Appearance of flower variegation in the mutable \textit{speckled} line of the Japanese morning glory is controlled by two genetic elements

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The \textit{speckled} mutant of the Japanese morning glory blooms pale yellow flowers with fine and round colored spots, or speckles, distributed over the corolla. Since it also occasionally produces flowers with colored sectors apparently due to somatic mutations, the mutable \textit{speckled} allele is thought to carry a transposable element. In this paper, we show that the appearance of the variegation phenotypes in the \textit{speckled} mutant was controlled not only by the recessive \textit{speckled} allele but also by a dominant genetic element, termed \textit{speckled-activator}. Our results also indicated that the recessive \textit{c-1} mutation affecting pigmentation of flowers and mapped near the \textit{speckled} locus causes a defect in a regulatory gene controlling the expression of structural genes for anthocyanin biosynthesis.

INTRODUCTION

Extensive genetic studies have been conducted on the Japanese morning glory (\textit{Pharbitis nil} or \textit{Ipomoea nil}) and more than 200 genetic loci, including colors and shapes of its flowers and leaves have been assigned to one of the 10 linkage groups (Imai, 1938; Hagiwara, 1956, 1977). According to the classical genetic studies (Yoneda and Takenaka, 1981), mutations generating white flowers are classified into four groups on the basis of pigmentation on corolla, flower-tube, stem and seed-coat. For example, mutations in the $C$ group genes produce flowers with white corolla and tube, red stems and pigmented seeds whereas those in $A$ genes confer white flowers, green stems and pigmented seeds.Genes for biosynthesis of the flower pigments, anthocyanins, can be classified into two groups; structural genes encoding enzymes in the pathway (Fig. 1) and regulatory genes acting on the structural genes (Dooner et al., 1991; Forkmann, 1993; Martin and Gerats, 1993). A mutation affecting the activity of more than one structural gene is likely to be a regulatory gene mutation. Among the $A$ group genes, we have shown that the gene $A-3$ is the structural gene encoding dehydroflavonol 4-reductase (DFR) because the mutable $a$-\textit{speckled} allele for flower variegation is the $DFR-B$ gene carrying the 6.4 kb transposable element $Tpn1$ (Inagaki et al., 1994, 1996; Hoshino et al., 1995). The variegation is caused by somatic excision of $Tpn1$ from the $DFR-B$ gene, restoring anthocyanin pigmentation in the flowers. The gene $A-3$ has been mapped on the linkage group V (Hagiwara, 1956, 1977).

The recessive \textit{speckled} mutant of the Japanese morning glory bears fine and round colored spots distributed over the corolla in a pale yellow background (Fig. 2; Imai, 1929, 1931, 1934). The variegation in the flowers has been attributed to a pattern-forming character controlled by the \textit{speckled} gene. Since the \textit{speckled} allele occasionally produces flowers with colored sectors apparently due to somatic mutations and since germinal revertants bearing fully colored flowers are also obtainable (Imai, 1931, 1934), the mutation is thought to be caused by an integration of a transposable element into a gene for pigmentation (Nevers et al., 1986). Both recessive alleles \textit{speckled} and \textit{c-1} have been mapped near the end of the linkage group III (Imai, 1929, 1938; Hagiwara, 1956, 1977).

During characterization of the lines of the Japanese morning glory bearing white and pale yellow flowers (Saito et al., 1994), we have realized that the line 54Y producing pale yellow flowers with green stems and pigmented seeds carries the mutable \textit{speckled} allele without an active element named \textit{speckled-activator} and that another line 78WW\textit{c-1} contains not only the \textit{c-1} mutation giving rise to white flowers with red stems but also the active \textit{speckled-activator}. Thus, the ordinary \textit{speckled} lines of the Japanese morning glory must contain two unlinked genetic components; the recessive \textit{speckled} allele and the

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dominant speckled-activator. Our results also indicate that the C-1 gene is a regulatory gene controlling the expression of at least three structural genes, F3H, DFR, and ANS in Fig. 1.

MATERIALS AND METHODS

Japanese morning glory lines. The line KK/ZSK-2 bearing fully colored flowers was described by Inagaki et al. (1994) and Hama-no-sora blooming fully blue flowers was purchased from Sakata Nursery, Yokohama. The authentic speckled lines Hatsushimo and Shigaki-touen were kindly provided by Shinji Suda in the Tokyo Association for the Japanese Morning Glory (Tokyo Asagao Kenkyuu-ka). The lines 51WWC-1 and 78WWC-1, previously described as 51WWC and 78WWC, respectively, bloom white flowers and the line 54Y produces pale yellow flowers (Saito et al., 1994). The line 51WWC-1 carrying the c-1 mutation (Hagiwara, 1956, 1977) was originated from the late T. Hagiwara’s collection. The line 78WWC-1 is derived from 51WWC-1 and also carries the c-1 mutation since all F1 hybrids between 51WWC-1 and 78WWC-1 bear white flowers.

Nucleic acids procedures. Preparation of plant DNA and DNA gel blot analysis were carried out as described before (Inagaki et al., 1994; Sambrook et al., 1989). The petunia DFR-A cDNA (Beld et al., 1989) and the ANS genomic DNA segment from the common morning glory (Ipomea purpurea or Pharbitis purpurea) carried by pDRA22 (Y. Hisatomi and S. Iida, unpublished), were used as probes. Preparations of total RNA from young flower buds and RNA gel blot analysis were performed according to the procedures previously described (Cox and Goldberg, 1988; Sambrook et al., 1989). The probes used for RNA gel blot hybridization were the Japanese morning glory DFR-B cDNA (Y. Inagaki and S. Iida, unpublished), the ANS genomic DNA segment on pDRA22 and the γ-subunit cDNA of the sweet potato mitochondrial F,F , ATP synthase (Morikami et al., 1993).

RESULTS AND DISCUSSION

Appearance of the speckled phenotypes. The line 54Y bears pale yellow flowers with green stems whereas the plant 78WWC-1 produces white flowers with red stems (Fig. 2A and B). All the F1 hybrids between these two lines bloomed red flowers with red stems (Fig. 2C), indicating that defects in these lines are complementing each other. The selfed F2 progeny gave the ratio of 1 white flowers: 2 red flowers: 1 pale yellow flowers (Table 1), showing that a mutation causing pale yellow flowers in 54Y and the c-1 mutation in 78WWC-1 are tightly linked (Fig. 3). Moreover, about three quarters of the F2 progeny bearing pale yellow flowers showed the typical speckled phenotypes; fine and round colored spots distributed over the corolla in a pale yellow background and fine red variegated spots in the green stem (Fig. 2D and E; Imai, 1931, 1934). We have occasionally noticed that fine red variegated spots in the stems bearing the speckled flowers were too small to be seen, giving rise to apparent green stems in a certain condition. Presumably, the growth conditions of the plants affect appearances of the variegated spots in the stems. Nonetheless, the F2 population displays a ratio of 8 plants bearing red flowers with red stems: 4 white flowers with red stems: 3 speckled flowers with variegated stems: 1 pale yellow flowers with green stems (Table 1). The results suggest that the flower variegation phenotype in the speckled flowers of the Japanese morning glory is controlled not only by the recessive speckled allele but also by a dominant allele termed speckled-activator. The line 54Y carries the mutable speckled allele in the homozygous condition without speckled-activator whereas the line 78WWC-1 contains both c-1 and speckled-activator homozygously (Fig. 3).

If our model in Fig. 3 is correct, the ordinary speckled lines described before (Imai 1934) must contain both the speckled allele and the speckled-activator in the homozygous conditions. In accordance with this notion, all F1 hybrids obtained by the cross between 54Y and the authentic speckled line Hatsushimo or Shigaki-touen gave the speckled phenotypes (Fig. 2G to J).
Flower variegation in the morning glory

Fig. 2. Pigmentation phenotypes of the Japanese morning glory lines. (A) The line 54Y blooms pale yellow flowers. (B) The line 78WWc-1 shows red stems and white flowers. (C) Fully red flower of F1 hybrid between the lines 54Y and 78WWc-1. (D) The speckled phenotype in an F2 plant showing fine and round colored spots in a pale yellow background. (E) Red spots in the stem of the speckled mutant observed in an F2 plant. (F) Colored sectors apparently due to somatic reversions. (G) The speckled phenotype in the line Hatsushimo. (H) Variegated flower of F1 hybrid between the lines 54Y and Hatsushimo. (I) The speckled phenotype in the line Shigakitouen. (J) Flower phenotype of F1 hybrid between the lines 54Y and Shigaki-touen.

Table 1. Flower phenotypes in the F2 population obtained by the cross between 54Y and 78WWc-1

<table>
<thead>
<tr>
<th>Cross combination (female × male)</th>
<th>red flowers</th>
<th>white flowers</th>
<th>speckled flowers</th>
<th>pale yellow flowers</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>78WWc-1 × 54Y</td>
<td>100 (100)</td>
<td>47 (50)</td>
<td>38 (38)</td>
<td>15 (13)</td>
<td>200</td>
</tr>
<tr>
<td>54Y × 78WWc-1</td>
<td>315 (308)</td>
<td>148 (154)</td>
<td>111 (115)</td>
<td>41 (38)</td>
<td>615</td>
</tr>
<tr>
<td>Total</td>
<td>415 (408)</td>
<td>195 (204)</td>
<td>149 (153)</td>
<td>56 (51)</td>
<td>815</td>
</tr>
</tbody>
</table>

The proportion of progeny with red, white, speckled, and pale yellow flowers were found to be 8: 4: 3: 1, respectively ($\chi^2 = 1.11, 0.90 > P > 0.75$). The numbers to be expected in each flower phenotype are shown in the parentheses. For interpretation of the results, see Fig. 3.
Segregation of plants bearing pale yellow flowers from the F2 plants with the speckled flowers in the F3 population. The model illustrated in Fig. 3 predicts that the speckled plants containing the recessive speckled allele in the homozygous state must carry the dominant speckled-activator either homozygously or heterozygously. Among the seven F2 plants bearing the speckled flowers examined, two gave only speckled progeny and the remaining five produced F3 progeny with a ratio of 3 speckled flowers and 1 pale yellow flowers (data not shown). We interpreted that the two former plants contain speckled-activator homozygously and the five latter heterozygously.

DNA and RNA gel blot analyses. Chemical analysis suggests that the line 54Y may carry a defect at the step involving the enzyme F3H in the anthocyanin biosynthesis pathway since an accumulation of the intermediate flavanone was observed (Saito et al., 1994). However, DNA and RNA gel blot analyses using the F3H cDNA from the wild type Japanese morning glory revealed that neither DNA rearrangement nor alteration of the mRNA expression in the F3H gene was detected in 54Y compared with the wild type KK/ZSK-2 while a significant reduction of the F3H mRNA was observed in the line 78WWc-1. The details of these analyses will be published elsewhere.

We have also examined the structure and expression of
the DFR and ANS genes in the lines 54Y and 78WWc-1. As Fig. 4 shows, significant reductions of both DFR and ANS mRNAs were observed in 78WWc-1 while expression of these genes in 54Y appears to be normal. The results indicate that the mutation c-1 affects the expression of at least three genes, F3H, DFR, and ANS in the anthocyanin biosynthesis pathway (Fig. 1). Since the line 78WWc-1 produces flowers with white corolla and tube, red stems and pigmented seeds, the C-1 gene is likely to be a regulatory gene controlling the expression of at least these structural genes in flower pigmentation.

No DNA rearrangement was detected in the DFR gene region among 54Y, 78WWc-1, and the wild type KK/ZSK-2 as well as in the ANS gene region among 54Y, 78WWc-1, and the wild type Hama-no-sora (data not shown). Although DNA rearrangements were observed in the DFR gene region of Hama-no-sora (A. Hoshino and S. Iida, unpublished), they should not affect the DFR-B gene expression because the plant bears fully blue flowers.

**Nature of the alleles speckled and speckled-activator.** The appearance of fine variegated colored spots in flowers is the typical speckled phenotype (Fig. 2D) and it has been attributed to a pattern-forming trait controlled by the speckled gene (Imai, 1931). Since the speckled allele occasionally produces flowers with colored sectors apparently due to somatic mutations (Fig. 2F) and since germinal revertants bearing fully colored flowers are also obtainable, the speckled mutation has been believed to be caused by an integration of a transposable element into a gene for pigmentation (Nevers et al., 1986). Although the structure of the mutable speckled allele remains to be elucidated, one can postulate that the putative transposable element in the speckled allele contains a non-autonomous element which lacks its transposase activity and that the dominant speckled-activator is an autonomous element acting in trans on the non-autonomous element (Fedoroff, 1989; Saedler and Gierl, 1996). If this is the case, one can further hypothesize that the action of the autonomous element results in not only excisions of the non-autonomous element giving rise to both somatic and germinal revertants but also activation of the speckled allele producing fine and round colored spots in pale yellow flowers. A close association of gene activation and mutator activity was observed in the autonomous maize transposable element Enl/Spm acting a certain mutable allele carrying its non-autonomous element (Fedoroff, 1989). For example, the autonomous Spm element acts the mutable allele am2-7995 carrying a non-autonomous Spm derivative, dSpm-7995, to result in the uniformed expression of the a-1 gene encoding DFR and causes the excision of the element to generate mutations (Masson et al., 1987; Fedoroff, 1989). It should be emphasized that the activation of the speckled allele by speckled-activator results in characteristic variegations, i.e., fine spots in both flowers and stems.

It should also be mentioned that the 6.4 kb transposable element Tpn1 found in the mutable a-3flecked allele for flower variegation of the Japanese morning glory belongs to the Enl/Spm family and that Tpn1 and its relatives are present in multiple copies in the genome of the Japanese morning glory (Inagaki et al., 1994; Hoshino et al., 1995). It remains to be examined whether Tpn1 and its relatives also do something to the mutable speckled allele.

Imai (1929, 1931) described a recessive modifier element, speckled-reduced, which affects the speckled allele to reduce the flower and stem variegations. Relations between speckled-activator and speckled-reduced and their interaction remain to be studied. Nevertheless, identification of the mutable speckled allele which is likely to be a mutation in a gene for pigmentation would be the first step to elucidate molecular mechanisms for the formation of the interesting variegations shown in Fig. 2. We are currently attempting to identify the speckled allele by employing the lines 54Y and 78WWc-1 described here.

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**REFERENCES**


Cox, K.H. and Goldberg R.B. (1988) Analysis of plant gene expres-


