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ISOLATION AND CHARACTERIZATION OF AN *ARABIDOPSIS THALIANA* SELF-INCOMPATIBILITY MUTANT INDUCED BY HEAVY-ION BEAM IRRADIATION

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Self-incompatibility (SI) is a genetic system that promotes outcrossing by rejecting self-pollen. In the Brassicaceae the SI response is mediated by the pistil S-locus receptor kinase (SRK) and its ligand, pollen S-locus cysteine-rich (SCR) protein. Transfer of *SRK-SCR* gene pairs to self-fertile *Arabidopsis thaliana* enabled establishment of robust SI, making this transgenic self-incompatible *A. thaliana* an excellent platform for SI analysis. Here we report isolation of a novel *A. thaliana* self-incompatibility mutant, AtC24 SI mutant, induced by heavy-ion beam irradiation. We show that the AtC24 SI mutant exhibits breakdown of SI, with pollen hydration, pollen tube growth and seed set resembling the corresponding processes in wild-type (self-fertile) *A. thaliana*. Further reciprocal crosses indicated that some perturbed SI factor in the stigmatic cell of the AtC24 SI mutant is responsible for the observed phenotype, while the pollen response remained intact. Our results demonstrate successful application of heavy-ion beam irradiation to induce a novel *A. thaliana* self-incompatibility mutant useful for SI studies.

Key words: *Arabidopsis thaliana* accession C24, heavy-ion beam, pollen, pollen tube, self-incompatibility.

INTRODUCTION

In many angiosperms, outcrossing is enforced by genetic self-incompatibility (SI), which allows the pistil to recognize and specifically reject self-pollen (Tantikanjana and Nasrallah, 2012). In the Brassicaceae (crucifer) family, the SI reaction is based on allele-specific interactions between two highly polymorphic proteins encoded by the S-locus haplotype: S-locus receptor kinase (SRK) displayed on the surface of stigma epidermal cells (Takasaki et al., 2000), and its ligand S-locus cysteine-rich (SCR) protein localized in the pollen coat (Schopfer et al., 1999; Takayama et al., 2000). Under one current model of SI signalling, contact between a pollen grain and stigma epidermal cell allows binding of SCR to its cognate SRK receptor to take place and activate the downstream SI cascades, leading to rejection of self-pollen.

Recently, the natural self-compatible (self-fertile) model plant *Arabidopsis thaliana* has been made to express SI upon transformation with *SRKb-SCRb* gene pairs derived from its self-incompatible sister species *A. lyrata* (Nasrallah et al., 2002). This finding implies that *A. thaliana* has retained all the required downstream components of SI signalling, making this self-incompatible *A. thaliana* (hereafter SI *Arabidopsis*) an excellent platform for SI analysis. Several SI molecules have been suggested to function downstream of *Brassica* SI (Stone et al., 1999; Murase et al., 2004; Samuel et al., 2009) but it has also been suggested that *A. thaliana* orthologs of *Brassica* SI molecules are not required for the SI response of *A. thaliana* (Kitashiba et al., 2011). Hence the molecular events precipitated by activation of SRK in *A. thaliana* SI are still unknown. Further studies through mutagen mutagenesis and subsequent mapping of the target gene are needed to identify the downstream SI molecules of *A. thaliana*.

Heavy-ion beam irradiation has often been employed as an efficient mutagenic technique to induce a desired mutant for functional gene analy-

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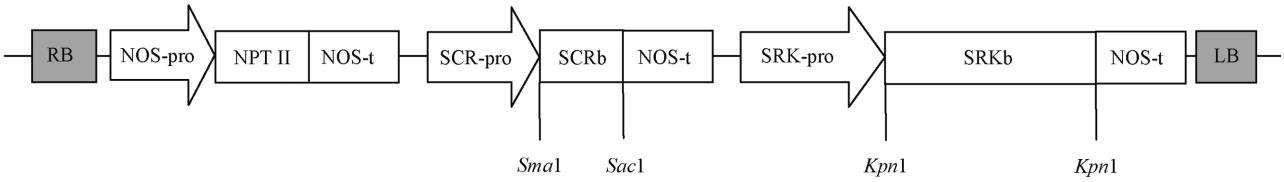

Fig. 1. Schematic map of *SRKb-SCRb*/pBI121 expression plasmid. RB – right border; NOS-pro – nopaline synthase promoter; NPT II – neomycin phosphotransferase; NOS-t – nopaline synthase terminator; SCR-pro – S-locus cysteine-rich promoter; SRK-pro – S-locus receptor kinase promoter; LB – left border.

sis. Successful isolation of target mutants induced by heavy-ion beam irradiation has been reported for varieties of ornamental plants such as dahlia (Hamatani et al., 2001), petunia (Miyazaki et al., 2002), torenia (Miyazaki et al., 2006) and verbena Kanaya et al., 2008). This powerful technique has also been used to induce mutation in rice (Hayashi et al., 2007), *Arabidopsis* (Kazama et al., 2008b) and tobacco (Kazama et al., 2008a). Unlike gamma rays and X-rays, heavy-ion beams have high linear energy transfer (LET), which exerts stronger biological effects and can produce more DNA damage, leading to mutation and inactivation of single or multiple genes, inducing stable knockout mutants (Kazama et al., 2007). Since a heavy-ion beam induces mutations with high frequency at a relatively low dose, it produces mutants with less cell damage, no severe growth inhibition, and a broad spectrum of mutations (Kazama et al., 2007).

Here we present a preliminary report on our attempt to induce an *A. thaliana* SI mutant (hereafter AtSI mutant) using heavy-ion beam irradiation. We describe a successful application of heavy-ion beam irradiation for isolation and characterization of a novel AtSI mutant which can serve as a platform for downstream SI gene analysis in *A. thaliana*.

MATERIALS AND METHODS

PLANT MATERIAL

Arabidopsis thaliana accession C24 was used throughout this study. Stable SI *Arabidopsis* was generated by transforming the construct harboring *SRKb-SCRb* gene pairs isolated from *A. lyrata* (Fig. 1) into *A. thaliana* C24 using the floral dip method as previously described (Clough and Bent, 1998). Genomic PCR and segregation analysis were performed to confirm the homozygous SI *Arabidopsis*. Gene-specific primers are listed in Table 1.

PLASMID CONSTRUCTION

The expression vector used for *A. thaliana* C24 transformation was constructed by cloning the *SRKb-SCRb* gene pairs from *A. lyrata* into the pBI121 plasmid driven by its own native promoter (Fig. 1). The *SRKb* gene fragment was inserted into the *Kpn*1 site, and the *SCRb* gene fragment was inserted between *Sma*l-*Sac*1 sites, producing *SRKb-SCRb*/pBI121 plasmid (Fig. 1). Gene-specific primers are listed in Table 1.

HEAVY-ION BEAM IRRADIATION

Dry SI *Arabidopsis* seeds (~5000 seeds) were packed with Hybri-Bag Hard (95 μm thickness, Cosmo Bio, USA) to provide a monolayer of seeds for homogenous irradiation. Heavy-ion beam irradiation was performed according to the previously described protocol (Kazama et al., 2008b) at the RIKEN Nishina Center Radioactive-Isotope Beam Factory (RIBF) Facility, Saitama, Japan.

ISOLATION OF AtSI MUTANT

The irradiated SI *Arabidopsis* seeds (~1100 seeds) were surface-sterilized and sown on 0.8% (w/v) agar containing 1/2 Murashige and Skoog (MS) medium (Wako, Japan) supplemented with MS vitamins (Sigma Aldrich, USA) and incubated at 25°C under a 16 h photoperiod. A total 1000 seedlings were grown in Arasystem trays (Betatech, Ghent, Belgium) to generate the M_1 population. To obtain seeds for the M_2 population, M_1 plants were incubated with dry ice for 3 h to induce self-fertilization. A total 255 independent M_2 lines were successfully recovered for AtSI mutant screening. To screen for AtSI mutants, silique length of the M_2

TABLE 1. Primers used in the study

Primer name	Sequence (5'-3')
SCRb-F1	CCCGGGATGAGGAATGCTACTTTCTTC
SCRb-R1	GAGCTCTAGCAAAATCTACAGTCGCATA
SRKb-F1	AGGTACCATGAGAGTTGTAGTACCAAACTG
SRKb-R1	TGGTACCTTACCGAGGGTCGATGGCCGA
SRKb-RT-F1	AAAGAACAGGGGATTCTACAACTCAAA
SRKb-RT-R1	ACTTCATGTGGTCGAAACGC
GAPDH-F1	GACCTTACTGTCAGACTCGAG
GAPDH-R1	TTAGGCCTTTGACATGTGGACGA

lines was measured as previously described (Lai et al., 2012a).

CHARACTERIZATION OF AtC24 SI MUTANT

A pollination assay was performed by hand-pollinating pre-emasculated stigmas with pollen grains. Pollen hydration was evaluated after 30 min pollination on papilla cells with pollen grains. Pollen tube growth was monitored on pollinated stigmas using aniline blue staining as described by Sumie et al. (2001). Stained pistils were observed and photographed with a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

GENOMIC PCR ANALYSIS AND DNA SEQUENCING

Genomic DNA was extracted from leaves using the DNeasy Plant Mini kit (Qiagen, USA). Genomic PCR analysis was performed according to the protocol described by Lai et al. (2011b, 2012c). DNA sequencing was performed using an ABI 3100 DNA Sequencer (ABI, USA), following the manufacture's protocol. Primers are listed in Table 1.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from stigmas by the previously described protocol (Lai et al., 2011). The concentration and purity of total RNA was determined by spectrophotometric analysis. Quantitative real-time PCR was performed according to the protocol described by Lai et al. (2012b, 2013). GAPDH was the control. Gene-specific primers are listed in Table 1.

RESULTS AND DISCUSSION

As a first step toward isolating the AtSI mutant we established SI *Arabidopsis* suitable for use in heavy-ion beam irradiation. Previously, transformation of *A. thaliana* C24 with *SRK-SCR* gene pairs was shown to produce stable SI *Arabidopsis* expressing robust SI indistinguishable from the naturally self-incompatible *A. lyrata* (Nasrallah et al., 2002). Hence, the *A. thaliana* C24 genetic background was used in this study. An expression vector harboring the *SRKb-SCRb* gene pairs was constructed (Fig. 1) and transformed into *A. thaliana* C24. Homozygous SI *Arabidopsis* exhibiting stable SI was screened using genomic PCR and confirmed through segregation analysis and pollination assay (data not shown). The resulting SI *Arabidopsis* exhibiting developmentally stable SI was used in subsequent heavy-ion beam irradiation.

SI *Arabidopsis* seeds (~5000 seeds) were heavy-ion beam irradiated at the RIBF Facility, Saitama, Japan (Fig. 2). Consistent with previously reported data (Kazama et al., 2008b), the irradiated M_1 seeds achieved 92.1% germination (data not shown). This result was expected, as heavy-ion beams induce mutations with high frequency at a relatively low dose without significantly affecting seed germination and plant survivability. A total 1000 M_1 seedlings were grown to generate the M_2 population (Fig. 2). To induce self-fertilization and obtain M_2 seeds the M_1 lines were incubated with dry ice (CO_2 source). Previously, adding carbon dioxide to the environment has been shown to induce seed set in SI *Brassica* genotypes (O'Neill et al., 1988). Carbon dioxide has been suggested to overcome SI by enhancing pollen activity during germination and tube growth (Dhaliwal et al., 1981), blocking the callose response in the stigmatic papilla cell in expression of SI (O'Neill et al., 1984), and increasing the rate of pollen adhesion (Palloix et al., 1985). Of the 1000 M_1 lines incubated, we successfully recovered and grew 255 individual M_2 lines for use in AtSI mutant screening (Fig. 2).

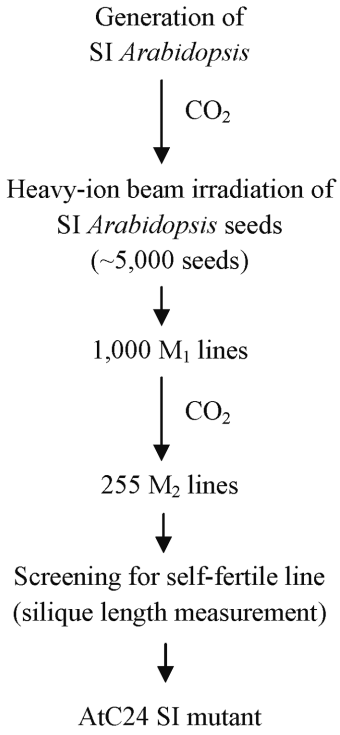

Fig. 2. Outline of screening for AtSI mutant from heavy-ion beam irradiated mutant population.

To screen for AtSI mutants, silique length of the M_2 lines was measured. From the 255 M_2 lines screened, we isolated a mutant line with well-developed siliques (mean length 11.0 mm, vs 12.5 mm in wild type; Fig. 3a). This mutant line, the AtC24 SI mutant, produced silique length similar to that seen

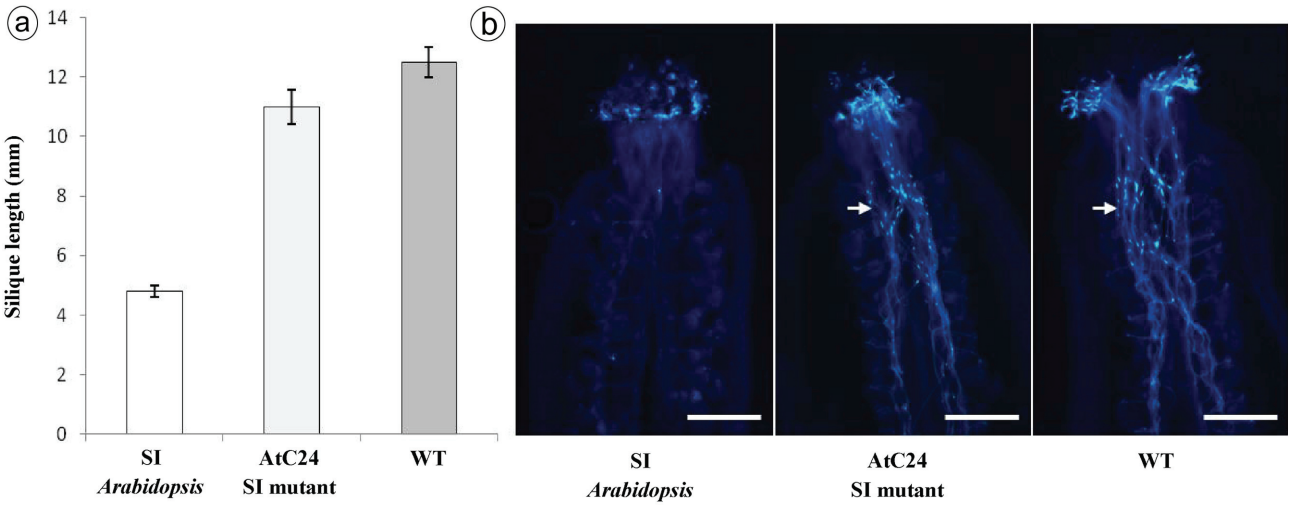

Fig. 3. AtC24 SI mutant silique length and pollen tube growth. (**a**) Length of AtC24 SI mutant siliques. For each sample, 5 siliques were measured in 3 biological replicates, (**b**) Aniline blue staining of pistils from AtC24 SI mutant. Arrows point to pollen tubes. Bars = 200 μm.

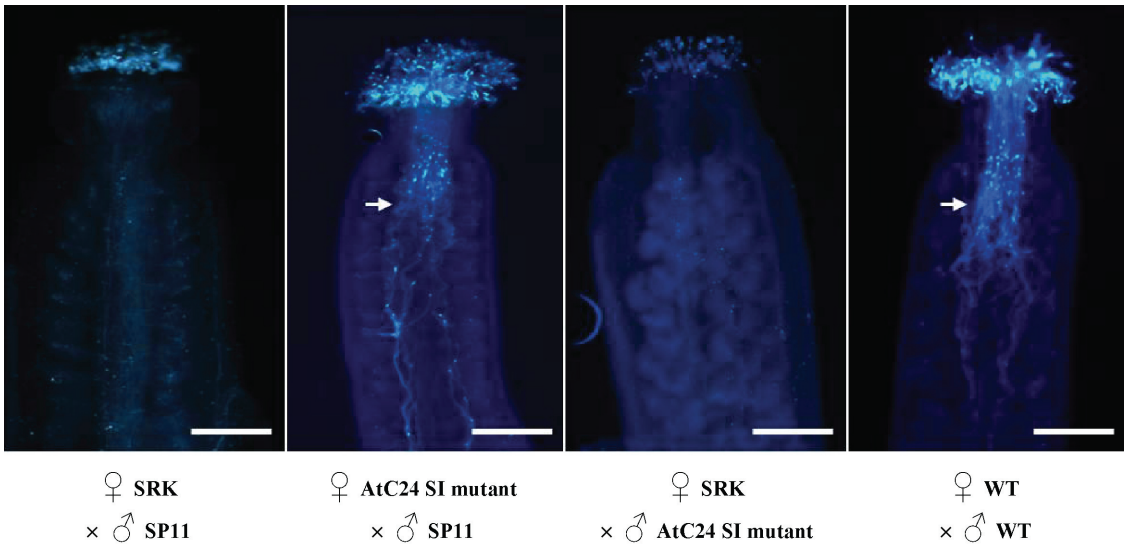

Fig. 4. Aniline blue staining of pistils from reciprocal crosses between SI *Arabidopsis* and AtC24 SI mutant. Arrows point to pollen tubes. Bars = 200 μm.

in self-fertile WT plants (Fig. 3a). No other obvious vegetative growth defects were observed in the AtC24 SI mutant. Further aniline blue staining revealed pollen tube growth in the AtC24 SI mutant (Fig. 3b). Pollen tubes were also observed growing into the style and ovary of the AtC24 SI mutant (Fig. 3b). In the aniline blue staining process, typically only accepted pollen grains will adhere and remain on the stigmatic papillae. All rejected pollen grains failed to adhere to the stigmatic papillae and as a result were washed away during fixation and processing for microscopic examination in the aniline blue staining protocol. Both silique length measurements and pollen tube monitoring suggest that a factor essential to the SI response was perturbed in the AtC24 SI mutant.

To determine the nature of this SI factor, reciprocal crosses between SI *Arabidopsis* and AtC24 SI mutants were performed. Pollination of SI *Arabidopsis* pistils expressing SRK (hereafter SRK-pistil) with AtC24 SI mutant pollen grains resulted in no pollen tube growth and short siliques (Fig. 4). In contrast, pollination of AtC24 SI mutant pistils with SI *Arabidopsis* pollen grains expressing SCR (hereafter SCR-pollen) resulted in pollen tube growth and well developed siliques (Fig. 4). This

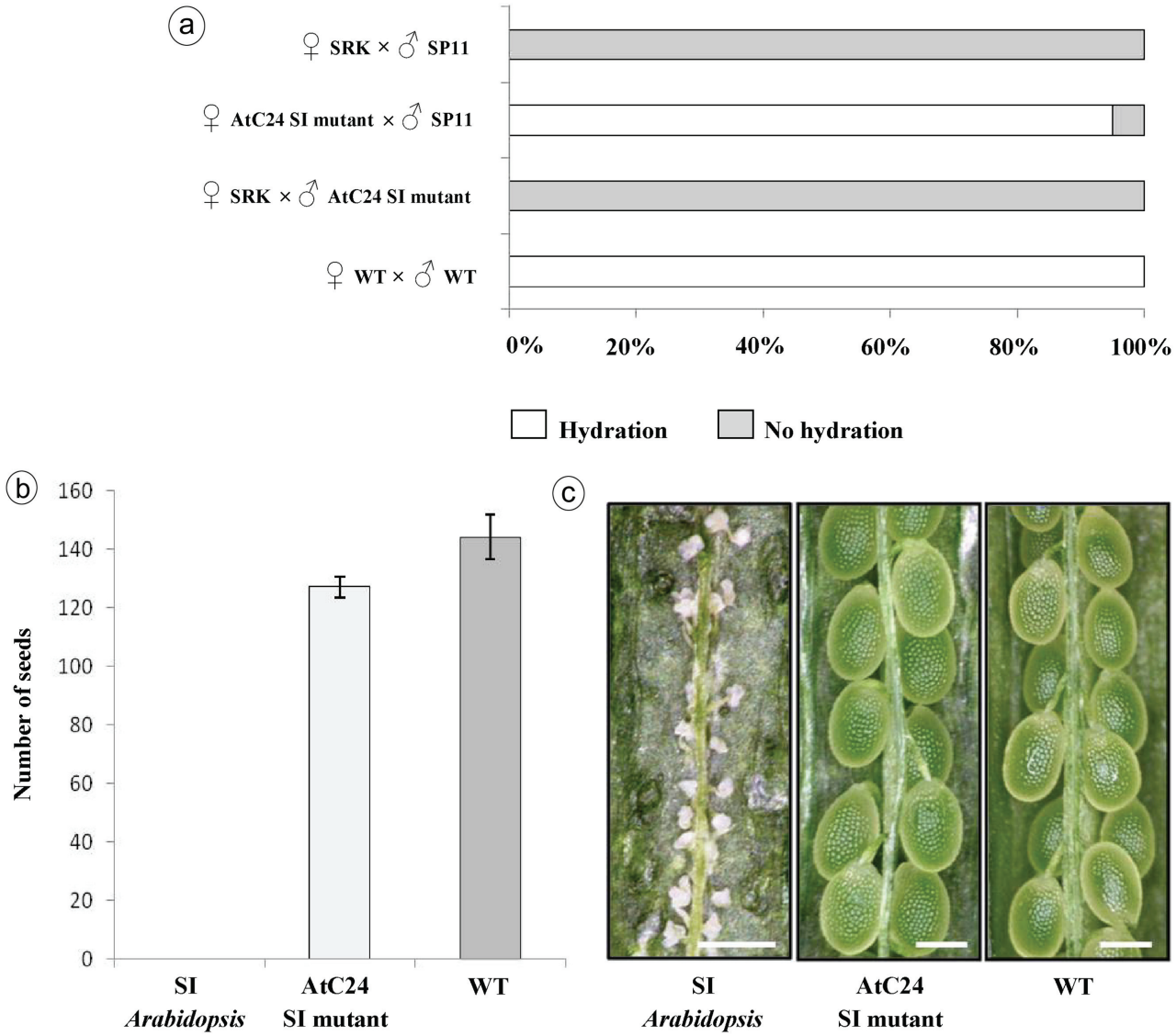

Fig. 5. Characterization of AtC24 SI mutant. (**a**) Pollen hydration assays of AtC24 SI mutant. For each sample, 25 pollen grains were tested, (**b**) Seed count of AtC24 SI mutant. For each sample, 5 siliques were counted in 3 biological replicates, (**c**) Seed set analysis of AtC24 SI mutant. Bars = 1 mm.

result indicates that the SI factor of the female organ (stigma) is compromised in the AtC24 SI mutant. To further determine the extent of pollen acceptance in the AtC24 SI mutant, pollen hydration assay and seed set analysis were performed to assess the degree of SI breakdown. Approximately 96% of the SCR-pollen grains pollinated deposited on AtC24 SI mutant papillae showed successful pollen hydration and germination in the pollen hydration assay (Fig. 5a). This result indicates strong pollen acceptance in the AtC24 SI mutant, further suggesting that the perturbed SI factor is crucial to this SI response. The loss of this SI factor resulted in total breakdown of SI observed in the AtC24 SI mutant. Similar observations were reported when SI molecules acting downstream of *Brassica* SI signalling were disrupted (Stone et al., 1999; Murase et al., 2004; Samuel et al., 2009). The pollen factor was apparently intact, as no pollen hydration and germination were detected when SRK-pistils were pollinated with AtC24 SI mutant pollen grains (Fig. 5a). In accord with the pollen hydration assay, AtC24 SI mutants with a well developed silique also produced seed set indistinguishable from that of self-fertile WT plants (Fig. 5b,c). No seeds were produced in SI *Arabidopsis* (positive control) (Fig. 5b,c).

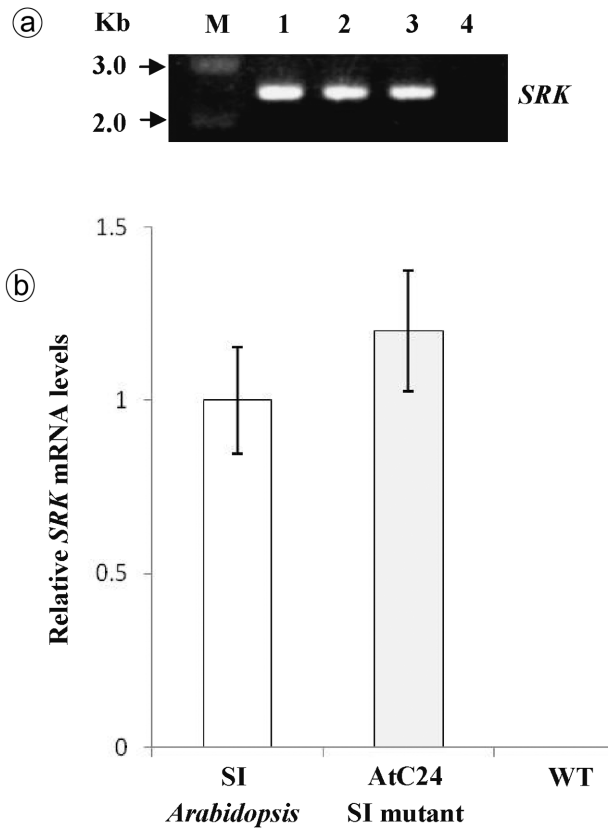

Fig. 6. Detection and expression analysis of *SRKb* in AtC24 SI mutant. (**a**) Genomic PCR of *SRKb* in AtC24 SI mutant. Lane M – 1.0 Kb DNA ladder; lane 1 – *SRKb-SCRb*/pBI121 plasmid; lane 2 – SI *Arabidopsis*; lane 3 – AtC24 SI mutant; lane 4 – WT plant, (**b**) Relative *SRKb* mRNA levels in stigmas of AtC24 SI mutant.

In *Arabidopsis*, heavy-ion beam irradiation has been shown to induce a broad spectrum of mutations, including base changes, small DNA deletions involving a few bases, and large DNA alterations such as inversions, translocations and deletions (Tanaka, 1999). To rule out the possibility that a mutation or deletion in the *SRKb* gene fragment might be responsible for the observed phenotype in the AtC24 SI mutant, genomic PCR analysis and DNA sequencing were performed (Fig. 6). Based on genomic PCR analysis, a ~2.5 Kb fragment coinciding with the size of the *SRKb* gene was detected (Fig. 6a). DNA sequencing did not detect any mutation or deletion in the *SRKb* gene of the AtC24 SI mutant. We also measured the level of the *SRKb* transcript in the AtC24 SI mutant, using qRT-PCR analysis (Fig. 6b). Previously a reduced level of SRK in stigmatic cells has been suggested to cause breakdown of SI (Liu et al., 2007). Quantitative analysis of the *SRKb* transcript level in the AtC24 SI mutant revealed no significant differences or reduction of *SRKb* expression versus SI *Arabidopsis* (positive control) (Fig. 6b). Thus the breakdown of SI observed in the AtC24 SI mutant was not caused by a mutation or deletion that disrupted the *SRKb* level. Instead, an important novel stigmatic SI factor acting downstream of SRK might be responsible for the breakdown of SI in the AtC24 SI mutant.

Here we reported successful isolation of a novel AtC24 SI mutant induced by heavy-ion beam irradiation. To our knowledge there are no reports on isolation of AtSI mutants induced by heavy-ion beam irradiation. The obtained AtC24 SI mutant will serve as valuable material for SI gene analysis and examination of the downstream SI molecules of *Arabidopsis*.

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