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 (cruicer) 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-
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 KpnI site, and the SCRb gene fragment was inserted between SmaI-SacI 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 M1 population. To obtain seeds for the M2 population, M1 plants were incubated with dry ice for 3 h to induce self-fertilization. A total 255 independent M2 lines were successfully recovered for AtSI mutant screening. To screen for AtSI mutants, silique length of the M2

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>SCRb-F1</td>
<td>CCCCGGGATGAGGAAATCATTTTCTTC</td>
</tr>
<tr>
<td>SCRb-R1</td>
<td>GAGCTCTAGCAAAATCTACGTCGATA</td>
</tr>
<tr>
<td>SRKb-F1</td>
<td>AGGTACCATGAGATGGTAGATCAAAAACGT</td>
</tr>
<tr>
<td>SRKb-R1</td>
<td>TGGTACCTTTACCGGGGTGGTGGCCGA</td>
</tr>
<tr>
<td>SRKb-RT1</td>
<td>AAGAACCGGGGATTTACAAACTCAA</td>
</tr>
<tr>
<td>GAPDH-F1</td>
<td>GACCTTACTGTCAGAAGTGGG</td>
</tr>
<tr>
<td>GAPDH-R1</td>
<td>TTAGGCTTTGACATGGACG</td>
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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 manufacturer’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 M1 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 M1 seedlings were grown to generate the M2 population (Fig. 2). To induce self-fertilization and obtain M2 seeds the M1 lines were incubated with dry ice (CO2 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., 1981), and increasing the rate of pollen adhesion (Palloix et al., 1985). Of the 1000 M1 lines incubated, we successfully recovered and grew 255 individual M2 lines for use in AtSI mutant screening (Fig. 2).

To screen for AtSI mutants, silique length of the M2 lines was measured. From the 255 M2 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
Arabidopsis thaliana self-incompatibility mutant

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. 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.
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).
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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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.


