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Deletion Mutations of the Self-incompatibility (*S*) Locus Induced by Gamma Irradiation in a Wild Diploid Species of Sweet Potato, *Ipomoea trifida*

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Abstract

Gamma irradiation was used to induce mutations affecting self-incompatibility (SI) in diploid *Ipomoea trifida*. Plants (S_1 homozygote) with developing flower buds were exposed with gamma rays of 10 and 20 Gy. Mature pollen from the irradiated plants was used for crosses with S_3 and S_{10} homozygotes. By pollination test and Southern blot analysis of the M_1 progenies, we screened 2 mutant plants carrying a deletion of the *S* locus derived from irradiated pollen, designated ΔS_1. In both mutant alleles, the deleted region was found to exceed a genetic distance of 12 cM around the *S* locus. Progeny test of both mutant plants showed that neither female nor male gametes carrying the ΔS_1 allele were fertile probably due to the lethal effect of the chromosomal deletion spanning the *S* locus.

Discipline: Genetic resources/ Plant breeding
Additional key words: Convolvulaceae, *S* gene, sporophytic self-incompatibility

Introduction

Cultivars of the hexaploid sweet potato (*Ipomoea batatas*) show several cross-incompatibility groups[1,7]. The presence of these incompatibility groups has been a barrier in crossbreeding of sweet potato due to the limitations in the choice of parental cultivars. The cross-incompatibility in sweet potato is likely to occur between plants of the same self-incompatibility (SI) phenotype, but the genetic system of the SI remains to be clarified due to its hexaploid nature. The diploid relative *I. trifida*, a species thought to be the wild ancestral species of sweet potato[12-14], has instead been used for studying the genetic control of the SI in the genus *Ipomoea*. Earlier studies demonstrated that the SI in *I. trifida* is sporophytically controlled by a single *S* locus with multiple alleles[4]. Approximately 50 *S* alleles (also called *S* haplotypes) have so far been genetically identified and their dominance relationships have also been determined[5]. Molecular and genomic approaches have since been made to clone the *S* gene and investigate mechanisms of the SI in this species[6,10,11,15,16]. Mutants affecting the SI are useful for analyzing functions of the *S* gene and for elucidating mechanisms of the SI. A spontaneous self-compatible mutant was found in a plant population of *I. trifida* and shown to have loss-of-function mutation(s) in male and/or female *S* gene(s)[2]. Since a wide variety of SI mutants are useful for further molecular studies, we have also tried to screen mutants affecting the SI by selfing X-irradiated plants and/or pollinating the irradiated pollen on the stigma of the same *S* phenotype. However, none of these attempts eventually resulted in obtaining any mutants (unpublished results). In this study, we designed a new mutant screening system using pollen from gamma-irradiated plants and successfully detected the deletion mutations spanning the *S* locus in *I. trifida*.

Materials and methods

1. Plant materials and gamma irradiation

Diploid *Ipomoea trifida* plants carrying a variety of *S* genotypes have been produced by crossings between different individuals and maintained in our laboratory[4,5]. In this study, S_1, S_3 and S_{10} homozygotes (carrying S_1S_1, S_3S_3 and $S_{10}S_{10}$ genotypes, respectively) were used. We also used S_c homozygote, where S_c is a self-compatible allele that was found in a spontaneous mutant plant[2]. The dominance relationship among these *S* alleles was determined to be $S_1 > S_c > S_{10} > S_3$ in the earlier studies[2,5], although the relationship between S_c and S_{10} ($S_c > S_{10}$) has been recently

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examined (data not shown).

A single plant (H45-3) homozygous for S_1 (S_1S_1 genotype) was chosen for gamma irradiation and the vegetatively propagated clones were grown in pots to flowering. The plant (clone) with developing flower buds was exposed with gamma rays of either 10 or 20 Gy using the ^{60}Co radiation facility in Kyoto University. Anthers (pollen) were collected from open flowers of the irradiated plants and were used for crosses during a 3-week period from 1 day after the irradiation. Pollen from the plants exposed with 20- and 10-Gy radiations was pollinated with S_3 and S_{10} homozygotes, respectively, as pistillate parents to obtain the M_1 progeny. During the period of crossing, pollen fertility was checked everyday using two anthers by counting 500 pollen grains stained with cotton blue. Seeds were harvested at maturity, germinated and grown in pots to flowering.

2. Pollination test and Southern blot analysis for determination of *S* genotypes

All the M_1 progeny plants grown to flowering were examined for the *S* phenotype and the self-compatibility by pollination test[2]. S_1S_3, S_3S_3 and S_3S_{10} genotypes (dominance relationship: S_1>S_{10}>S_3 in both pollen and stigma) were used as testers of S_1, S_3 and S_{10} phenotypes, respectively. Determination of the compatible or incompatible reaction in pollination was based on the observation of germination of pollen on stigmas after staining with aniline blue. To determine *S* genotypes, Southern blot analysis was performed using several DNA markers located around the *S* locus according to Tomita et al.[15]. Genomic DNA digested with each of 9 restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Hin*dIII, *Pvu*II, *Sac*I, *Xba*I, and *Xho*I) were blotted and hybridized using each marker as a probe. Progenies of mutant plants were produced by crossing them with S_3 and S_c homozygotes as well as by their reciprocal crossings, and were also analyzed by pollination test and Southern hybridization as described above.

Results

1. Screening of mutants derived from crosses with the pollen of gamma-irradiated plants

One hundred thirty-six flowers (pistils) of S_3 homozygote were pollinated with pollen produced from an S_1 homozygous plant irradiated with gamma rays of 20 Gy. A total of 129 seeds (in 79 pods) were obtained from the cross and designated R31 line (Table 1). Pollen from the plant exposed with 10-Gy radiation was used for pollination of 210 flowers (pistils) of S_{10} homozygote, and a total of 310 seeds (in 151 pods) were obtained and designated R101 line (Table 1). Pollination test was carried out using 101 and 288 M_1 plants grown to flowering stages in R31 and R101 lines, respectively (Table 2). All these plants were found to be fully self-incompatible, indicating that no dominant self-compatible mutation was inherited from irradiated pollen. From the result of reciprocal crosses of 101 individuals in the R31 line with either the [S_3] or [S_1] tester ([S_x] indicates the phenotype S_x hereafter), we found 3 plants (R31-1, -2 and -3) exhibiting the phenotype [S_3] on both male and female sides, whereas all the rest (98 plants) exhibited the phenotype [S_1] (Table 2a). Likewise, the change in *S* phenotype from [S_1] to [S_{10}] was found in one of 288 plants for the R101 line (R101-1; Table 2b). These results suggest that the loss-of function mutation(s) altering the expression of phenotype [S_1] may be induced in the irradiated pollen parents of the 4 plants (R31-1, -2, -3, and R101-1), because the pistil-derived S_3 and S_{10} alleles are both recessive to S_1 (S_1>S_{10}>S_3)[5].

DNA markers mapped around the *S* locus were used for genotyping of the above-described 4 plants. Based on the genetic map[15], we selected 7 markers covering a total of 12.1 cM flanking the *S* locus (Fig. 1). Of these, AAM-68 is most closely linked to *S* at 0 cM (complete linkage). By Southern blot hybridization probed with each marker, we examined RFLPs (restriction fragment length polymorphisms) between parental plants (S_1, S_3 and S_{10} homozygotes) used for crosses of R31 and R101 lines. We successfully detected RFLPs with 4 markers SAM-51, SAM-23, AAM-68, and SAM-12. Two markers, AAM-12 and AF-51, did not exhibit any RFLPs among S_1, S_3 and S_{10} homozygotes (shown as 'ND' in Fig. 1), whereas AF-13 showed the RFLP only between S_1 and S_{10}. When using AAM-68 as a probe, none of the 4 plants (R31-1, -2, -3, and R101-1) were found to carry hybridizing bands corresponding to the allele derived from the pollen parent (S_1 homozygote; Fig. 2 (upper panel)). Similarly, the pollen parent-derived allele was absent from the 4 plants for any

Table 1. M_1 progenies derived from crosses using gamma-irradiated pollen

Line	Pistillate parent (♀)		Irradiated pollen parent (♂)	Dose of γ-ray	No. of crosses	No. of pods bearing seeds	Total no. of seeds obtained
R31	S_3S_3	×	S_1S_1	20 Gy	136	79	129
R101	$S_{10}S_{10}$	×	S_1S_1	10 Gy	210	151	310

other markers flanking the *S* locus (Fig. 1). In addition to the *S*-linked markers, we randomly chose polymorphic DNA markers independent of the *S* locus and used those for Southern analysis. Results of hybridizations with these markers (AAM-41, AAM-43, SAM-21, SAM-31, etc.) all showed that 2 plants (R31-2 and R101-1) carried the pollen parent-derived allele, while the other 2 plants (R31-1 and R31-3) did not, as shown in Fig. 2 (lower panel) as an example of the hybridization pattern probed with SAM-31. These findings and the above-mentioned pollination tests strongly suggest that R31-2 and R101-1 carry a mutated allele derived from irradiated pollen, in which a genomic region encompassing the S_1 haplotype is deleted. On the other hand, R31-1 and R31-3 may be generated by self-fertilization of the pistillate parent (S_3 homozygote) possibly due to a kind of mentor effect of irradiated pollen, although no selfed progeny has been found in diploid *I. trifida* except for a spontaneous self-compatible mutant[2]. Thus, we have obtained two SI mutants R31-2 and R101-1, and hereafter designate the mutant allele as ΔS_1, the genotype of R31-2 as ΔS_1S_3 and that of R101-1 as ΔS_1S_{10}.

Table 2. Pollination responses of M_1 progeny plants

a. R31 line

Plant no.	Self	× ♂ [S_3]	× ♀ [S_3]	× ♂ [S_1]	× ♀ [S_1]
R31-1	−	−	−	+	+
R31-2	−	−	−	+	+
R31-3	−	−	−	+	+
Others[1)]	−	+	+	−	−

b. R101 line

Plant no.	Self	× ♂ [S_{10}]	× ♀ [S_{10}]	× ♂ [S_1]	× ♀ [S_1]
R101-1	−	−	−	+	+
Others[2)]	−	+	+	−	−

[S_1], [S_3] and [S_{10}] indicate phenotypes S_1, S_3 and S_{10}, respectively.
+: Compatible, − : Incompatible.
1): 98 plants examined.
2): 287 plants examined.

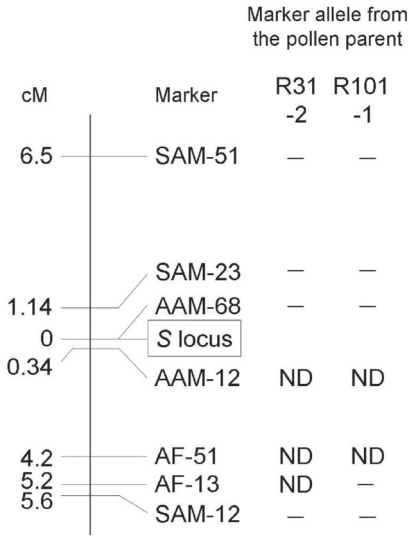

Fig. 1. DNA marker analysis in 2 mutant plants R31-2 and R101-1

A genetic linkage map of DNA markers around the *S* locus in *I. trifida*[15] is shown. On the right of the map, the presence or absence (−) of respective marker alleles derived from the irradiated pollen parent in 2 mutant plants is indicated. 'ND' indicates that the presence or absence of those was not determined because no polymorphism was detected between parental plants for the markers.

2. Analysis of progenies of SI mutants

We conducted progeny tests of the 2 mutant plants to examine the inheritance of mutant alleles and also to obtain plants homozygous for respective alleles. Since both mutant plants were fully self-incompatible, they were first crossed with S_c homozygote. Since S_c is a self-compatible allele (dominance relationship: $S_1>S_c$), it should be useful for producing plants homozygous for ΔS_1 allele by enabling the selfing of ΔS_1S_c heterozygote (or S_c hemizygote) if S_c is dominant over ΔS_1 ($S_c>\Delta S_1$). From the crosses of S_c homozygote with R31-2 and R101-1, we obtained 12 and 11 progeny individuals, respectively (Table 3). All of them exhibited the self-compatibility phenotype [S_c] as expected. However, genotyping using AAM-68 marker (Fig. 1) revealed that they were either S_3S_c or $S_{10}S_c$, while no ΔS_1S_c genotype was detected in either progeny.

Next, R101-1 was crossed with S_3 homozygote. Reciprocal crosses produced a total of 23 progeny individuals (Table 3), in which S_3S_{10} and ΔS_1S_3 are expected to appear in a ratio of 1:1. However, all exhibited the phenotype [S_{10}] and were further found to be S_3S_{10} based on the genotyping with the AAM-68 marker. Finally, we

Table 3. Progeny test of R31-2 and R101-1plants

♀	×	♂	No. of progeny	*S* phenotype	*S* genotype*
R31-2	×	S_cS_c	12	[S_c]	S_3S_c
R101-1	×	S_cS_c	11	[S_c]	$S_{10}S_c$
R101-1	×	S_3S_3	12	[S_{10}]	S_3S_{10}
S_3S_3	×	R101-1	11	[S_{10}]	S_3S_{10}
R31-2	×	R101-1	3	[S_{10}]	S_3S_{10}
R101-1	×	R31-2	16	[S_{10}]	S_3S_{10}

S genotypes of R31-2 and R101-1 are predicted to be ΔS_1S_3 and ΔS_1S_{10}, respectively.
*: Determined based on genotyping using AAM-68 marker.

Table 4. Pollen fertilities in deletion mutants and other plants

Plant	Pollen fertility ± S.E.(%)
R31-2	38.6 ± 8.8
R101-1	42.7 ± 3.7
Average in R31 line[1)]	76.4 (44.1–99.1)*
Average in R101 line[2)]	52.5 (5.2–90.6)*
Control[3)]	89.7 (76.1–99.6)*

1): 10 plants except R31-2 were randomly selected and examined.
2): 10 plants except R101-1 were randomly selected and examined.
3): 7 control plants (not irradiated) were examined.
*: A range of individual pollen fertilities is shown in parenthesis.

made reciprocal crosses between R31-2 and R101-1, and obtained a total of 19 progeny individuals. Again, all of these were found to exhibit the phenotype [S_{10}] and carry S_3S_{10} genotype (Table 3). Thus, the results of progeny tests suggest that neither male nor female gametes carrying ΔS_1 were fertile.

We examined pollen fertilities of 2 mutant plants as well as 20 plants randomly chosen from R31 and R101 lines (Table 4). R31-2 and R101-1 had 38.6% and 42.7% pollen fertilities, respectively. Pollen fertilities of plants randomly chosen from both lines greatly varied (5.2-99.1%) compared with those of control (wild-type) plants (89.7% on average). Thus, pollen fertilities of 2 mutant plants could be interpreted as semisterility, where half the pollen carrying ΔS_1 would be sterile.

Discussion

In the present study, we screened 2 mutant plants (R31-2 and R101-1) that lost the *S* specificity (S_1) from the irradiated pollen parent (Table 2). Southern analysis using *S* locus-flanking markers (Fig. 2) revealed that both mutants had a deletion spanning the *S* locus in the S_1 haplotype, which corresponds to the genetic distance of more than 12.1 cM. The genomic region between 2 flanking markers SAM-23 and AAM-12 (1.48 cM in genetic distance; Fig. 1) is known to cover approximately 500 kb in physical distance[16]. If this ratio (338 kb/cM) is applied, the physical size of the deletion is estimated to be more than approximately 4 Mb.

A large portion of seeds in R31 and R101 lines (76% and 53%, respectively) were obtained from the crosses within the first 1 week after irradiation. In fact, deletion mutations detected in this study occurred in the pollen parent within 3 days after irradiation. We observed that pollen fertilities of 2 gamma-irradiated plants remarkably dropped at 1 week after irradiation and then gradually recovered

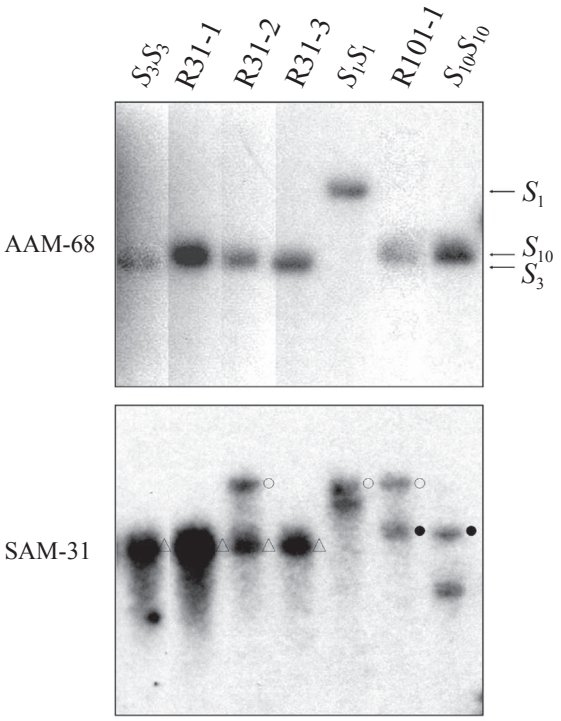

Fig. 2. Southern blot analysis of plants obtained from R31 and R101 lines

Three plants (R31-1, -2 and -3) from R31 line and one plant (R101-1) from R101 line are used for hybridization together with their parental *S* homozygotes. S_3 and S_{10} homozygtes were crossed with the pollen produced from an irradiated S_1 homozygous plant for R31 and R101 lines, respectively. (Upper panel) A DNA marker AAM-68 that cosegregates with the *S* locus (linked at 0 cM) was used as a probe in the hybridization using genomic DNA digested with *Eco*RI. Arrows indicate positions of hybridization bands from 3 *S* homozygotes. (Lower panel) A DNA marker SAM-31 that is independent of the *S* locus was used as a probe in the hybridization using genomic DNA digested with *Hin*dIII. Open and closed circles indicate hybridization bands derived from one of two marker alleles in S_1 and S_{10} homozygotes, respectively. Open triangle indicates the hybridization band from S_3 homozygote.

until 3 weeks. These findings suggest that crossing using pollen at early stages after irradiation is effective to obtain mutations affecting SI and that lower-dose irradiation may help to narrow the deletion region.

Sporophytic SI is more difficult to detect in pollen-part mutations at the *S* locus than gametophytic SI[9]. On the other hand, a recessive mutation of the modifier (*m*) gene unlinked to the *S* locus has been reported to be induced by gamma irradiation in the sporophytic SI of Brassicaceae[8]. To screen such recessive mutants in *I. trifida*, however, an efficient seed production system

needs to be developed, because no artificial procedures to permit selfing (bud-pollination, CO_2 treatment, etc.) can be applied in this species due to the stable SI reaction. Thus, there remain many obstacles to successfully use mutagenesis-based approaches in further studies on the SI of *I. trifida*. We are progressing in the use of transformation-based approaches for the functional analysis of *S* locus genes, since *Agrobacterium*-mediated transformation has recently been available in *I. trifida*[3]. In this approach, deletion mutants detected in this study may be useful as recipient plants to be transformed with a range of genomic DNA fragments around the *S* locus to assess the effect of these fragments on phenotypic expression.

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