response that persists throughout stigma development (Nasrallah et al., 2004; Boggs et al., 2009a).

Twelve independent transformants were analyzed by pollination assays and microscopic monitoring of pollen tube growth (Supplemental Table S2; “Materials and Methods”). As shown in Figure 2B, when transgenic AtS1pr::SRKb(C463W) stigmas were pollinated with “self” SCRb-expressing pollen from a C24[SRKb-SCRb] plant, a robust SI response was observed that was indistinguishable from that observed in the stigmas of C24 plants expressing the wild-type AtS1pr::SRKb transgene. These results demonstrate that the C463W mutation neither abolished nor weakened SRKb function and that all 12 AtS1pr::SRKb(C463W) transformants expressed the SRKb(C463W) protein at levels sufficient to confer SI. By contrast, when the same AtS1pr::SRKb(C463W) stigmas were pollinated with wild-type untransformed pollen (i.e. pollen lacking SCRb; Fig. 2B; Supplemental Table S2), we observed a compatible response that was identical, both in terms of pollen tube number and rate of tube growth, to that observed when the stigmas of C24 plants expressing the wild-type AtS1pr::SRKb transgene were pollinated with wild-type untransformed pollen. This result is inconsistent with the previously proposed notion that disrupting the SRK-thioredoxin interaction in stigmas expressing a functional SRK would result in a constitutive incompatibility response that inhibits both self- and cross polination (Cabrillac et al., 2001; Haffani et al., 2004).

**Loss-of-Function Mutations in the AtTRX3 and AtTRX4 Genes Do Not Affect Pollination Responses in A. thaliana SRKb-Expressing Stigmas**

In a second test of the potential role of AtTRX3 and AtTRX4 in pollination responses, we assessed the consequences of eliminating expression of these proteins in the self-incompatible stigmas of A. thaliana SRKb-SCRb plants carrying transfer DNA (T-DNA) insertions in these genes. Because T-DNA insertion lines are available in the Columbia (Col-0) accession but not in the C24 accession, we used Col-0 plants for this experiment. We had previously shown that Col-0 [SRKb-SCRb] transformants express transient SI, whereby the stigmas of young floral buds, stage 13 and early stage 14 according to Smyth et al. (1990), express as intense an SI response as observed in the naturally self-incompatible A. lyrata, with breakdown of SI at later stages of flower development (Nasrallah et al., 2002; Liu et al., 2007). Therefore, all pollination assays described below were performed using stage 13 bud stigmas.

We obtained two strains, hereafter designated trx3-1 and trx4-1, which carry T-DNA insertions in AtTRX3 and AtTRX4, respectively. We determined that the T-DNA in trx3-1 was inserted 28 bp before the second exon of the AtTRX3 gene (Fig. 3A) and that AtTRX3 transcripts were undetectable in the stigmas of trx3-1 homozygous plants (Fig. 3B). These results demonstrate that trx3-1 is a null mutation, as previously reported by Park et al. (2009), who failed to detect TRX3 protein in trx3-1 homozygotes. Similarly, we found that the stigmas of trx4-1 homozygotes, in which the T-DNA was inserted within the first intron of the AtTRX4 gene (Fig. 3A), lacked AtTRX4 transcripts (Fig. 3B), demonstrating that trx4-1 is also a null mutation.

To assess the potential effects of the trx3-1 and trx4-1 null mutations on SRK activity, we crossed a trx3-1 homozygote and a trx4-1 homozygote with a Col-0 [SRKb-SCRb] transformant. For each cross, we identified F2 plants that carried the SRKb-SCRb transgenes and were homozygous for trx3-1 or trx4-1. These plants are hereafter designated trx3-1[SRKb-SCRb] and trx4-1[SRKb-SCRb].

Figure 3B shows that the stigmas of trx3-1[SRKb-SCRb] plants expressed SRKb but not AtTRX3, while the stigmas of trx4-1[SRKb-SCRb] plants expressed SRKb but not AtTRX4. Pollination assays were performed on trx3-1[SRKb-SCRb] and trx4-1[SRKb-SCRb] stigmas using SCRb-expressing pollen from a Col-0 [SRKb-SCRb] plant and pollen from an untransformed wild-type Col-0 plant. As controls for these assays, we used the same pollen to pollinate the stigmas of Col-0 [SRKb-SCRb] and wild-type untransformed Col-0. As shown in Figure 3C, SCRb-expressing pollen grains failed to germinate on the stigmas of both trx3-1[SRKb-SCRb] and trx4-1[SRKb-SCRb] plants. The fact that this SI response was as intense as that triggered in Col-0 [SRKb-SCRb] stigmas by SCRb-expressing pollen demonstrates that neither the trx3-1 nor the trx4-1 mutation diminishes SRKb function. In contrast to Col-0 [SRKb-SCRb] pollen, wild-type Col-0 pollen produced pollen tube growth on trx3-1[SRKb-SCRb] and trx4-1[SRKb-SCRb] stigmas that was as profuse as on Col-0 [SRKb-SCRb] stigmas (Fig. 3C), indicating that neither the trx3-1 nor the trx4-1 mutation results in SCRb-independent constitutive activity of SRK.

Our observation that null mutations in either AtTRX3 or AtTRX4 have no effect on the pollination responses of Col-0[SRKb-SCRb] stigmas might be due to functional
redundancy of the two genes. Therefore, we crossed trx3-1[SRKb-SCRb] and trx4-1[SRKb-SCRb] to generate SRKb-SCRb plants that were homozygous for both trx3-1 and trx4-1. Reverse transcription (RT)-PCR demonstrated that the stigmas of these trx3-1 trx4-1 [SRKb-SCRb] plants lacked AtTRX3 or AtTRX4 transcripts (Fig. 3B), and pollination assays showed that these stigmas exhibited an intense SI response toward pollen from Col-0[SRKb-SCRb] and a fully compatible response toward pollen from untransformed wild-type Col-0 (Fig. 3C). Because the pollination responses of trx3-1 trx4-1[SRKb-SCRb] were indistinguishable from those of wild-type Col-0[SRKb-SCRb] plants, we conclude that loss of AtTRX3 and AtTRX4 expression in A. thaliana stigmas has no effect on the catalytic activity of SRKb, either in the presence or absence of SCRb.

**DISCUSSION**

Reversible thiol-based redox modifications have emerged as a major mechanism for regulating the activity of proteins involved in many biological processes, ranging from transcription to signal transduction. In particular, studies in mammalian systems have shown that receptor kinase-mediated signaling can be either stimulated or inhibited by the disulfide bond-reducing activity of thioredoxins (Truong and Carroll, 2013). Consequently, the proposal that SRK catalytic activity is inhibited by thioredoxin h proteins in the absence of its cognate SCR ligand and is stimulated when binding of cognate SCR disrupts the SRK-thioredoxin h interaction (Cabrillac et al., 2001) provides an attractive mechanism for explaining the tight control of SRK-mediated signaling in the stigmas of self-incompatible crucifers.

However, our results do not support the hypothesis that SRK is redox regulated by thioredoxin h proteins. Certainly, any regulation of SRK by these thioredoxins, if it occurs at all, does not apply to all SRKs. We found no evidence that AtTRX3/AtTRX4 and their proposed interaction with SRKb contribute to the outcome of pollinations, be they incompatible or compatible. Neither mutating the TM-localized Cys residue that was reportedly required for the interaction of Brassica spp. SRKs with thioredoxin h proteins nor introducing null mutations in the AtTRX3 and AtTRX4 genes produced qualitative or quantitative changes in the pollination responses of SRKb-expressing A. thaliana stigmas toward either SCRb-expressing pollen or wild-type pollen. Despite the total lack of AtTRX3 and AtTRX4 transcripts in the stigmas of trx3-1 trx4-1[SRKb-SCRb] plants, we were unable to reproduce the constitutive incompatibility reported for the stigmas of B. napus antisense THL1/THL2 transformants, in which THL1 and THL2 transcripts were reduced but not eliminated (Haffani et al., 2004).
We conclude that the AtTRX3 and AtTRX4 thioredoxin h proteins do not regulate SRKb or its downstream effectors through their disulfide-reducing activity, nor do they regulate SRKb signaling independent of their catalytic activity, as described for the tomato (Solanum lycopersicum) signaling pathway that is mediated by the Cladosporium fulvum resistance-9 (Cf-9) protein and confers resistance to pathogen strains expressing the Avirulence gene9 (Avr9) elicitor. In this pathway, the Cf-9-interacting thioredoxin CITRX is thought to function as a negative regulator, not via its reducing activity, but by acting as an adaptor protein that recruits the cytosolic kinase Avr9/Cf9 induced kinase1 to the Cf-9 cytoplasmic domain (Rowland et al., 2005; Nekrasov et al., 2006).

Importantly, our results are not due to some peculiar property of the SRKb variant or SRKs from A. thaliana species, because the TM-localized Cys residue thought to be essential for the SRK-THL and SRK-AtTRX3/AtTRX4 interactions is missing in SRK variants from Brassica spp. and C. grandiflora as well as A. lyrata and A. halleri. This observation underscores the conclusion that this Cys residue is not required for proper regulation of the activity of all SRKs.

How then might our results be reconciled with published reports on the role of thioredoxin h proteins in redox regulation of SRK? Regarding the observed interaction in yeast between SRK and THLs via the TM-localized Cys residue of SRK, one possibility is that this interaction is an artifact of the yeast two-hybrid assays used to reveal and subsequently analyze the SRK-THL interaction. Thioredoxins reduce proteins by Cys thiol-disulfide exchange, a process that involves the formation of a disulfide bond between a Cys in the thioredoxin active site and a Cys in the target protein. Thus, the notion that a cytosolic thioredoxin can interact with a Cys residue that is buried in the TM domain of SRK is inherently problematic. Interestingly, the SRK fragments that were used as bait for yeast two-hybrid screening contained the kinase domain and an N-terminal extension consisting of six amino acids derived from the TM region (Fig. 2A; Bower et al., 1996; Mazzurco et al., 2001). In these fragments, the TM-localized Cys residue would not be embedded in a membrane and would be available for disulfide bridge formation with thioredoxin in the yeast nucleus, a situation that does not occur in plants.

As for the in vitro experiments showing that THL1 inhibits SRK activity and that this inhibition is relieved by addition of pollen coat proteins from “self” pollen (Cabrillac et al., 2001), another in vitro study of SRK activation (Takayama et al., 2001) had questioned the involvement of thioredoxins. While both studies concluded that “self” SCR caused activation of SRK as measured by an increase in SRK autophosphorylation activity, this activation was observed in the presence of THL1 in the first study (Cabrillac et al., 2001) but did not require the presence of thioredoxin in the second (Takayama et al., 2001). We suggest that THL1, which is phosphorylated by SRK in vitro (Bower et al., 1996), acts as an SRK pseudosubstrate that competes with SRK for autophosphorylation, leading to the apparent inhibition of SRK autophosphorylation activity upon addition of THL1.

Finally, with regards to the low-level constitutive incompatibility response observed by antisense suppression of THL1/THL2 (Haffani et al., 2004), several factors complicate interpretation of this phenotype. The antisense construct used in this study contained the full-length THL1 or THL2 sequence, raising the possibility that the antisense silencing effect might not have been specific for these THL genes or even other thioredoxin genes. Importantly, the study did not use a strain whose stigmas express SI. Rather, it was performed using a self-fertile strain of B. napus in which the basis of self-fertility was not known and the functionality of the endogenous SRK was not established. Furthermore, no changes in SRK kinase activity or complex formation were observed in the THL1/THL2 antisense plants relative to wild-type plants. As a result, the authors acknowledged that the pollination phenotype observed in antisense THL1/THL2 stigmas does not provide conclusive evidence for the negative regulation of SRK by THL1/THL2 (Haffani et al., 2004).

Our results leave open the question of how the ligand-independent SRK oligomers observed in unpollinated stigmas are maintained in an inactive state. Although AtTRX3, and to a lesser extent AtTRX4, are the major thioredoxins in the A. thaliana stigma, we cannot rule out the possibility that another thioredoxin might function as a negative regulator of SRK catalytic activity by reducing one of several disulfide bridges that link Cys residues located outside the TM domain. However, it is possible that the ligand-independent SRK oligomers are inactive, not only due to the effect of some inhibitory factor, but also because they assume an autoinhibitory conformation in the absence of the SCR ligand. Ligand-activated receptor kinases are typically regulated by multiple mechanisms (Lemmon and Schlessinger, 2010; Endres et al., 2011). A prime example is the A. thaliana BRASSINOSTEROID-INSSENSITIVE1 (BRI1) receptor, which is subject to two modes of inhibition, cis-inhibition by its C-terminal domain and trans-inhibition by the BRI1 Kinase Inhibitor1 protein, both of which are relieved by binding of the brassinolide ligand to the BRI1 extracellular domain (Wang et al., 2005; Wang and Chory, 2006; Jaillais et al., 2011a, 2011b). Further studies of SRK, including resolution of crystal structures for the receptor in its ligand-unbound and ligand-bound forms, are required to understand how tight regulation of SRK activity is achieved to prevent the spontaneous activation of SRK-mediated signaling and the illegitimate inhibition of compatible pollen.

MATERIAL AND METHODS

Plant Materials

The Arabidopsis thaliana C24 and Col-0 plants used in this study were grown at 22°C under continuous light. Col-0[SKKb-SCRb] transformants containing the Arabidopsis lyrata SRKb and SCRb genes with their native 5ʹ and 3ʹ regulatory regions were previously described (Nasrallah et al., 2002, 2004).
The T-DNA insertion mutants (Alonso et al., 2003) in A. thaliana were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Plant genotypes were determined by PCR using the primers listed in Supplemental Table S3.

Transgenes and Plant Transformation

The pCAMBIA derivative containing the AtS1pr::SRKb chimera gene, in which the A. lyrata SRKb transcriptional unit is placed downstream of the 5′-untranslated region of the AtS1pr promoter, was previously described (Boggs et al., 2009a). To generate the SRKb(C463W) mutant transgene, the AtS1pr::SRKb-SCRb plasmid served as template for recombinant PCR using the SRKb(C463W) forward (5′-GGT CCA TCA TGT TCT GGG TTT GGA GAA GGA-3′) and SRKb(C463W) reverse (5′-TCC TTC TCC AAA CCC AGA ACA TGA TGG AAC-3′) primers. All plasmids were sequenced at the Cornell University Life Sciences Core Laboratories Center to exclude the presence of PCR-generated errors. The plasmids were introduced into C24 wild-type plants by the floral dip method (Clough and Bent, 1998), and transformants were selected on Murashige and Skoog medium (Sigma-Aldrich) containing 50 μg mL−1 hygromycin.

Pollination Assays

One day before performing the assays, stage 12 flower buds were emasculated (for staging of flower development, see Smyth et al., 1990). The stigmas of these buds, now at stage 13 of development, were manually pollinated with pollen grains from mature postanthesis flowers under a stereomicroscope. Two hours after pollination, the stigmas were fixed, stained with decolorized aniline blue, and examined by epifluorescence microscopy as previously described (Kho and Bear, 1968). Each pollination assay was performed in triplicate. In these assays, an incompatible response is manifested by the growth of fewer than 10 pollen tubes per pollinated stigma, a partially incompatible response by growth of 10 to 29 pollen tubes per pollinated stigma, and a compatible response by growth of more than 30 pollen tubes per pollinated stigma.

Expression Analysis

Total RNA was extracted from 25-stage 12 stigmas using the TRIzol reagent (Invitrogen). The RNA was treated with DNaseI (Invitrogen), and 0.5 μg of this RNA was used to synthesize first-strand complementary DNA using an oligo (dT) primer and the First-Strand cDNA Synthesis Kit for Real-Time PCR (Biorad). RT-PCR was performed using the gene-specific primers listed in Supplemental Table S1 under the following amplification parameters: 94°C for 30 s, 55°C for 45 s, and 72°C for 2 min.

Sequence Analysis

The TMpred Server was used to predict the TM region of SRK protein variants (Hofmann and Stoffel, 1993). Sequences were aligned using ClustalW (Larkin et al., 2007). The thioredoxin variants (Hofmann and Stoffel, 1993). Sequences were aligned using ClustalW. The phylogenetic tree was constructed with the neighbor-joining method using the MEGA5.1 program (Tamura et al., 2007). The thioredoxin

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** AtTRX gene expression patterns based on expression data available in public databases.

**Supplemental Table S1.** Comparison of A. thaliana AtTRX3, AtTRX4, and AtTRX5 and their closest B. rapa relatives.

**Supplemental Table S2.** Plobination phenotypes of stigmas from C24 plants expressing the SRKb(C463W) mutant or the wild-type SRKb control.

**Supplemental Table S3.** Primers used for genotyping, real-time PCR, and RT-PCR.

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