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Linkage analysis of a fertility restoring mutant generated from CMS rice

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Abstract DNA polymorphism between a cytoplasmic male-sterile rice line II-32A, the male-fertile maintainer counterpart II-32B, a fertile revertant (T24), as well as two commercial indica restorers, was analyzed with randomly amplified polymorphic DNA (RAPD). A very low degree of polymorphism was found between the revertant T24 and II-32A compared with that of indica rice varieties. This result, together with agronomic and genetic evidence, suggests the revertant to be a product of a nuclear mutation. An analysis of polymorphism between II-32A and the revertant T24 with 510 RAPD decamer primers identified the co-segregating markers $OPB07_{640}$ and $OPB18_{1000}$ to be linked to a sterile allele of the restoring locus in the revertant T24, at a distance of 5.3 cM. RAPD analysis of a mapping population of Tesanai2/CB with primer OPB07 revealed linkage of $OPB07_{640}$ with RG374 (10.8 cM) and RG394 (8.8 cM) on chromosome 1. Thus the restorer gene, designated Rf_5, was tentatively localized between RG374 and RG394 on chromosome 1 and appears to be independent of other mapped restorer genes in rice.

Key words Rice (*Oryza sativa*. L) · CMS · Fertile revertant · Restorer gene · RAPD marker · Mapping

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Introduction

The molecular basis of rice cytoplasmic male sterility (CMS) has not yet been elucidated. CMS has been assumed to result from the interaction of cytoplasmic components with the substituted alien nucleus. Numerous studies have focused on differences in mitochondrial genomes between CMS lines and their maintainer counterparts (Kadowaki et al. 1990; Iwahuchi et al. 1993). However, the high degree of polymorphism between the CMS line and its maintainer mitochondrial genomes makes it difficult to identify the mitochondrial factor controlling male sterility.

On the other hand, some progress has been made in the study of the role of the nuclear genome in fertility restoration. Genetic analyses have made it clear that two genes are generally involved in fertility restoration of the WA-type (carrying a cytoplasm of wild rice encoding abortive pollen) CMS lines, whereas one gene is required to restore fertility to the BT-type (with the Chinsura Boro cytoplasm) CMS lines (Shinjyo 1968; Gao 1981; Yang and Ru 1984; Virmani et al. 1986; Govinda Raj and Virmani 1988). The restorer gene for the BT-type CMS line (Rf_1) has been mapped to chromosome 10 (Shinjyo 1984). This map position was later confirmed by RFLP analysis (Fukuta et al. 1992; Yu et al. 1995). Recently, Shinjyo and Sato (1994) localized another restorer gene (Rf_2) of the BT- and Lead-type (carrying the cytoplasm of Myanmar Lead rice) CMS lines to chromosome 2 using trisomics and genetic marker lines. Zhang et al. (1994) identified an introgressed chromosome segment containing Rf_4 (previously named Rf_2) of IR24 in near-isogenic lines (NILs) on chromosome 1, between RZ382 and RG458. They also postulated that Rf_3 (previously named Rf_1) of IR24 was located on chromosome 7 based on RFLP between NILs (also see Kinoshita 1995). By using primary trisomics, Bharaj et al. (1995) determined the locations of two restorer genes for the WA-type CMS lines contained in IR36. The stronger restorer gene (*Rf*-WA-1) is

on chromosome 7, while the weaker restorer gene (*Rf*-WA-2) is on chromosome 10. However, there is no evidence to indicate identity between Rf_3 and *Rf*-WA-1 (both located on chromosome 7) or between Rf_1 and *Rf*-WA-2 (both on chromosome 10). Mapping of these restorer genes with molecular markers will facilitate restorer breeding by marker-aided selection.

Studies of fertility reversion of CMS lines may help to elucidate the mechanism of CMS. We have reported the mutagenic induction of a fertile revertant, T24, from indica CMS line II-32A (Shen et al. 1996). The revertant T24 is able to restore not only the fertility of its parental CMS line, but also that of other indica CMS lines such as Zhenshan 97A and XieqingzaoA. This restoration is conditioned by a single nuclear gene, which is different from restoration by the restorer genes so far identified, such as those found in Minghui 63 and 20964 (Shen et al. 1996). Genetic characterization of the revertant should provide insight into the mechanism of CMS and fertility restoration. Tagging and mapping of the new restorer gene may clarify its relations with the other restorer genes mentioned above and facilitate breeding for restorer lines with the revertant as a new restorer-gene source. In addition, identification of tightly linked markers to the restorer gene will enable map-based cloning of the gene and eventually elucidate the molecular basis of CMS.

In the present study, we have compared polymorphisms among the revertant, its parent and other restorers using randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al. 1990), identified markers linked to the restorer gene, and localized the restorer gene by linkage analysis.

Materials and methods

Plant materials

In 1991, a fertile revertant, T24, was selected from the M_3 generation of the cytoplasmic male-sterile indica rice line II-32A, the dry seeds of which had been irradiated with ^{60}Co γ-rays. This mutant is able to restore the fertility of indica CMS lines with one nuclear gene (Shen et al. 1996). Two of the derivatives of the revertant after seven generations of selfing, G3 and E15, together with the parent II-32A, the maintainer line II-32B and two indica restorer lines Minghui 63 and IR24, were used in the present study. Seeds of these lines were provided by the Jinghua Seed Co., Zhejiang, China. To set up a mapping population, a cross was made between II-32A and E15. The F_2 population of 95 individuals, and all other materials, were planted in a rice field in the early season (April–August) of 1995. When plants grew to peak tillering stage, 2–3 tillers from each plant were collected and stored at − 70°C. The male sterility and fertility of F_2 plants were distinguished at the flowering and grain-filling stage. The fertile F_2 individuals were advanced to F_3 to determine their homozygosity and heterozygosity for fertility.

Rice DNA isolation

Total rice DNA for Southern hybridization was extracted according to McCouch et al. (1988). DNA concentration was determined spectrophotometrically. DNA for RAPD analysis was isolated basically according to Zheng et al. (1995). After extraction, the supernatant was treated with RNase (1 μg/μl).

Rapd analysis

For RAPD analysis with bulked DNA samples, the DNA concentration of each sample was first adjusted to 100 ng/μl. An equal amount of DNA from ten sterile and ten homozygous fertile F_2 individuals was bulked separately. To search for RAPDs between II-32A and its revertant, DNA samples of II-32A and the revertant were used in a PCR reaction. RAPD procedures were conducted according to Williams et al. (1990) with some modifications. Approximately 100 ng of DNA was used as a template in a final reaction volume of 12.5 μl. The DNA amplification was performed with RAPD decamer primer kits A–Z (Operon Technologies) in either a Perkin Elmer PCR System 9600 or a 2400 Thermocycler programmed as follows: 2 min at 94°C for pre-denaturation; 40 cycles of 10 s at 94°C, 15 s at 36 °C, 30 s at 72°C followed by 2 min at 72°C for extension. Amplification products were resolved by electrophoresis in a 1.4% agarose gel containing 0.5 μg/ml of ethidum bromide. Polymorphism between the bulks or between II-32A and the revertant was confirmed by repeated amplification. Linkage of the polymorphic DNA fragment with the restorer gene was analyzed with 95 F_2 individuals of II-32A/E15.

For marker mapping, the parents of a mapping population, Tesanai2/CB (Lin et al. 1996), were analyzed with primers producing markers linked to the restorer gene. The polymorphic DNA fragments amplified from Tesanai2 and CB were analyzed with Southern hybridization for their homology to the markers linked to the restorer gene. After confirmation of the homology, 91 F_2 individuals of Tesanai2/CB were analyzed with one of the polymorphic primers

Table 1 Comparison of RAPD markers among II-32A (CMS line), II-32B (maintainer), two revertant lines, G3 and E15, and two restorer lines, Minghui63 (MH63) and IR24

Rice lines in comparison	No. of polymorphic primers	Percent polymorphic primers	No. of RAPD fragments	Total No. of amplified products	RAPD rate (%)
II-32A/G3	20	28.6	30	420	7.1
II-32A/E15	19	27.1	27	423	6.4
II-32B/G3	25	35.7	39	421	9.3
II-32B/E15	25	35.7	44	426	10.3
II-32A/MH63	44	62.9	94	439	21.1
II-32A/IR24	45	64.3	105	445	23.6
II-32A/II-32B	24	34.3	43	422	10.2
G3/E15	9	12.9	10	414	2.4

for the linkage of the RAPD marker with RFLP markers. Rice RFLP markers were obtained from Dr. S. Tanksley, Cornell University, Ithaca, N.Y.

Southern-blot analysis

Polymorphic DNA fragments linked to the restorer gene were directly recovered from gels with a Sephaglas™ Bandprep Kit (Pharmacia). Alternatively, the polymorphic DNA was re-amplified with about 5 µl of sliced gel containing the DNA fragment as a template. Amplification was performed in a reaction volume of 50–100 µl. The recovered or re-amplified DNA fragments were labelled with ^{32}P-ATP (Amersham) by random priming (BRL). For confirmation of the homology of polymorphic DNA fragments amplified between Tesanai2 and CB with RAPD markers linked to the restorer gene, the amplification products were resolved in an electrophoresis gel and blotted to a Hybond N^+ membrane (Amersham) by capillary action. Pre-hybridization, hybridization, washing and exposure were done as described in Sambrook et al. (1989).

Linkage analysis

Linkage of RAPD markers to the target gene and to RFLP markers of the rice RFLP map was estimated using Mapmaker software (Lander et al. 1987). Linkage was considered significant if the logarithm of odds (LOD) score was $\geqslant 3.0$. The Kosambi mapping function was used to convert the recombination fraction to map distances among markers and the target gene (Kosambi 1944).

Results

Polymorphism between the revertant and its parent

In a preliminary experiment, 200 RAPD primers were employed to amplify the DNA of II-32A and the revertant line E15. Thirty four primers generated polymorphic fragments between the lines, which accounts for 17% of the total primers used. Of about 1000 amplification products, 40 were polymorphic. Among them, 16 bands were present only in the male-sterile II-32A, while 24 were found in the revertant. RAPD distribution between II-32A and the revertant line was not significantly different ($\chi^2 = 1.225$, $0.25 < P > 0.5$). The occurrence of RAPDs indicated that there were changes in genomes between II-32A and E15. To assess the genomic differences between II-32A and E15, further experiments were carried out to analyze the genetic diversity among II-32A, II-32B, two revertant lines, G3 and E15, and two commercial restorers, Minghui 63 and IR24, using RAPD analysis. Of 70 primers employed, 69 generated amplified products. Each primer produced from 1 to 12 bands ranging from 300 bp to 3000 bp in size. The results gave some indication of the genetic differences between the genotypes used (Table 1). As in the preliminary experiment, 7.1% and 6.4% of the amplified bands were polymorphic between II-32A and G3, and II-32A and E15, respectively, while 9.3% and 10.3% of the amplified bands were polymorphic between II-32B and G3, and II-32B and E15. In contrast, the RAPD rates between II-32A and Minghui 63, and between II-32A and IR24 were as high as 21.1% and 23.6%, respectively. Obviously, the genomic difference between the revertant and II-32A was far less than that between II-32A and the commercial restorers and even smaller than that between II-32A and II-32B (10.2%). Figure 1 shows some typical electrophoresis patterns of the amplified products displaying the similarity of the revertant and II-32A(B), and their dissimilarity to the commercial restorers.

Identification of RAPD markers linked to the restorer gene

In an initial experiment, DNAs of the bulked sterile and f ertile F_2 segregants of a cross between II-32A and E15 were used as templates. After RAPD analysis with 512 single primers and 860 primer pairs, no polymorphism was found between the DNA pools. An alternative approach was adopted to analyze directly the polymorphism between II-32A and the revertant E15 in an attempt to identify RAPD markers which might be less closely linked to the restorer gene. Besides the 200 primers employed in the preliminary experiment, 310 more primers were used. Over 50 primers were found to

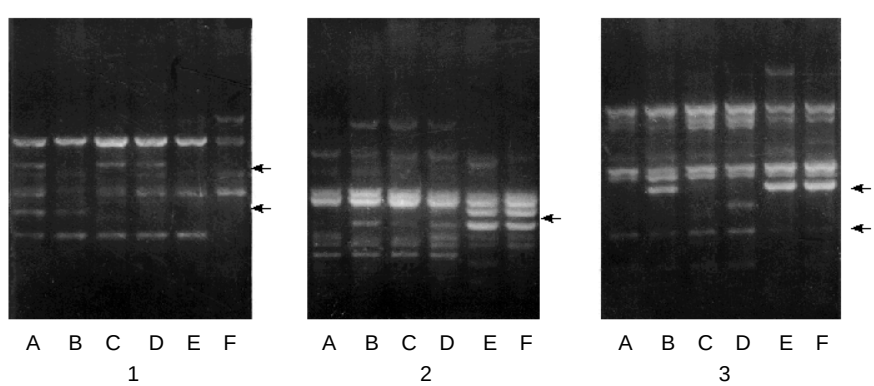

Fig. 1 Some typical RAPD patterns generated with primers (**1**) OPB02 (ACCCCGCCAA), (**2**) OPJ16 (CTGCTTAGGG), and (**3**) OPK09 (CCCTACCGAC) among the parental II-32A (*A*), II-32B (*B*), the revertant restorer lines G3 (*C*) and E15 (*D*) and two restorer lines Minghui 63 (*E*) and IR24 (*F*). *Arrows* indicate RAPDs differentiating the similiarity of the revertants with II-32A (*B*) and their disimiliarity with commercial restorers

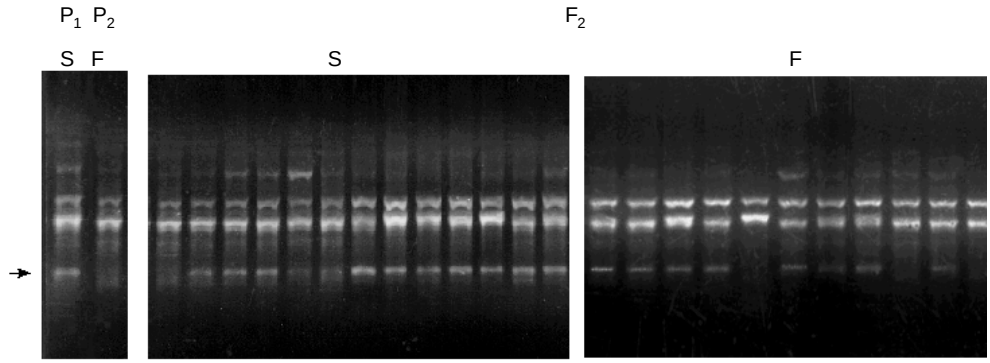

Fig. 2 RAPD analysis of II-32A (P_1), the revertant line E15 (P_2) and some of the F_2 sterile (*S*) and fertile (*F*) individuals with primer OPB07

amplify polymorphic DNA fragments between II-32A and E15. These polymorphic primers were further tested by amplifying the DNA of one randomly selected homozygous sterile segregant and one homozygous fertile segregant, and 16 primers reproduced polymorphic DNA fragments. These 16 primers were then used to amplify the DNA of 22 sterile F_2 segregants of II-32A/E15. OPB07 (GGTGACGCAG) and OPB18 (CCACAGCAGT) generated RAPDs of 640 bp and 1000 bp, respectively, in II-32A and in 21 out of 22 sterile F_2 segregants of II-32A/E15, indicating a possible linkage of the RAPDs with the recessive allele of the restorer gene. The rest of the primers produced RAPDs which did not show linkage to the restorer gene or its allele. OPB07 and OPB18 were then used to analyze 73 fertile F_2 segregants. $OPB07_{640}$ and $OPB18_{1000}$ completely co-segregated in the F_2 population (data not shown). Among 73 fertile F_2 segregants, 55 had $OPB07_{640}$ and $OPB18_{1000}$ (Fig. 2). Therefore, out of 95 segregants, 76 amplified these RAPDs and 18 did not, indicating that they were unbiasedly segregating in a Mendelian fashion (3:1, $\chi^2 = 1.2385$, $0.25 < P > 0.5$). A two-point test showed that $OPB07_{640}$ and $OPB18_{1000}$ were linked in repulsion phase to the recessive allele of the restorer gene with a genetic distance of 5.3 cM.

$OPB07_{640}$ and $OPB18_{1000}$ were cross hybridized and no homology was detected between the two fragments indicating that, although tightly linked, they are not identical (data not shown).

Linkage analysis and chromosomal localization of the restorer gene

Hybridization of the $OPB07_{640}$ and $OPB18_{1000}$ probes with genomic DNA of II-32A and E15 revealed that these two fragments have many homologous DNA sequences in their genomes (data not shown), indicating that these fragments may contain repetitive sequences. This ruled out the possibility of mapping the fertility restorer gene with these RAPD markers by Southern hybridization.

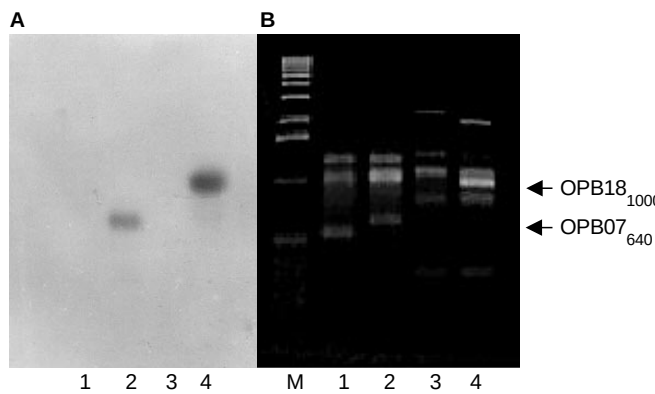

Fig. 3 RAPD analysis of Tesanai2 (*1, 3*) and CB (*2, 4*) with primers OPB07 (*1, 2*) and OPB18 (*3, 4*) (**B**), and the autoradiogram from the Southern hybridization of the RAPD product blot with $OPB07_{640}$ and $OPB18_{1000}$ as probes (**A**). *M* molecular-weight marker (1-kb ladder)

Instead, OPB07 and OPB18 were used to amplify DNA from parents of the mapping population Tesanai2/CB. Interestingly, these two primers generated two RAPD fragments in CB with the same size as $OPB07_{640}$ and $OPB18_{1000}$ amplified in II-32A. To examine whether the fragments of the same size were homologous, electrophoretically separated PCR products were blotted and hybridized with mixed probes of $OPB07_{640}$ and $OPB18_{1000}$, collected and re-amplified from RAPD products of II-32A. The $OPB07_{640}$ and $OPB18_{1000}$ probes hybridized only to the fragments of 640 bp and 1000 bp in CB (Fig. 3). This result confirmed that Tesanai2 and CB are polymorphic at the $OPB07_{640}$ and $OPB18_{1000}$ loci and that their F_2 generation can be used to analyze the linkage of the RAPD markers with RFLP markers. Ninety one F_2 individuals were used for linkage analysis with primer OPB07. Sixty nine individuals amplified the $OPB07_{640}$ fragment while 22 individuals did not (3:1, $\chi^2 = 0.0036$, $0.95 < P > 0.975$). The linkage analysis with Mapmaker revealed that $OPB07_{640}$ was linked to RG374 and RG394 on chromosome 1, 10.8 cM from

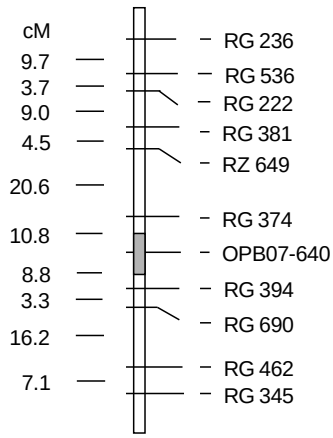

Fig. 4 Partial linkage map of chromosome 1 developed from the mapping population Tesanai2/CB showing the location (*shaded part*) of fertile restorer gene

RG374 and 8.8 cM from RG394, respectively. Because the genetic distance between $OPB07_{640}$ and the restorer gene was estimated at 5.3 cM, the restorer gene may, therefore, be located between RG374 and RG394 (Fig. 4).

An attempt to fine-map the restorer gene was made by RFLP analysis of E15, II-32A and their F_2 population using RG374, RG394 and RZ649 as probes. Unfortunately, no polymorphism was found between II-32A and E15 with DNA digested with 16 restriction enzymes.

Discussion

In the selection of fertile revertants from the M_1 generation of CMS lines irradiated with γ-rays, mechanical contamination by the maintainer and cross-pollination could not be completely excluded. Previous agronomic and genetic analyses of the revertant have provided evidence of a bona fide mutant (Shen et al. 1996). In the present study, RAPD analysis was used as an effective way to detect differences among genomes. Comparison between the revertant and II-32A, as well as other restorer lines, may add strong evidence to the above conclusion. Polymorphism between II-32A and the revertant was found. However, the RAPD rates between II-32A and the revertant (6.4%–7.1%) were much less than those between II-32A and the two restorers Minghui 63 and IR24 (21.1% and 23.6%), the latter being in the range of typical RAPD rates among indica varieties (Fukuoka et al. 1992). The results also showed that the level of polymorphism between II-32A and the revertant was even smaller than that between II-32B and the revertant. This might be partly due to cytoplasmic DNA amplification because total DNA was used as the template in the present experiment. Although II-32B is the recurrent parent in the backcross for maintenance of II-32A, the origin of different cytoplasms of II-32A and II-32B may contribute to the difference of polymorphism levels between II-32A or II-32B and the revertant. The above results, together with morphological, agronomic and genetic evidence obtained in previous experiments, indicated that the fertility restoring revertant is a product of a nuclear mutation and is unlikely to be a physical contamination or to result from cross-pollination. However, whether the mutation is a point mutation or a chromosome deletion is not yet know. Because the revertant has strong restoring ability for several types of indica CMS lines (Shen et al. 1996), it can serve as a useful new restorer gene source. In addition, it contains the cytoplasm of II-32A, so that it provides an effective system for restorer selection by simply using it as the maternal parent in crosses with the other desired lines and then isolating progeny individuals with normal seed setting, so avoiding the need for testcrossing to assess progeny fertility. Moreover, because the revertant and its parent II-32A are near-isogenic lines, they provide excellent experimental materials for CMS and fertility restoration studies.

In the present study, DNA polymorphism (6–7%) was found between the revertant and its parent. This result suggests that γ-rays induced other chromosome alterations in addition to the mutation causing fertility reversion. It is well known that γ-rays often cause various kinds of mutations including chromosome deletions (Gustafsson and Ekberg 1977). The presence versus absence of lost regions may contribute to the polymorphism. On the other hand, II-32A may remain heterozygous at numerous loci in some recalcitrant regions during its maintenance by repeated backcrosses, which is reflected by a RAPD rate of about 10% between II-32A and its maintainer II-32B (Table 1), whereas E15 has become a homozygous line after seven generations of selfing. The heterozygosity versus homozygosity in some loci will also add to the polymorphism in RAPD analysis for it is a dominant marker.

The present study initially adopted a method of bulked segregant analysis (Michelmore et al. 1991) for RAPD marker identification in an attempt to tag the mutated gene or to find markers closely linked to the target gene. Unfortunately, the use of many primers failed to reveal any polymorphism. In a subsequent experiment, DNA comparisons were made between II-32A and the revertant leading to the identification of markers linked to the target gene (5.3 cM). Although γ-rays caused DNA alterations, the polymorphism between II-32A and the revertant is still low. Moreover, the alterations may be scattered throughout the genome, and perhaps are less densely located near the target gene. Therefore, it is difficult to screen for more closely linked markers by this strategy. An alternative strategy for identifying tightly linked markers for a target gene contained in the revertant may be to use bulked segregants of a cross of the revertant with a

distantly related allelic line. In this way, abundant polymorphism between the parents is likely to increase the probability of finding very closely linked markers.

Genetic analysis of the fertile revertant has shown that the restoring ability of the revertant for indica CMS lines is conditioned by one nuclear gene. This genetic mode is different from that of other indica restoring lines such as Minghui 63, 20964 (Shen et al. 1996) and IR24 (Gao 1981). The latter restorers behave in a two-gene inheritance mode. The restorer genes of most indica restorers currently used in China were derived from IR lines, such as IR24, IR26 and IR36, which can be traced back to Cina and SLO17 (Li and Yuan 1986; Govinda Raj and Virmani 1988). Allelic analysis of the restorer genes by the segregation of cross and backcross generations of the revertant and typical restorers is now underway. In the present study, the restorer gene resulting from induced mutation of the CMS line was tagged and mapped to chromosome 1, between RG374 and RG 394. Although one of the restorer genes of IR24, Rf_4, is also present on chromosome 1 (Zhang et al. 1994), these genes are located far apart. This result suggests that the restorer gene present in the revertant line T24 is different from those present in IR24 and IR36. The mechanism of restoration may also be distinct. The restorer gene present in the fertile revertant T24 is designated Rf_5. The present study is a first step towards map-based cloning of this gene. Further work is planned to identify tightly linked molecular markers in order to isolate BAC clones containing the restorer gene Rf_5.

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