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Y. Inagaki · Y. Hisatomi · S. Iida

Somatic mutations caused by excision of the transposable element, *Tpn1*, from the *DFR* gene for pigmentation in sub-epidermal layer of periclinally chimeric flowers of Japanese morning glory and their germinal transmission to their progeny

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Abstract Pigmentation in flowers of Japanese morning glory is intense in the epidermal layer, lighter in the sub-epidermis, and much lighter in the internal tissues; by contrast coloration in stems occurs only in the sub-epidermal layer. The *a-3^f* mutant of Japanese morning glory bears white flowers with normal-colored flecks and sectors, and its variegation also occurs in leaves and stems. The mutable line can produce chimeric flowers pigmented uniformly in the sub-epidermal tissue and variegated in the epidermal layer, and stems of these flowers are also pigmented. Since they give selfed progeny that segregate to give a ratio of three germinal revertants bearing fully colored flowers to one flecked mutant, it has been [OR Imai (1934) has] postulated that somatic mutations in the sub-epidermal layer can be transmitted to the next generation and that the germ cells in the reproductive organs must form from the cells of the sub-epidermal layer. Recently, we found that the 6.4-kb *En/Spm*-related transposable element, *Tpn1*, resides within the *DFR-B* gene for anthocyanin biosynthesis in the mutable *a-3^f* line. To test whether somatic mutations caused by *Tpn1* excision from the *DFR-B* gene in the sub-epidermis of periclinally chimeric flowers are transmissible to their progeny, we have examined the structure of the *DFR-B* region in the germinal revertants derived from the chimeric flowers and compared the sequences generated by the somatic excision of *Tpn1* in periclinally chimeric flowers with those in their germinal revertants. Our results confirm that somatic mutations caused by *Tpn1* excision from the *DFR-B* gene in the sub-epidermal tissue of chimeric flowers can be transmitted to their progeny, which results in the generation of germinal revertants.

Key words Transposable elements · Periclinal chimera · Somatic mutation · Germinal transmission · Japanese morning glory

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Y. Inagaki[1] · Y. Hisatomi[2] · S. Iida[2] (✉)
Department of Biological Science and Technology, Science University of Tokyo, Yamazaki, Noda-shi, Chiba 278, Japan

Y. Inagaki
Department of Health Science, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Present addresses:
[1] Department of Bacteriology, National Institute of Health, Toyama, Shinjuku-ku, Tokyo, 162, Japan
[2] Division of Gene Expression and Regulation I, National Institute for Basic Biology, Okazaki, 444, Japan

Introduction

Although mutable alleles concerned with flower variegation have been described in various plants (Nevers et al. 1986; Forkmann 1993), detailed genetic and molecular analyses of their variegated pigmentation phenotypes, which are caused by transposable elements, have been studied mainly in snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), and Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*). They include *nivea^{recurrens}*, *pallida^{recurrens}*, and *delila-602* in *Antirrhinum* (Coen et al. 1989; Goodrich 1992), *rt-vu15* or *rt** in *Petunia* (Brugliera et al. 1994; Kroon et al. 1994), and *a-3^{flecked}*, or *a-3^f*, in *Pharbitis* (Inagaki et al. 1994). The patterns of flower variegation in *Pharbitis* are more complicated than those in *Antirrhinum* and *Petunia*. This is because pigmentation in *Pharbitis* flowers is intense in the epidermal layer, lighter in the sub-epidermis and much lighter in the internal tissues, whereas coloration of flowers in *Antirrhinum* or *Petunia* occurs only in the epidermal layer. Therefore *Pharbitis* can produce periclinally chimeric flowers pigmented uniformly either in the sub-epidermal tissue or in the internal tissues and flecked in the epidermal layer (Imai 1931, 1934, 1935; Kihara 1934), whereas such chimeric flowers are not seen in *Antirrhinum* and *Petunia*.

The shoot meristems of many angiosperms consist of three cell layers, termed L1, L2 and L3; the L1 cells give rise to the epidermal layer, the L2 to the sub-epidermis, and the L3 to the internal tissues (Tilney-Bassett 1986; Huala and Sussex 1993). Since the cell lineages of the three layers are usually distinct, a somatic mutation in earlier meristem

development may give rise to a branch in which one of the layers consists of cells carrying the mutation (see Fig. 1 A). If such a somatic mutation occurs in the L2 layer of a branch, for example, it will result in a mosaic plant having a periclinally chimeric branch in which the sub-epidermal cells contain the somatic mutation (see Fig. 1 B).

The *Pharbitis* line carrying the mutable allele *a-3^f* in the *A-3* locus for anthocyanin pigmentation bears white flowers with normal-colored flecks and sectors, and its variegated pigmentation also occurs in leaves, stems and hypocotyls (Imai 1931, 1934, 1935; Kihara 1934; Inagaki et al. 1994; see also Fig. 1 C). This variegation is caused by recurrent somatic mutation from the homozygously recessive white to the dominant pigmented allele in the heterozygous condition, and germinal revertants have been reported to appear with frequencies of around 5% (Imai 1934). In the *a-3^f* line, the 6.4-kb transposable element *Tpn1* was found within the *DFR-B* gene encoding dihydroflavonol-4-reductase (DFR) for anthocyanin biosynthesis (see Fig. 2). Moreover, excision of *Tpn1* from the *DFR-B* gene (*DFR-B**) appeared to coincide with reversion of the *a-3^f* mutation in a few germinal revertants and their progeny (Inagaki et al. 1994). Among the three copies of the *DFR* gene in the *Pharbitis* genome, *Tpn1* resides within the second intron 9 bp upstream of the third exon of the *DFR-B* gene (*DFR-B::Tpn1*). This element carries 28-bp perfect terminal inverted repeats, the outer 13-bp sequences of which are identical to those of the maize element *En/Spm*, and is flanked by a 3-bp direct duplication (Inagaki et al. 1994; Hoshino et al. 1995). Like other plant transposable elements (Coen et al. 1989; Fedoroff 1989; Gierl and Saedler 1992), *Tpn1* generates characteristic small DNA rearrangements, called empty donor sequences (EDSs); upon excision (Inagaki et al. 1994; see also Table 1).

In the flowers of Japanese morning glory, pigmentation occurs not only in the epidermal layer but also in the subepidermis and internal tissues whereas coloration in its stems is restricted to the sub-epidermal layer (Imai 1931, 1934, 1935; Kihara 1934). It is thus easier to distinguish some types of chimera from others by observing pigmentation in the flowers and stems. The mutable *a-3^f* line can produce not only the variegated flowers but also periclinally chimeric flowers, such as those pigmented only in the epidermal or sub-epidermal layer, as well as mericlinally chimeric flowers and fully colored flowers (Imai 1931, 1934; Kihara 1934). Pigmentation in the epidermal or sub-epidermal layer was detected by examining cross-sections of the periclinally chimeric flowers microscopically (Kihara 1934).

In the mutable *a-3^f* line, Imai (1931, 1934) noticed four easily distinguishable types of periclinally chimeric flowers: type N, both stem and flower colored (pigmented in at least epidermal and sub-epidermal layers); type I, green stem and colored flower which was only slightly less intense than type N (pigmented only in epidermis); type II, colored stem and light colored flower with intense flecking (only sub-epidermis pigmented uniformly and epidermis flecked); and type III, green stem and flecked flower with faintly colored rays (pigmented equally in internal tissues) (Fig. 1 B–C). The type-N and -II flowers with colored stems gave selfed progeny that segregated in a ratio of three germinal revertants bearing fully colored flowers to one flecked mutant, whereas progeny of types I and III were predominantly flecked. Based on these observations in the *a-3^f* line, Imai (1934) was one of the first investigators to realize that somatic mutations in the sub-epidermal layer derived from L2 can be transmitted to the next generation and that the germ cells in the reproductive organs must form from cells in the sub-epidermal layer (Tilney-Bassett 1986).

We have repeated the old experiments conducted by Imai (1931, 1934) in order to test the presumption that somatic mutations caused by *Tpn1* excision from the *DFR-B* gene in the sub-epidermis of periclinally chimeric flowers in a branch of mosaic plants are transmissible to their progeny by molecular means. Sequences generated by somatic excisions of *Tpn1* in periclinally chimeric flowers were found to be identical to those in their germinal revertants, supporting this presumption. The results also firmly confirm the notion that the mutable *a-3^f* allele is indeed the *DFR-B* gene carrying *Tpn1* and that the variegation phenotypes in flowers, leaves and stems are due to somatic reversion of the *DFR-B* gene by *Tpn1* excision.

Materials and methods

Two *a-3^f* lines of Japanese morning glory, KK/SSB-3 and KK/SSB-4, were described previously (Inagaki et al. 1994). Plants were grown in pollinator-free green houses and all the progeny described here were produced by self-fertilization. Plant DNAs were isolated from young leaves and excision of *Tpn1* from the *DFR-B* gene was detected by Southern hybridization (Sambrook et al. 1989; Inagaki et al. 1994) using the petunia *DFR* cDNA as a probe (Beld et al. 1989).

To determine the EDSs generated by *Tpn1* excision, genomic DNA from young leaves or from chimeric flowers was isolated and a fragment of about 250-bp containing EDSs was amplified by the polymerase chain reaction (PCR) with the primers DI2 and DE3 described previously by Inagaki et al. (1994). The PCR-amplified fragment was cloned into the Bluescript vector SK$^-$ (Stratagene) and DNA sequencing was performed by the chain-termination method (Sambrook et al. 1989).

Results and discussion

Flower phenotypes observed in descendants of mosaic variants

Imai (1931, 1934) noted that mosaic variants of the *a-3^f* line produce four types of periclinally chimeric flowers: types N, I, II and III. While it was relatively easy to find flowers of types I, II and N, it was rather difficult to identify type-III variegated flowers with faintly colored rays and green stems. Since we were able to detect such type-III flowers only on rare occasions, we have examined only the flowers of types I, II and N and their progeny (Fig. 1 B–C). In types-I and -II flowers, we observed microscopically that pigmentation indeed occurred in the epidermal or subepidermal layer.

A mosaic variant often produces more than two branches bearing chimeric flowers of different types (Imai 1931,

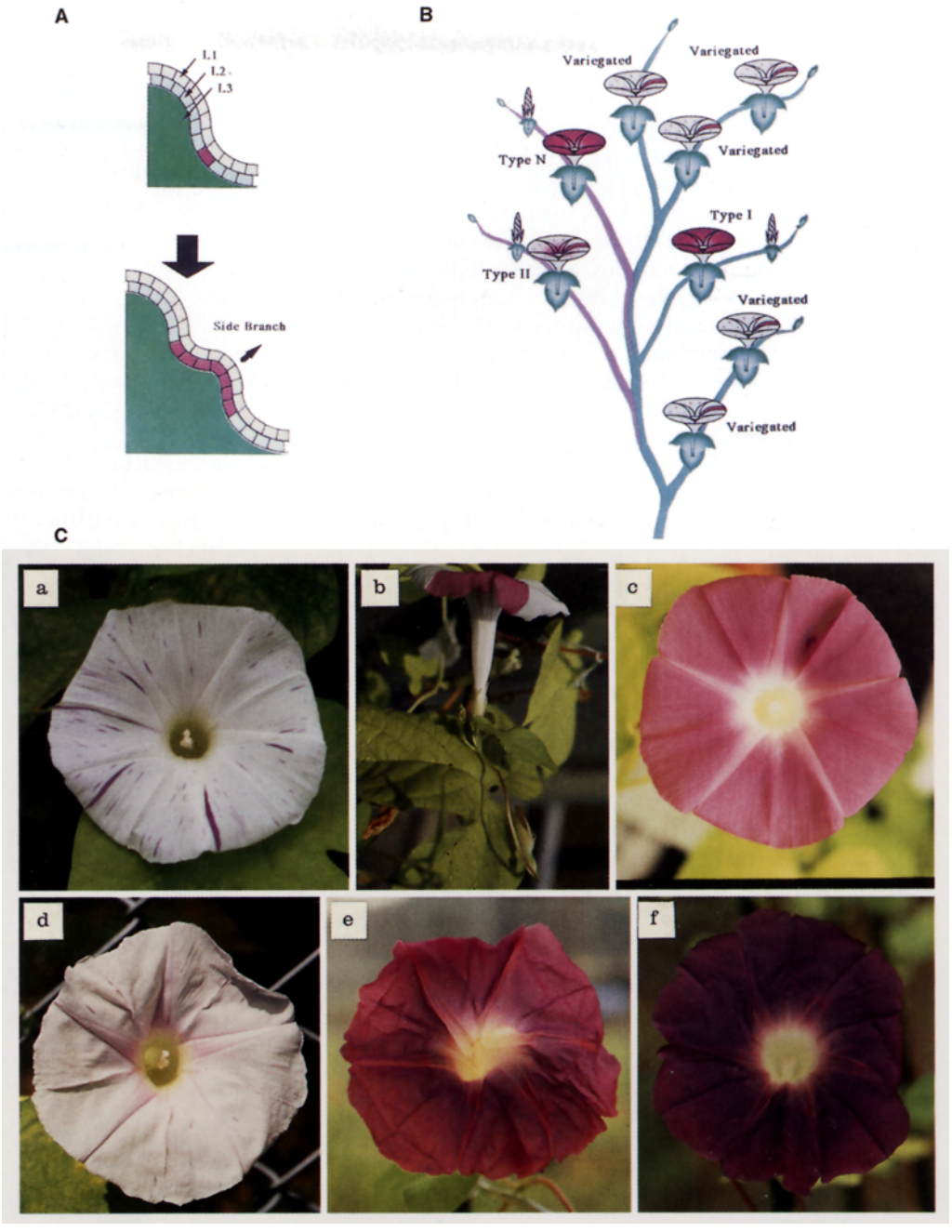

Fig. 1 A–C A schematic representation of a mosaic variant and periclinal chimeras. **A** Origin of periclinal chimeras in a meristem. An L2 cell in which a somatic mutation occurred divides anticlinally and results in a periclinal chimera in a developing side branch. **B** A schematic representation of a mosaic variant producing branches with periclinally chimeric flowers. **C** Flower phenotypes: **(a)** variegated flower, **(b)** variegated flower and stem, **(c)** type-I periclinally chimeric flower, **(d)** type-II periclinally chimeric flower, **(e)** type-N periclinally chimeric flower, **(f)** fully colored flower in a germinal revertant

1934; Kihara 1934). As Fig. 3 shows, we chose three mosaic variants from the *a-3*^{f} line KK/SSB-3: one mosaic plant bearing both flecked and type-II flowers, and two with types I, II and flecked flowers in different branches. We also chose two mosaics from KK/SSB-4: one producing type-I and -II flowers and another bearing type-N flowers.

Since full pigmentation is observed in the hypocotyls of the germinal revertants, they can be easily distinguished from the variegated mutants in seedlings. Out of five different branches bearing type-II flowers, three gave progeny with colored hypocotyls (3-5II-a, 3-5II-i and 3-6II-c) and they were indeed germinal revertants since they bore fully colored flowers (see Fig. 3). A germinal revertant (4-8N-g) was also obtained from a branch with type-N flowers.

From the remaining two branches bearing type-II flowers, we were able to obtain a few seeds, some of which failed to germinate. While a flower of Japanese morning glory can give a maximum of six seeds, flowers of these two branches often produced only one or two seeds. Only three out of five seeds were able to germinate from the mosaic plant 3-4 and unfortunately one of the progeny showing a colored hypocotyl died before flowering. We examined one of the remaining progeny with green hypocotyls and found it to be a mosaic having type-N flowers (3-4N-f). From two seeds obtained from the branch with type-II flowers in the mosaic 4-6, the plant 4-6II-e survived and bore variegated flowers.

No germinal revertant was derived from type-I flowers. All the 11 progeny, obtained from three different lines

of type-I flowers, produced green hypocotyls. Nine out of the eleven progeny plants were shown to bear variegated flowers, one was a mosaic plant with type-II and -N flowers (3-5I-l) and another was a white variant in which only white flowers bloomed (4-6I-h).

Most of the progeny derived from four germinal revertants bore fully colored flowers whereas the mutable plant 4-6II-e gave two progeny with variegated flowers and one mosaic plant bearing type-I flowers. Six additional germinal revertants and three mosaics, together with one plant with variegated flowers, were obtained from two mosaics, 3-4N-f and 3-5I-l. Interestingly, the plant e-2 produced one germinal revertant, one mosaic, and one white variant in addition to four progeny with flecked flowers. These results are consistent with the notion that the branches with type-N or type-II flowers tend to produce germinal revertants.

Structure of the *DFR* regions in the germinal revertants and their progeny

To examine the structure of the *DFR-B* regions, genomic DNAs isolated from young leaves were cleaved with *Bgl*II and were subjected to Southern hybridization using the petunia *DFR* cDNA as a probe (Beld et al. 1989; Inagaki et al. 1994). As Fig. 2 shows, presence or absence of *Tpn1* at the *DFR-B* gene can be easily distinguished by restriction fragment length polymorphisms (RFLPs), and the results are summarized in Fig. 3. For RFLPF analysis in the mosaic plants, we chose branches with variegated flowers at relatively young stages and their DNAs were isolated from young leaves with green stems. All ten mosaic plants examined in this way were found to carry the *DFR-B* genes homozygously and with *Tpn1* inserted (*DFR-B*::*Tpn1*/*DFR-B*::*Tpn1*).

Seven out of eleven germinal revertants obtained were subjected to RFLP analysis, and all of them carried the *DFR-B* genes with *Tpn1* excised either in a heterozygous (*DFR-B**/*DFR-B*::*Tpn1*) or in a homozygous (*DFR-B**/*DFR-B**) condition. Since the somatic excision of *Tpn1* from the *DFR-B* gene that results in the heterozygous (*DFR-B**/*DFR-B*::*Tpn1*) condition must be sufficient to generate a pigmented sector in mosaic plants, the appearance of the germinal revertants in both the heterozygous and the homozygous conditions is likely to be due to segregation. The production of progeny bearing variegated flowers (*DFR-B*::*Tpn1*/*DFR-B*::*Tpn1*) from the branches with flowers of types II and N (3-4, 4-6 and 3-5I-l) is consistent with this notion.

Nine of the eleven progeny, derived from three different lines with type-I flowers, bore variegated flowers and all of them carried *DFR-B* genes with *Tpn1* present homozygously (*DFR-B*::*Tpn1*/*DFR-B*::*Tpn1*). The structure of the *DFR* regions in the white variants remains to be elucidated. Figure 3 also summarizes the results of the RFLP analysis of the *DFR* regions in additional 34 selfed progeny of various lines, five with variegated flowers and 29 with fully colored flowers. The results of co-segregation between *DFR-B*::*Tpn1*/*DFR-B*::*Tpn1* and the appearance of variegated flowers confirm our previous conclusion that the *a-3*^{f} allele is in fact the *DFR-B* gene carrying *Tpn1*, and that the mutable phenotype is due to somatic reversion of the *DFR-B* gene by *Tpn1* excision (Inagaki et al. 1994).

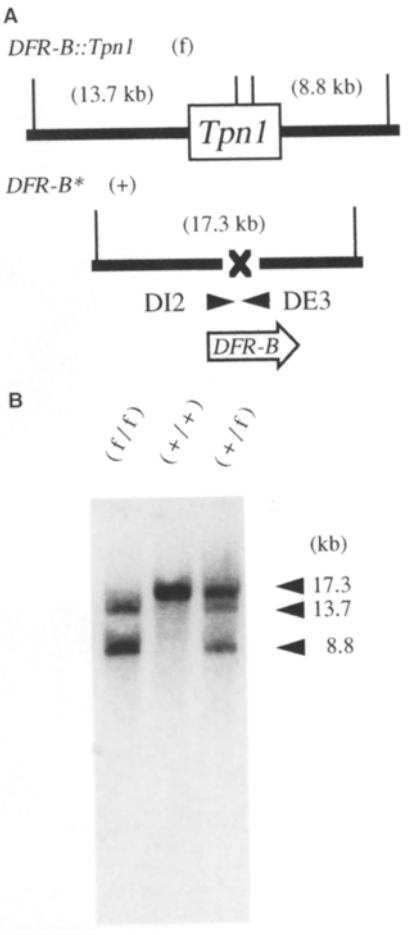

Fig. 2 A, B RFLP analysis of *DFR-B* region. **A** Restriction maps of the *DFR-B* regions in *DFR-B*::*Tpn1* and *DFR-B**. *Short vertical bars* above the maps represent *Bgl*II sites and the lengths of the *Bgl*II fragments are indicated in *parentheses*. The *box* and the *horizontal arrow* below the maps indicate *Tpn1* and the *DFR-B* gene, respectively. The *cross* and the *horizontal arrowheads* DI2 and DE3 indicate the position of EDSs and the sites of primers for PCR amplification, respectively (Inagaki et al. 1994). **B** Typical RFLP patterns observed in *DFR-B* regions: (*f/f*), *DFR-B*::*Tpn1*/*DFR-B*::*Tpn1*; (+/+), *DFR-B**/*DFR-B**; (+/*f*), *DFR-B**/*DFR-B*::*Tpn1*. The lengths of hybridizing fragments are indicated in kb

Transmission of empty donor sequences found in type-II or type-N flowers of mosaic plants to their germinal revertants

In type-II and -N flowers, somatic reversions of the mutable *a-3*^{f} allele occur at least in the sub-epidermis, and these flowers produce germinal revertants having *Tpn1* excised from the *DFR-B* gene. Since the EDSs generated by *Tpn1* excision may vary in each excision event, the EDS found in a germinal revertant is not necessarily identical to that produced by somatic reversion in a parental mutable plant.

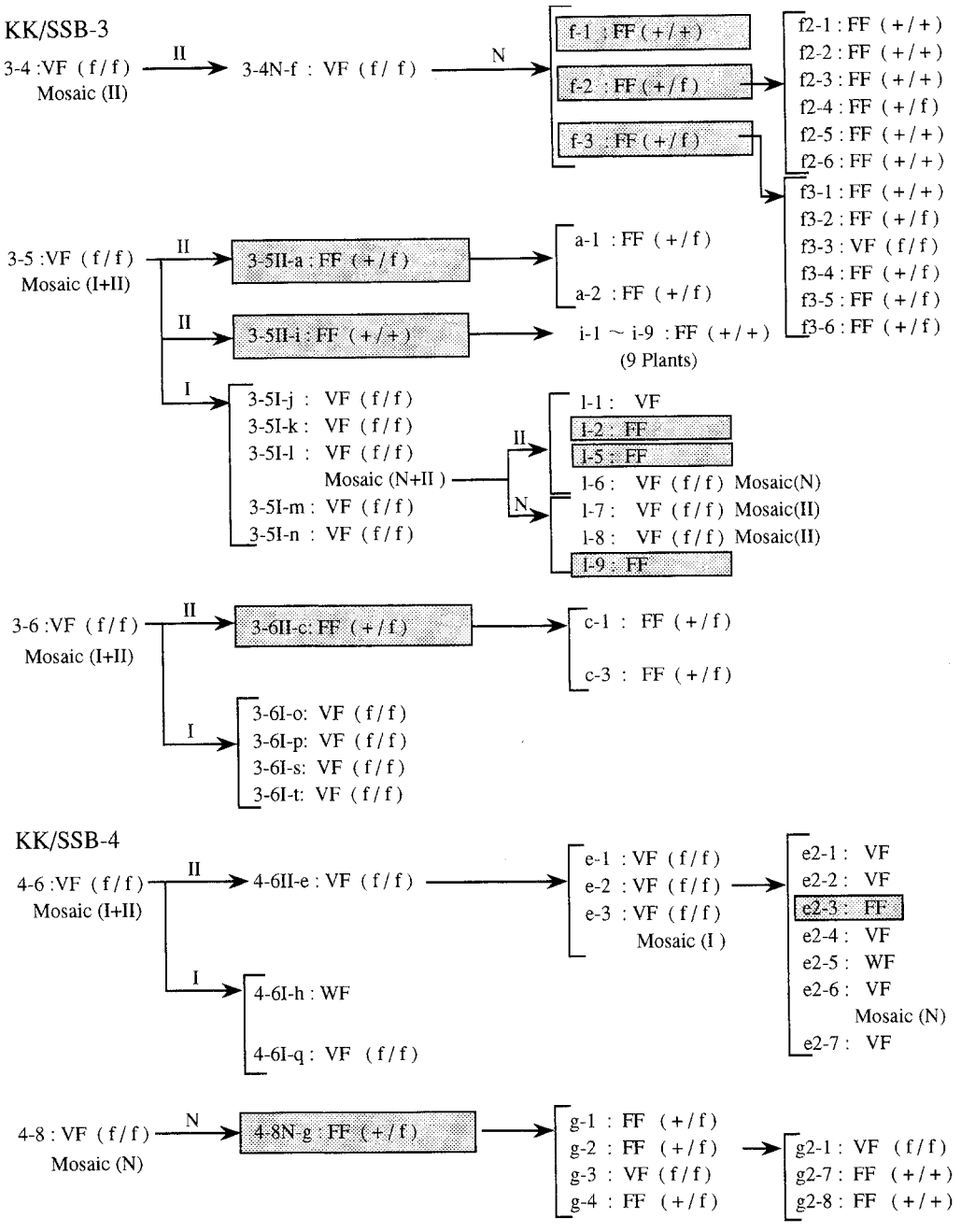

Fig. 3 Pedigree of descendants of mosaic variants. All the progeny were produced by self-fertilization. Flower phenotypes are: *VF* variegated flower; *FF* fully colored flower; *WF* white flower. The symbols *I, II and N* indicate branches of mosaics bearing types-I, -II and -N flowers, respectively. Symbols for the structures of the *DFR-B* regions determined by RFLP analysis are as in Fig. 2. Germinal revertants are indicated by *shadowed boxes*

If the somatic reversions which occurred in the sub-epidermis of periclinally chimeric flowers were transmissible to germinal revertants in the next generation, then the EDSs detected in the sub-epidermis of type-II flowers should be identical to those found in their germinal revertants. To test this hypothesis, genomic DNAs were extracted from type-II flowers in four different branches and their EDSs, determined by sequencing PCR-amplified fragments with the appropriate primers (Fig. 2), were compared with those of their germinal revertants (Table 1). We also compared EDSs from type-N chimeric flowers in one branch with those of their germinal revertants.

Chimeric flowers may consist of cells containing many different somatic revertants, e.g. a type-II flower contains at least an identical EDS common to all the sub-epidermal cells as well as various other sequences due to flecking in the epidermal cells. Since one branch in a mosaic plant bears periclinally chimeric flowers of the same type, such as type II, due to a somatic mutation prior to shooting the branch (Fig. 1), these flowers must contain the same EDSs. We thus isolated the genomic DNA from around three chimeric flowers of the same type in each branch. In each genomic DNA, fragments containing EDSs were amplified by PCR at least twice and were cloned separately. At least four different cloned fragments were sequenced, and usually the EDSs obtained were found to be identical even from the type-N flowers examined. Perhaps, sub-epidermal cells exceeded epidermal cells in number or else the same EDSs were generated in these cells of the type-N flowers sequenced. Alternatively, cells in both epidermal

Table 1 Empty donor sequences (EDSs) found in parental chimeric flowers and germinal revertants. The sequences at the wild-type allele and the mutable *a-3^f* allele are GTGT **AAA** TTGT and GTGT **AAA**: *Tpn1*: **AAA** TTGT, respectively (Inagaki et al. 1994). The 3-bp sequence duplicated at the *Tpn1* integration site, and its sequence alterations generated by *Tpn1* excision, are shown in *boldface*

EDSs (in chimeric flowers)	EDSs (in germinal revertants)
Type-II flowers	
GTGT **AAATTAAA** TTGT (3-5)	GTGT **AAATTAAA** TTGT (3-5II-a)
GTGT **AAATAAA** TTGT (3-5)	GTGT **AAATAAA** TTGT (3-5II-i)
GTGT **AAA** TTGT (3-6)	GTGT **AAA** TTGT (3-6II-c)
GTGT **AAATTAA** TTGT (3-5I-1)	GTGT **AAATTAA** TTGT (1-2)
Type-N flowers	
GTGT **AAATTAAA** TTGT (3-4N-f)	GTGT **AAATTAAA** TTGT (f-1; f-2; f-3)

and sub-epidermal layers of the type-N flowers may have carried an identical EDS as a consequence of the replacement of one cell layer by another layer of cells in which somatic excision of *Tpn1* had occurred. Such a replacement is known to occur occasionally (Tilney-Bassett 1986). Identical EDSs were also detected in PCR-amplified fragments in the genomic DNA from the leaves of the same branch bearing type-II flowers (3-5I-1, in Fig. 3). The results clearly confirm the prediction that EDSs generated by somatic excision of *Tpn1* from the *DFR-B* gene in the sub-epidermis of the periclinally chimeric flowers can be transmitted to their germinal revertants (Table 1).

By observing the flower chimeras and their progeny in the mutable *a-3^f* line of Japanese morning glory, Imai (1934, 1935) realized that somatic mutations in the sub-epidermal layer derived from L2 can be transmitted to the next generation and that the germ cells in the reproductive organs must form from the cells in the sub-epidermal layer. Using the same mutable *a-3^f* line, we have shown that the somatic mutation is caused by excision of *Tpn1* from the *DFR-B* gene and that EDSs generated by *Tpn1* excision are indeed transmissible to the next generation. The results presented here have confirmed and further expanded the previous notion that the mutable *a-3^f* allele is the *DFR-B* gene carrying *Tpn1* in its second intron and that flower variegation is caused by the somatic excision of *Tpn1* (Inagaki et al. 1994). Consistent with this notion, we were able to demonstrate that the *DFR* gene expressed in the fully pigmented flowers is the *DFR-B* gene and that *DFR-B* transcripts are scarcely present in the variegated flowers (Inagaki Y, Takahashi S and Iida S, unpublished results).

In recent years considerable attention has been paid to elucidating the molecular interactions between the cell layers in reproductive meristems using chimeras caused by transposable elements (Huala and Sussex 1993; Carpenter and Coen 1995). Since *Pharbitis* has an extensive history of genetic studies and over 200 genetic loci (including several mutable alleles that condition unstable flower phenotypes in their pigmentation and shape formation) have been localized on the ten linkage groups (Imai 1934, 1938; Hagiwara 1956), *Pharbitis* may also prove to be an excellent model with which to elucidate the interactions between the cell layers by molecular means.

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