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Two Members of the Thioredoxin-h Family Interact with the Kinase Domain of a Brassica *S* Locus Receptor Kinase

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To determine potential targets of the *S* locus receptor kinase (SRK) during the Brassica self-incompatibility response, a yeast two-hybrid library was screened with the SRK-910 protein kinase domain. Two thioredoxin-h-like clones, THL-1 and THL-2, were found to interact specifically with the SRK-910 protein kinase domain and not to interact with the protein kinase domains from the Arabidopsis receptor-like protein kinases (RLK) RLK4 and RLK5. The interaction between THL-1 and the SRK-910 protein kinase domain was confirmed using coimmunoprecipitation experiments with fusion proteins produced in *Escherichia coli*. THL-1 has thioredoxin activity based on an insulin reduction assay, and THL-1 is weakly phosphorylated by the SRK-910 protein kinase domain. THL-1 and THL-2 are both expressed in a variety of tissues but show some differences in steady state mRNA levels, with THL-2 being preferentially expressed in floral tissues. This indicates a more general biological function for these thioredoxins in addition to a potential role as effector molecules in the self-incompatibility signal cascade.

INTRODUCTION

In the Brassica family, a self-incompatibility system is present that prevents self-fertilization (reviewed in Nasrallah et al., 1994a; Dickinson, 1995; Goring and Rothstein, 1996) and is controlled by the multiallelic *S* locus (Bateman, 1955). When the pollen parent shares the same *S* allele as the pistil on which the pollen has landed, the cells at the top of the pistil (stigma papillae) recognize this pollen and prevent it from germinating. Thus, only pollen originating from a plant having different *S* alleles can germinate and fertilize the ovule.

To date, two different genes that are tightly linked to the *S* locus appear to be required for the self-incompatibility reaction, the *S* locus glycoprotein (SLG) and the *S* receptor kinase (SRK). Loss of *SLG* gene expression (Toriyama et al., 1991; Nasrallah et al., 1992; Shiba et al., 1995) or mutations in the *SRK* gene (Goring et al., 1993; Nasrallah et al., 1994b) have been associated with self-compatibility. SLG is a secreted glycoprotein present throughout the cell wall of the stigma papilla cell (Kandasamy et al., 1989), whereas SRK is a membrane-bound glycosylated protein present in the stigma (Delorme et al., 1995; Stein et al., 1996). The putative extracellular domain of the SRK protein shows a high degree of sequence similarity to SLG (Stein et al., 1991; Goring and Rothstein, 1992; Glavin et al., 1994; Delorme et al., 1995), and the protein kinase domain has been shown to encode a functional serine/threonine kinase (Goring and Rothstein, 1992; Stein and Nasrallah, 1993).

Given these results, the simplest explanation for the self-incompatibility reaction is that there is a pollen component (ligand) present on the incompatible pollen that is responsible for activating the SRK, which in turn is thought to activate a signaling pathway in the stigma leading to the rejection of the incompatible pollen. Because classic genetic studies (Bateman, 1955) have shown that Brassica self-incompatibility is governed by a single genetic locus, this putative pollen ligand must also be tightly linked to the *S* locus. Although the pollen component has not been definitively identified, candidates are being investigated (Doughty et al., 1993; Boyes and Nasrallah, 1995; Hiscock et al., 1995). One role that has been proposed for the SLG is aiding the putative pollen ligand in crossing the cell wall and allowing it to come into contact with the extracellular domain of the membrane-bound SRK (Goring and Rothstein, 1992; Stein et al., 1996).

Very little is known about the molecular nature of the signal transduction pathway acting through the SRK that leads to an inhibition of pollen germination. Treatment of pistils with okadaic acid has led to a breakdown in Brassica self-incompatibility, suggesting that type 1 or type 2A phosphatases are involved in this pathway (Rundle et al., 1993; Scutt et al., 1993). In the Papaver self-incompatibility system, a small stigma S protein has been identified that can inhibit pollen tube growth from incompatible pollen (Foote et al., 1994). This inhibition of pollen

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tube growth in the self-incompatibility response by the S protein appears to involve calcium signaling (Franklin-Tong et al., 1993, 1995). We have examined whether calcium signaling is involved in the Brassica self-incompatibility system and found that calcium fluxes could be detected in the stigma papilla cell with both self-compatible and self-incompatible pollinations (J.D.W. Dearnaley, N.N. Levina, R.R. Lew, I.B. Heath, and D.R. Goring, submitted manuscript).

As a first step toward dissecting the Brassica self-incompatibility signaling pathway, we identified two proteins that interact with the SRK-910 protein kinase domain. The *SRK-910* gene was isolated from the functional *S* locus in the self-incompatible *Brassica napus* ssp *oleifera* line called W1 (Goring et al., 1992; Goring and Rothstein, 1992). Using the yeast two-hybrid system, we isolated two members of the thioredoxin-h family that interact specifically with the SRK-910 protein kinase domain.

RESULTS

Screening of the Brassica Pistil Two-Hybrid Library

For the yeast two-hybrid system, the region of the *SRK-910* cDNA encoding the protein kinase domain was cloned into a yeast vector to produce a protein fusion with the LexA DNA binding domain (LexAkinase^{910}), and a W1 pistil cDNA library was constructed in the pVP16 activation domain plasmid (Vojtek et al., 1993). The LexAkinase^{910} plasmid and VP16 pistil cDNA library were then transformed into the yeast L40 strain containing two reporter genes, *HIS3* and *lacZ* (Vojtek et al., 1993). Interactions between LexAkinase^{910} and VP16 pistil proteins were identified by histidine prototrophy and β-galactosidase activity (Vojtek et al., 1993). Secondary screening of positive clones involved testing for interactions in the presence of several different LexA fusions: (1) LexAkinase^{910}, used to screen the library; (2) LexAmukinase^{910}, with a lysine-to-alanine substitution at position 557 that abolished catalytic activity; (3) LexAkinase^{RLK4}, containing the protein kinase domain from the Arabidopsis receptor-like kinase (RLK) *RLK4* gene; (4) LexAkinase^{RLK5}, containing the protein kinase domain from the Arabidopsis *RLK5* gene; and (5) LexAlamin, used to detect nonspecific interactions.

An initial screening of the W1 pistil cDNA library resulted in the isolation of several THL (for thioredoxin-h–like)-1 clones. In subsequent screenings in which THL-1 clones were partially eliminated, several THL-2 clones were isolated. In both cases, the interaction as detected by growth in the absence of histidine and β-galactosidase activity was only detected in the presence of LexAkinase^{910} (Figures 1A and 1B). Cotransformation of LexAkinase^{910} with pVP16 alone resulted in very weak activation of the reporter genes (Figure 1C), but the activation levels were considerably lower than that seen with either VP16:THL-1 or VP16:THL-2. No interaction was detected between the mutated form of LexAkinase^{910} and either VP16:THL-1 or VP16:THL-2, suggesting that the interaction required a phosphorylated form of the SRK-910 protein kinase domain (Figures 1A and 1B). In addition, no interaction was detected with LexAlamin and the two Arabidopsis kinases (Figures 1A and 1B). The Arabidopsis RLK4 is related to the SRKs but is expressed primarily in root tissue and is likely to have a quite different role from the SRKs (Walker, 1993). RLK5 belongs to the leucine-rich repeat family of receptor-like kinases (Walker, 1993). Immunoblot analysis of the yeast extracts by using an anti-LexA antibody confirmed that the LexA fusion proteins were present in the transformed cells at levels at least equal to that seen for LexAkinase^{910} (W. Sulaman and D.R. Goring, unpublished results).

Sequence Analysis and Expression Patterns for THL-1 and THL-2

Sequence analysis of the THL-1 and THL-2 clones revealed that they are related to each other and belong to the thioredoxin family (Figures 2A and 2B). THL-1 and THL-2 share 61% amino acid identity, and their alignment is shown in Figure 2A. The highest levels of sequence identities were found in the Arabidopsis thioredoxin-h family, for which five members have been characterized (Rivera-Madrid et al., 1995). THL-1 is most similar to TRX3, and THL-2 is most similar to TRX4 (Figure 2B and Table 1). Thioredoxins from *Escherichia coli* to mammals have been characterized with a consensus active site of Cys-Gly-Pro-Cys, in which the flanking cysteines are involved in the reducing function (Figure 2B; Holmgren, 1989). Such an active site is present in two members of the Arabidopsis thioredoxin-h family, TRX1 and TRX2 (Rivera-Madrid et al., 1995). However, THL-1 and THL-2 show a variant active site consisting of Cys-Pro-Pro-Cys (Figure 2A), which is also found in the remaining three Arabidopsis members, TRX3, TRX4, and TRX5 (Rivera-Madrid et al., 1995). The internal two amino acids can influence the redox potential of a thioredoxin protein, with Cys-Gly-Pro-Cys being more reducing than Cys-Pro-Pro-Cys (Grauschopf et al., 1995).

To determine the expression patterns of THL-1 and THL-2, RNA gel blot analysis was performed with RNA samples from root, stem, leaf, petal, anther, and pistil (Figure 3). In both cases, transcripts of ~600 nucleotides were detected. THL-1 was highly expressed in all tissues tested (Figure 3). THL-2 mRNA could also be detected in all of these tissues; however, higher levels of THL-2 steady state mRNA were present in the floral tissues (Figure 3, lanes 4 to 10). Thus, both THL-1 and THL-2 are expressed in the pistil, but they are also expressed in a variety of other tissues and appear to be ubiquitous proteins. Given the sequence similarity between THL-1 and THL-2, further analysis was performed only with THL-1.

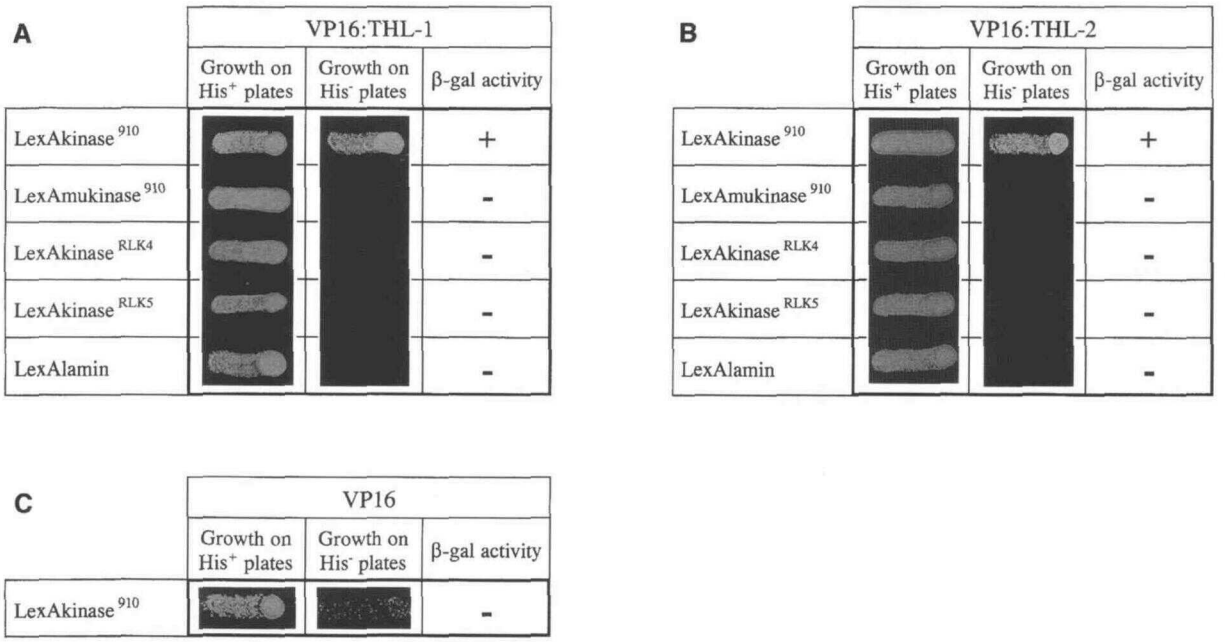

Figure 1. Interaction of the SRK-910 Kinase Domain with THL-1 and THL-2.

(A) and **(B)** THL-1 and THL-2, respectively, were tested for interactions with different LexAkinase constructs and LexAlamin. Interactions between proteins were assayed by growth on synthetic dextrose plates lacking histidine (activation of the *HIS3* reporter gene) and β-galactosidase (β-gal) activity (activation of the *lacZ* reporter gene). Only the combination of THL-1 or THL-2 with LexAkinase^{910} resulted in growth in the absence of histidine after 2 days. In addition, β-galactosidase activity could be detected within 30 min of incubating filter lifts with X-gal. (+) indicates the presence of β-galactosidase activity as detected by the formation of a blue color. (−) indicates the absence of β-galactosidase activity.
(C) Interaction of LexAkinase^{910} with the pVP16 vector (no insert). LexAkinase^{910} with VP16 alone has very weak reporter gene activation, with slight growth in the absence of histidine after 2 days. No β-galactosidase activity was detected after several hours of incubating filter lifts with X-gal. Very weak activity (faint blue color) was detected only after an overnight incubation.

The Reducing Activity of THL-1

We were interested in determining whether THL-1 has thioredoxin activity, especially given that its active site contained the sequence Cys-Pro-Pro-Cys. A THL-1 fusion protein containing six histidine residues and a T7 epitope tag at the N terminus (6-His • Tag/T7 • Tag:THL-1) was produced in *E. coli* and purified by affinity chromatography. Thioredoxin activity was measured by the ability of the THL-1 protein to reduce insulin in the presence of DTT (Holmgren, 1979). Insulin is composed of two chains (A and B) held together by two disulfide bridges. The reduction of insulin results in the separation of the two chains, and the insulin B chain forms a white precipitate detected at OD_{650}. The assay was performed by mixing bovine pancreas insulin and DTT either with THL-1 or in a control lacking THL-1; OD_{650} readings were taken at intervals of 2 to 3 min (Figure 4). In the presence of THL-1, insulin B chain precipitation occurred at a much faster rate than it did in the control, thereby showing that THL-1 has reducing activity.

Phosphorylation of THL-1

Phosphorylation of substrates has been demonstrated in many kinase–substrate interactions (reviewed in Hunter, 1995). To determine whether the interaction between the SRK-910 kinase domain and THL-1 results in phosphorylation of THL-1, two different 6-His • Tag/T7 • Tag:THL-1 proteins were produced. THL-1(C1) starts two amino acids after the initiation methionine, whereas THL-1(A31) has 10 amino acids added from the 5′ untranslated region (Figure 2A); thus, A31 produces a larger fusion protein than does C1. The SRK-910 protein kinase domain was produced as glutathione *S*-transferase (GST) kinase fusion in both the functional (GSTkinase^{910}) and mutant (GST-mukinase^{910}) forms (Goring and Rothstein, 1992). The GST fusions were then mixed with either THL-1(C1) or THL-1(A31) in a kinase assay containing γ-^{32}P-ATP (Goring and Rothstein, 1992). The proteins were separated by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography (Figure 5).

A

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     A31          C1
      ▽           ▽
THL-1 TKSKRREREKRMAATAEVIPAGEVIACHTVEDWNNKLKAAKESNKLIVIDFTAVWCPPCRFIAPIFVE  67
THL-2                MAAEE-Q--G--EIDV-AVQ-DT--Q----------S------M---V-AD  52
                          △

THL-1 LARKHL DVVFFKVDVDELATVAQEFDVQAMPTFVYMKGEEKLDKVVGAAKEEIEAKLLKHSQVAAA 133
THL-2 ----FMSSAI---------QN-----G-E------LI-DGNVV------R--DLH-TIA--TG--T- 119
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B

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THL-1  MAATAEVIPAGEVIACHTVEDWNNKLKAAKESNKLIVIDFTAVWCPPCRFIAPIFVELAKKHL    63
TRX3         MAAE------------TE-----N--K---------T-----------V-AD------    57
TRX1        MASEE-Q---------T--EQ-QK-N--KT-V-V----S--G--------F-AD----LP    58
S1      IVEDKLDFKG-N-HVIT-K---DQ-IAE-NKDG-IV-AN-S-S--G---V---VYA-MS-TYP   224
TXN              MVKQ-ESK-AFQEA  -D-- GD --V-V--S-T--G--KM-N-F-HS-SE-YS    50
TRXA             MSDK- I-L T-DSFDTDVL-AD GA-LV--W-E--G--KM----LD-I-DEYQ    51

THL-1   DVVFFKVDVDELATVAQEFDVQAMPTF VYMKGEEKLDKVVGAA KEEIEAKLLKHSQ VAAA  123
TRX3    ------------N---E--K------- IF--EG-IKET----- ----I-N-E--KTV----  118
TRX1    N-L-L---T---KS--SDWAI------ MFL-EGKI-------K -D-LQSTIA--LA       114
S1      QLM-LTI---D-VDFSSTW-IR-T--- FFL-NGQQI--L---N -P-L-K-VQALGD GS    282
TXN     N-I-LE----DCQD--S-CE-KCT--- QFF-KGQ-VGEFS--N --KL--TINELV        105
TRXA   GKLTVA-LNI-QNPGT-PKYGIRGI--LLLFKN--VASATK---LS-GQLKEF-DANLA       110
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Figure 2. Amino Acid Sequence Alignments of THL-1 and THL-2.

(A) The predicted amino acid sequences for the THL-1 and THL-2 cDNAs isolated from the two-hybrid library are shown. For THL-1, two different cDNAs were isolated: A31 and C1. THL-1(A31) has part of the 5′ untranslated region translated to produce a fusion with VP16, whereas THL-1(C1) is missing the first two amino acids (positions are marked by arrowheads). The initiation methionine for THL-1 is in boldface. For THL-2, all cDNAs isolated started at the same position and are missing the first few amino acids (marked by the arrowhead). THL-1 and THL-2 share 61% amino acid sequence identity. The nucleotide sequences have been submitted to GenBank as accession numbers U59379 for THL-1 and U59380 for THL-2. **(B)** The predicted amino acid sequence for THL-1 is aligned with two different thioredoxin-h clones from Arabidopsis (TRX1 and TRX3), the C-terminal thioredoxin domain in the *Phalaris coerulescens* S1 pollen gene product (S1), human thioredoxin (TXN), and *E. coli* thioredoxin (TRXA). The thioredoxin active sites, CPPC or CGPC, are underlined. Gaps are represented by spaces, and dashes denote conserved amino acids.

The autophosphorylated GSTkinase^{910} produces a 72-kD phosphoprotein (Figure 5, lanes 1 and 4). The bands between 29 and 53 kD (Figure 5, lanes 1 and 4) are GSTkinase^{910} degradation products and were also detected when the GSTkinase^{910} was incubated with control extracts lacking THL-1 (data not shown). No phosphorylation of THL-1 could be detected in the presence of the GSTmukinase^{910} (Figure 5, lanes 2 and 3). However, in the presence of the functional GSTkinase^{910}, a weakly phosphorylated band could be detected that comigrates with the THL-1(C1) protein (Figure 5, lane 1) or with the THL-1(A31) protein (Figure 5, lane 4). Thus, THL-1 is phosphorylated by the SRK-910 protein kinase domain, suggesting a direct interaction between the two proteins.

Coimmunoprecipitation of THL-1 and the SRK-910 Kinase Domain

To verify that a direct interaction is occurring between THL-1 and the SRK, binding of THL-1 to the SRK-910 kinase domain was studied in vitro. Maltose binding protein (MBP) kinase fusions were produced in *E. coli*, immunoprecipitated with an anti-MBP antibody, and then incubated with extracts containing the 6-His • Tag/T7 • Tag:THL-1 protein. After several washes, the proteins were separated by SDS-PAGE, and the 6-His • Tag/T7 • Tag:THL-1 protein was detected by immunoblot analysis using an anti-T7 • Tag antibody (Figure 6). We found that THL-1 is coimmunoprecipitated with the MBPkinase^{910} and MBPmukinase^{910} (Figure 6, lanes 3 and 4),

Table 1. Amino Acid Sequence Identity of THL-1 and THL-2 to the Arabidopsis Thioredoxin-h Family

Arabidopsis	THL-1 (%)	THL-2 (%)
TRX1	59	58
TRX2	44	46
TRX3	75	59
TRX4	63	85
TRX5	69	61

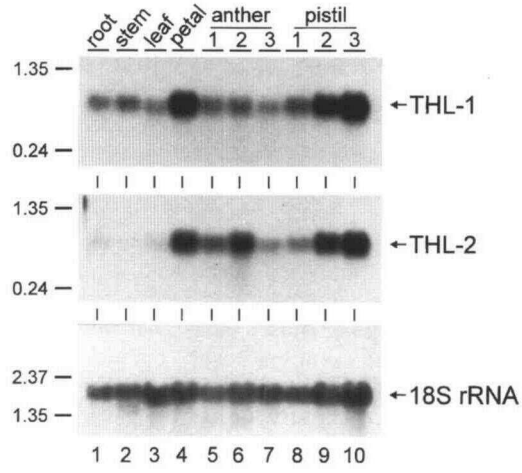

Figure 3. Distribution of THL-1 and THL-2 mRNAs.

THL-1, THL-2, and 18S rRNA probes were hybridized with total RNA extracted from various tissues from the self-incompatible *B. napus* ssp *oleifera* line W1. For the anther and pistil tissue (lanes 5 to 10), 1 to 3 indicate increasing bud sizes, where 1 is 1- to 2-mm buds (lanes 5 and 8), 2 is 3- to 4-mm buds (lanes 6 and 9), and 3 is 5- to 7-mm buds (lanes 7 and 10). The numbers at left indicate an RNA marker in kilobases (Gibco BRL). For both THL-1 and THL-2, a transcript of ~600 nucleotides was detected in all tissues tested. THL-2 transcripts in the root, stem, and leaf were detected after a longer exposure (data not shown). The 18S rRNA serves as a control to show that approximately equal amounts of total RNA were loaded in each lane.

but not with MBP alone (Figure 6, lane 2) or with the MBPkinase^{RLK5} (Figure 6, lane 5). No signal was present when the anti-T7 • Tag antibody was preincubated with the THL-1 protein, verifying that the THL-1 protein had been detected (data not shown). Although a specific interaction was detected with the SRK-910 protein kinase domain, THL-1 was able to bind both the functional and mutant forms of the SRK-910 kinase domain (Figure 6, lanes 3 and 4), suggesting that phosphorylation is not required for this interaction. This is in contrast to the interaction in the yeast two-hybrid system in which THL-1 only interacted with LexAkinase^{910} and not with LexAmukinase^{910} (Figure 1).

DISCUSSION

Little is known about the role of the SRK in the rejection of incompatible pollen during the Brassica self-incompatibility response. In animals, the receptor tyrosine kinase signaling pathway has been well studied, and several substrates have been found to bind directly to the tyrosine kinase domain (reviewed in Fantl et al., 1993; Marshall, 1995). Upon receptor activation that leads to autophosphorylation of the tyrosine kinase domain, substrates bind to the protein kinase domain through their SH2 domains in a phosphorylation-dependent manner (reviewed in Cohen et al., 1995). These signaling molecules then go on to regulate other substrates, resulting in a signal cascade. As a first step in characterizing the Brassica self-incompatibility signaling cascade, the yeast two-hybrid system was used to isolate proteins that interact with the SRK-910 kinase domain.

Two different thioredoxin-h members, THL-1 and THL-2, were found to interact specifically with the SRK-910 protein kinase domain. In the yeast system, these did not interact with the RLK4 protein kinase domain, the RLK5 protein kinase domain, lamin, or the mutant SRK-910 protein kinase domain. Further, under in vitro conditions, THL-1 is phosphorylated by the SRK-910 kinase domain and is able to bind to this protein. However, the in vitro THL-1 also binds to the mutant SRK-910 kinase domain. The discrepancy between the yeast two-hybrid data and the coimmunoprecipitation data may simply be due to the differences in the assays used. However, the mutation of the conserved lysine in the catalytic domain is thought not only to knock out kinase activity but also to cause a conformational change (Carrera et al., 1993). Thus, THL-1 binding may be influenced by the conformation of the kinase domain, and LexAmukinase^{910} may have a slightly different conformation

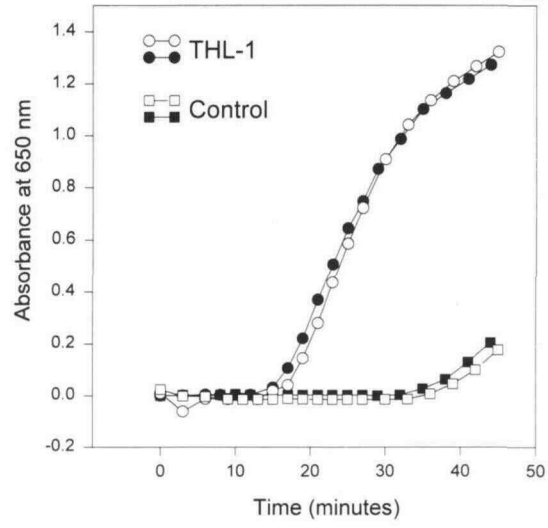

Figure 4. Reduction of Insulin by Recombinant THL-1.

Thioredoxin reducing activity was determined by the rate of insulin B chain precipitation (OD_{650}) resulting from the reduction of insulin. Bovine pancreatic insulin and DTT were added to extract containing THL-1 (circles) or to a control extract lacking the THL-1 protein (squares). The results from two separate experiments are shown. The reaction containing THL-1 reduces insulin at a much greater rate compared with the control. Activity is recorded in the control because DTT can also reduce insulin but at a much slower rate than thioredoxin.

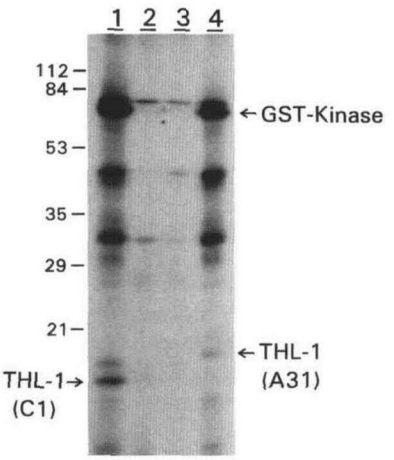

Figure 5. Phosphorylation of THL-1 by the SRK-910 Kinase.

Autoradiography of an SDS–polyacrylamide gel with two different THL-1 fusion proteins (A31 and C1) incubated with either the active SRK-910 kinase domain (GSTkinase^{910}) or the inactive form of the same kinase (GSTmukinase^{910}) in the presence of γ-^{32}P-ATP is shown. Lane 1 contains GSTkinase^{910} plus THL-1(C1); lane 2, GSTmukinase^{910} plus THL-1(C1); lane 3, GSTmukinase^{910} plus THL-1(C1) and THL-1(A31); and lane 4, GSTkinase^{910} plus THL-1(A31). The positions of the molecular mass markers in kilodaltons are indicated at left. The major phosphorylated form of THL-1 (lanes 1 and 4, marked by the arrows) comigrated with the Coomassie blue–stained THL-1. A weaker band can also be detected migrating just above the major band and can be seen for both THL-1(C1) (lane 1) and THL-1(A31) (at a longer exposure). This weaker band is most likely due to phosphorylation at multiple sites that results in an altered migration. The full-length autophosphorylated GSTkinase^{910} (GST-kinase) is marked by an arrow (lanes 1 and 4). The bands between 29 and 53 kD are degradation products from the GSTkinase^{910}.

to the MBPmukinase^{910}. In either case, the binding of THL-1 to the MBPmukinase^{910} suggests that THL-1 interactions may be influenced by conformation rather than phosphorylation. In this context, note that conformational changes are involved in receptor tyrosine kinase activation (Heldin, 1995). By analogy, structural changes in the SRK upon ligand binding might lead to the binding of the THL proteins to the kinase domain during the signal transduction process.

THL-1 and THL-2 are ubiquitously expressed and thus are likely to have a general role in the function of the plant outside of their potential role in Brassica self-incompatibility. The standard role for thioredoxin is to modulate enzyme activity by reducing disulfide bridges (reviewed in Holmgren, 1989). In plants, there are three types of thioredoxins: thioredoxin-f, thioredoxin-h, and thioredoxin-m. Thioredoxin-f and thioredoxin-m are found in the chloroplasts and play a role in regulating photosynthetic enzymes (reviewed in Buchanan, 1991). However, the thioredoxin-h family, to which THL-1 and THL-2 belong, is found in the endoplasmic reticulum, cytosol, and mitochondria (Buchanan, 1991). Two genes for thioredoxin-h have been cloned from tobacco (Brugidou et al., 1993), whereas five thioredoxin-h genes have been isolated from Arabidopsis (Rivera-Madrid et al., 1995). THL-1 and THL-2 are most similar to the Arabidopsis *TRX3* and *TRX4* genes, respectively. Interestingly, when Li et al. (1994) isolated three different self-incompatibility alleles for the pollen *S* gene in *Phalaris coerulescens*, a thioredoxin-h domain was found in the C-terminal region of the predicted amino acid sequence. This domain was found to have thioredoxin activity when produced in *E. coli* (Li et al., 1995). Thus, a protein with thioredoxin activity appears to represent the pollen component of the *P. coerulescens* self-incompatibility system.

In addition to regulating enzyme activity, thioredoxins have also been implicated in a wide variety of other roles. In mammals, thioredoxin converts the glucocorticoid receptor from a nonbinding to a steroid binding state (Grippo et al., 1985) and regulates the DNA binding activity of the NF-κB transcription factor (Matthews et al., 1992). Human thioredoxin has been found to be the same as the adult T-cell leukemia-derived factor, and its redox activity is required for the stimulation of cell proliferation (Gasdaska et al., 1994; Oblong et al., 1994; Powis et al., 1994). Human thioredoxin has also been implicated in interferon-γ–mediated growth arrest of HeLa cells (Deiss and Kimchi, 1991). In Chlamydomonas cells, reduced thioredoxin can regulate the binding of a protein complex to the 5′ untranslated region of the chloroplast *psbA* mRNA and, as a result,

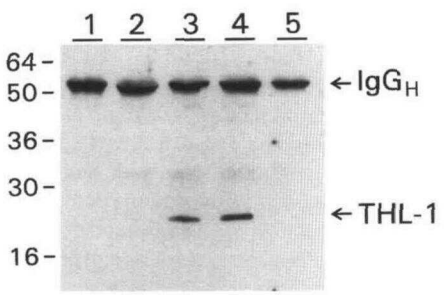

Figure 6. Coimmunoprecipitation of THL-1 with the SRK-910 Kinase Domain.

Immunoblot analysis of immunoprecipitated MBP fusion proteins mixed with 6-His • Tag/T7 • Tag:THL-1(A31) protein extracts. The THL-1 protein was detected using an anti–T7 • Tag antibody. Lane 1 contains cell extract with no MBP fusion; lane 2, MBP alone; lane 3, MBPkinase^{910}; lane 4, MBPmukinase^{910}; and lane 5, MBPkinase^{RLK5}. The positions of the molecular mass markers in kilodaltons are indicated at left. THL-1, as detected by the anti–T7 • Tag antibody, can only be detected in lanes 3 and 4; thus, THL-1 is only associated with the MBPkinase^{910} and MBPmukinase^{910}. The large band migrating at ~50 kD is a result of the cross-reactivity of the secondary antibody (peroxidase-labeled goat anti–mouse IgG antibody) to the IgG heavy chain (IgG$_H$) in the rabbit anti-MBP antibody used to immunoprecipitate the MBP fusion proteins.

confers light-regulated translation of this mRNA (Danon and Mayfield, 1994). For most of these examples, thioredoxin redox activity has been found to be important for its function. However, for bacteriophage T7, which uses *E. coli* thioredoxin as an essential subunit of the T7 DNA polymerase, the reducing activity of thioredoxin is not required for polymerase activity. Thioredoxin mutants with amino acid substitutions in the active site had no effect on the polymerase activity. However, these mutant thioredoxins do have a lower affinity for the polymerase, indicating that the active site region is involved in binding to the polymerase (Huber et al., 1986).

Given the diversity of roles that exist for thioredoxins, there are several possibilities for the nature of the interaction between the SRK-910 protein kinase domain and THL-1 or THL-2. During the self-incompatibility response, the THL proteins may regulate the SRK-910 protein kinase through their redox activity, leading to increased or decreased catalytic activity. However, if the THL proteins represent the next step in the SRK-910–mediated signaling cascade, the SRK-910 protein kinase may regulate thioredoxin activity, possibly through phosphorylation, and then the THL proteins may regulate the next step of the pathway through their reducing activity. Finally, as seen for T7 DNA polymerase, the redox activity of the THL proteins may not even be required, and they may be some type of adapter molecule for the next substrate. By looking at the interactions between THL-1 and the functional and mutated forms of the SRK-910 protein kinase domain, the nature of the interaction does not appear to be phosphorylation dependent. This may not be so surprising given that THL-1 and THL-2 do not contain other domains attached to the thioredoxin-h region for mediating phosphorylation-dependent binding. For another plant receptor kinase, Arabidopsis RLK5, Stone et al. (1994) have demonstrated that a type 2C protein phosphatase (KAPP) interacts directly with the RLK5 kinase domain in a phosphorylation-dependent manner. In this case, the interaction is mediated through the N-terminal KI domain of KAPP, whereas the C-terminal region encodes the type 2C protein phosphatase (Stone et al., 1994). Although we have demonstrated that the THL proteins interact with the SRK-910 protein kinase domain both in vitro and in the yeast system, evidence is still required for a role in the Brassica self-incompatibility system.

METHODS

Screening of the Yeast Two-Hybrid Library

The yeast two-hybrid vectors and library construction methods were as described by S. Hollenberg (Vojtek et al., 1993). The library was constructed in the pVP16 vector using polymerase chain reaction–amplified cDNA synthesized from poly(A)$^+$ pistil mRNA. The pistils were collected from the self-incompatible W1 line (Goring et al., 1992). The total size of the library transformed into *Escherichia coli* was ~6 × 10^6 colonies. The colonies were scraped off the plates, and plasmid DNA was then extracted for yeast transformations. The L40 yeast strain (Vojtek et al., 1993) was first transformed with the LexAkinase^{910} plasmid and then transformed with the VP16 pistil cDNA library by using the yeast transformation protocols described by Schiestl and Gietz (1989) and Gietz et al. (1992).

With each screen, ~10^6 to 7 × 10^6 yeast transformants were plated on synthetic dextrose (SD) minimal medium plates supplemented with 20 mg/L adenine sulfate (SD_{Ade}). On these plates, a few thousand colonies grew in the absence of histidine (activation of the *HIS3* reporter gene). β-Galactosidase assays were then performed on filter lifts of these colonies (Vojtek et al., 1993), which resulted in a few hundred clones positive for β-galactosidase activity (activation of the *lacZ* reporter gene). For ~100 positives, total yeast DNA was extracted and electroporated into *E. coli* HB101 cells to rescue the library plasmid (Cell-Porator Electroporation System with the Cell-Porator Voltage Booster; Gibco BRL). To verify positive interactions, the library plasmid was then retransformed in the yeast L40 strain in combination with LexAkinase^{910}, LexAmukinase^{910}, LexAkinase^{RLK4}, LexAkinase^{RLK5}, or LexAlamin and plated on SD plates supplemented with 20 mg/L adenine sulfate and 20 mg/L L-histidine HCl ($SD_{Ade,His}$). Transformants were then tested for growth on $SD_{Ade,His}$ plates and SD_{Ade} plates and for β-galactosidase activity. THL-1 clones were partially eliminated in some screens by either yeast colony hybridization or restriction enzyme mapping of rescued library plasmids.

Plasmid inserts were initially sequenced using primers flanking the polylinker in the pVP16 vector, then completely sequenced by subcloning regions of the cDNAs. Sequencing was performed using either manual sequencing with Sequenase Version 2.0, as recommended by the supplier (U.S. Biochemical), or automated sequencing with ABI Prism Dye Terminator Cycle Sequencing (Perkin-Elmer) and the Applied Biosystems 373 DNA Sequencer (Molecular Biology Core Facility, York University, North York, Canada). DNA sequences were analyzed using DNASIS and PROSIS software (Hitachi Software Engineering, Inc., San Bruno, CA) or by GenBank data base searches.

RNA Isolation and Gel Blot Analysis

Total RNA from vegetative tissues and floral tissues from the W1 line was extracted according to the procedure of Jones et al. (1985). Approximately 20 μg of RNA for each sample was denatured by heating in 50% formamide and then subjected to electrophoresis through 1.2% agarose gel containing 2.2 M formaldehyde. The fractionated RNA was transferred to the Zeta probe GT membranes (Bio-Rad), according to Sambrook et al. (1989). The THL-1 and THL-2 cDNA fragments were labeled with α-^{32}P-dATP, according to Feinberg and Vogelstein (1983), and used to probe the RNA blots. A 1.5-kb EcoRI fragment from a *Brassica rapa* cDNA encoding the 18S rRNA (Da Rocha and Bertrand, 1995) was used as a control probe. Hybridization was performed at 42°C in 50% formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M sodium phosphate, and 0.001 M EDTA), 0.5% SDS, and 10% dextran sulfate. The hybridized filters were washed twice in 0.1 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 65°C for 30 min.

Insulin Reduction and Protein Phosphorylation Assays

Two different THL-1 cDNAs (C1 and A31) were cloned into the pTrcHis vector to produce 6-His • Tag/T7 • Tag:THL-1 proteins (Invitrogen, San Diego, CA). THL-1(C1) starts two amino acids after the initiation methionine, whereas THL-1(A31) has 10 amino acids added from the 5′

untranslated region. Purification was performed using the Ni-NTA agarose, according to the manufacturer's instructions (Qiagen, Chatsworth, CA). To control for the potential effects of contaminating proteins, the same purification was performed with *E. coli* extracts lacking THL-1 (control extract). Approximately 30% (A31) to 50% (C1) of the THL-1 extracts were composed of the THL-1 protein. The insulin reduction assay was performed as described in Holmgren (1979), using the control and the THL-1(C1) extracts.

For the phosphorylation assay, the THL-1(A31) and THL-1(C1) proteins were transferred from the extraction buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 50 mM imidazole, 10% glycerol) to the kinase buffer (20 mM Pipes, pH 7.0, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 20 μg/mL aprotinin, 10% glycerol) by using Centricon-10 microconcentrators, according to the manufacturer's instructions (Amicon, Oakville, Canada), and stored at −80°C until use. The GSTkinase fusion proteins were purified as previously described (Goring and Rothstein, 1992). Fifty microliters of glutathione agarose beads with bound GSTkinase proteins was resuspended in 50 μL of kinase buffer containing 20 μCi of γ-^{32}P-ATP and 2.5 μg of the THL-1 extracts. The reactions were incubated at room temperature with gentle agitation for 30 min. Upon completion, 25 μL of 5× sample buffer was added, and the proteins were separated on a 13.5% SDS–polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R 250, dried, and exposed to x-ray film to visualize the phosphorylated proteins.

Coimmunoprecipitation

For the production of maltose binding protein (MBP) fusion proteins, the kinase domains were cloned into the pMAL vectors (New England Biolabs, Mississauga, Canada). Overnight, cultures were diluted 1:25, grown to an OD_{600} of 0.5, and then induced for 2 hr with 1 mM isopropyl-β-D-thiogalactopyranoside. For the 6-His • Tag/T7 • Tag:THL-1(A31) fusion protein, the overnight culture was diluted 1:100, grown to an OD_{600} of 0.2, and then induced for 4 hr with 1 mM isopropyl-β-D-thiogalactopyranoside. All cells were spun down at 6000*g* for 10 min, and the pellets were resuspended in 1.4 mL of PLC lysis buffer (Reith et al., 1991) per 35 mL of culture and frozen overnight. The next day, the samples were thawed, sonicated, and spun at 11,000*g* for 15 min. One microliter of rabbit preimmune serum and 100 μL of 10% (v/v) protein A–Sepharose (Pharmacia) were added to each supernatant, mixed for 2 hr at 4°C, and spun at 11,000*g*. The THL-1 supernatant was stored on ice until use.

To the MBP supernatants, 5 μL of rabbit anti-MBP antibody (New England Biolabs, Mississauga, Canada) was added and mixed for 1 hr at 4°C, and 100 μL of a 10% protein A–Sepharose was then added and mixed for 1 hr at 4°C, followed by two washes with PLC lysis buffer and two washes with kinase buffer (20 mM Pipes, pH 7.0, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 20 μg/mL aprotinin). The immunoprecipitated proteins were resuspended in 25 μL of kinase buffer containing 40 μM ATP, incubated for 15 min at room temperature, followed by one wash with the PLC lysis buffer, and resuspended in 400 μL of PLC lysis buffer. To each sample, 400 μL of the THL-1 extract was added, mixed for 2 hr at 4°C, and followed by three washes with HNTG buffer (Reith et al., 1991). The immunoprecipitated proteins were then resuspended in SDS-PAGE sample buffer, separated on a 13.5% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. The THL-1 protein was detected by immunoblotting using a primary antibody of mouse T7 • Tag antibody (Novagen), a secondary antibody of peroxidase labeled goat anti–mouse IgG antibody, and the LumiGLO Chemiluminescent substrate kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

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