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The self-incompatibility (SI) response in *Brassica* allows recognition and rejection of self-pollen by the stigmatic papillae. The transmembrane *S* locus receptor kinase (SRK), a member of the receptor-like kinase (RLK) superfamily in plants, mediates recognition of self-pollen on the female side[1], whereas the *S* locus cysteine-rich protein (SCR) is the male component of the SI response[2]. SCR is presumably located in the pollen coat and is thought to be the SRK ligand[2,3]. Although many RLKs have been isolated in plants, the mechanisms of signal transduction mediated by these molecules are largely unknown. Here we show that SRK is phosphorylated *in vivo* within one hour following self-pollination. We also demonstrate that, *in vitro*, autophosphorylation of SRK is prevented by the stigma thioredoxin THL1 in the absence of a ligand. This inhibition is released in a haplotype-specific manner by addition of pollen coat proteins (PCPs). These data indicate that SRK is inhibited by thioredoxins and activated by PCPs.

To investigate the phosphorylation status of SRK during the self-incompatibility reaction, we labelled pistils from self-incompatible S_3 homozygous *Brassica oleracea* plants with ^{32}P-orthophosphate, and phosphorylation of the receptor was monitored during a self-pollination time course. Physiological studies have shown that pollen hydrates 30-90 min after its deposition on the stigmatic papillae[4]. Figure 1a shows that SRK was phosphorylated about 60 min after self-pollination, but as with pollen

hydration, the time course of SRK phosphorylation varied between 45-60 min depending on the experimental conditions. No phosphorylation of SRK was detected up to 90 min following compatible pollination (data not shown). Only a fraction of the SRK protein was phosphorylated *in vivo* (compare Fig. 1a with Fig. 1b left panel). Stigmatic papillae are able to discriminate between a self and a cross pollen deposited on the same cell[5], indicating that the SI reaction is highly localised at the subcellular level. Our data are consistent with SRK being activated only within regions of the stigma papillar cells that contact the pollen grains.

In contrast to these results obtained *in vivo*, recombinant SRK, expressed in a baculovirus-insect cell expression system, was constitutively active *in vitro*[6] (Fig. 1b). This difference was not due to overexpression because SRK was also constitutively active in microsomes isolated from stigmas (Fig. 1b). One possible explanation for these conflicting results was the existence of an inhibitory factor *in vivo* that was absent from the microsome fraction used in the *in vitro* assay. To test this hypothesis, different amounts of a soluble stigma extract were added to recombinant SRK in an *in vitro* phosphorylation assay. Under these conditions, SRK phosphorylation decreased in a dose dependant manner (Fig. 2a) suggesting that the soluble stigma extract contained a negative regulator of SRK phosphorylation. A similar result was obtained when stigma-extracted SRK was used instead of recombinant SRK (data not shown). The inhibitory effect of the soluble stigma fraction was lost when it was boiled or treated with trypsin but not following treatment with denatured trypsin, indicating that the active component was a protein (data not shown). We tested soluble extracts from stigmas of *Brassica* lines carrying different *S* haplotypes and extracts from different tissues. All extracts had an inhibitory effect although the various tissue extracts varied in their ability to inhibit SRK activity, despite the fact that equal amounts of protein were added (Fig. 2b). These experiments showed that the inhibitory activity was present in a wide range of tissues, albeit at different concentrations, and was not haplotype-specific.

Although phosphatase inhibitors were used in the phosphorylation buffer, it was nonetheless possible that the observed activity might be the result of a non-inactivated phosphatase. To test this hypothesis, the soluble stigma extract was added either immediately or 30 min after the beginning of the phosphorylation reaction (at which point SRK is already phosphorylated, see Fig. 1b) and the reactions were incubated for an additional 30 min. As shown in Fig. 2c, inhibition of SRK activity occurred only if the soluble extract was added at the beginning of the reaction indicating that it prevents SRK phosphorylation rather than dephosphorylates this protein.

To test whether the decrease in SRK activity was due to direct binding of the inhibitor to SRK, we performed competition experiments in an *in vitro* phosphorylation reaction. To the phosphorylation reaction medium, we added an excess of an inactive recombinant form of SRK (^{m}His), together with the functional recombinant HA-tagged SRK, and soluble stigma extract. Figure 2d shows that when the ^{m}His competitor was added in a 3.6 fold excess, SRK-HA kinase activity was not inhibited by the stigma extract. This experiment indicated that the inhibitor bound to both the wild-type and mutant forms of SRK in a stoechiometric and phosphorylation-independant manner.

ARC1 (Arm Repeat Containing 1) interacts specifically with the cytoplasmic domain of SRK[7], but it was unlikely to correspond to the inhibitor because it interacts only with the phosphorylated form of SRK and is expressed specifically in stigmas. Furthermore, reduced levels of ARC1 in transgenic plants are correlated with an attenuation of the SI response[8]. Two other proteins, THL1 and THL2 (Thioredoxin-h-like), were better candidates because they bind the cytosolic domain of SRK in a phosphorylation-independant manner[9]. Moreover, in mammals, thioredoxin (TRX) binds and regulates the activity of cytosolic kinases such as ASK1 (Apoptosis Signal-regulating Kinase 1)[10] and p38 MAP Kinase[11].

We looked at the possible regulatory effect of thioredoxin on SRK activity by adding purified *Spirulina* thioredoxin (SpTRX) to SRK phosphorylation assays. SpTRX mimicked the effect of the stigma extract (Fig. 3a). DTT, which can be used by thioredoxins as an electron donor, is necessary for the inhibitory effect of TRX on ASK1[10]. Figure 3b shows that SpTRX lost its inhibitory activity when DTT was omitted from the buffer or when SpTRX was treated with H_2O_2. SRK phosphorylation was not affected by these treatments when SpTRX was omitted (data not shown). The effect of DTT was dose dependant but excess DTT (5 mM) suppressed the action of SpTRX, possibly by preventing disulphide bridge formation between the enzyme and SRK. Moreover, specific depletion of thioredoxins from the stigma extract by repeated incubation with Thiobond^{TM} resin abolished the inhibitory effect of the soluble stigma extract (Fig. 3c upper panel). Inactivated resin did not deplete the inhibitory activity. The *S* Locus Glycoprotein (SLG, a soluble glycoprotein expressed in stigmas[12]) was not significantly depleted from the extract, indicating that loss of inhibitory activity was not due to a general depletion of protein (Fig. 3c, lower panel). Taken together, these results indicated that the inhibitory effect of the stigma extract was due to the activity of a thioredoxin.

We confirmed that THL1 is capable of inhibiting SRK activity by adding a recombinant form of THL1, expressed in *Escherichia coli*, to phosphorylation reactions *in vitro* (Fig. 3d). To get further insight into the mechanism of action of this enzyme, we produced two mutated forms of THL1 in which one or both of the catalytic cysteines were replaced by serines (THL1^{C45S} and THL1$^{C45S/C48S}$). These mutations suppress thioredoxin activity of the enzyme by impairing its catalytic mechanism[13]. The two mutant forms of THL1 did not inhibit SRK phosphorylation *in vitro*, suggesting that the catalytic activity of THL1 is necessary for it to act as an inhibitor.

In *Brassica*, the male component of the SI response is a PCP[3]. When PCPs extracted from S_3 or S_{15} pollen were added to recombinant SRK in the presence of the

inhibitor, SRK was phosphorylated in a dose dependant and haplotype-specific manner (Fig. 4a). This result indicates that PCPs are capable of activating SRK in the presence of the inhibitor. In animal receptor kinases, ligands activate their receptor by inducing their oligomerisation and transphosphorylation[14]. This activation can be mimicked by the addition of bivalent antibodies that create bridges between receptor molecules[15]. Interestingly, in the presence of the antibody anti-SRK_3N-ter, recombinant SRK was phosphorylated in a dose-dependant and saturable manner (as expected for an antigen/antibody interaction) mimicking the action of PCPs (Fig. 4b). Similar results were obtained with stigma extracted SRK (data not shown). By analogy with the studies carried out on animal receptors, these data suggest that the activation of SRK by PCPs involves receptor oligomerisation. This is consistent with a previous study carried out in our group, which demonstrated transphosphorylation between SRK molecules[6]. To our knowledge, this is the first demonstration that a plant receptor-like kinase is phosphorylated in response to a specific stimulus.

Taken together, the data presented here strongly support a model in which THL1 (and THL2) interacts in a reversible manner with SRK in stigmas and prevents spontaneous activation of the SI signalling pathway. In this model, SRK thioredoxin inhibition is released by PCPs following self-pollination. In the absence of this regulation, it is probable that the SI system would be constitutively active, blocking both self and cross-pollinations and hence preventing seed set.

Methods

Extraction and analysis of stigma and recombinant SRK. Insect cell microsomes expressing recombinant, HA-tagged and kinase-inactive (K553R), 6His-tagged SRK_3 proteins have been described[6]. Crude stigma extracts, plant microsomes and soluble fractions from *B. oleracea* lines were prepared as described[16], except that extraction buffer used for microsome preparation was 50 mM Hepes pH7.4, 0.5 M Sucrose, 1 mM

DTT, 10 mM EDTA, 10 mM NaF, 5 mM β-Glycerophosphate plus a protease inhibitor cocktail (Boehringer). After ultracentrifugation, the microsome pellet was resuspended in 50 mM Hepes pH7.4 and stored, with soluble fraction (supernatant), at –80°C before use. Proteins were separated through a 7.5 % SDS-PAGE gel, electroblotted and analysed by autoradiography or immunodetected with anti-SRK_3N-ter (MAb 85-36-71) as described[16,17].

Immunoprecipitation of SRK. SRK was immunoprecipitated in 1 ml of TBST buffer (20 mM Tris pH8, 150 mM NaCl, 0.05% w/v Tween 20) complemented with 10 mM EDTA, a protease inhibitor cocktail (Boehringer) and phosphatase inhibitors (5 mM Orthovanadate, 10 mM β-Glycerophosphate, 10 mM NaF). Immunoprecipitation was carried out with monoclonal anti-SRK_3N-ter (MAb 64-32-30)[17] or monoclonal anti-HA (MAb 12CA5, Boehringer) and Protein G Agarose (Tebu) according to the instructions of the manufacturer.

***In vivo* radioisotope labelling.** Pistils from S_3 homozygous *Brassica oleracea* plants were radiolabelled at room temperature in a microplaque well in 10 mM MES pH6, complemented with 25 µCi per pistil of ^{32}P-orthophosphate (ICN, specific activity 285 Ci/mg, 1.25 µCi/µl). After a 4 h incubation, incubation buffer was replaced by 10 mM MES buffer pH6 and pistils were self-pollinated. SRK was immunoprecipitated from crude protein extracts as described above.

Phosphorylation *in vitro*. Typically, stigma microsomes or insect cell microsomes expressing SRK (10 µg protein) were labelled for 30 min at room temperature using 5 µCi of γ^{32}P-ATP (4 µCi/µl, Amersham-Pharmacia, specific activity 3,000 Ci/mmol) as described[6]. For stigma microsomes, ATP was removed at the end of the reaction by desalting on a hand made column containing 600 µl of P6DG Gel (Biorad) equilibrated in 20 mM Tris pH8, 2.5% SDS. SRK was immunoprecipitated prior to SDS-PAGE analysis. PCPs added to phosphorylation reactions were prepared as described[18].

Bacterial and recombinant stigma thioredoxins. *Spirulina* thioredoxin (Sigma) was resuspended in microsome extraction buffer and incubated for 30 min on ice. For the redox experiments (Fig.3b) DTT was omitted, added at different concentrations or replaced by H_2O_2. *THL1* was amplified from a *B. oleracea* stigma cDNA library[19] using oligonucleotides TH1 (5'-GGAGGGATCCATGGCCGCAACAGCAGAAGTG-3') and TH2 (5'-CTCAGGATCCGTCGGCACCACAACACAAAGG-3') by the polymerase chain reaction. Mutant forms of THL1 ($THL1^{C45S}$ and $THL1^{C45S/C48S}$) were obtained using PCR mutagenesis with oligonucleotides TH3 (5'-CACAGCAGTGTGGTGCCCACCTAGCCGTTTCATTG-3'), TH4 (5'-CACAGCAGTGTGGAGCCCACCTAGCCGTTTCATTG-3') and TH5 (5'-AAGAAGAAGGATAATCAAGCGGCAGCAACT-3'). In Fig. 3 wild-type THL1, $THL1^{C45S}$ and $THL1^{C45S/C48S}$ are referred to as C-C, S-C and S-S respectively. The three *THL1* constructs were verified by DNA sequencing and inserted into pQE30 (Qiagen) for expression in *E. coli*. Expressed proteins were purified on a Ni-NTA affinity column under native conditions using the Ni-NTA spin Kit (Qiagen) according to the manufacturer's instuctions. Imidazole was removed from eluates by desalting as described above except that the column was equilibrated in microsome extraction buffer.

Thioredoxin depletion on Thiobond™ resin. One bed volume of Thiobond™ resin (Invitrogen) was reduced in two bed volumes of 50 mM Hepes pH7.4, 20 mM β-Mercaptoethanol for 30 min at room temperature. Inactive resin was produced by incubating one bed volume of reduced resin in two bed volumes of 20 mM 4-Vinyl Pyridine[20] after reduction. Active and inactive resins were washed and resuspended in 50 mM Hepes pH7.4 before use. Thioredoxin depletion by active and alkylated resins was carried out for 30 min at room temperature under shaking, using 4 µl of bed resin per µg protein. After incubation, unbound proteins were recovered by decantation of the resin and were incubated two more times with fresh resin. An aliquot of each fraction

was tested in an *in vitro* phosphorylation assay. Non-specific adsorbtion of protein on the beads was monitored by immunodetection of SLG with anti-SLG_3 N-ter[16].

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LEGENDS

FIGURE 1. SRK is phosphorylated 60 min after self-pollination *in vivo*, but is constitutively active *in vitro*. **a** Time course of SRK phosphorylation *in vivo*. SRK was immunoprecipitated from extracts of radiolabelled stigmas and detected either by autoradiography (upper panel) or immunodetection (lower panel). **b** SRK is constitutively active *in vitro*. *In vitro* phosphorylation reactions were carried out with stigma microsomes containing SRK (left panel) and with microsomes from insect cells expressing either functional, HA-tagged SRK (HA) or kinase-inactive, His-tagged SRK (^{m}His) (right panel). Rec.SRK=Recombinant SRK.

FIGURE 2. Phosphorylation of SRK *in vitro* is inhibited by a soluble protein. **a** Dose dependant inhibition of SRK phosphorylation by a soluble stigma extract. **b** The soluble inhibitor of SRK phosphorylation is neither haplotype-specific nor tissue-specific. **c** SRK is not dephosphorylated by soluble stigma proteins added 30 min after the beginning of the phosphorylation reaction. **d** Kinase-inactive SRK (^{m}His) competes with functional, HA-tagged SRK (HA) for the binding of the inhibitor. In **b**-**d**, 0.6µg of soluble protein was added, no extract was added to controls.

FIGURE 3. Thioredoxin inhibits SRK phosphorylation *in vitro.* **a** *Spirulina* thioredoxin (SpTRX, 20ng) has the same inhibitory effect as soluble stigma proteins (0.6µg). **b** The inhibitory activity of *Spirulina* thioredoxin is redox dependant. SpTRX (0.6µg) was pretreated with redox compounds as indicated. NT = Not Treated. **c** Depletion of thioredoxin from the stigma extract by functional Thiobond^{TM} resin abolishes the inhibitory activity of the extract. **d** *In vitro* phosphorylation of SRK is inhibited by 15ng of *Brassica* THL1 (C-C), but

not by the inactive mutant forms (S-C and S-S) nor by an extract from an antisense control (AS).

FIGURE 4. SRK is phosphorylated following addition of an agonist in the presence of thioredoxin. **a** Following inhibition of SRK phosphorylation in the presence of soluble stigma extract, addition of S_3, but not S_{15}, PCPs induces SRK kinase activity. **b** Under the same conditions, addition of a bivalent antibody (anti-SRK_3N-ter) also induces phosphorylation of SRK.