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The *S* receptor kinase determines self-incompatibility in *Brassica* stigma

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The self-incompatibility possessed by *Brassica* is an intraspecific reproductive barrier by which the stigma rejects self-pollen but accepts non-self-pollen for fertilization. The molecular/biochemical bases of recognition and rejection have been intensively studied. Self-incompatibility in *Brassica* is sporophytically controlled by the polymorphic *S* locus[1]. Two tightly linked polymorphic genes at the *S* locus, *S* receptor kinase gene (*SRK*) and *S* locus glycoprotein gene (*SLG*), are specifically expressed in the papillar cells of the stigma[2–4], and analyses of self-compatible lines[5–7] of *Brassica* have suggested that together they control stigma function in self-incompatibility interactions. Here we show, by transforming self-incompatible plants of *Brassica rapa* with an SRK^{28} and an SLG^{28} transgene separately, that expression of SRK^{28} alone, but not SLG^{28} alone, conferred the ability to reject self (S^{28})-pollen on the transgenic plants. We also show that the ability of SRK^{28} to reject S^{28} pollen was enhanced by SLG^{28}. We conclude that SRK alone determines *S* haplotype specificity of the stigma, and that SLG acts to promote a full manifestation of the self-incompatibility response.

S receptor kinase is a membrane-spanning receptor kinase that consists of an extracellular domain (called the S domain), a transmembrane domain and a cytosolic domain with serine/threonine kinase activity[8]. *S* locus glycoprotein is a secreted glycoprotein[3,4] whose amino-acid sequence is highly similar, but not identical, to the S domain of SRK (ref. 2). It has been proposed that SRK and SLG function together as the receptor for a pollen ligand, which determines the *S* specificity of pollen, and that this receptor–ligand interaction sets off a cascade of biochemical reactions leading to the self-incompatibility response[9,10]. A cysteine-rich gene located at the *S* locus, called *SP11* (ref. 11) or *SCR*[12], has been shown by gain-of-function and loss-of-function experiments to encode the potential pollen ligand[12]. However, the proposed role of SRK and SLG in self-incompatibility interactions has so far been based entirely on circumstantial evidence[2–7]. The most direct way to show the function of SRK or SLG would be to transform *Brassica* plants with the *SLG* or *SRK* gene of a different *S* haplotype and show that the transgenic plants acquire the *S* haplotype specificity of the transgene. However, all transformation experiments reported so far have resulted in the breakdown of self-incompatibility in the transgenic plants because of co-suppression between the endogenous *SLG* and/or *SRK* gene and the *SLG* and/or *SRK* transgene[13–15].

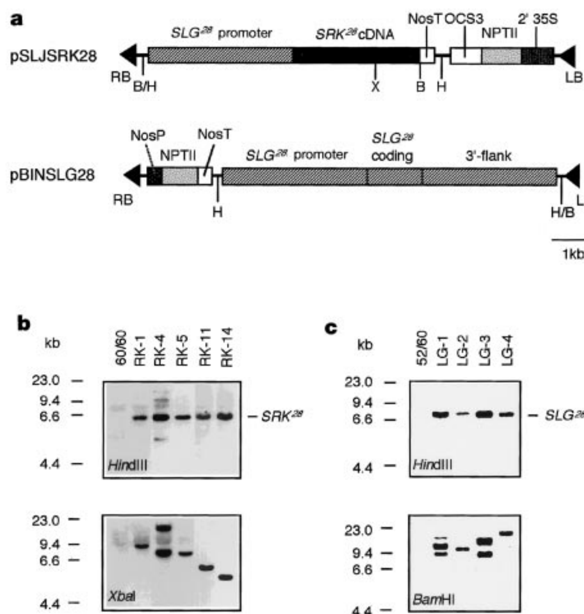

Figure 1 Vector construction and detection of transgenes. **a**, Representation of the T-DNA region of transformation vectors pSLJSRK28 and pBINSLG28. H, *Hin*dIII; X, *Xba*I; B, *Bam*HI; NosT, *nos* terminator; OCS3, the 3′ region of *ocs*; 2′, *mas2′* promoter; 35S, cauliflower mosaic virus 35S promoter; NosP, *nos* promoter. RB, right border; LB, left border. **b**, Representative DNA gel blot analysis of the presence (upper panel) and its copy numbers (lower panel) of the SRK^{28} transgene in an S^{60} homozygote (60/60) and five independent transgenic plants (RK-1, RK-4, RK-5, RK-11 and RK-14) using the cDNA encoding the kinase domain of SRK^{28} (SRK^{28}-KD) as a probe. **c**, DNA gel blot analysis of the presence (upper panel) and its copy numbers (lower panel) of the SLG^{28} transgene in an $S^{52}S^{60}$ heterozygote (52/60) and four independent transgenic plants (LG-1, LG-2, LG-3 and LG-4) using SLG^{28} cDNA as a probe.

We chose to examine the function of SRK by introducing an *SRK* gene of a class I *S* haplotype, SRK^{28}, into plants homozygous for a class II *S* haplotype, S^{60}. This classification of *S* haplotypes is based on amino-acid sequence similarities between their SLG proteins and between their SRK proteins[16]. An *S* haplotype can be dominant over, recessive to, or co-dominant with another *S* haplotype, but class II *S* haplotypes are, in most cases, recessive to class I *S* haplotypes in pollen[17]. We considered that the lower degree of nucleotide sequence similarity between *SRK* genes of different classes of *S* haplotypes than between *SRK* genes of the same class might minimize the problem of co-suppression encountered in all the previous transformation experiments. We constructed a transformation vector, pSLJSRK28 (Fig. 1a), and introduced it into S^{60} homozygotes of *B. rapa* by *Agrobacterium*-mediated transformation. To examine the function of SLG, we constructed another transformation vector, pBINSLG28 (Fig. 1a), and introduced it into $S^{52}S^{60}$ heterozygotes of *B. rapa* by the same transformation method (S^{52} is a class I *S* haplotype). The SLG^{28} promoter used in both constructs had previously been shown to be active in the stigma but not in the anther[18,19].

In the case of pSLJSRK28 transformation, the SRK^{28} transgene was detected in 17 independent transgenic plants by DNA gel blot

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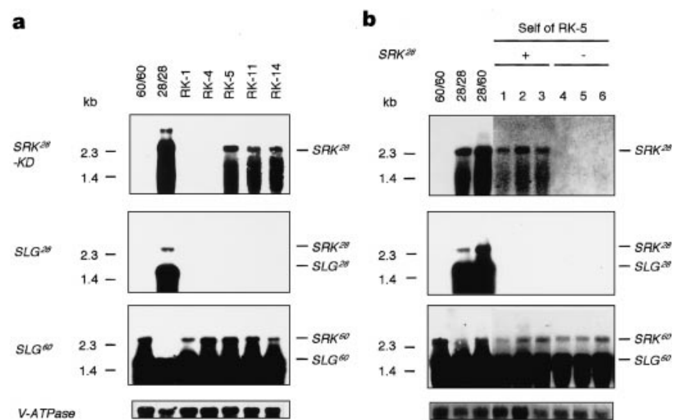

Figure 2 RNA blot analysis of transcription of the endogenous *SRK*^{28} gene and the *SRK*^{28} transgene. The probes used are shown to the left of the blots. **a**, Transcription of *SRK*^{28} in an S^{60} homozygote (60/60) and an S^{28} homozygote (28/28), and five primary transgenic plants (RK-1, RK-4, RK-5, RK-11 and RK-14). **b**, Transcription of *SRK*^{28} in three control plants, S^{60} homozygote (60/60), S^{28} homozygote (28/28) and $S^{28}S^{60}$ heterozygote (28/60), and six plants of the self-pollinated progeny (self) of RK-5. The presence (+) or absence (–) of the *SRK*^{28} transgene is indicated above the blots.

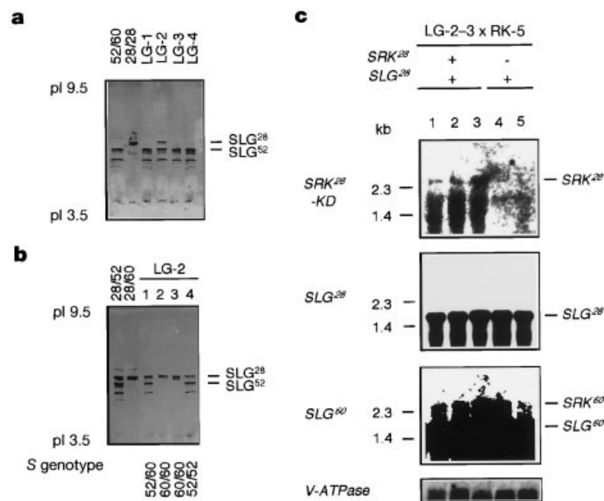

Figure 3 Expression of the *SRK*^{28} and *SLG*^{28} transgenes. **a**, Isoelectric focusing immunoblot analysis of the expression of the endogenous *SLG*^{28} gene in an $S^{52}S^{60}$ heterozygote (52/60) and an S^{28} homozygote (28/28), and the expression of the *SLG*^{28} transgene in four primary transgenic plants (LG-1, LG-2, LG-3 and LG-4). **b**, Isoelectric focusing immunoblot analysis of the expression of the endogenous *SLG*^{28} gene in an $S^{28}S^{52}$ heterozygote (28/52) and an $S^{28}S^{60}$ heterozygote (28/60), and the expression of the *SLG*^{28} transgene in four of the plants (1–4) obtained from bud self-pollinating of LG-2. The genotypes of the latter four plants are shown below the blot. **c**, Transcription of the *SRK*^{28} and *SLG*^{28} transgenes in the LG-2–3 × RT-5 progeny. The presence (+) or absence (–) of the *SRK*^{28} and *SLG*^{28} transgenes is indicated above the blots. The probes are indicated to the right of the blots.

analysis (Fig. 1b). All of them were self-incompatible; three (RK-5, RK-11 and RK-14) rejected both S^{28} and S^{60} pollen, whereas the other 14 plants rejected only S^{60} pollen. The pollen of the former three plants was rejected by S^{60} but not by S^{28} stigmas. These results suggest that the self-incompatibility phenotype of the stigmas of RK-5, RK-11 and RK-14 had changed from that of the parental S^{60} homozygotes, but the self-incompatibility phenotype of the pollen had not. Further, the self-incompatibility phenotype of the other 14 transgenic plants was not altered.

To determine whether the new self-incompatibility phenotype of the stigmas of RK-5, RK-11 and RK-14 resulted from the expression of the *SRK*^{28} transgene, we first pollinated these three plants with pollen from S^{24}, S^{43}, S^{45} and S^{52} homozygotes (all being class I *S* haplotypes), and S^{29} and S^{44} homozygotes (class II *S* haplotypes) of *B. rapa*[17]. In all pollinations, pollen tubes penetrated the stigma, indicating that the new self-incompatibility phenotype was specific to the S^{28} haplotype. We then carried out RNA gel blot analysis and detected the *SRK*^{28} transcript in the stigmas of these three plants, but not in the stigmas of the transgenic plants that did not acquire the ability to reject S^{28} pollen (Fig. 2a). The expression level of *SRK*^{28} in these three plants averaged 32–35% of that in S^{28} heterozygote. The endogenous *SRK*^{60} and *SLG*^{60} were expressed at normal levels in all the transgenic plants, and, as expected, the *SRK*^{28} transcript was not detected in the anther of any of these transgenic plants (data not shown).

We examined whether the S^{28} haplotype specificity acquired by the stigmas of RK-5 co-segregated with the *SRK*^{28} transgene in the progeny. As this plant was self-incompatible (with the stigma and pollen phenotypes being $S^{28}S^{60}$ and S^{60}, respectively), we carried out self-pollination at immature bud stages when self-incompatibility was not manifested. Polymerase chain reaction (PCR) analysis showed that 16 out of the 20 plants in the progeny inherited the *SRK*^{28} transgene and DNA gel blot analysis (Fig. 1b) indicated that RK-5 carried a single copy of the *SRK*^{28}. Thus, the segregation ratio of the *SRK*^{28} transgene in the self-pollinated progeny of RK-5 was consistent with mendelian inheritance of a single copy of the transgene ($\chi^2 = 0.1$, $P > 0.7$, degrees of freedom (d.f.) = 1). As was the case for RK-5, the stigmas of all the 16 progeny plants that carried the *SRK*^{28} transgene rejected both S^{28} and S^{60} pollen, and

Table 1 Self-incompatibility (SI) phenotypes of plants in self-pollinated and crossed progeny of RK-5

Progeny	*S* genotype	SI phenotype		No. of plants	No. of flowers	Seeds/flower
		Stigma	Pollen			
Selfing of RK-5	S^{60}/S^{60}, SRK^{28}/SRK^{28} or –, –/–	$S^{28}S^{60}$	S^{60}	16		
	S^{60}/S^{60}, –/–, –/–	S^{60}	S^{60}	4		
S^{60} × RK-5	S^{60}/S^{60}, SRK^{28}/–, –/–	$S^{28}S^{60}$	S^{60}	6	375	1.9
	S^{60}/S^{60}, –/–, –/–	S^{60}	S^{60}	5	104	15.3
S^{60} homozygotes	S^{60}/S^{60}, –/–, –/–	S^{60}	S^{60}	6	54	10.9
S^{52} × RK-5	S^{52}/S^{60}, SRK^{28}/–, –/–	$S^{28}S^{52}S^{60}$	S^{52}	6	312	1.1
	S^{52}/S^{60}, –/–, –/–	$S^{52}S^{60}$	S^{52}	5	118	14.9
$S^{52}S^{60}$ heterozygotes	S^{52}/S^{60}, –/–, –/–	$S^{52}S^{60}$	S^{52}	4	42	15.9
LG-2-3 × RK-5	S^{60}/S^{60}, SRK^{28}/–, SLG^{28}/–	$S^{28}S^{60}$	S^{60}	5	383	0.3
	S^{60}/S^{60}, –/–, SLG^{28}/–	S^{60}	S^{60}	4	259	14.5
$S^{28}S^{60}$ heterozygotes	S^{28}/S^{60}, –/–, –/–	$S^{28}S^{60}$	S^{28}	4	194	0.2

No. of plants, number of plants examined; no. of flowers, number of flowers pollinated with S^{28} pollen; seeds/flower, average number of seeds per flower. Three crossed progeny, S^{60} × RK-5, S^{52} × RK-5 and LG-2–3 × RK-5, were obtained by bud-pollinating an S^{60} homozygote, an S^{52} homozygote and LG-2–3 (S^{60}/S^{60}, –/–, SLG^{28}/SLG^{28}), respectively, with pollen from RK-5 (S^{60}/S^{60}, SRK^{28}/–, –/–). S^{60} homozygotes, $S^{52}S^{60}$ heterozygotes and $S^{28}S^{60}$ heterozygotes were used as controls for compatible or incompatible pollinations. *S* genotypes were determined by PCR. SI phenotypes of all progeny plants were determined by monitoring pollen behaviour on the stigmatic surface after pollination.

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they all produced the *SRK*^{28} transcript (Fig. 2b). Together these results indicate that the S^{28} haplotype specificity acquired by the three primary transgenic plants, RK-5, RK-11 and RK-14, may have arisen from the expression of the *SRK*^{28} transgene.

In the case of pBINSLG28 transformation, the *SLG*^{28} transgene was detected in four independent transgenic plants, LG-1, LG-2, LG-3 and LG-4, by DNA gel blot analysis (Fig. 1c). All of them were self-incompatible (that is, rejecting S^{52} and S^{60} pollen). Isoelectric focusing (IEF)-immunoblot analysis was carried out using an anti-SLG^{43} monoclonal antibody that we had previously shown to crossreact with 14 of 16 class I SLGs examined (including SLG^{28} and SLG^{52}), but not with 4 of class II SLGs (such as SLG^{60})[13]. All these four plants produced the endogenous SLG^{52} protein at a normal level, but only LG-2 produced a high level of SLG^{28} from the transgene (Fig. 3a). However, LG-2, as well as the other three transgenic plants, failed to reject S^{28} pollen, suggesting that the stigma phenotype of these transgenic plants remained the same as that of their parental plants ($S^{52}S^{60}$). Through bud pollination, self-pollinated progeny was raised from LG-2, and on the basis of PCR analysis, 16 out of the 18 plants carried the transgene. This segregation ratio can be explained by the mendelian inheritance of a single copy of the *SLG*^{28} transgene ($\chi^2 = 1.7, P > 0.1$, d.f. = 1) carried by LG-2 (see the DNA gel blot shown in Fig. 1c). As was the case for LG-2, all 16 progeny plants that carried the *SLG*^{28} transgene failed to reject S^{28} pollen, even though they all produced high levels of SLG^{28}.

Among these 16 plants, LG-2–3 produced the highest level of SLG^{28} protein and it was homozygous for the *SLG*^{28} transgene (Fig. 3b). The genotype of LG-2–3 was thus designated S^{60}/S^{60}, –/–, *SLG*^{28}/*SLG*^{28}, with '–/–' indicating that this plant did not carry the *SRK*^{28} transgene. LG-2-3 was crossed as female with RK-5 (S^{60}/S^{60}, *SRK*^{28}/–, –/–, with '–/–' indicating the absence of the *SLG*^{28} transgene) by bud pollination (as both plants were homozygous for the S^{60} haplotype and would be incompatible if crossed at the mature flower stage). The self-incompatibility phenotypes of this progeny were compared with those of the progeny from two other crosses, S^{60} × RK-5 and S^{52} × RK-5. All these three progeny contained plants hemizygous for the *SRK*^{28} transgene, because, as stated earlier, RK-5 carried one copy of the *SRK*^{28} transgene. Without exception, the self-incompatibility phenotypes of the plants in each progeny, as determined by pollen tube behaviour, were precisely those predicted on the basis of their genotypes determined by PCR analysis (Table 1). For example, when the plants were pollinated with S^{28} pollen, those that did not inherit the *SRK*^{28} transgene yielded an average of more than 10 seeds per flower, a number comparable to that obtained from compatible crosses. In contrast, plants that inherited the *SRK*^{28} transgene yielded an average of less than two seeds per flower.

We compared the ability of the plants in each of these three progeny to reject S^{28} pollen to assess any effect that the differences in the genetic background of these three families might have on rejection of S^{28} pollen. As shown in Table 1, plants of the S^{60} × RK-5 progeny that carried the *SRK*^{28} transgene set a few seeds (an average of 1.9 seeds per flower). Plants of the LG-2–3 × RK-5 progeny that expressed both the *SRK*^{28} and the *SLG*^{28} transgene (Fig. 3c) showed very strong incompatibility with S^{28} pollen: an average of 0.2 seeds per flower, a number comparable to that obtained from incompatible pollination of $S^{28}S^{60}$ heterozygotes with S^{28} pollen. Plants of the S^{52} × RK-5 progeny that carried the *SRK*^{28} transgene and the endogenous *SLG*^{52} and *SLG*^{60} showed an intermediate level of incompatibility with S^{28} pollen: an average of 1.1 seeds per flower.

Thus, the degree of S^{28} pollen rejection by the plants carrying the *SRK*^{28} transgene appears to be enhanced by the presence of the *SLG*^{28} transgene, and to a lesser extent by the presence of the endogenous *SLG*^{52} gene, when compared with the plants carrying the *SRK*^{28} transgene and the endogenous *SLG*^{60} gene. Notably, both S^{28} and S^{52} belong to class I *S* haplotypes and S^{60} belongs to class II *S* haplotypes, and the degree of S^{28} pollen rejection by SRK^{28} appears to correlate with the degree of amino-acid identity between its S domain and the SLGs (98% identity with SLG^{28}, 76% identity with SLG^{52} and 65% identity with SLG^{60}): the higher the identity, the stronger the rejection. As SLGs are secretory proteins and are abundantly present in the cell wall of the stigma[20], it is possible that an SLG has a role in the binding of its cognate SRK with the pollen ligand by forming a complex with the S domain of the SRK and facilitating the process of the recognition reactions. The ability of the complex formation between SLGs and SRKs might then decrease as the sequence identity between them decreases.

In conclusion, our study provides the first direct evidence to our knowledge that the *S* haplotype specificity of the stigma in self-incompatibility recognition reactions of *Brassica* is solely determined by SRK and that SLG, although not involved in *S* haplotype specificity, can enhance the process of self-incompatibility recognition reactions. These findings, coupled with the identification of the potential pollen ligand, SCR, in self-incompatibility interactions[12] and the finding that ARC1, a protein that interacts with the kinase domain of SRK, is required for stigma function in self-incompatibility interactions[21], will open up opportunities for the molecular/biochemical characterization of SRK/SCR interactions and the SRK-mediated signalling pathway, which both lead to pollen rejection. In addition, our study shows that, by judicious selection of an *SRK* transgene and an *S* genotype of the recipient, it is feasible to confer a new self-incompatibility specificity on the stigma of *Brassica* plants. □

Methods

Construction of transformation vectors

SRK^{28} (same as *SRK*^{9}) complementary DNA (ref. 18) and *SLG*^{28} (same as *SLG*^{9}) genomic DNA (ref. 22) were isolated from *B. rapa* plants homozygous for the S^{28} haplotype (a class I haplotype). A chimaeric gene, comprising the promoter region (3.2 kilobases (kb)) of *SLG*^{28}, the coding region of *SRK*^{28} cDNA and the nopaline synthase transcription terminator, was inserted into a binary vector pSLJ491 (ref. 23) to yield pSLJSRK28. A 7.3-kb *Hin*dIII fragment of *SLG*^{28} genomic DNA containing the promoter region (3.2 kb), the coding region, and the downstream flanking sequence (2.8 kb), was inserted into a binary vector pBin19 to generate pBINSLG28 (ref. 19).

Plant transformation

$S^{52}S^{60}$ heterozygotes[13] used in the transformation with pBINSLG28 were a commercial hybrid variety cv. Osome (Takii Seed Co.) of self-incompatible *B. rapa*. S^{60} homozygous plants used in the transformation with pSLJSRK28 were obtained from bud self-pollination of Osome. We previously determined that S^{52} belongs to class I *S* haplotypes and S^{60} to class II *S* haplotypes. We also established the dominant/recessive relationships among S^{28}, S^{52} and S^{60} as follows. S^{52} was dominant over S^{60} in pollen; S^{52} and S^{60} were co-dominant in stigma[13]; S^{28} was co-dominant with S^{52} and dominant over S^{60} in pollen; S^{28} was co-dominant with S^{52} and S^{60} in stigma. The hypocotyl explants were transformed with *Agrobacterium tumefaciens* strain pCIB542/A136 harbouring pSLJSRK28 or pBINSLG28 according to published methods[24].

DNA gel blot analysis

Total DNA was extracted from young leaves. Two µg DNA digested with *Hin*dIII, *Bam*HI or *Xba*I were electrophoresed on 0.8% agarose gels and transferred to nylon membranes for hybridization with digoxigenin (Dig)-labelled cDNA encoding the kinase domain of *SRK*^{28} (*SRK*^{28}-KD) or *SLG*^{28} (ref. 18), according to the manual of the Dig Nucleic Acid Detection Kit (Boehringer Mannheim). After hybridization, the membranes were washed twice in 0.1% SSC, 0.1% SDS at 65 °C for 20 min.

Pollination test

The pollination data are based on pollen tube counts that were determined by ultraviolet-fluorescence microscopy[25] with more than 30 flowers in respective pollinations.

RNA gel blot analysis

For RNA gel blot analysis, poly(A)$^+$ RNA was isolated from stigmas and anthers at one day before anthesis by using the Micro Fast Track messenger RNA Isolation Kit (Invitrogen). After denaturation in glyoxal, the RNA was loaded on 1% agarose gels in 10 mM sodium phosphate buffer pH 7.0 for electrophoresis. For the S^{28} homozygote, 1 µg of poly(A)$^+$ RNA was loaded; for all the other plants, 2 µg of poly(A)$^+$ RNA was loaded. The RNA was transferred to nylon membranes and hybridized with Dig-labelled *SRK*^{28}-KD, *SLG*^{28} cDNA, *SLG*^{60} PCR fragment[13] and vacuolar H$^+$ *ATPase* (*V-ATPase*) fragment. The

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V-ATPase fragment was amplified from the genomic DNA of Osome plants by PCR using the primers of its conserved region[26]. Washing and detection were carried out as described for DNA gel blot analysis.

Detection of transgene by PCR

S-genotypes of self-pollinated and crossed progeny were determined as follows. Genomic DNA was prepared from young leaves. The SLG^{28} transgene was amplified by PCR with SLG^{28} specific primers, PS18 and PS15 (ref. 27). The SRK^{28} transgene was amplified by PCR with SRK^{28} specific primers, PK28 (5′-CCTCTTATATTTTTCTGCCTCTGG-3′) and PK4 (ref. 28). PK28 was designed on the basis of the nucleotide sequence of the transmembrane domain of SRK^{28}, and PK4 was designed on that of exon 4 of SRK^{28}. The expected PCR product of the SRK^{28} gene was 648 bp. To discriminate between the SRK^{28} transgene and the endogenous SRK^{52} gene, PCR-RFLP was conducted by using the primers, PS18 and B (ref. 29) to obtain PCR products, which were then digested with *Mbo*I and electrophoresed on 5% polyacrylamide gels; the fragments were visualized by silver-staining. The endogenous SLG^{52} and SLG^{60} genes were amplified by PCR with PS5 and PS15, and PS3 and PS21, respectively[13,27].

Immunoblot analysis

Total protein was extracted from five stigmas for the S^{28} homozygote and ten stigmas for all the other plants in 50 mM Tris-HCl pH 7.5. The extract was subjected to thin-layer polyacrylamide gel IEF (Ampholine PAG Plate, pI 3.5–9.5; LKB Pharmacia) and transferred to PVDF membranes (Millipore) by electroblotting. SLG proteins were detected with the anti-SLG^{43} (a class I SLG) monoclonal antibody which crossreacts with most of class I SLGs (ref. 30).

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Structure of the winged-helix protein hRFX1 reveals a new mode of DNA binding

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Regulatory factor X (RFX) proteins are transcriptional activators that recognize X-boxes (DNA of the sequence 5′-GTNRCC(0–3N)RGYAAC-3′, where N is any nucleotide, R is a purine and Y is a pyrimidine) using a highly conserved 76-residue DNA-binding domain (DBD). DNA-binding defects in the protein RFX5 cause bare lymphocyte syndrome or major histocompatibility antigen class II deficiency[1]. RFX1, -2 and -3 regulate expression of other medically important gene products (for example, interleukin-5 receptor α chain, IL-5Rα)[2]. Fusions of the ligand-binding domain of the oestrogen receptor with the DBD of RFX4 occur in some human breast tumours[3]. Here we present a 1.5 Å-resolution structure of two copies of the DBD of human RFX1 (hRFX1) binding cooperatively to a symmetrical X-box[4,5]. hRFX1 is an unusual member of the winged-helix subfamily of helix–turn–helix proteins[6] because it uses a β-hairpin (or wing) to recognize DNA instead of the recognition helix typical of helix–turn–helix proteins. A new model for interactions between linker histones and DNA is proposed.

We used multiwavelength anomalous dispersion (MAD) to determine the structure of the hRFX1 DBD (Fig. 1) recognizing a symmetrical X-box (see Methods). Unexpectedly, the hRFX1 DBD proved to be a member of the winged-helix hepatocyte nuclear factor (HNF)-3/*forkhead*-related subfamily of HTH transcription factors (Fig. 2, reviewed in ref. 7). The DBD consists of three α-helices (H), three β-strands (S) and three connecting loops (L), arranged in the order H1-S1-H2-L1-H3-L2-S2-W1-S3. The third loop, connecting β-strands S2 and S3, forms wing W1 of the winged-helix motif. Residues forming the hydrophobic core of the DBD originate from the secondary structural elements H1, S1, H2,

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