

A Versatile and Highly Reliable Method of Identifying Genes Responsible for Mutations Caused by Highly Active Transposable Elements

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Abstract

Active transposition of the DNA transposon *Tif1* in the *Torenia* mutable line “Flecked” generates new mutants, including those with imperfect penetrance. However, when a gene responsible for a mutation is identified using the transposon display method, the high transposition activity of *Tif1* generates false-positive bands, thus making identification difficult. In this study, we designed a transposon display method that uses the F₂ mutant-type population obtained from a cross between the mutant and another line harboring *Tif1* in a different genomic location. When this method was applied to the “Flecked” mutation, only the band containing *TfMYB1*, which is responsible for the mutation, was commonly detected in the mutant-type F₂ population, while the unspecific bands were segregated. Further, this method effectively identified the gene responsible for the “Petaloid” mutation, which displays imperfect penetrance. Thus, this method can be potentially used in various other crops to identify the mutations caused by highly active transposons, including those with imperfect penetrance.

Discipline: Plant breeding

Additional key words: imperfect penetrance, torenia (*Torenia fournieri* Lind. ex Fourn.), transposon display

Introduction

Transposable elements, which are effective endogenous crop mutagens, have been used to impart improved agricultural traits. For example, the Japanese morning glory (*Ipomoea nil*) underwent a burst of mutagenesis caused by transposable elements in the late Edo era (1806-1860) (Nitasaka 2007). This burst of mutagenesis produced various morphological mutants called “mutant morning glories” (*henka asagao* in Japanese), which display a variety of flower and leaf shapes, as well as flower colors and patterns. A gene mutated by the integration of a transposable element can be identified by using the element’s nucleotide sequence as a tag. This method (called transposon tagging) has made a remarkable contribution to the discovery of genes involved in flower color and morphology (Gierl & Saedler 1992).

Transposable elements are classified into two groups (Wicker 2012). Class I elements (called retrotransposons)

transpose following a “copy and paste” mechanism, in which the transposon is not excised once integrated into genomic DNA. These retrotransposons cause genetically stable mutations. Class II elements (called DNA transposons or simply “transposons” in the following text) transpose following a “cut and paste” mechanism, which sometimes causes reverse mutation. However, if the transposon is a non-autonomous element encoding no transposase, the mutation is stabilized by segregating the non-autonomous element and the corresponding transposase gene (Shimamoto et al. 1993). Excision of a transposon can cause incorrect repair of the flanking sequence, which is called a “footprint” (Coen et al. 1986). If a footprint occurs in an exon, stable mutation can be induced by a frameshift or small change in the amino acid sequence. As can be seen from the discussion above, both retrotransposons and transposons are effective endogenous mutagens that generate stable mutations that can be useful for crop breeding.

Transposon display, which is based on amplified

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fragment length polymorphism (AFLP) (Van den Broeck et al. 1998, Frey et al. 1998, Fukuda-Tanaka et al. 2001), has been used as a standard and highly sensitive method of detecting the integration of transposable elements. In this method, genomic DNA is digested with a restriction enzyme, and the resultant DNA fragments are ligated with an artificially synthesized oligomeric DNA adapter. Next, the DNA fragments that include a transposable element and flanking genomic DNA are amplified and detected through polymerase chain reaction (PCR) by using a primer set consisting of one primer that binds to the adapter and another fluorescently labeled primer that binds to the transposable element. Thus, transposon display is a dominant marker.

When using transposon display to identify the gene responsible for a mutation, the transposon display band detected in a mutant but not in the parent line should be from the target gene. If the parent line is not specified, reverse mutation is useful as a control (Fukuda-Tanaka et al. 2001); the band detected in a mutant but not in the fixed revertant should be the target gene. This is because alleles of the target gene, from which the transposable element was excised, are homozygous in the fixed revertant. In this method, S₁ plants of the revertant type should be phenotypically examined to confirm the fixation of reverse mutation.

The authors isolated a *Torenia* (*Torenia fournieri* Lind. ex Fourn.) mutant named “Flecked” that harbors the active *En/Spm* transposon *Tf1* (Nishijima et al. 2013). In this mutant, *Tf1* is integrated into *TfMYB1*, which encodes the *R2R3-MYB* transcription factor that regulates anthocyanin biosynthesis. The presence of *Tf1* at this locus results in the downregulation of *TfMYB1* and faded petal color from violet to pale violet (Fig. 1). In “Flecked,” highly active

transposition of *Tf1* induces petals that are densely spotted or fully colored in violet, as well as causing new mutations. One such new mutation, the double-flowered mutant “Petaloid” in which stamens have been converted into petals, has been reported (Nishijima et al. 2016). In this mutant, *Tf1* is integrated in the class C floral homeotic gene *TfFAR*. Dozens of other mutations with varying morphology, color, and color pattern of flowers and shoots have been isolated from the selfed descendants of “Flecked” (Nishijima, unpublished data).

In “Flecked,” highly active transposition of *Tf1* sometimes causes extra bands in the transposon display, thereby making identification of the target gene difficult. Meanwhile, when we identify the target gene using a fixed revertant, the substantial period of time necessary for fixation sometimes causes the excision of *Tf1*. This results in the loss of some bands unspecific to the target gene in the revertant, which, in turn, generates false-positive bands in the mutant.

A highly active transposon has also been reported in *Petunia* (Gerats et al. 1989), suggesting that the presence of a highly active transposon is a relatively common phenomenon. Even if this is the case, mutant-type plants are expected to have transposon integration within the target allele. In contrast, some plants exhibiting a revertant phenotype should be homozygous for the revertant alleles without transposon integration. Thus, the transposon display band commonly detected in mutant-type plants and undetected in some revertant-type plants may be from the target gene. This method has been used to successfully identify genes responsible for *Petunia* mutations induced by highly active transposons (Van den Broeck et al. 1998). However, if the



Fig. 1. *Torenia* (*Torenia fournieri*) lines used in the experiments.

“Common Violet,” is the parent line of “Flecked.” “Petaloid” was isolated from selfed descendants of “Flecked.” Photographs are reproduced from Nishijima et al. (2013 and 2016).

penetrance of a mutation is not sufficiently high, similar to the quantitative agricultural traits seen in various crops, a homozygote of the mutant alleles exhibiting no phenotype or only a mild one may be recognized as a revertant. Mutations of this type are also frequently observed in selfed descendants of *Torenia* “Flecked” (e.g., Nishijima 2016). Since this makes the selection of “true” revertants difficult, it may be unreasonable to identify the target gene based on loss of the band in some revertant-type plants.

From the concerns voiced above, the most reliable identification of the target gene should be based on the phenomenon whereby mutant-type plants always have the mutant allele, unless penetrance of the mutation is extremely low. Using this concept, a band commonly observed in the mutant-type population should be from the target gene. Thus, unspecific bands generated by the integration of transposon copies at sites other than that of the target gene should not commonly appear in the mutant-type population. To attain this condition, the use of an F₂ population obtained from a cross between the mutant and another line harboring copies of the transposon in different genomic locations may be effective (Fig. 2). In this method, all transposon-integrated sites are heterozygotes in the F₁ generation, and will

be segregated in the F₂ generation. Thus, transposon copies unspecific to the mutation will disappear in some mutant F₂ plants, unless closely linked to the target gene.

In the present study, we applied this method to two *Torenia* mutants in which the genes responsible for the mutations are already known —“Flecked” (described above) and an imperfect penetrance mutant “Petaloid” (Nishijima et al. 2016) — in order to assess the effectiveness of the method with mutations caused by a highly active transposon and also with mutations with imperfect penetrance.

Materials and Methods

1. Plant Material

This study used three *Torenia* lines: “Flecked” (Nishijima et al. 2013), “Petaloid” (Nishijima et al. 2016), and S₄ plants of a “Flecked” revertant (abbreviated as “rFlecked”). “Petaloid” was isolated from the selfed progeny of “Flecked” (Nishijima et al. 2016). Genomic sites of *Tf1* integration in “rFlecked” were expected to be different from those in “Flecked” and “Petaloid” due to active *Tf1* transposition spanning four generations of reproduction. It should be noted that all three lines used in the experiments

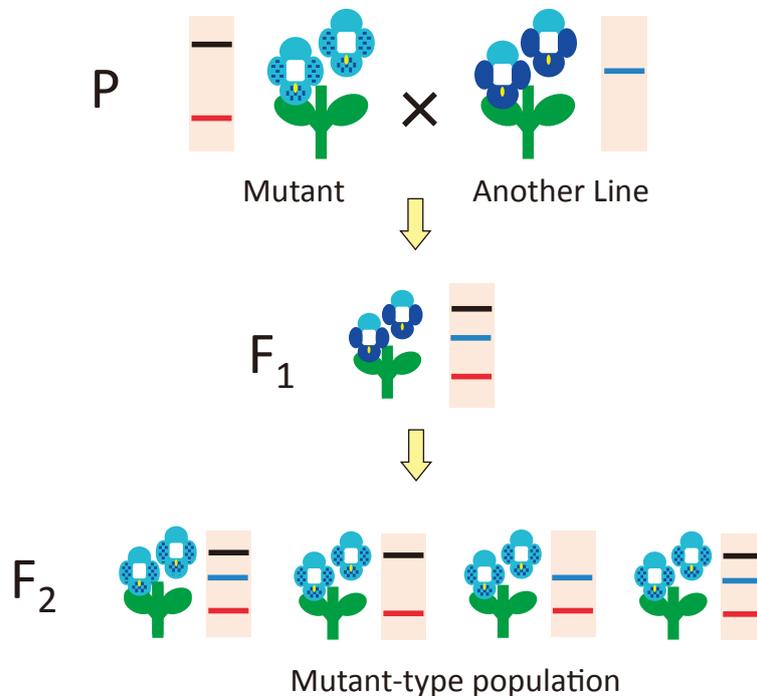


Fig. 2. Scheme depicting the method of identifying the gene responsible for a mutation caused by a highly active transposable element.

Beige rectangles beside the plants indicate the band pattern of transposon display. The red band indicates the target band generated by the gene responsible for the mutation, while the black and blue bands indicate unspecific bands from each parent. All mutant-type F₂ segregants are expected to harbor the target band, while the unspecific bands are segregated.

are a mixture of plants with different genomic *Tf1* integration sites, except for those of the target gene, because the lines were maintained by the self-pollination of a number of plants in each generation. However, one plant per line was used as the parent of a cross. Methods of growing and cross-pollination have been described elsewhere (Nishijima et al. 2013). “rFleked” was crossed as a pollen parent with “Fleked” and “Petaloid” (Fig. 1). One flower per F₁ plant was self-pollinated. The F₂ seeds from six F₁ flowers were mixed and planted to raise F₂ populations. Mutant-type plants were selected based on the expression of mutant traits, and 12-16 mutant-type plants were subjected to transposon display. As a control, seeds derived from the self-pollination of six mutant-type flowers of a “Fleked” plant were mixed, and the mutant-type S₁ plants were subjected to transposon display.

2. Transposon Display

Corollas at an early stage of pigmentation (10-15 mm in length) were collected and frozen in liquid nitrogen. The samples were then stored at -80°C. Genomic DNA was extracted as previously described (Nishijima et al. 2016), and then subjected to simplified transposon display (Fukuda-Tanaka et al. 2001) with slight modification. In brief, genomic DNA was digested using Taq I at 65°C for 6 h, and then the adapter shown in Table 1 was ligated to the digested DNA fragments using T₄ DNA ligase at 16°C for 6 h. Nested PCR was conducted using the primer sets shown in Table 1 to amplify the sequence between the right terminal region of *Tf1* and the ligated adapter. The first PCR reactions were performed using a preincubation step of 2 min. at 95°C followed by 30 cycles of 40 s at 95°C, 60 s at 58°C, and 60 s at 72°C. The second PCR reactions were performed with basically the same program, but the annealing temperature was lowered stepwise from 72 to 59°C (-1°C per cycle), and then kept at 58°C in the following 30 cycles. GoTaq (Promega) was used as the DNA polymerase. PCR products were subjected to denaturing polyacrylamide gel electrophoresis (5% (w/v) polyacrylamide gel contain-

ing 36% (w/v) urea; 300 mm in length and 0.5 mm in thickness). Fluorescent PCR products including the *Tf1* sequence were detected using a fluorescence laser scanner (Typhoon 9400, GE Healthcare Science) with an excitation wavelength of 532 nm. The band estimated as that of the target gene was excised and then extracted using distilled water at 95°C for 20 min. The extract was subjected to PCR employing the same primer set and temperature program used for the second PCR of transposon display, although the primer was not fluorescently labeled. The amplified DNA fragment was subcloned and sequenced using the usual method.

Results and Discussion

1. Phenotypic Segregation

All F₁ plants were the normal type both in crosses between “Fleked” and “rFleked” or between “Petaloid” and “rFleked” (Table 2). F₂ plants exhibited ratios of normal-type plants higher than the theoretically expected ratio (i.e., normal-type:mutant-type = 3:1 in both mutants), suggesting that *Tf1* was excised *de novo* during development of the population.

2. Transposon display

Seed parents, pollen parents, and 12-16 F₂ mutant-type plants were subjected to transposon display. As expected, the band patterns of seed and pollen parents were completely different in both cross combinations (Figs. 3 and 4).

As described above, mutant-type plants are expected to be homozygous relative to the *Tf1*-integrated allele of the target gene. Thus, a band commonly detected in the mutant-type F₂ population should be that of the target gene. A band was commonly detected in the F₂ mutant-type population of “Fleked” (Fig. 3, arrowhead), while the other bands were not detected in some plants. The size of the former band coincided with that of *TfMYB1* containing a *Tf1* insertion (347 bp), which is responsible for “Fleked” mutation. Based on cloning and sequencing analysis, we

Table 1. Adapter and PCR primers used for transposon display.

Oligo DNA species	Binding site	Base sequence
Adapter	–	5'-CTGTGAATGCTGCGACTACGATG-3' 3'-CTTACGACGCTGATGCTACGC-5'
Primer set for 1st PCR	Adapter	5'-CTGTGAATGCTGCGACTACGAT-3'
	<i>Tf1</i>	5'-CGGCGACAGAGGAGAATTTAGCT-3'
Primer set for 2nd PCR	Adapter	5'-AATGCTGCGACTACGATGCGA-3'
	<i>Tf1</i>	5'-GACCGTTTCTGTGCGCAATTC-3' ^z

^z 5' end labelled with 5(6)-carboxy-X-rhodamine (ROX).

Table 2. Trait segregation of F₁ and F₂ plants derived from crosses between mutant-type flower of “Flecked” or “Petaloid” and S₄ selfed descendant from somatic revertant flower of “Flecked” (rFlecked).

Cross	Generation	Plant only with NT flowers	Plant with MT flowers ^z
		(No. of plants (%))	(No. of plants (%))
Flecked × rFlecked	F ₁	30 (100)	0 (0)
	F ₂	75 (82)	16 (18)
Petaloid × rFlecked	F ₁	30 (100)	0 (0)
	F ₂	68 (85)	12 (15)

^z Plants with mutant-type flower include those bearing both mutant-type and somatic revertant flowers within a plant.

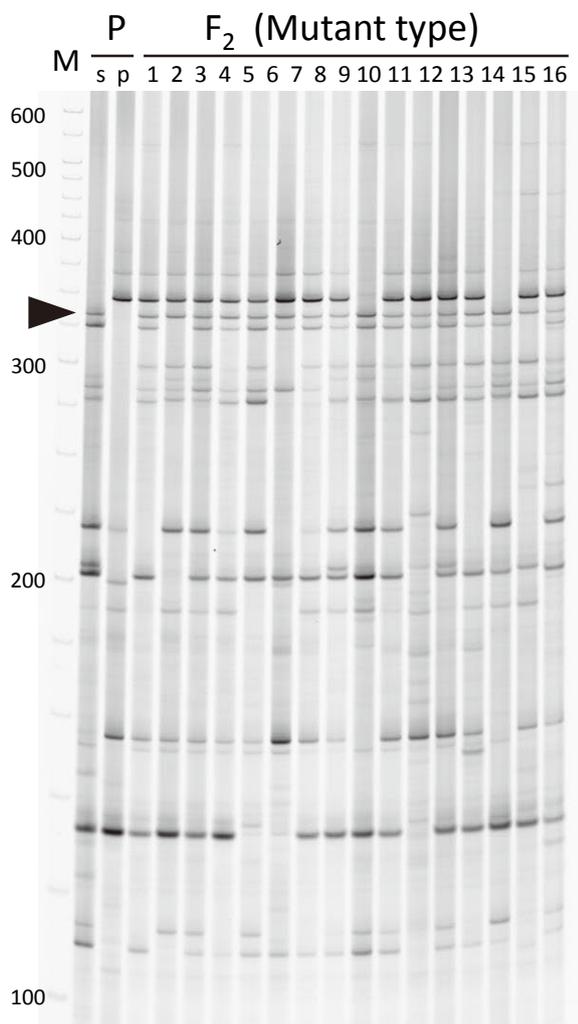


Fig. 3. Transposon display of the mutant-type F₂ population from a cross between “Flecked” and “rFlecked.” The black arrowhead indicates the band generated by the target gene (i.e., *TfMYB1* containing a *Tf1* insertion). M: molecular weight marker; P: parents; s: seed parent; p: pollen parent; F₂, F₂ plants. Numbers beside the vertical axis represent the length of the molecular weight markers in base pairs.

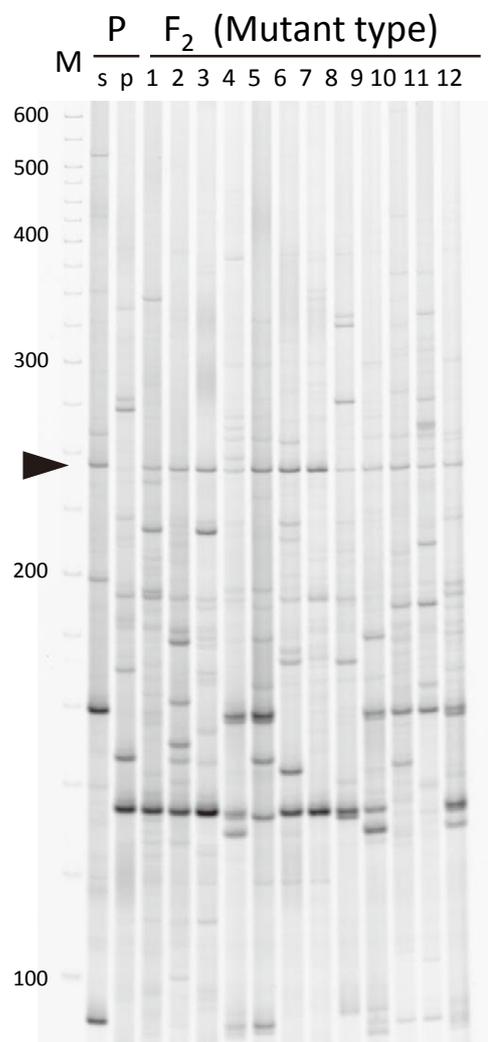


Fig. 4. Transposon display of the mutant-type F₂ population from a cross between “Petaloid” and “rFlecked.” The black arrowhead indicates the band generated by the target gene (i.e., *TfFAR* containing a *Tf1* insertion). M: molecular weight marker; P: parents; s: seed parent; p: pollen parent; F₂, F₂ plants. Numbers beside the vertical axis represent the length of the molecular weight markers in base pairs.

further confirmed that this band is *TfMYB1* containing a *Tf1* insertion.

A band was also commonly detected in the F₂ mutant-type population of “Petaloid” (Fig. 4, arrowhead), while the other bands were not detected in some plants. The size of the commonly detected band coincided with that of *TfFAR* containing a *Tf1*-insertion (247 bp), which is responsible for “Petaloid” mutation. Based on cloning and sequencing analysis, we further confirmed that this band is *TfFAR* containing a *Tf1* insertion. Phenotypic expression is not completely stable in “Petaloid,” causing 5-10% false somatic revertant flowers with no genetic reversion (Nishijima et al. 2016). However, our method successfully identified the target gene, thus demonstrating the applicability of this method to mutations with imperfect penetrance.

The bands other than that of the causal gene were segregated among F₂ mutant-type plants in both “Flecked” and “Petaloid” populations. Moreover, additional bands that were not detected in the parents appeared in the F₂ plants, illustrating the active *de novo* transposition of *Tf1*.

Meanwhile, mutant-type S₁ segregants of “Flecked” generated seven bands that were commonly detected in the mutant-type population, one of which was from *TfMYB1* (Fig. 5, arrowhead). This redundant band pattern clearly makes identification of the target gene difficult, and, in turn, demonstrates the advantage of the F₂ population described above.

It should be noted that copy number of *Tf1* was 3-13 when estimated from the band number detected in transposon display (Figs. 3-5), while the copy number was around 3 when analyzed with southern-blot analysis (Nishijima et al. 2012). Since our experimental conditions employed in transposon display hardly produce any false positive bands (data not shown), the higher copy number detected in transposon display was probably due to much higher sensitivity of the method as compared to southern-blot (Van den Broek et al. 1998).

In conclusion, the effectiveness of the method described above was proven in terms of its application to mutations caused by a highly active transposon and also to mutations with imperfect penetrance. It should be noted that this method is effective when the transposon is integrated in a genomic site other than the coding region, which causes genetic reversion. Meanwhile, non-specific bands commonly seen in the F₂ mutant-type population might appear when the copy number of the transposon is high. If this is the case, and penetrance of a mutation is sufficiently high, analysis of the normal-type F₂ population in addition to the mutant-type population may provide useful clues regarding the identity of the target gene. The band not seen in some normal-type segregants may be used as a second condition with which to narrow the candidates. In addition, the simultaneous use of selective fluorescent-labelled primers with

selective nucleotide in their 3' end will be effective in cases where the band number is too large to be clearly separated (Fukuda-Tanaka et al. 2001).

3. Future Prospects

In this report, only recessive mutants (i.e., “Flecked” and “Petaloid”) were used to examine the performance of our method. However, our method will also be effective for dominant and semi-dominant mutations. This is because mutant-type plants are expected to have an allele mutated through transposon integration, and unspecific copies of the transposon will be segregated in the F₂ generation regard-

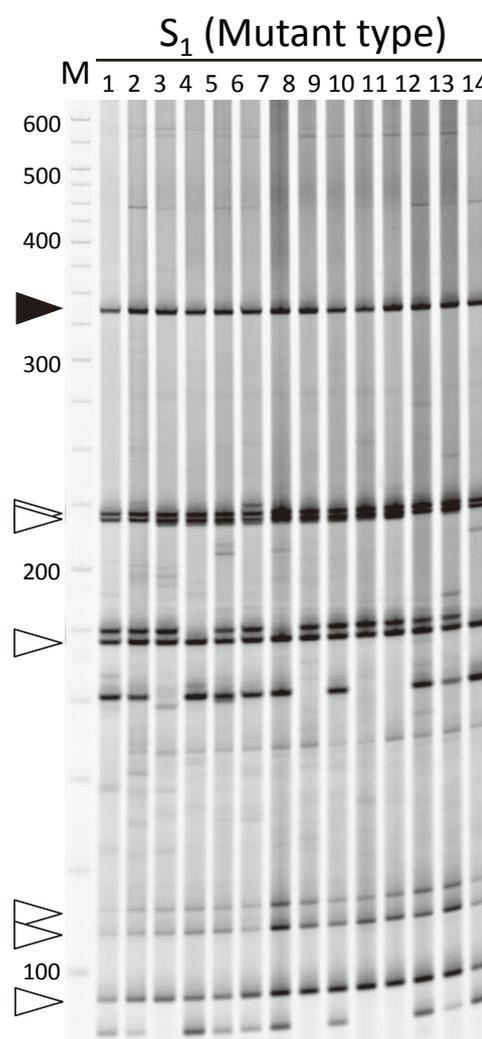


Fig. 5. Transposon display of the mutant-type S₁ population of “Flecked.” The black arrowhead indicates the band generated by the target gene (i.e., *TfMYB1* containing a *Tf1* insertion), while the open arrowheads indicate unspecific bands commonly seen in mutant-type plants.

M: molecular weight marker; S₁, S₁ plants. Numbers beside the vertical axis represent the length of the molecular weight markers in base pairs.

less of whether the mutation is recessive.

In the field of crop breeding and production, unstable germinal or somatic mutations, typically variegation of leaf and flower, are often observed depending on the species and cultivars. These mutations are usually recognized as “useless sports” when too unstable to be used as breeding materials. At least some of these mutations may be caused by highly active transposons. “Flecked” was first found among such “useless sports” (Nishijima et al. 2013). However, we cautiously maintained this mutant through the repeated self-pollination of mutant-type flowers and the selection of mutant-type plants in the next generation. In the process of line maintenance, various new mutations have appeared with probability comparable to that of radiation and chemical mutagenesis (Nishijima, unpublished data). Some new mutations are unstable, causing reverse mutations where the gene responsible for the mutation may be identified by employing our method. Other mutations are stable and possibly caused by a footprint generated by *Tf1*. These stable mutants may be used as breeding materials when agriculturally valuable. Thus, if “useless” unstable mutants could be carefully maintained, such a situation could become a “treasure island.” In such a situation, the method proposed in the present report will be useful to identify genes responsible for mutations, which will be of great help in elucidating the molecular mechanisms involved in agriculturally valuable mutations.

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