An Aquaporin-Like Gene Required for the *Brassica* Self-Incompatibility Response

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Self-incompatibility in *Brassica* refers to the rejection of self-related pollen and is mediated by a receptor protein kinase localized to the plasma membrane of the stigma epidermis in the flower. The recessive mutation *mod* eliminates self-incompatibility in the stigma. In *mod* mutants, self-compatibility was shown to be associated with the absence of transcripts encoded by an aquaporin-related gene. This observation suggests that a water channel is required for the self-incompatibility response of *Brassica*, which is consistent with the concept that regulation of water transfer from the stigma to pollen is a checkpoint in the early events of pollination in the crucifer family.

In many species of flowering plants, self-fertilization is prevented or reduced by the mechanism of self-incompatibility (*1*). In the crucifer *Brassica*, this genetic system ensures that, in a field of wild mustard plants, every instance of self-pollination is inhibited whereas most cross-pollinations are not. This mating control is achieved within minutes after capture of pollen by the papillar cells of the stigma epidermis (Fig. 1) and is manifested by the disruption of pollen tube development (*2*). The recognition of pollen in self-incompatible crucifers is controlled by haplotypes of the *S* locus (designated S_1, S_2, ... S_n), each of which consists of a cluster of genes that are inherited as a unit (*3*). Two genes within the cluster encode papillar cell–specific surface receptors that are required for the stigma to discriminate against self-related pollen (*3*–*6*). It is thought that, on self-pollination, the *S* locus–encoded and plasma membrane–localized receptor kinase SRK detects a pollen-borne ligand and initiates an intracellular phosphorylation cascade within the stigmatic epidermal cell that blocks the development of incompatible pollen (*3*). The specific events of the self-incompatibility signal transduction pathway are not known. Products encoded by two members of the thioredoxin gene family interact with the kinase domain of SRK in a yeast two-hybrid screen, but their function in self-incompatibility is unclear (*7*).

Loci that are not linked to the *S* locus are also required for self-incompatibility in the stigma (*8*) and may encode proteins of the SRK signaling pathway. We analyzed one such genetic modifier or self-incompatibility, the spontaneous *mod* mutation of *Brassica campestris* (*8*). Reciprocal crosses (*9*) between the self-incompatible (SI) S_8/S_8 homozygote and the self-compatible (SC) USDA C634 strain indicate that the *mod* mutation compromises stigma function but not pollen function. The F_1 plants derived from these crosses are SI, which indicates that self-compatibility is the recessive trait. We forced self-fertilization of one F_1 plant by manual pollination of immature buds to produce an F_2 population of 260 plants. Pollination analysis of the F_2 plants revealed that the ratio of SI:SC plants approximated a modified dihybrid ratio of 9:7 in the entire population and a ratio of 3:1 among S_8/S_8 homozygotes. These results are consistent with the segregation of a nonfunctional *S* haplotype derived from strain C634 (*6*) and of a recessive mutation (*mod*) that is epistatic and not linked to the *S* locus.

To identify RNA transcripts encoded by or controlled by the *MOD* locus, we used the technique of differential display combined with reverse transcription–polymerase chain reaction analysis (DDRT-PCR) (*10*) with RNA isolated from the stigmas of 10 S_8/S_8 SI plants (the SI pool, expected to be genotypically *MOD/MOD* or *MOD/mod*) and RNA isolated from the stigmas of 10 S_8/S_8 SC plants that exhibited >500 pollen tubes per stigma on self-pollination (the SC pool, expected to be genotypically *mod/mod*). This strategy minimizes interference from irrelevant polymorphisms of individual plants, because the pooled samples should be randomized for genomic sequences with the exception of those located near or at the target locus.

We analyzed the RNA pools by DDRT-PCR (*11*) and obtained 29 cDNA differences in PCR reactions with 240 primer combinations. The DDRT-PCR products were used to identify restriction fragment length polymorphisms (RFLPs) genetically linked to the *MOD* locus. We detected a 119–base pair (bp) fragment, DD33, that was amplified from the SI pool but not from the SC pool. In SC plants, DD33 hybridized to a 1.6-kb Bgl II fragment, whereas in SI plants it hybridized to an 8-kb Bgl II fragment or to both 8- and 1.6-kb fragments (Fig. 2A). A 1.1-kb cDNA was isolated by screening an S_8/S_8 stigma cDNA library with DD33 and was shown to hybridize to the 8- and 1.6-kb restriction fragments (designated $DD33^{MOD}$ and $DD33^{mod}$, respectively) identified by the DD33 probe, as well as to a family of related genes (Fig. 2B). Sequence analysis revealed that the DD33 sequence was identical to the 3′ untranslated region of the 1.1-kb cDNA and to the corresponding genomic sequences contained on the 8-kb $DD33^{MOD}$ and 1.6-kb $DD33^{mod}$ restriction fragments.

To determine whether DD33 was derived from or linked to *MOD*, we extended RFLP analysis to other plants in the F_2 population (79 S_8/S_8 and 79 S_8/S_{f2} plants were tested). Although several restriction fragments identified by the 1.1-kb cDNA probe were polymorphic in this group of plants, only the $DD33^{MOD}$ and $DD33^{mod}$ fragments cosegregated with pollination phenotype (Fig. 2B). All SI plants contained the $DD33^{MOD}$ fragment, and all plants were homozygous for $DD33^{mod}$ were SC. The cosegregation of the DD33 RFLP with pollination phenotype was also observed in the F_3 generation (Fig. 2C) and in backcrosses to the C634 strain. The 10 plants of the SC pool produced only SC progenies (17 plants in each of 10 families). Two F_2 plants of the SI pool, predicted to be *MOD/mod* on the basis of DD33 poly-

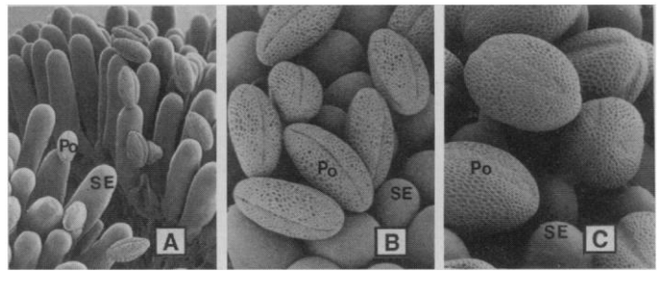

Fig. 1. Hydration of pollen in a compatible pollination in *Brassica*. The scanning electron micrographs show the changes in shape and volume of pollen grains at 5 min (**A** and **B**) and 45 min (**C**) after self-pollination. In (C), pollen grains are fully hydrated and show a three- to fourfold increase in volume relative to the unhydrated pollen grains in (A) and (B). Po, pollen; SE, stigma epidermal cell. Magnification, ×135 (A) and ×325 (B and C).

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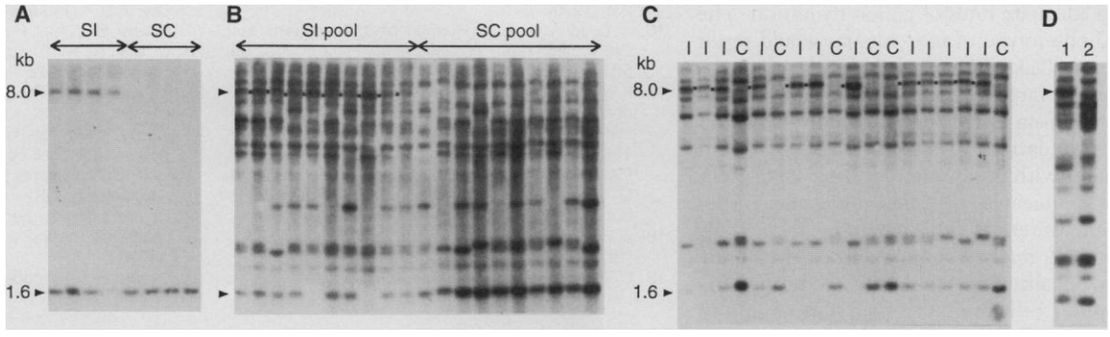

Fig. 2. DNA gel blot analysis of DD33 and related sequences in SI and SC plants. Gel blots of DNA digested with Bgl II were probed with either the 119-bp DD33 fragment (**A**) or the 1.1-kb DD33 cDNA (**B** through **D**). (A) DNA from four SI and four SC plants used for DDRT-PCR. The SC plants exhibit a 1.6-kb restriction fragment (and are $DD33^{mod}/DD33^{mod}$). One SI plant exhibits an 8-kb fragment (and is $DD33^{MOD}/DD33^{MOD}$), and three SI plants exhibit both 8- and 1.6-kb fragments (and are $DD33^{MOD}/DD33^{mod}$). (B) DNA from the 10 SI and 10 SC plants of the RNA pools. Arrowheads indicate the positions of the 8- and 1.6-kb fragments, and small squares mark the 8-kb fragment in each of the SI plants. The sixth lane from the left represents the plant whose progeny is shown in (C). (C) DNA from SI (I) and SC (C) F_3 progenies. (D) DNA from an SI undeleted *MOD/mod* plant (lane 1) and an SC γ-irradiated mutant plant (lane 2) that is missing the 8-kb $DD33^{MOD}$ fragment (arrowhead).

morphism, yielded expected ratios of 1:1 and 3:1 SI:SC plants for backcrossed and selfed progenies (*12*). Independent evidence for the linkage of the DD33 sequence to the *MOD* locus was also provided by analysis of SC mutants generated by pollinating a *mod/mod* strain with γ-irradiated *MOD/MOD* pollen (*13*); in this screen, eight independent SC mutants lacked the $DD33^{MOD}$ fragment (Fig. 2D).

The 119-bp DD33 probe detected a 1.1-kb transcript in polyadenylated [poly(A)$^+$] RNA (*14*) from stigmas, leaves, and anthers of *MOD/MOD* plants, but only a weak signal was apparent after prolonged exposures of blots with RNA from *mod/mod* plants (Fig. 3A), indicating that the $DD33^{mod}$ allele is hypomorphic. The steady-state amounts of DD33 transcripts in *MOD* stigmas are at least 30 times those in *mod* stigmas. No change in the abundance of the DD33 transcript was observed in stigma samples from *MOD* or *mod* plants in response to self-pollination (Fig. 3B) or cross-pollination.

The 1.1-kb cDNA contained an open reading frame that encodes 286 amino acids (Fig. 4) similar in sequence to the MIP (major intrinsic protein) membrane proteins from plants, mammals, yeasts, and bacteria (*15*). MIP proteins form channels with six membrane-spanning domains and are thought to facilitate the transport of water and other small molecules across membranes. The DD33 coding region is most similar to those of the aquaporin genes, whose products transport water; in plants, these include genes that encode tonoplast intrinsic proteins (*16*) as well as plasma membrane intrinsic proteins such as *Arabidopsis* RD28-PIP, PIP1A, PIP1B, and PIP2 (*17*). The DD33 gene contains residues conserved in the MIP gene family (*15*). It shows the greatest sequence identity (98.6%) to PIP1B and it clearly belongs in the PIP1 subgroup of genes. Therefore, it is likely that the MOD protein forms a channel in the plasma membrane and transports water.

Hydration is important for pollen germination and subsequent pollen tube development. Pollen grains, which are released from the anther in a relatively dessicated state, must draw water and perhaps other substances from the receptive stigmatic surface (Fig. 1). This stigma-to-pollen transfer of material is a regulated process in plants such as *Brassica* and other crucifers whose dry stigmas lack surface secretions (*2*, *18*). Incompatible pollinations in this family are often manifested by the failure of pollen hydration (*18*), and certain male-sterile *Arabidopsis* mutants produce pollen that fails to hydrate on the stigma (*19*). Therefore, water transfer from stigmatic papillar cells to pollen grains may be a checkpoint at which the outcome of pollen-stigma interactions in the crucifer family is determined.

We propose that the MOD protein functions in the regulation of water availability at the papillar cell surface. The water channel would be required for self-incompatibility as a component of the SRK signaling pathway. The activation of SRK on self-pollination would result in MOD activation and an increase in the flow of water into the papillar cell away from pollen, thus prevent-

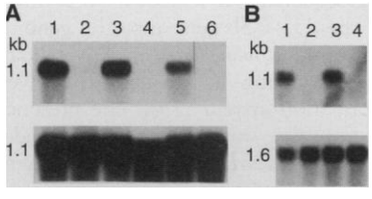

Fig. 3. Gel blot analysis of DD33 transcripts in SI (*MOD/MOD*) and SC (*mod/mod*) plants. Polyadenylated RNA was isolated from (**A**) SI stigmas (lane 1), SC stigmas (lane 2), SI leaves (lane 3), SC leaves (lane 4), SI anthers (lane 5), and SC anthers (lane 6), as well as from (**B**) unpollinated SI (lane 1) and SC (lane 2) stigmas and self-pollinated SI (lane 3) and SC (lane 4) stigmas. Blots were hybridized first with the 119-bp DD33 probe (upper panels) and subsequently with the 1.1-kb DD33 cDNA [lower panel in (A)] or with an actin probe [lower panel in (B)].

Fig. 4. Sequence alignment of the predicted $DD33^{MOD}$ (MOD) protein (GenBank accession number AF004293) with three *Arabidopsis* plasma membrane aquaporins: PIP1B (GenBank Z17424), PIP1A (GenBank X75881), and PIP2A (GenBank X75883). Dashes represent gaps introduced to optimize alignment and dots indicate residues in the PIP sequences that are identical to the MOD sequence. The residues that are conserved in members of the MIP family (*15*) are underlined and the serine residues that are potential targets for phosphorylation are indicated by arrowheads. Residue numbers are indicated on the right.

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MOD     MEGKEEDVRVGANKFPERQPIGTSAQSDKDYKEPPPAPLFEPGELASWSFWRAGIAEFIATF   62
PIP1B   ..........................................L.....................   62
PIP1A   ..........................................F......S..............   62
PIP2A   .AKDV.A.PGEG--.QT.----------..QD.....FIDGA..KK...Y..V....V..L   45

MOD     LFLYITVLTVMGVKRSPSM------CASVGIQGIAWAFGGMIFALVYCTAGISGGHINPAVT   118
PIP1B   ................N.------.....................................   118
PIP1A   ................N.------.....................................   114
PIP2A   .........I.Y.IQSDTDAGGVD.GG...L...........I.................   103

MOD     FGLFLARKLSLTRAVYYIVMQCLGAICGAGVVKGFQPKQYQALGGGANTVAPGYTKGSGLGA   180
PIP1B   ..........................................................I.H.....   180
PIP1A   ............L.....................................H..........   172
PIP2A   ........V..P..LL..IA........V.F..A..SSY.TRY.....SL.D..ST.T..A.   161

MOD     EIIGTFVLVYTVFSATDAKRNARDSHVPILAPLPIGFAVFLVHLATIPITGTGINPARSLGA   242
PIP1B   ..........................................................I.H.....   242
PIP1A   ..........................................................A.....   230
PIP2A   ................P..S.......V...........M.................F..   219

MOD     AIIFNKDNAWDDHWVFWVGPFIGAALAALYHVIVIRAI----PFKSRS                286
PIP1B   ..........................................----.....               286
PIP1A   ...Y...HS.....................V.....----.....                     270
PIP2A   .V.Y..SKP.....I..........I..F..QF.L..SGSKSLGSF..AANV               267
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ing adequate rates of pollen hydration. The SC phenotype of *mod* stigmas would result from a lack of channel molecules that can serve as targets of SRK activity. Although a mechanism of self-incompatibility based on the regulation of water availability is consistent with pollination biology, some MIPs are permeable to small molecules other than water (*15*). Therefore, the MOD channel might promote either the efflux and localized accumulation at the pollen–papillar cell interface of substances inhibitory to pollen germination and tube ingress, or the influx, and therefore localized depletion from the cell wall, of substances required for pollen germination and tube growth. The rapid modulation of membrane permeability in response to self-pollination could be brought about by reversible phosphorylation of MOD channel proteins, resulting in an increase in their transport activity (*20*) or in their rapid recruitment to the plasma membrane (*21*).

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Aluminum Tolerance in Transgenic Plants by Alteration of Citrate Synthesis

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Aluminum when in soluble form, as found in acidic soils that comprise about 40 percent of the world's arable land, is toxic to many crops. Organic acid excretion has been correlated with aluminum tolerance in higher plants. Overproduction of citrate was shown to result in aluminum tolerance in transgenic tobacco (*Nicotiana tabacum*) and papaya (*Carica papaya*) plants.

When solubilized in acid soils, Al (primarily in the form of Al^{3+}) is toxic to many crops and is the major limiting factor for plant productivity on these soils. Soil acidification accelerated by certain farming practices and by acid rain affects about 40% of the arable land worldwide (*1*, *2*). Although crop production on acid soils can be sustained by application of lime, runoff pollution is an undesirable side effect. Thus, the production of Al-tolerant plant varieties either by conventional breeding or genetic engineering appears to be the best solution.

Symptoms of Al toxicity are similar to those of nutrient deficiency (*3*, *4*), probably owing to the inhibition of root development caused by the action of Al at the root tip (*2*, *5*). In simple nutrient solutions, micromolar concentrations of Al can begin to inhibit root growth within 60 min (*6*).

Plants show a range of natural resistance to Al toxicity (*4*, *7*). Tolerance may occur by Al exclusion from the root tip (*8*) and in several cases has been closely correlated with an increased capacity to release organic acids, such as citric acid (*9*, *10*), which may chelate Al^{3+} outside the plasma membrane, thereby preventing its uptake (*10*). In vitro, organic acids do indeed decrease the toxic effect of Al, citrate being more effective than succinate or malate (*11*).

To examine whether increased citrate production and release in transgenic plants could confer Al tolerance, we produced transgenic tobacco and papaya plants that overexpress a citrate synthase (CS) gene from *Pseudomonas aeruginosa* in their cytoplasm. We targeted the bacterial CS to the cytoplasm rather than the mitochondria to avoid redistribution of carbon from citrate synthesis to other components of the Krebs cycle.

We constructed a chimeric gene with the coding sequence of the *P. aeruginosa* CS gene (CSb) (*12*) fused to the 35*S* promoter from the cauliflower mosaic virus and *nos* 3′-end sequences (35S-CSb). We introduced the 35S-CSb gene into the genome of tobacco (*Nicotiana tabacum* L var. xanthi) plants using a Ti plasmid–derived transformation system (*13*). The presence of the transgene was confirmed in 30 independent transgenic lines by Southern (DNA) blot hybridization analysis (*14*). The T2 progeny of homozygous plants harboring a single copy of the transgene were selected for further analysis. No obvious phenotypical differences were observed between the plants harboring the 35S-CSb construct and the controls when grown under greenhouse conditions. Four lines expressing between two- to threefold higher levels of CS (*15*) than the control were further analyzed (Fig. 1).

To determine whether the cytoplasmic

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