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Antisense suppression of thioredoxin *h* mRNA in *Brassica napus cv.* Westar pistils causes a low level constitutive pollen rejection response

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Abstract

In *Brassica*, the thioredoxin *h* proteins, THL1 and THL2, were previously found to be potential inhibitors of the *S* receptor kinase (SRK) in the *Brassica* self-incompatibilty response. To investigate the biological roles of THL1 and THL2 in pollen–pistil interactions, the stigma-specific *SLR1* promoter was used to drive antisense *THL1/2* expression in *Brassica napus* cv. Westar. This cultivar is normally compatible, but antisense suppression of *THL1/2* led to a low level constitutive rejection of all *Brassica napus* pollen tested. Fluorescence microscopy revealed that the pollen rejection was a typical *Brassica* self-incompatibility rejection response with reduced pollen adhesion, germination and pollen tube growth. In addition, Westar was found to express the SLG_{15} and SRK_{15} proteins which may be the target of regulation by THL1 and THL2. Thus, these results indicate that the THL1 and THL2 are required for full pollen acceptance in *B. napus* cv. Westar.

Abbreviations: ARC1, arm repeat containing-1; KAPP, kinase associated protein phosphatase; MLPK, *M* locus protein kinase; SCR, *S* locus cysteine-rich protein; SLG, *S* locus glycoprotein; SLR, *S* locus related; SP11, *S* locus protein 11; SRK, *S* receptor kinase; THL, thioredoxin-like

Introduction

In *Brassica*, the initial stages of pollination involve cell–cell interactions between pollen and stigmatic papillae at the surface of the pistil which can then lead to the acceptance or rejection of the pollen. Typically, when a compatible pollen grain lands on the stigmatic surface, the surrounding pollen coat flows to the area of contact between the pollen grain and the stigmatic papilla to facilitate pollen adhesion, hydration, and germination. The outer surface of the pollen plays a critical role in this initial adhesion event as well as the subsequent expansion of the stigmatic papilla cell wall in the contact region (reviewed in Dickinson, 1995; Wheeler *et al.*, 2001). On the stigmatic side, the *S* locus-related (SLR)-1 protein has also been found to be required for pollen adhesion (Luu *et al.*, 1999). Following adhesion and germination, the emerging pollen tube penetrates the expanded cell

wall of the stigmatic papilla and grows down to the transmitting tissue in the pistil. The male gametes are carried within the growing pollen tube and are released at the ovule for fertilization (reviewed in Dickinson, 1995; Wheeler *et al*., 2001).

Brassica also has a self-incompatibility system which permits rejection of 'self' pollen and, hence, avoidance of inbreeding (reviewed in Hiscock and McInnis, 2003). Following a self-incompatible pollination, the rejection response is very rapid, occurring shortly after the pollen grain has landed on the stigmatic surface. The fertilization process is typically blocked at the adhesion, hydration or germination stage. Any pollen tubes that are formed are usually unable to penetrate the stigmatic surface and remain curled on the surface. This system is regulated by three tightly-linked polymorphic loci collectively referred to as *S* haplotypes and encoding the *S* locus glycoprotein (SLG), the *S* receptor kinase (SRK), and the *S* locus protein 11 or *S* locus cysteine-rich protein (SP11/SCR) (reviewed in Hiscock and McInnis, 2003). Both SLG and SRK are localized to the stigma, and while SRK is essential for this response, SLG may only have an enhancing effect for some *S* haplotypes (Kandasamy *et al*., 1989; Delorme *et al*., 1995; Suzuki *et al*., 2000, 2003; Takasaki *et al*., 2000; Silva *et al*., 2001). SP11/SCR determines the *S* haplotype specificity in the pollen and represents the ligand for SRK (Schopfer *et al*., 1999; Takayama *et al*., 2000; Shiba *et al*., 2001). An *S* haplotype-specific interaction between SCR/SP11 and SRK has been demonstrated leading to SRK phosphorylation (Cabrillac *et al*., 2001; Kachroo *et al*., 2001; Takayama *et al*., 2001). SRK is then predicted to activate a signalling pathway in the stigma causing pollen rejection. SRK may work in conjunction with a second kinase, the *M* locus protein kinase (MLPK), which was recently identified as an essential component of the *Brassica* self-incompatibility system (Murase *et al*., 2004).

Screens for proteins which bind to the SRK kinase domain have identified the thioredoxin *h* proteins, THL1 and THL2; the kinase associated protein phosphatase, KAPP; an E3 ubiquitin ligase, ARC1; a calmodulin; and a sorting nexin (Bower *et al*., 1996; Braun *et al*., 1997; Gu *et al*., 1998; Vanoosthuyse *et al*., 2003). Of these various interactors, ARC1 appears to be the only known downstream component of the SRK signalling pathway (Gu *et al*., 1998; Stone *et al*., 1999). ARC1 is proposed to promote the ubiquitination and degradation of stigmatic proteins following a self-incompatible pollination and SRK activation (Stone *et al*., 2003). In contrast, THL1/2 does not appear to be part of the SRK activated signalling pathway as this interaction does not require SRK kinase activity (Bower *et al*., 1996). However, thioredoxin was identified as a soluble inhibitor in stigmatic extracts repressing SRK kinase activity *in vitro* (Cabrillac *et al*., 2001). The addition of *S* haplotype-specific pollen coat proteins overcame this inhibitory effect and resulted in increased SRK phosphorylation (Cabrillac *et al*., 2001). Thus, thioredoxins were proposed to negatively regulate SRK in the absence of self-incompatible pollen, while the SP11/SCR ligand activates SRK following a self-incompatible pollination.

In this study, we have investigated the biological functions of THL1 and THL2 during pollen–pistil interactions *in vivo*. Transgenic *Brassica napus* cv. Westar plants were generated harbouring antisense *THL1* or *THL2* transgenes under the control of the stigma-specific SLR1 promoter. Transgenic plants with reduced levels of *THL1* and *THL2* mRNA in the stigma showed reduced seed production following pollination with 'compatible' pollen with a rejection response very similar to self-incompatibility. These results indicate that THL1 and THL2 are required for successful compatible pollinations in *B. napus* cv. Westar.

Materials and methods

Plant material and the generation of transgenic Westar plants

The *Brassica napus* W1 and Westar lines have been described in Goring *et al*. (1992, 1993) and the *Brassica oleracea* S_3 and P57Sc lines have been described in Delorme *et al*. (1995) and Cabrillac *et al*. (1999). Various *Brassica napus* cultivars (Tribute, Drakkar, Tanto, Global, Bronowski, Chine 32 and Yudal) were obtained from INRA Le Rheu (France).

The *THL1* and *THL2* cDNAs were respectively cloned in antisense orientation under the control of the *SLR1* promoter, that directs high-level of expression in the stigma (Franklin *et al*., 1996).

For transformation, the *SLR1*:antisense *THL1* and *SLR1*:antisense *THL2* constructs were cloned into the *Hind*III and *Sac*I sites of pCAMBIA2301 (CAMBIA, Australia), and then individually transformed into Westar using *Agrobacterium*-mediated transformation. Regenerating shoots of transgenic Westar clones were selected on kanamycin (50 μg/ml) and tested for the presence of the β-glucuronidase (GUS) reporter gene by performing GUS staining using X-GLUC (BioVectra™). The resulting antisense *THL1* transgenic plants (denoted AT1) and antisense *THL2* transgenic plants (denoted AT2) were selfed to produce the T2 generation for further analysis.

RNA isolation and RNA blot analysis

Total RNA was isolated from stigmas following the procedure described in Cock *et al*. (1997). Ten micrograms of total RNA was fractionated on formaldehyde-agarose gels and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). Hybridization was conducted overnight at 68 °C using single-stranded riboprobes in a hybridization buffer (Ambion). Filters were washed extensively, with a final wash at 68 °C in 0.1% SDS and 0.1 × SSC. Hybridization signals were detected by autoradiography for 7 days at −80 °C using X-OMAT AR (Kodak Scientific Imaging Film) film with an intensifying screen (NEF-490).

Full-length riboprobes with the entire *THL1* or *THL2* coding regions were cloned into pBluescript KS(+) (Stratagene) in the antisense orientation under the control of T7 promoter. Single-stranded antisense THL1 or THL2 transcripts were synthesized *in vitro* and labelled with [α-^{32}P]dUTP using the MAXIscript™ kit (Ambion), as recommended by the manufacturer. Labelled riboprobes were then separated from unincorporated nucleotides using MicroSpin G-25 columns (Amersham Pharmacia Biotech).

RT-PCR, cloning and analysis of RT-PCR products

In order to reconstruct the SRK_{15} cDNA, RT-PCR was performed using primers to generate overlapping fragments. Two micrograms of total RNA extracted from stigmas of Westar cultivar, were treated with one unit of *DNase* I (Amplification Grade, GIBCO BRL) at room temperature for 15 min. The *DNase* I was then inactivated by heating at 65 °C for 10 min in 2 mM EDTA. RT-PCR was performed using the first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) using the *Not*I-d$(T)_{18}$ adaptor primer supplied. Five μl of the first-strand cDNA synthesis reaction was added to 20 μl of amplification cocktail [10 mM Tris–HCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 1% DMSO, 0.2 mM each dATP, dGTP, dCTP and dTTP], and 1.25 U of *Tsg* DNA polymerase (BioBasics) in a 25 μl volume for PCR amplification. The first-strand cDNA was used to amplify specific DNA fragments using the following pairs of oligonucleotides: PS3 and SG17 (5′-TGTTCCGTCTCTCAAGTCCCACTGCTGCGG-3′); SG71 (5′-GTGATAGCAGAGCTTCTTCCCAACG-3′) and SK30 (5′-TTCTCGCCCTCATAAACACAACAG-3′); SK32 (5′-ATGCAACACCTATTGTGGGAAATCAAGTTC-3′) and SK37 (5′-CGACCGAAGGGTTTCGCACGCATTGTCACTCA-3′) (Nishio *et al*., 1996; Cabrillac *et al*., 1999). These primers were added to a final concentration of 200 nM. PCR conditions were 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C, with a final extension for 10 min at 72 °C. The RT-PCR products obtained from Westar and W1 were purified on a QIAquick column (Qiagen), and cloned into pTOPO (Invitrogen). Two RT-PCR clones were sequenced (at the Core Molecular Facility, York University, Toronto) and the deduced sequences were aligned with the previously reported SRK_{15} sequence (Cabrillac *et al*., 1999).

Brassica pollination assay

One day before anthesis, large flower buds were emasculated and covered with pollination bags. Tagged pistils were hand-pollinated 1 day later, covered for 5–7 days for seed production, and then left uncovered to maturity. For fluorescence microscopy, 24 h after pollination 8–12 whole pistils were cut at the peduncle, fixed in 3:1 (v/v) ethanol:glacial acetic solution for 15 min. The pistils were then softened in 1 N NaOH for 1 h at 60 °C, washed three times with water, and stained with 0.1% (w/v) decolorized aniline blue in 0.1 M K3PO4 (pH 7) buffer for 1 h at 60 °C. Whole pistils were gently squashed onto a slide glass by placing the cover glass on the pistils. Mounted samples were examined under a fluorescence microscope (Zeiss).

For treatment with 6-aminonicotinamide (6-AN), *Brassica napus* Westar flower buds were collected one day before anthesis and incubated overnight with their pedicels submerged in 6-AN (100 μM and 200 μM diluted in water from a 1 M 6-AN stock in DMSO; Sigma–Aldrich). For the control treatment, the same amount of DMSO was added. The pistils were then pollinated with untreated pollen, left for 3 h, and analyzed as described above.

Protein analysis of SLG_{15} and SRK_{15}

The immunoprecipitation and immunoblots used one of three different monoclonal antibodies: Mab 85-36-71 (Gaude *et al.*, 1993), Mab 64-32-30 (Delorme *et al.*, 1995), or Mab 157-35-50 (Giranton *et al.*, 1995). All three monoclonal antibodies were raised against a peptide corresponding to 10 amino acids at the N-terminus of SRK_3; and strongly recognize SRK_3, as well as the SRK proteins from the Class II *S* haplotypes, S_2, and S_{15}, and the SLG proteins from S_2, S_5 and S_{15} (Cabrillac *et al.*, 1999). For the analysis of SLG proteins, the protein extractions, isoelectric focusing, and immunoblotting with Mab 157-35-50 was done as previously described (Giranton *et al.*, 1995; Cabrillac *et al.*, 1999).

For the analysis of SRK_{15}, 10 stigmas of unpollinated freshly opened flowers from *B. oleracea* P57 Sc S_{15} and S_3, and five or 10 stigmas from *B. napus* Westar were collected (Gaude *et al.*, 1993; Delorme *et al.*, 1995). Total stigma proteins were extracted with SDG buffer (65 mM Tris–HCl pH 6.5, SDS 2.5% (w/v), DTT 2% (w/v), Glycerol 10% (w/v)), boiled 5 min at 100 °C and centrifuged at 18000 × *g* for 20 min at 4 °C. The soluble proteins were immunoprecipitated in 1 ml of TBS–Tween buffer (20 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% (w/v) Tween-20, 10 mM EDTA, one tablet of protease inhibitor cocktail (Boehringer) per 10 ml and phosphatase inhibitors (5 mM β-glycerophosphate, 10 mM NaF) with 40 μl protein G Agarose (Santa Cruz) and a 1/1000 dilution of Mab 64-32-30 for 1 h at room temperature (Delorme *et al.*, 1995; Cabrillac *et al.*, 2001). After several washes, the proteins were separated through a 7.5% SDS–PAGE gel, electroblotted and immunodetected with a 1/1000 dilution of Mab 85-36-71 (Gaude *et al.*, 1993; Cabrillac *et al.*, 2001).

Results

Antisense suppression of endogenous THL1 *and* THL2 *mRNA in the stigmas of Brassica napus cv. Westar*

In order to study the function of THL1 and THL2 in the stigma during pollen–pistil interactions, antisense *THL1* or *THL2* transgenic plants were generated in *Brassica napus* cv. Westar. The *THL1* and *THL2* cDNAs were placed in the antisense orientation under the control of the strong stigma-specific SLR1 promoter (Franklin *et al.*, 1996). This promoter has been successfully used to express or suppress other *Brassica* self-incompatibility genes with the pollination phenotype being altered to cause either a breakdown in self-incompatibility leading to increased pollen adhesion and seed production, or conferring pollen rejection with decreased pollen adhesion and seed production (Stahl *et al.*, 1998; Stone *et al.*, 1999; Silva *et al.*, 2001). *THL2* is a floral specific thioredoxin *h* gene and is highly expressed in the pistil while *THL1* is more ubiquitously expressed, but at lower levels in the pistil relative to *THL2* (Bower *et al.*, 1996). Using the *SLR1* promoter, RNA suppression of *THL1* and *THL2* only occurs in the stigma, the site of pollen–pistil interactions. The Westar cultivar is normally fully compatible following both self- and cross-pollination, and was initially tested due to ease of transformation. Since pollen rejection and reduced seed production was detected in the primary transformants (see below), these lines were then fully characterized.

Primary Westar transformants carrying either the antisense *THL1* (AT1) or the antisense *THL2* (AT2) constructs were generated and self-pollinated to produce subsequent generations for further analysis. Three independent transgenic lines, obtained from the self-pollination of three different primary transformants, were analyzed in detail: one antisense *THL1* transgenic line (AT1a) and two antisense *THL2* (AT2a and AT2b) lines. DNA blot analyses revealed that these transformants each carry a single copy of either the antisense *THL1* or *THL2* transgene (data not shown).

To examine the antisense suppression of endogenous *THL1* and *THL2* mRNA, mature stigmas were collected for RNA extraction from two transgenic sibling plants in the antisense *THL1* line (AT1a-1, 2), and from three transgenic sibling

plants in each of the two independent antisense *THL2* lines (AT2a-1,2,3 and AT2b-1,2,3). Total RNA was hybridized with ^{32}P-labeled *THL1* or *THL2* riboprobes which specifically detect endogenous *THL1* or *THL2* mRNAs. RNA blot analyses revealed that either construct resulted in the suppression of both endogenous *THL1* and *THL2* mRNA (Figure 1). The *THL1* and *THL2* cDNAs are both approximately 550 bp in length and share 60% sequence identity. However, there is an internal region of 200 bp with approximately 77% sequence identity which may be responsible for the antisense suppression of both transcripts. Thus, as compared to the Westar control, there was a large decrease in both the endogenous levels of *THL1* and *THL2* mRNA in all three transgenic lines (Figure 1).

Seed production in antisense THL1 *and* THL2 *Transgenic Westar Lines*

To examine seed production, pistils from the three transgenic Westar lines were pollinated with self-pollen, or pollen from untransformed Westar, *Brassica napus cv*. W1 or *Brassica napus cv*. Topas. The W1 line is a self-incompatible line, generated in the Westar background that rejects self-pollen, but is fully compatible with Westar in reciprocal crosses (Goring *et al*., 1992). Topas is a fully compatible line that accepts both self- and cross-pollen. Control pollinations were also carried out on sibling plants that did not carry the transgene (non-transgenic or NT). The Westar and Topas control pollinations and non-transgenic siblings typically showed full seed production following self-pollination as well as in crosses with the other pollen donors (Figure 2a–d). Control W1 pollinations following self-pollination or with pollen from another W1 plant resulted in no seed production as expected for this self-incompatible line. Compatible pollinations of W1 with Westar and Topas pollen result in good seed production (Figure 2a–d).

When seed production was examined in the transgenic AT1a, AT2a, and AT2b Westar lines, reduced seed production was detected following all pollinations tested. Following self-pollination, the

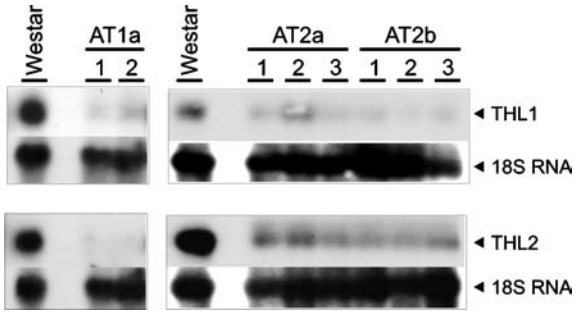

Figure 1. RNA blot analysis of endogenous THL1 and THL2 mRNA levels in the antisense THL1 and THL2 transgenic Westar plants. Ten micrograms of total stigmatic RNA extracted from Westar and the three transgenic lines were loaded in each lane. Mature stigmas were collected just prior to anthesis from two transgenic sibling plants in the antisense THL1 line (AT1a-1, 2), and from three transgenic sibling plants in each of the two independent antisense THL2 lines (AT2a-1, 2, 3 and AT2b-1, 2, 3). The RNA blots were initially hybridized to THL1 and THL2 riboprobes, respectively; and then hybridized to the 18S rRNA probe as a control for the even loading. The probes used are shown to the right of the panels.

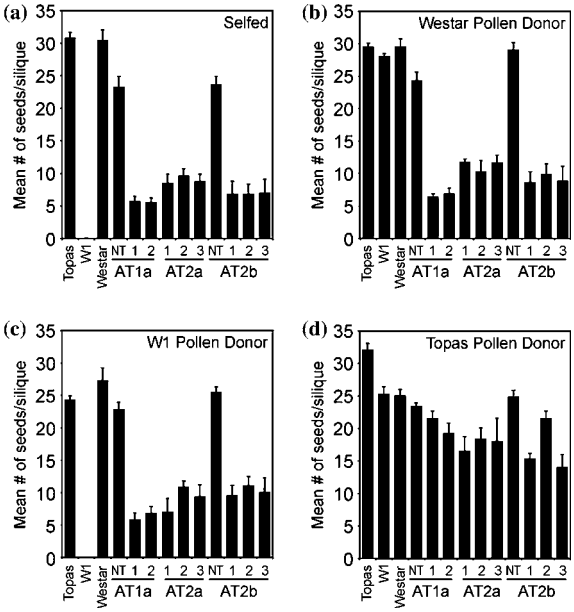

Figure 2. Seed production analysis in the antisense THL1 and THL2 transgenic Westar plants. The average number of seeds per silique was measured from 6 to 12 seed siliques per plant with the exception of two plants where 4–5 siliques were used. Plants tested include two transgenic sibling plants in the antisense THL1 line (AT1a-1, 2), three transgenic sibling plants in each of the two independent antisense THL2 lines (AT2a-1, 2, 3 and AT2b-1, 2, 3), and two non-transgenic siblings (AT1a-NT and AT2b-NT). Topas and Westar are control compatible lines, and W1 is a control self-incompatible line. Error bars designate ± Standard error (SE). (a) Self-pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 lines. (b) Pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 pistils with Westar pollen. (c) Pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 pistils with W1 pollen. (d) Pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 pistils with Topas pollen.

transgenic AT1 and AT2 lines showed a significant reduction in seed production compared to the compatible controls, Westar, Topas, and the non-transgenic siblings, with a 60%–80% reduction (Figure 2a). However, the level of seed set was not as low as seen following the self-incompatible pollination in W1, the latter resulting in essentially no seeds being produced. Reciprocal crosses with untransformed Westar indicated that the defect was not due to male sterility in the transgenic lines (data not shown). Thus, there was a partial reduction in seed production associated with the antisense suppression of THL1 and THL2 mRNAs in the stigmas.

A similar trend was observed following Westar and W1 pollinations on the transgenic AT1 and AT2 pistils with again a 60%–80% reduction in seed production compared to the compatible controls (Figure 2b, c). Therefore, the transgenic AT1 and AT2 Westar lines were displaying a more generalized reduced seed production in comparison to the *S* haplotype-specific W1 control. Statistical analysis of this data indicated that the reduced seed production in the *THL1*- or *THL2*-antisense transgenic Westar lines was statistically significant in comparison to the non-transgenic siblings, Westar and W1 compatible pollinations. Since both Westar and W1 are highly related cultivars, we also tested another canola cultivar, Topas, as a pollen donor to determine the extent of the reduced seed production. There was some decrease in seed production observed though not as dramatic as with the other crosses (Figure 2d).

Pollen adhesion, germination, and pollen tube growth in antisense THL1 *and* THL2 *Westar lines*

The seed production studies suggested that the antisense *THL1* and *THL2* Westar plants were producing less seed following various pollinations and that the transgenic effect was only present on the female side. In order to determine where the block was occurring leading to reduced seed production, the pollinations were examined more closely for pollen adhesion, germination and pollen tube growth. This assay is also more sensitive in the detection of any potential pollen rejection response as the assay examines a large number of pollen grains placed on the stigmatic surface. In comparison, seed production assays only requires a small subset of these pollen grains to germinate, form pollen tubes, and fertilize the ovules.

The transgenic AT1a, AT2a, and AT2b Westar lines were self-pollinated and crossed with pollen from W1, Westar, and Topas. The pistils were then stained with aniline blue for the detection of callose and examined by fluorescence microscopy. W1, Westar and Topas pistils were selfed and crossed to compatible and incompatible pollen donors and used as controls for all pollinations. Following compatible pollinations, Westar, Topas and W1 pistils typically have between 150 and 250 pollen grains adhered to the stigmatic papillae (Figure 3) and these pollen grains have formed pollen tubes which are growing through the transmitting tissue of the pistil (Figure 4a).

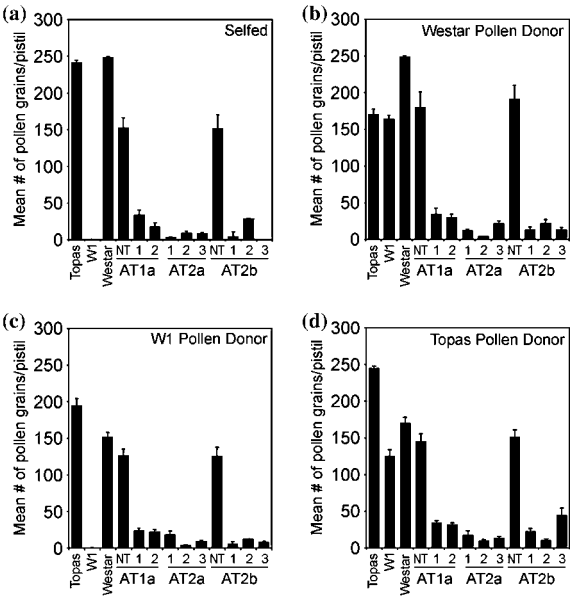

Figure 3. Pollen grain adhesion in the antisense THL1 and THL2 transgenic Westar plants. The average number of pollen grains per pistil was measured from 7 to 16 pistils per plant. Plants tested include two transgenic sibling plants in the antisense THL1 line (AT1a-1, 2), three transgenic sibling plants in each of the two independent antisense THL2 lines (AT2a-1, 2, 3 and AT2b-1, 2, 3), and two non-transgenic siblings (AT1a-NT and AT2b-NT). Topas and Westar are control compatible lines, and W1 is a control self-incompatible line. Error bars designate ± standard error (SE). (a) Self-pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 lines. (b) Pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 pistils with Westar pollen. (c) Pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 pistils with W1 pollen. (d) Pollinations of Westar, W1, Topas, and the transgenic AT1 and AT2 pistils with Topas pollen.

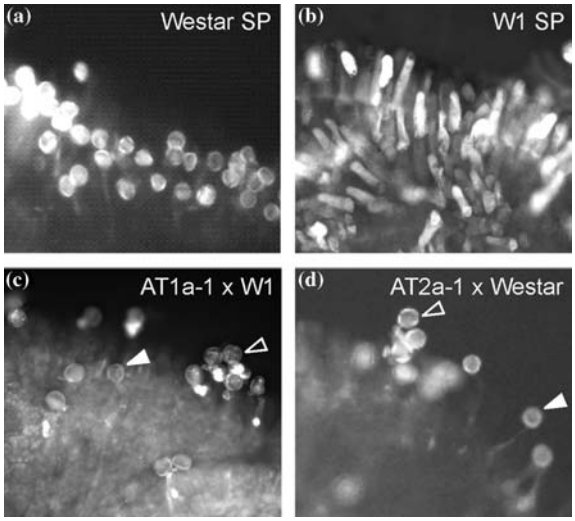

Figure 4. Aniline blue staining and fluorescence microscopy of pollinated pistils, 24 h after pollination. (a) Self-pollination of a Westar pistil. In this compatible pollination, there are large numbers of pollen grains adhered on the surface which have produced pollen tubes growing through the pistil. (b) Self-pollination of a W1 pistil. In this incompatible pollination, the rejected pollen grains have been washed off during staining, and the stigmatic papillae show fluorescence due to callose deposition. (c) Pollination of an AT1a-1 pistil with W1 pollen. (d) Pollination of an AT2a-1 pistil with Westar pollen. For (c) and (d), most of the pollen grains have been rejected and washed off, very few pollen grains have produced pollen tubes. Closed arrowheads indicate growing pollen tubes that have successfully penetrate the stigmatic surface. Open arrowheads indicate pollen grains with curled pollen tubes.

However, self-incompatible pollinations with W1 pistils typically have few pollen grains on the surface (Figures 3a and c and 4b). The pollen grains do not adhere tightly to the surface as a result of the self-incompatibility rejection response and wash off during the aniline blue staining.

All three transgenic AT1a, AT2a, and AT2b Westar lines were found to have decreased numbers of pollen grains on the pistil surface following all pollinations tested (Figures 3 and 4 c and d). Westar, Topas, and W1 pollen, which would normally be compatible on Westar pistils, were now being rejected. Another sign of a typical *Brassica* self-incompatibility response was the presence of pollen grains which adhered to stigma, germinated to produce pollen tubes, but whose pollen tubes were unable to penetrate the stigmatic surface and become curled on the stigmatic surface. This phenotype was also seen on the transgenic AT1a, AT2a, and AT2b pistils following Westar, W1 and Topas pollinations (Figure 4c and d). The number of pollen grains present on the surface was higher than with the self-incompatible line, W1, suggesting that the pollen was not rejected as strongly in the transgenic AT1a, AT2a, and AT2b Westar. In addition, some pollen tubes could be seen growing down the transmitting tissue which would explain why some seed production was detected (Figures 2 and 4c and d). In the case of Topas, the higher level of seed production observed in Figure 2 suggests that more pollen grains successfully formed pollen tubes which were then able to penetrate the stigmatic barrier to effect fertilization. Reciprocal crosses with the transgenic Westar pollen on Westar, W1 and Topas pistils showed good pollen adhesion and pollen tube growth confirming that the transgenic pollen was unaffected and the defect was only in the transgenic pistils as expected (data not shown). These results indicated that the compatibility phenotype of the transgenic AT1a, AT2a, and AT2b Westar pistils had been altered resulting in a partial self-incompatibility phenotype.

The effect of inactivating thioredoxin in pistils was also tested using the inhibitor 6-aminonicotinamide (6-AN) (Mou *et al*., 2003). The target of 6-AN is 6-phosphogluconate dehydrogenase in the pentose phosphate pathway which is responsible for generating cytosolic NADPH (Hothersall *et al*., 1998). Both THL1 and THL2 belong to the cytosolic thioredoxin *h* family which is dependent on NADPH for reduction, and therefore, would become oxidized in the presence of 6-AN (Schurmann and Jacquot, 2000). Wild-type Westar pistils which were treated with 100 or 200 μM 6-AN showed a similar phenotype to the antisense transgenic AT1a, AT2a, and AT2b Westar lines (Figure 5). A reduction in the number of pollen grains and pollen tubes detected in the aniline blue stained pistils was observed when compared to wild-type Westar pistils treated only with a control solution lacking 6-AN (Figure 5). This further supports the role for a redox control during pollen–pistil interactions.

Expression of SLG_{15} and SRK_{15} in the Brassica napus cultivar Westar

Given the rejection response present in the transgenic AT1a, AT2a, and AT2b Westar lines, we then investigated whether Westar may contain a

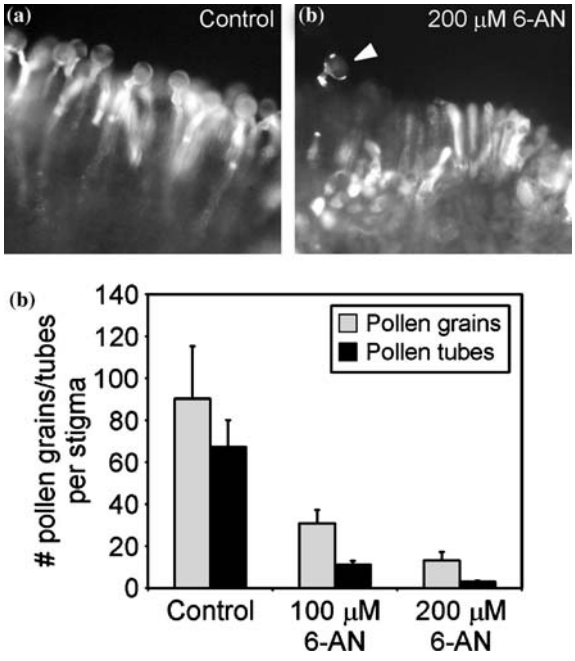

Figure 5. Effect of the 6-AN inhibitor on Westar pollinations. (a) Aniline blue staining of a pollinated Westar pistil, treated with a control solution (no 6-AN), showing many germinated pollen grains. (b) Aniline blue staining of a pollinated Westar pistil, treated with 6-AN, showing very few pollen grains. The closed arrowhead indicates a growing pollen tube that has successfully penetrated the stigmatic surface. (c) The average number of pollen grains or pollen tubes detected in Westar pistils following treatment with 6-AN.

functional SRK responsible for this low level constitutive rejection response. Westar is an allotetraploid and while self-compatible, does carry *S* haplotype genes which are no longer capable of eliciting a self-incompatibility response. Previously, two expressed *SLG* and *SRK* genes, called SLG_{A10} and SRK_{A10} were characterized (Goring *et al*., 1993). SLG_{A10} is predicted to be functional while SRK_{A10} was found to contain a 1 bp deletion predicted to cause premature termination producing a non-functional truncated protein (Goring *et al*., 1993).

Westar was also found to express another *SLG* gene, named SLG_{WS2} (Robert *et al*., 1994) which was first described as *SLG-Sc* in the self-compatible *Brassica oleracea* P57Sc line (Gaude *et al*., 1993; Pastuglia *et al*., 1997), and more recently was identified as the $SLGB_{15}$ gene characterized in the *Brassica oleracea* P57Sc line (Cabrillac *et al*., 1999). The S_{15} haplotype present in the *B. oleracea* P57Sc line consists of two different SLG_{15} genes, $SLGA_{15}$ and $SLGB_{15}$, as well as the SRK_{15} gene (Cabrillac *et al*., 1999). P57Sc is a self-compatible line; however, genetic crosses with closely related *B. oleracea* var. botrytis lines and tester lines from the Horticulture Research International (Wellesbourne, UK) have indicated that the self-compatible trait is due to a defect in pollen, whereas the female recognition component, SRK_{15}, is functional (Pastuglia *et al*., 1997; Cabrillac *et al*., 1999). For this reason, we investigated if Westar expresses the SRK_{15} gene. Using RT-PCR, the SRK_{15} cDNA was successfully amplified from Westar pistil mRNA, and found to have an identical nucleotide sequence to the SRK_{15} cDNA isolated from the P57Sc S_{15} line (data not shown). Similarly, the SRK_{15} cDNA was isolated by RT-PCR in W1 and Topas.

The $SLGA_{15}$, and $SLGB_{15}$ proteins in the *B. oleracea* P57Sc S_{15} line can be resolved by isoelectric focusing (IEF) gel electrophoresis into four main glycoforms (Gaude *et al*., 1993; Cabrillac *et al*., 1999; Figure 6a). These bands were previously identified by MALDI-TOF-MS to consist of $SLGA_{15}$ for the most basic band and $SLGB_{15}$ for the other three bands (Cabrillac *et al*., 1999; Figure 6a). Using IEF followed by immunoblotting with an SRK_3 N-terminal antibody, which also recognizes SLG_{15}, we analyzed eleven self-compatible varieties of *B. napus* (Global, Topas, Tribute, Westar, Drakkar, Tanto, Bronowski, Chine 32, and Yudal), and the *B. oleracea* Rapid cycling and P57Sc S_{15} lines. Westar was the only cultivar that shared a similar banding profile to the P57Sc S_{15} line (Figure 6a). This indicates that Westar produced the SLG_{15} proteins previously detected in stigmas of the P57Sc S_{15} line (Cabrillac *et al*., 1999). With the exception of Yudal, the remainder of the *Brassica* lines also expressed the $SLGB_{15}$ protein (Figure 6a).

To examine the presence of the SRK_{15} protein, the SRK_3 N-terminal antibody, Mab 64-32-30, which was previously found to detect SRK_3, SLG_{15}, and SRK_{15} but not SLG_3, was used to immunoprecipitate SLG and SRK proteins (Delorme *et al*., 1995; Cabrillac *et al*., 1999, 2001). A second monoclonal antibody to the same SRK_3 N-terminal region, Mab 85-36-71, was then used for immunodetection (Giranton *et al*., 1995). As previously seen for the S_3 *B. oleracea* line, a single 120 kDa band representing SRK_3, and faint bands at around 62 kDa representing the extracellular domain of

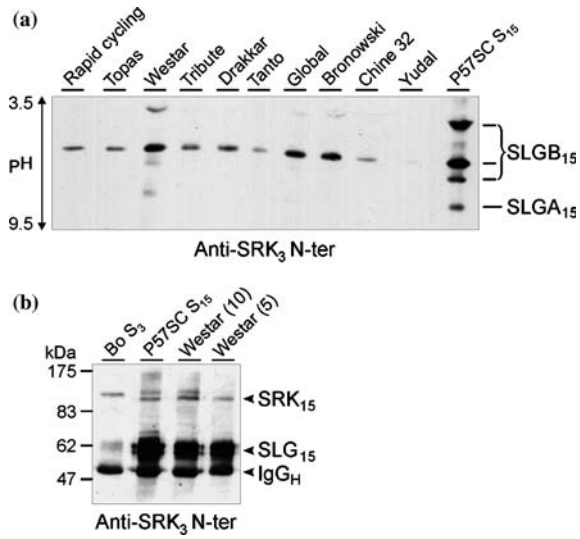

Figure 6. Analysis of SLG_{15} and SRK_{15} Proteins in *B. napus* and *B. oleracea* stigmas. (a) Isoelectric focusing immunoblot analysis of $SLGA_{15}$ and $SLGB_{15}$ proteins in Westar and other self-compatible varieties of *Brassica*. Soluble stigmatic proteins were resolved by IEF gel electrophoresis and immunodetected with the SRK_3 N-terminal antibody, Mab157-35-50 which is also known to detect SLG_{15} (Cabrillac *et al*., 1999). Westar shows a similar banding to that seen for $SLGA_{15}$ and $SLGB_{15}$ in the *B. oleracea* P57Sc S_{15} line. (b) SLG and SRK proteins were immunoprecipitated with the SRK_3 N-terminal antibody, Mab64-32-30, from stigmas of *B. oleracea* S_3, P57Sc S_{15} and the *B. napus* cultivar Westar (either 5 or 10 stigmas as indicated). Immunodetection was carried out with the SRK_3 N-terminal antibody, Mab 85-36-71. These antibodies detect SRK_3, SLG_{15} and SRK_{15}, but do not detect SLG_3; and SRK_{15} migrates slightly below SRK_3, as previously seen (Cabrillac *et al*., 1999). Westar shows the same pattern of SLG and SRK as seen in the *B. oleracea* P57Sc S_{15} line.

SRK_3 ($eSRK_3$) are detected (Figure 6b; Giranton *et al*., 1995; Cabrillac *et al*., 1999). SLG_3 is not detected with this antibody (Figure 6b; Giranton *et al*., 1995; Cabrillac *et al*., 1999). In the P57Sc S_{15} line, there is a doublet running just below 62 kDa representing SLG_{15} and a band around 105 kDa which represents SRK_{15} (Figure 6b; Cabrillac *et al*., 1999). In the two Westar lanes using either 5 or 10 pistils for the extractions, the banding pattern is the same as predicted for the P57Sc S_{15} line (Figure 6b; Cabrillac *et al*., 1999). In the P57Sc S_{15} and Westar (10) lanes there is one additional band migrating just above the 105 kDa SRK_{15} which may be a more extensively glycosylated form of SRK_{15} that is less abundant as it is not detected in the Westar (5) lane (Figure 6b). Since we have detected both the *SRK_{15}* cDNA and a protein of the correct size, we conclude that Westar produces a full-length SRK_{15} protein.

Discussion

We have demonstrated that the antisense suppression of two thioredoxin *h* genes, *THL1* and *THL2*, in the stigmas of *B. napus* Westar plants causes a low level of a constitutive self-incompatibility-type response. This response resulted in rejection of what would normally be compatible pollen, both self-pollen and cross-pollen, causing reduced pollen adhesion and germination, reduced pollen tube growth and reduced seed production. Based on this analysis, we conclude that the thioredoxin *h* proteins, THL1 and THL2, are required for full pollen acceptance in *B. napus* cv. Westar.

Thioredoxins are small proteins that are involved in the redox regulation of disulphide bridges in target proteins (reviewed in Schurmann and Jacquot, 2000; Dietz, 2003). In their reduced form, thioredoxins donate electrons to the disulphide bridge resulting in a conversion to the dithiol state. This redox reaction can be reversed through oxidation by, for example, reactive oxygen species (ROS) (Dietz, 2003). Therefore, thioredoxins participate in a reversible modification that can regulate the activity of target proteins. Our data presented in this paper analysing the antisense *THL1* and *THL2* Westar lines supports that thioredoxin *h* proteins are important for successful pollen–pistil interactions. The require-

ment for redox regulation was also confirmed when Westar pistils treated with the 6-AN inhibitor also displayed reduced pollen adhesion, germination and pollen tube growth. The question then arises what is THL1 and THL2 regulating in *B. napus* Westar that would lead to increased pollen rejection following the RNA suppression of the *THL1* and *THL2* genes?

Several studies indicate that THL1 and THL2 influence the self-incompatibility response via SRK. Bower *et al.* (1996) showed that THL1 and THL2 interact with the kinase domain of SRK in the yeast two-hybrid assay. A cysteine at the C-terminal end of the SRK transmembrane domain was found to be necessary for this interaction (Mazzurco *et al.*, 2001). This cysteine was found to be conserved in all the SRK alleles characterized to date, but not in other closely related receptor kinases suggesting that the thioredoxin regulation is specific to SRK (Mazzurco *et al.*, 2001). Moreover, SRK_3 was constitutively phosphorylated in purified microsomal membranes from pistils or from a recombinant insect cell/baculovirus system, and stigmatic extracts contained a soluble inhibitor, identified as thioredoxin, were found to reduce this basal activity (Giranton *et al.*, 2000; Cabrillac *et al.*, 2001). The inhibitory effect of thioredoxin could be overcome by the addition of *S* haplotype-specific pollen coat proteins (Cabrillac *et al.*, 2001). In contrast, Takayama *et al.* (2001) observed an increase in SRK_8 phosphorylation, *in vitro*, following the addition of $SP11/SCR_8$ in the absence of thioredoxin. However, their data are consistent with a model of SRK being partially active in the absence of thioredoxin, and only becoming fully activated following ligand binding.

Following these studies, THL1 and THL2 may be mediating their effects via an SRK in the transgenic Westar lines, but only if a functional SRK protein is present in the stigmas. The Westar line is normally self-compatible and has been shown to carry an SRK allele, SRK_{A10}, with a 1 bp deletion that is predicted to cause premature termination (Goring *et al.*, 1993). Here we have confirmed the presence of the S_{15} haplotype in Westar (Cabrillac *et al.*, 1999), and shown that the SRK gene is identical to that found in *B. oleracea* and therefore potentially encodes a functional *SRK* protein. At first glance, these data may not appear to be consistent with the fact that wild-type Westar is self-compatible, but the self-compatible phenotype may be due to failure to produce an active S_{15} male component either due to the presence of a non-functional $SP11/SCR_{15}$ gene or as a result of the genetic dominance of a Class I *S* haplotype (Shiba *et al.*, 2002). Note that the otherwise functional, S_{15} haplotype has also been shown to be defective on the male side in some B. oleracea lines (Pastuglia *et al.*, 1997).

Based on these analyses, SRK_{15} is a prime target for regulation by THL1 and THL2; however, there are inconsistencies that do not fit with this model. There is evidence that the interaction of the THL proteins with SRK_{15} is not as strong as with other SRK alleles. In yeast two-hybrid assays, THL1 and THL2 were shown to interact with several SRK alleles, but an interaction with SRK_{15} could not be detected using this method (However, SRK_{15} may have been unstable in the yeast cells as the ARC1 interaction was also extremely weak; Mazurcco *et al.*, 2001). In addition, we were unable to detect significant SRK_{15} kinase activity or changes in complex formation which might be predicted to occur in the absence of THL1 and THL2 (data not shown). Therefore, it is unclear if SRK_{15} is the target of negative regulation by THL1 and THL2 in Westar. There may instead be other receptors or factors being regulated by THL1 and THL2 during pollen–pistil interactions, and we are continuing to investigate this.

Redox regulation by various thioredoxins has been well documented in plants, particularly in the chloroplast by thioredoxin *f* and *m* (reviewed in Dietz, 2003). However, less is known about the functions of the plant thioredoxin *h* family (reviewed in Gelhaye *et al.*, 2004). Roles for thioredoxin *h* have been proposed during seed germination (Wong *et al.*, 2002; Kim *et al.*, 2003), and a wide variety of potential targets has been described (reviewed in Gelhaye *et al.*, 2004; Yano *et al.*, 2001). Interestingly, a novel thioredoxin has recently been implicated in regulating another receptor, the tomato Cf-9 receptor-like protein (Rivas *et al.*, 2004). Cf-9 confers disease resistance to the fungal pathogen, *Cladosporium fulvum*. A yeast two-hybrid screen with the C-terminal tail of Cf-9 resulted in the identification of the thioredoxin protein, CITRX. Virus-induced gene silencing of CITRX resulted in an enhanced defense response against the fungal pathogen. This was also accompanied by increased resistance to *C. fulvum* indicating that CITRX acts as a negative

regulator of Cf-9. Thus, the negative regulation of receptors by thioredoxins may become a more frequent theme in plant signalling.

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