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Report

A Cryptic Modifier Causing Transient Self-Incompatibility in *Arabidopsis thaliana*

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Summary

Breakdown of the pollination barrier of self-incompatibility (SI) in older flowers, a phenomenon known as pseudo-self-compatibility or transient SI, has been described as an advantageous reproductive assurance strategy that allows selfing after opportunities for out-crossing have been exhausted [1–9]. Pseudo-self-compatibility is quite prevalent as a mixed mating strategy in nature, but the underlying molecular mechanisms are not known. We had previously shown that *Arabidopsis thaliana* exhibits cryptic natural variation for pseudo-self-compatibility, which is uncovered by transformation of different accessions with SI specificity-determining *SRK* and *SCR* genes from its self-incompatible sister species *A. lyrata* [10, 11]. Here, by using this transgenic *A. thaliana* model, we show that pseudo-self-compatibility is caused by a hypomorphic allele of *PUB8,* an *S*-locus-linked gene encoding a previously uncharacterized ARM repeat- and U box-containing protein that regulates *SRK* transcript levels. This is the first gene underlying pseudo-self-compatibility to be identified and the first report in which cryptic natural variation unveiled by a transgene enabled the cloning of a gene for a complex trait.

Results and Discussion

A. thaliana is a highly self-fertile species that has nonfunctional alleles of the *S*-locus receptor (*SRK*) and *S*-locus cysteine-rich protein (*SCR*) genes [12], which are, respectively, the stigma and pollen determinants of SI specificity in the Brassicaceae. In self-incompatible species, allele-specific interactions between SRK and SCR allow recognition of self pollen, activation of the receptor, and initiation of a signaling cascade that leads to rejection of self pollen at the stigma surface [13]. We previously showed that different geographical accessions of *A. thaliana* exhibited significant differences in the ability of their stigmas to sustain a strong SI response over the course of their development when transformed with *SRK* and *SCR* genes isolated from the *Sb* haplotype of self-incompatible *A. lyrata* [10, 11]. In C24, stigmas showed a strong SI phenotype that persisted throughout flower maturation, and these plants did not set seed [11]. In contrast, in Col-0 and RLD, stigmas exhibited pseudo-self-compatibility, with breakdown of SI at later stages of flower development resulting in full, albeit delayed, seed set [10, 11]. Because these differences were observed in several independent *SRKb-SCRb* transformants and are independent of transgene insertion site or copy number (Experimental Procedures), they must be due to polymorphisms among accessions.

Genetic analysis of a C24 *SRKb-SCRb* x RLD cross identified a recessive allele at one genetic locus as the major determinant of pseudo-self-compatibility in RLD, with minor effects exerted by recessive alleles at other loci. Preliminary mapping with 138 F2 plants located this major-effect locus to chromosome 4 between marker g3883 and SSLP marker D (Figure 1A, Table S1 in the Supplemental Data available online). A major locus underlying pseudo-self-compatibility in Col-0 was also mapped to the same region with 240 F2 plants from a C24 *SRKb-SCRb* x Col-0 cross. To eliminate the influence of minor-effect loci from the C24 *SRKb-SCRb* x RLD-derived population, a mapping population was created by selfing a recombinant inbred line (RIL) heterozygous for the major-effect locus. Fine-scale mapping with 3210 plants delimited this locus to an interval that encompasses the relic *A. thaliana S* locus [12] (Figure 1B). In Col-0, this interval is 34.1 kb and contains 6 annotated open reading frames (ORFs), including Ψ*SRK* (At4g21370) and Ψ*SCR* sequences [12], a small inverted repeat of part of the Ψ*SRK* kinase domain (At4g21366), two transposon-related ORFs (At4g21360 and At4tg21363), and an ARMADILLLO (ARM) repeat-/U box-containing ORF (At4g21350) (Figure 1B), all of which are also found in RLD. In C24, this genomic region is only 8.6 kb and lacks all of the aforementioned ORFs except At4g21350 (Figure 1B) [14].

A pseudo-self-compatible (PSC) *SRKb-SCRb* recombinant inbred line (RIL200; Experimental Procedures) was transformed with C24-derived genomic fragments containing At4g21350 (4K7) or At4g21340 (4K6) as control (Figure 1B). Although 4K6 transformants exhibited full seed set, 4K7 transformants exhibited phenotypes ranging from full to no seed set, likely because of positional effects and copy number of the transgenes. Self-pollinations of 13 randomly chosen 4K7 transformants revealed reduced pollen tube growth on late-stage stigmas relative to PSC *SRKb-SCRb* plants and 4K6 controls (Figure S1). Microscopic analysis of self-pollinated stigmas in two transformants exhibiting pronounced reduction in seed set and one transformant that failed to set seed established that reduced seed set was due to an expansion of the window of SI expression into late-stage stigmas relative to PSC *SRKb-SCRb* plants (Figure 2). This trait cosegregated with the transgene (Table S2) in T2 progenies of primary transformants. Thus, At4g21350, also known as *PLANT U-BOX8* (*PUB8*) [15, 16], is the gene responsible for the difference in age-dependent pseudo-self-compatibility between RLD/Col-0 and C24.

Consistent with previous observations that At4g21350 and its *A. lyrata* ortholog exhibit much higher levels of nucleotide diversity than other loci [17, 18], as

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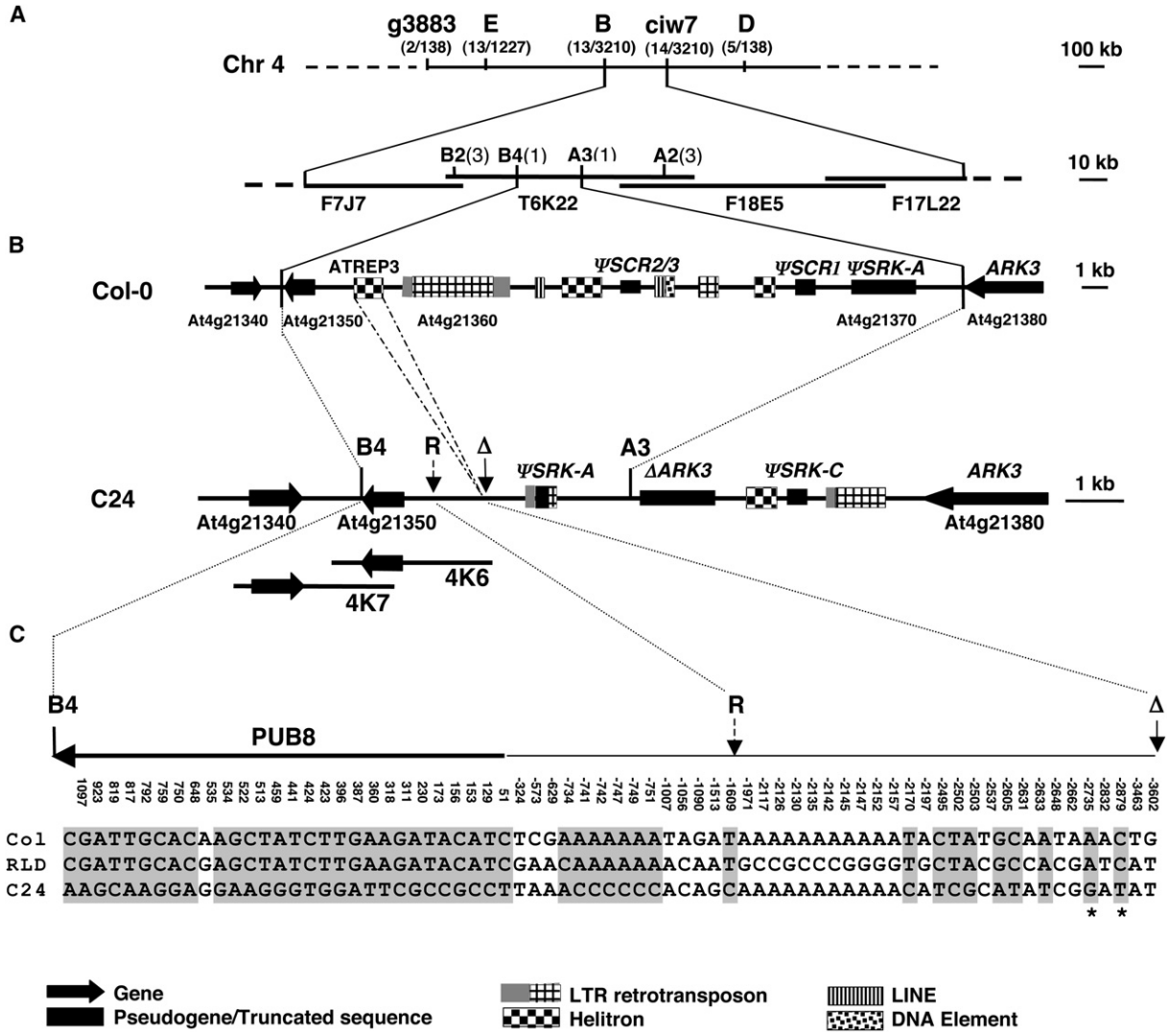

Figure 1. Map-Based Cloning of the *PUB8* Gene Underlying Variation for Pseudo-Self-Compatibility

(A) Mapping the target gene to a region of chromosome 4 within a BAC contig containing BAC T6K22, which spans the *S* locus. Molecular markers used for mapping are shown above the map (see Table S1 for amplification primers) along with the number of recombinant plants observed.

(B) Structural comparison of the *S*-locus region in Col-0 and C24, showing the annotated ORFs, the Ψ*SRK* and Ψ*SCR* sequences, and recombination breakpoints identified in fine mapping. The *S* haplotype of Col-0 and that of RLD, which has been determined to be very similar if not identical to Col-0 [14], are structurally very different from the C24 *S* haplotype as a result of deletions (ATREP3 helitron and LTR retrotransposon), a duplication (*ARK3*), and interhaplotypic recombination (presence of Ψ*SRK* remnants of two distinct alleles Ψ*SRK-A* and Ψ*SRK-C*). The recombination breakpoint (R), which defines one limit of the mapped region, and the site of the large deletion in C24 (Δ), which is located 3602 bp upstream of the At4g21350 coding region, are shown as landmarks for polymorphism studies. The DNA fragments 4K7 and 4K6 were derived from a C24 genomic clone and used for complementation of the pseudo-self-compatibility trait.

(C) Nucleotide polymorphisms in the *PUB8* coding region (thick line, with arrow indicating orientation) and 3602 bp of 5′ sequence (thin line) extending from the ATG initiating codon to deletion point Δ. Only variable nucleotides are shown, with numbers indicating positions relative to the "A" in the ATG initiating codon (position "1," with positions upstream of the ATG depicted as negative numbers and positions within the coding region downstream of the ATG depicted as positive numbers). Polymorphisms that distinguish C24 from Col-0 and RLD (shaded residues) may represent sites underlying pseudo-self-compatibility. Asterisks indicate two SNPs that create two transcription factor binding sites unique to C24: one for Dof (PLACE ID: DOFCOREZM) and another for MYB (PLACE ID: MYBCORE) proteins.

expected for genes tightly linked to the highly polymorphic *S* locus, the predicted PUB8^{C24} protein differs from PUB8^{RLD} and PUB8$^{Col\text{-}0}$ by 6 and 7 substitutions, respectively (Figure 1C and Figure S2). These polymorphisms are not responsible for differences in pseudo-self-compatibility between C24 and RLD or Col-0, however. Sequence analysis of a recombinant (R378) recovered in our mapping population determined that the recombination breakpoint occurred between 1175 bp and 1695 bp upstream of the *PUB8* initiating codon (Figures 1B and 1C), producing a chimeric segment consisting of the *PUB8^{C24}* protein-coding region and 5′ sequences from RLD (Figures 1C and 3A). Because the R378 recombinant and its progenies were PSC, the polymorphisms underlying differences for age-dependent pseudo-self-compatibility must lie within

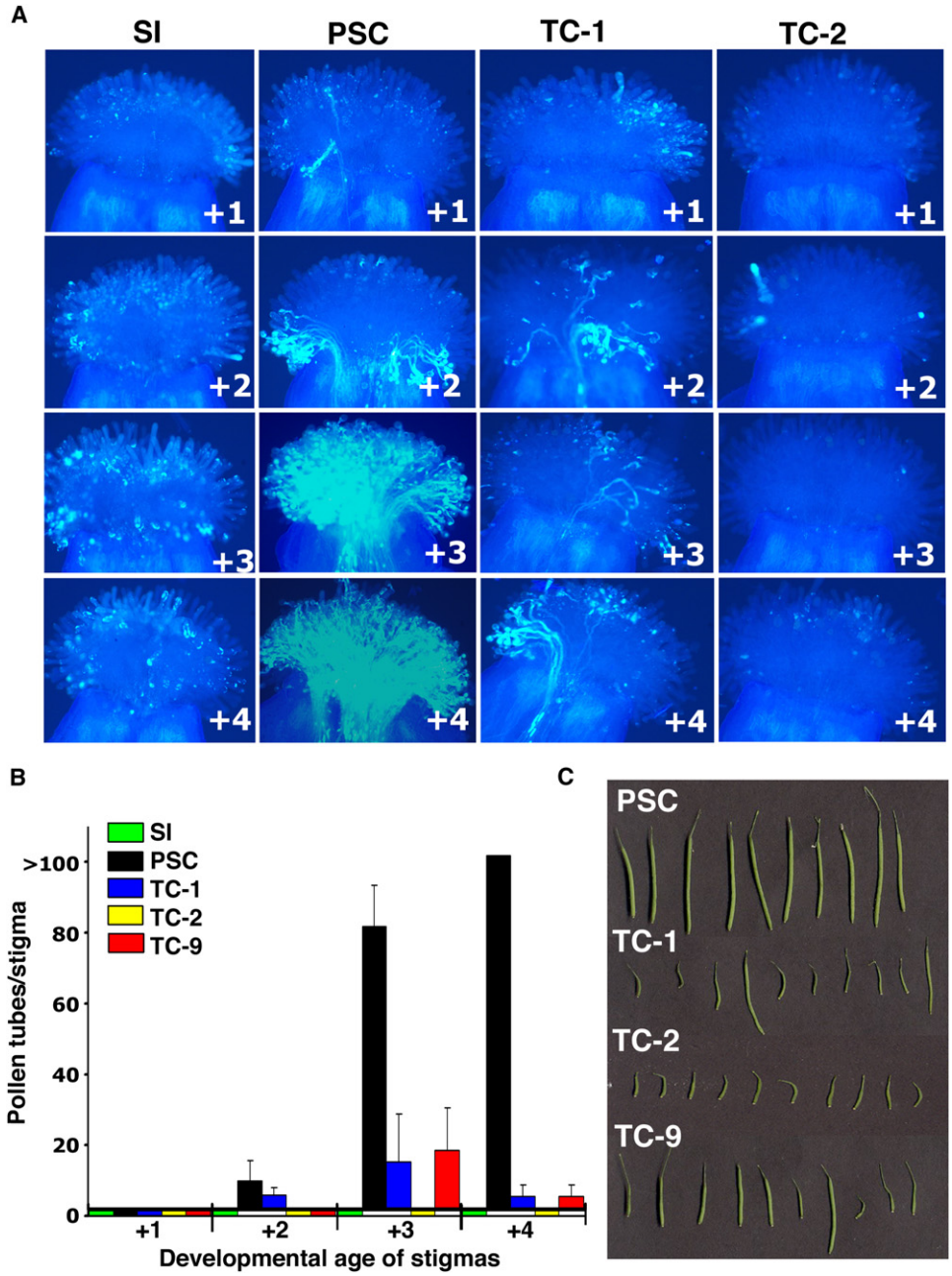

Figure 2. The Pseudo-Self-Compatibility Trait and Its Complementation with the *PUB8*^{C24} Transgene

(A) Micrographs of self-pollinated stigmas of *SRKb-SCRb* plants from just-opened flowers (+1) and progressively older flowers (+2, +3, and +4). Pollinations were performed with pollen from mature flowers and stigmas from developing floral buds and flowers along an inflorescence. For all plants, reciprocal pollinations to *SCRb*-expressing and wild-type plants established that stigmas and pollen were functional. SI, plants expressing developmentally stable SI; PSC, plants exhibiting age-dependent pseudo-self-compatibility (breakdown of SI starting from stage +2 stigmas); TC-1 and TC-2, plants transformed with the *PUB8*^{C24} allele and exhibiting complete (TC-2) or partial (TC-1) complementation of pseudo-self-compatibility.

(B) Quantitation of the SI response in developing stigmas from SI and PSC plants and from three independent *PUB8*^{C24}-complemented plants (TC-1, TC-2, TC-9). The number of pollen tubes formed per stigma is shown with standard errors.

(C) Seed setting in PSC and *PUB8*^{C24}-complemented plants. Note the fully expanded and seed-filled siliques of a PSC plant and the largely unexpanded (TC-1 and TC-2) or partially expanded (TC-9) siliques of *PUB8*^{C24}-complemented plants.

a 5.9 kb 5′ noncoding region between the two recombination breakpoints R and A3 (Figures 1B and 1C).

PUB8 is expressed ubiquitously (Figure 3B; transcriptional data at http://www.genevestigator.ethz.ch), suggesting that it may have multiple functions. It exhibits preferential expression in stigmas, however, especially in self-incompatible *A. lyrata* (Figure 3B). Previous genome-wide transcriptional profiling of *A. thaliana* stigmas [19] did not show *PUB8* to be more highly expressed in the stigma epidermis relative to other stigma cells, however. Quantitative real-time RT-PCR of stigmas revealed reduced *PUB8* transcript levels in

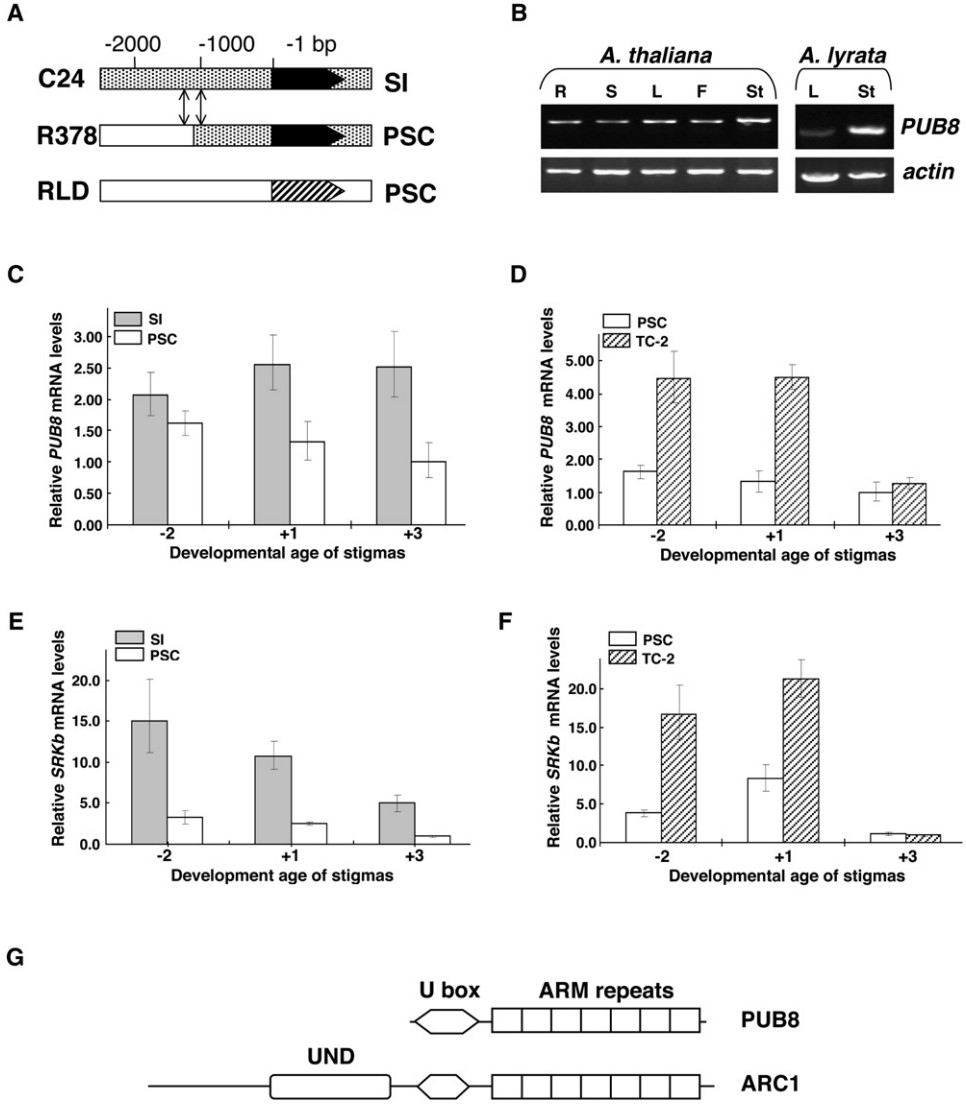

Figure 3. Analysis of *PUB8* Expression

(A) The recombination breakpoint that implicates the *PUB8* 5′ region in pseudo-self-compatibility. The *PUB8* coding region and its orientation are depicted by pentagons. Sequences 5′ and 3′ of the coding region are indicated by stippled boxes for C24 and white boxes for RLD, and the vertical arrows bracket the segment containing the recombination breakpoint. The phenotypes of plants carrying the parental and recombinant (R378) chromosomes are shown.

(B) Expression of *PUB8* in *A. thaliana* and *A. lyrata*. The left panel shows *PUB8* expression in *A. thaliana* roots (R), stem (S), leaves (L), flowers from which pistils were removed (F), and stigmas (St). The right panel shows *PUB8* expression in *A. lyrata* leaves (L) and stigmas (St).

(C–F) Quantitative real-time RT-PCR of stigmas at representative stages of development (−2, +1, and +3). The levels of *PUB8* and *SRKb* transcripts (with standard deviations based on three replicates) are compared between SI and PSC RILs (C and E) and between PSC RIL and $PUB8^{C24}$-complemented TC-2 plants (D and F).

(G) Diverged structures of the *A. thaliana* and *A. lyrata* PUB8 and *Brassica napus* ARC1 proteins.

PSC stigmas relative to SI stigmas, particularly at late stages of development (Figure 3C). Furthermore, restoration of SI in $PUB8^{C24}$-complemented stigmas was accompanied by increased *PUB8* transcript levels (Figure 3D). These results suggest that *PUB8* gene expression may be limiting in PSC stigmas, consistent with the change from developmentally stable SI to pseudo-self-compatibility being effected by modulation of *PUB8* gene expression. Another example of a spontaneous mutation to partial self-compatibility caused by reduced gene expression was previously described in *Brassica* [20]. For *PUB8,* the reduced expression associated with pseudo-self-compatibility might result from nucleotide substitutions in 5′ *cis*-regulatory sequences that distinguish RLD and Col-0 from C24, or from the influence of retroelement and helitron sequences that are found in RLD and Col-0 but are missing in C24 (Figures 1B and 1C).

Interestingly, at the early −2 and +1 stages, the stigmas of PSC plants also expressed reduced levels of *SRKb* transcripts relative to the stigmas of SI plants (Figure 3E), and *SRKb* transcripts were increased concomitant with increased *PUB8* transcripts in $PUB8^{C24}$-complemented stigmas (Figure 3F). However, no

significant differences were observed in *PUB8* and *SRKb* transcript levels between PSC and $PUB8^{C24}$-complemented stigmas at the late +3 stage (i.e., when SI breaks down in PSC stigmas), presumably because by this stage, stigmas would have already accumulated PUB8 and SRKb proteins to the levels necessary for sustained inhibition of self pollen. Taken together, these results suggest that PUB8 regulates the levels of *SRKb* transcripts and that the breakdown of SI associated with reduced *PUB8* expression is due in large part to reduced *SRKb* mRNA, causing suboptimal levels of SRKb protein at late stages of stigma development. PUB8 action cannot be specific to *SRKb,* however. Because expression of the *SRKb* transgene in *A. thaliana* C24 stigmas is a faithful recapitulation of *SRKb* expression in its native *A. lyrata Sb* stigma context, PUB8 must rather function as a general regulator of endogenous *SRK* transcript levels in the stigmas of self-incompatible *Arabidopsis* species.

Plant U-box proteins function in hormone signaling, defense, and cell death [21–24], but little is known about how they effect these biological roles [25]. The presence of a U-box domain having all of the canonical residues of functional U-box E3 ligases [15, 16] (Figure 3G and Figure S2) suggests that PUB8 functions in ubiquitination, whereas its ARM repeats suggest that it interacts with other proteins [20]. PUB8 might regulate *SRKb* transcript levels indirectly by ubiquitinating a factor required for transcription or for transcript stability, either causing its degradation or resulting in its subcellular redistribution [26]. In *Brassica napus*, the U-box/ARM protein ARC1 (BnARC1) is an E3 ligase that interacts with the SRK kinase domain and has been proposed to cause the degradation of a negative regulator of SI or of a factor required for successful pollen tube growth [27–29]. *PUB8* differs from *BnARC1* and its most closely related *A. thaliana* homolog At1g29340/*PUB17* [15, 16] in several respects. *PUB8* and *BnARC1* share only ~25% amino acid sequence identity; PUB8 lacks the UND domain (Figure 3G and Figure S2), which in BnARC1 contains a functional nuclear localization signal, a coil-coil domain, and a leucine zipper domain [29]; *PUB8* is expressed in all tissues analyzed (Figure 3A), unlike *BnARC1,* which is expressed specifically in stigmas [27]; and PUB8 does not interact with the SRKb kinase domain in yeast two-hybrid assays (data not shown). Thus, PUB8 represents a novel U-box/ARM repeat protein required for SI.

The location of *PUB8* at the *S* locus of *Arabidopsis* species is intriguing. There are few if any known examples of tight genetic linkage between a regulatory gene and its target. Furthermore, because of reduced recombination in the *S*-locus region, a mutation at a modifier locus tightly linked to the SI recognition genes is likely to have a significant impact on the distribution of *S* haplotypes in natural populations. Indeed, the *S* haplotype carried by RLD and Col-0 is the most prevalent in the species [11, 17]. Although maintenance of SI requires that matched alleles of the *SRK* and *SCR* genes remain in strict linkage disequilibrium, no such requirement dictates the linkage of other SI-related genes to each other or to the *S* locus. Indeed, the *Brassica S* locus, which occurs in a genomic context different from that in *Arabidopsis* species, is not flanked by a *PUB8*-like gene [30]. Although the linkage of *PUB8* to the *Arabidopsis S* locus may be fortuitous, it is tempting to speculate that the *SRK-SCR-PUB8* cluster represents a relic of a larger ancestral *S* locus that included not only the SI self-recognition genes but also other genes required for SI. Genes of this hypothetical SI supergene cluster would have subsequently been dispersed to different chromosomal locations by divergent genome rearrangements in the *Brassica* and *Arabidopsis* lineages.

Table 1. Association of $PUB8^{C24}$ and $PUB8^{RLD/Col\text{-}0}$ Alleles with Self-Incompatibility and Pseudo-Self-Incompatibility, Respectively, in *SRKb-SCRb* Transgenic Plants of Different *A. thaliana* Accessions

Accession	ABRC Number	*PUB8* Allele	Phenotype
C24	CS22620	$PUB8^{C24}$	SI
RLD	CS913	$PUB8^{RLD/Col\text{-}0}$	PSC
Col-0	CS22625	$PUB8^{RLD/Col\text{-}0}$	PSC
WS-0	CS22623	$PUB8^{RLD/Col\text{-}0}$	PSC
Mt-0	CS6799	$PUB8^{RLD/Col\text{-}0}$	PSC
Nd-1	CS22619	$PUB8^{RLD/Col\text{-}0}$	PSC
No	CS1394	$PUB8^{RLD/Col\text{-}0}$	PSC

The $PUB8^{C24}$ and $PUB8^{RLD/Col\text{-}0}$ alleles were identified with SSLP marker C (Table S1).

Another intriguing question is whether *PUB8*-mediated pseudo-self-compatibility produced a transitional phase of mixed mating in the evolutionary switch from outcrossing to inbreeding in the *A. thaliana* lineage, at least in some populations. This question is particularly important in view of empirical data [11, 14] and population genetic considerations [31] that do not support a previously proposed view [17] of the switch to self-fertility as resulting from a complete loss of SI effected by a selective sweep of the Col-0 Ψ*SCR1* allele (Figure 1B). There is increasing evidence for multiple origins of self-fertility in *A. thaliana* [14]. Because the *S*-locus recognition genes are the primary determinants of the outcrossing mode of mating, they are major targets of selection for self-fertility. Our results suggest that these genes were targeted directly via their mutational inactivation in some *A. thaliana* populations (e.g., C24) and indirectly via mutations at modifier loci that affect their expression, such as *PUB8,* in other populations. By using an SSLP marker (Table S1) within *PUB8* 5′ sequences that distinguishes $PUB8^{C24}$ from $PUB8^{RLD/Col\text{-}0}$, we found that the $PUB8^{RLD/Col\text{-}0}$ allele occurs in approximately one-third of 86 accessions tested (Table S3), including Mt-0, Nd-0, and Ws-0, all of which exhibit pseudo-self-compatibility when transformed with *SRKb-SCRb* [11] (Table 1). Furthermore, the $PUB8^{RLD/Col\text{-}0}$ allele is not restricted to the *S* haplotype found in Col-0 and RLD, but it also occurs in combination with the other two *A. thaliana S* haplotypes identified to date [14, 17]. Examination of *SRKb-SCRb* transformants in more accessions will be required to determine whether the wide geographical distribution of $PUB8^{RLD/Col\text{-}0}$ reflects a historic selective advantage of this allele.

The PUB8 protein and its yet-to-be identified targets are clearly important determinants of a stable SI phenotype. Despite decades of intensive study in naturally self-incompatible species, *PUB8* is the first gene underlying transient self-incompatibility to be cloned in any species. The cloning of this gene underscores the value

of using the transgenic *A. thaliana SRKb-SCRb* model and the cryptic natural variation it unveils for identifying novel factors required for SI and for understanding how the SI response is orchestrated and maintained.

Experimental Procedures

Plant Growth, Pollinations, Genetic Analysis, and Mapping

A. thaliana plants were grown at 22°C, 16 hr light/8 hr dark. A C24 *SRKb-SCRb* plant was crossed to wild-type RLD and to wild-type Col-0. The self-incompatible, kanamycin-resistant, and *SRKb-SCRb*-containing F1 plants were subjected to forced self-pollination in immature buds before stigmas acquired the ability to reject self pollen. Kanamycin-resistant F2 plants segregated for SI and pseudo-self-compatibility, as determined by pollination assays performed on pollen-free stigmas of developing floral buds and flowers [10, 11].

RILs homozygous for the *SRKb-SCRb* transgenes and heterozygous for the target locus were generated from the C24 *SRKb-SCRb* x RLD cross by repeated forced self-pollination for eight generations. The progenies of one of these RILs were used for fine mapping of the major-effect locus underlying pseudo-self-compatibility, and a PSC RIL, designated RIL200, was used for complementation with the $PUB8^{C24}$ gene. Furthermore, RIL200 was also crossed to an untransformed C24 plant. All F1 plants derived from this cross exhibited developmentally stable SI, providing further evidence that pseudo-self-compatibility is not caused by the position or copy number of the *SRKb-SCRb* transgenes.

DNA Analysis

Genomic DNA was prepared from leaves [32] and analyzed by PCR with newly generated markers (Table S1). The distribution of $PUB8^{Rld/Col-0}$ and $PUB8^{C24}$ alleles was analyzed with SSLP marker C (Table S3). The PLACE database [33] (http://www.dna.affrc.go.jp/PLACE/index.html) was queried with *PUB8* 5′ sequences for *cis*-acting regulatory motifs that distinguish $PUB8^{C24}$ from $PUB8^{RLD}$ and $PUB8^{Col-0}$.

Isolation of $PUB8^{C24}$ Fragments and Complementation of the PSC Trait

A λDASH II genomic library constructed from C24 DNA was screened with a *PUB8* probe amplified from Col-0 DNA. For complementation of pseudo-self-compatibility, the insert from a *PUB8*-positive clone (λ4) was used to generate two subclones (4K6 and 4K7, Figure 1) in pCAMBIA1300, which were introduced into *A. thaliana* by the floral dip method [10].

Because seed set is not correlated with reduced strength of SI when the number of germinated pollen tubes exceeds the number of available ovules, expansion of the SI window was assessed by self-pollination assays in randomly chosen $PUB8^{C24}$-containing primary transformants. Subsequently, two lines exhibiting reduced seed set and one line with no seed were selected for detailed association studies of the phenotype and transgene. The observed variability in strength of complementation is consistent with similar studies of other plant genes, especially those underlying natural variation [34].

RNA Analysis

50 stigmas were dissected from buds/flowers at different stages of development and combined into −2, +1, and +3 pools. Total RNA (1 μg) from each pool was reverse-transcribed with oligo(dT) primers and SuperScriptII (Invitrogen, Carlsbad, CA) and subjected to quantitative real-time RT-PCR with *PUB8*-specific primers and *SRKb* primers flanking the *SRKb* first intron (Table S1). Because the *PUB8* gene lacks introns, total RNA was treated with RNase-free DNase I, and the effectiveness of DNase-treatment was confirmed by RT-PCR via intron-flanking primers for actin (Table S1) and no-reverse transcriptase control. Real-time PCR was performed with SYBR green fluorescence and the iCycler iQ5 system (Bio-Rad, Hercules, CA). The relative amount of transcripts, calculated from three replicates by the comparative CT (theshold cycle) method and normalized to the endogenous reference UBC (Table S1) and relative to a calibrator (any one of the samples), is given by $2^{-\Delta\Delta CT}$, where

ΔΔCT = [CT(*PUB8* or *SRKb*) − CT(*UBC*)] − [CT(calibrator) − CT(*UBC*)].

Supplemental Data

Supplemental Data include two figures and three tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/17/8/734/DC1/.

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