Cutting Leaves and Plant Growth Regulator Application Enhance Somaclonal Variation Induced by Transposition of \textit{VGs1} of \textit{Saintpaulia}

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For determination of the endogenous and exogenous causes of somaclonal variation in \textit{in vitro} culture, a bioassay system was developed using the variegated \textit{Saintpaulia} (African violet) ‘Thamires’ (\textit{Saintpaulia} sp.), having pink petals with blue splotches caused by transposon \textit{VGs1} (\textit{Variation Generator of Saintpaulia 1}) deletion in the promoter region of \textit{flavonoid 3',5'-hydroxylase}. Not only true-to-type but also many solid blue and chimeric plants regenerate \textit{in vitro}-cultured explants of this cultivar. Using multiplex PCR that enables the determination of these variations, we attempted to evaluate the effects of four candidate triggers of mutation: pre-existing mutated cells, shooting conditions \textit{in vitro} or \textit{ex vitro}, cutting treatment of explants, and addition of plant growth regulators (PGRs) to the medium. The percentages of somaclonal variations among total shoots regenerated from leaf segments and stamens were 46.6 and 56.5, which were higher than the percentages expected from pre-existing mutated cells (3.6 and 1.4, respectively). These results indicate that pre-existing mutated cells are not a main cause of somaclonal variations. The percentage of somaclonal variation was independent of culture conditions for mother plants; the mutation percentages of adventitious shoots regenerated from \textit{ex vitro-} and \textit{in vitro}-grown leaves were 9.2\% and 8.5\%, respectively. In addition, the percentage of somaclonal variations of adventitious shoots regenerated under \textit{in vitro} conditions from the \textit{in vitro} grown mother plants was also low, at 4.9\%. This indicates that the \textit{in vitro} condition itself is not a main cause of somaclonal variation. However, when adventitious shoots were regenerated from 10 × 5-mm cut-leaf laminas on a PGR-free medium, the percentage of somaclonal variation was 26.4\%. In addition, the percentage of somaclonal variations dramatically increased when PGRs were added to the medium for both leaves and leaf segments (39.9 and 46.6, respectively). The bioassay system using \textit{Saintpaulia} ‘Thamires’ will enable the screening of many environmental factors because of its rapidity and ease of use and will facilitate the development of a new tissue culture technology for avoiding mutation.

Key Words: adventitious shoot, African violet, \textit{F3′5′H}, transposon.

Introduction

Tissue culture often induces mutations called somaclonal variation (Larkin and Scowcroft, 1981). Because somaclonal variation is sometimes beneficial for mutation breeding, environmental stimuli are sometimes combined with tissue culture for efficient induction of somaclonal variation. However, in the clonal propagation of horticultural crops or in the generation of trans-
including chromosomal doubling, point mutation in DNA, and transposition of transposable elements (TEs) (Bairu et al., 2011).

Environmental/exogenous or endogenous factors may induce somaclonal variation in vitro and many researchers have attempted to identify the causes of such variation. One of the endogenous factors is mutated cells pre-existing in explants. Usually, the contribution of pre-existing mutations to the occurrence of somaclonal variation can be evaluated by conducting further rounds of shoot regeneration using explants from first-round regenerants (Broertjes, 1969). When the variation in the second- or later-round regenerants is much lower than that in the first-round regenerated shoots, the somaclonal variations observed in later rounds of regenerants can be concluded to be newly generated, and the first-round explants may have contained mutated cells contributing to somaclonal variation. In addition, phenotypic separation caused by chimeric separation via adventitious shoot regeneration is believed to result from the same mechanism of somaclonal variation arising from pre-existing mutated cells. For determination of the exogenous factors that induce somaclonal variation, it is important to calculate the percentage of pre-existing mutated cells in explants and to evaluate the percentages of somaclonal variations in vitro. The exogenous factors causing somaclonal variations have also been well discussed; in vitro culture environments can be mutagenic and regenerated plants sometimes exhibit phenotypic changes (Bouharmont, 1994). For example, plant growth regulators (PGRs) have been proposed as candidate exogenous agents that induce somaclonal variation (Bairu et al., 2011). In addition, traumatic stress induced by explant cutting has been proposed as another candidate (McCIntock, 1984).

It is very difficult to evaluate mutation percentages induced under various culture conditions from only the phenotypes of regenerants because of the large space required for growing many plants. In addition, the existence of more than one mechanism for somaclonal variation complicates the experimental design. Therefore, genetic markers for somaclonal variations are a powerful tool capable of bypassing large-scale phenotypic evaluation and enabling the quantitative evaluation of each environmental stress implicated in somaclonal variation.

Saintpaulia sp. is a suitable model plant to study somaclonal variation because adventitious shoots can easily regenerate from any organ and regeneration from leaves can occur independently of PGR addition. ‘Thamires’ has pink flowers caused by the pigment pelargonidin with blue splotches due to malvidin (Sato et al., 2011a,b). The purple variegations result from the deletion of a hAT superfamily TE, VGs1 (Variation Generator of Saintpaulia 1), inserted in the promoter region of flavonoid 3’5’-hydroxylase (F3’5’H), such that the transcription of F3’5’H is suppressed by the inactivation of a promoter by the inserted VGs1, belonging to the DNA-mediated TEs called “class II”), such as Ac/Ds in Zea mays (Müller-Neumann et al., 1984) and Tam3 in Antirrhinum majus (Hehl et al., 1991), resulting in the suppression of malvidin synthesis (Sato et al., 2011b). In previous studies, we determined that the pre-existing mutation percentage in the vegetative organ of this cultivar was <3%, but that the percentages of somaclonal variations induced via adventitious shoots were much higher than 3% (Sato et al., 2011b). This finding indicates that the high levels of somaclonal variation did not occur in mutated but in wild-type cells, and VGs1 was activated in in vitro culture. We hypothesized that the use of this cultivar would allow evaluation of each exogenous candidate factor for somaclonal variation using genetic markers. In this experiment, we attempted to evaluate the effects of four reported candidate factors of mutation using the F3’5’H genotypes: pre-existing mutated cells as an endogenous factor, in vitro culture conditions, cutting treatment for explants, and PGRs added to the medium as exogenous factors.

Materials and Methods

Mother plant preparation

Saintpaulia ‘Thamires’ plants were obtained from Royal Green Inc. (Gifu, Japan). Plants were grown in a greenhouse on the experimental farm of Kyoto University (Kyoto, Japan). The greenhouse was shaded to allow a light intensity of 140–200 μmol·m−2·s−1, and maintained at an appropriate temperature below 35°C with mist cooling in summer and above 10°C with oil heating in winter. For in vitro plant preparation, meristems were cut from those plants where the flowers had average numbers of splotches and cultured on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 1/10 strength NH4NO3. In vitro plants from meristem culture were cut and propagated to 50 plants in modified MS medium with normal-strength NH4NO3. Both media were supplemented with 3% (w/v) sucrose and 0.3% (w/v) gellan gum (Wako, Osaka, Japan). The medium pH was adjusted to 5.8–6.0 prior to autoclaving at 121°C for 15 min. All plantlets were grown in a growth chamber maintained at 25°C under 16-h photoperiod conditions, and a light intensity below 40 μmol·m−2·s−1 provided by cool white fluorescent lights. To avoid using mutated plants as mother plants for the experiments, real-time PCR was performed according to Sato et al. (2011a) to discard blue, chimeric, and too frequently transposing mutants, and plants with mutated cell percentages of whole leaves below 5% as determined by real-time PCR were chosen for subsequent experiments.

Real-time PCR for evaluation of mutation percentage in explants

For real-time PCR used for appropriate mother plant selection and evaluation of the percentage of pre-
existing mutated cells in the explants, DNA was extracted from in vitro-grown plants using a DNeasy Plant Mini Kit (Qiagen, Hamburg, Germany). On the basis of sequence differences, primers were designed for detecting a post-transposon excision sequence ("target") in the promoter region of F3’5’H, following Sato et al. (2011b). Chalcone synthase A (CHS-A: DQ788862) was used as a reference gene. Standard curves were obtained with a dilution series of DNA from solid blue mutants. For the ΔΔCt (comparative Ct) method, the primers used to detect the post-transposon excision sequence were Up-73 Fw (5’-ACACATGCAATGCTGCTCTATTA-3’) and Code-84 Rev (5’-ACAGAAATTTCCGGATGATGAAG-3’), and those used to detect the CHS-A reference gene were CHS-A Fw (5’-CTCTTGAAGGATGTCCCTTCTTT-3’) and Code-84 Rev (5’-TCCAACCTCAACCTGATCAGGAAG-3’). Real-time PCR was performed using a 7900HT thermal cycler (Applied Biosystems, CA, USA) with SDS 2.1 software (Applied Biosystems). The PCR mixture contained 2 μL of template gDNA (corresponding to 1–2 ng), 10 μL of SYBR Premix Ex Taq II (Takara, Otsu, Japan), and 0.2 μM forward and reverse primers in a final volume of 20 μL. The program was set at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 10 s; melting curves were visually inspected in the subsequent dissociation steps (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s). For the quantification of mutated cell percentages, the number of PCR cycles (Ct) required to reach the threshold level was determined by the ΔΔCt method according to ABI User Bulletin #2, Bloch et al. (2001), and Ben-Shahar et al. (2002). As calibration samples, genomic DNAs from solid blue mutants were used because these comprised 100% mutated cells. For the calculation of mutated cell percentages in the leaves of in vitro plants, ΔΔCt of variegated petals was compared with that of blue petals.

Multiplex PCR for evaluation of the occurrence of somaclonal variation

DNA was extracted from young, expanded small leaves in vitro and ex vitro by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980) with modifications. Samples frozen in liquid nitrogen were ground with stainless beads in plastic tubes (Safe-Lock Tubes; Eppendorf, Basel, Switzerland) and powdered using Shake Master neo (Biomedical, Osaka, Japan) for 2 min, and 300 μL of CTAB solution was added to each tube followed by incubation at 55°C for 20 min. Chloroform (600 μL) was added to the incubated sample and, after centrifugation at 13000 × g, the supernatant was mixed with ethanol (EtOH). The residue from centrifugation was rinsed with 70% EtOH and dissolved in 200 μL of water. DNA concentrations were below the level detectable using a spectrometer, owing to the very small amounts of DNA contained in fresh samples.

For multiplex PCR, primers able to amplify both pink and blue cells were designed using post- and pre-VGs1-deleted nucleotide sequences (AB596834 and AB596833, respectively); multiplex F1: 5’-TCACACATGCAATGCTGCTCTATTA-3’ and multiplex F2: 5’-GATCCCACGGAAGTTATGTA-3’, and multiplex R: 5’-GAATCTCGACTGCCAGGTCTTTA-3’, as shown in Figure 1a. The primer set of multiplex F2 and multiplex R amplifies 705 bp of VGs1 non-deleted sequences, and the combination of multiplex F1 and multiplex R amplifies 316 bp of VGs1-deleted sequences. Multiplex PCR was performed using a KAPA 3G Plant PCR kit (Kapa Biosystems, MA, USA) containing 1 μL of template gDNA, 0.3 μM each of multiplex 714 and multiplex-Blue specific-F, and 0.2 μM BSP_R-new primer in a final volume of 10 μL according to the manual instructions. The program was set at 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s. PCR products were electrophoresed in 1% agarose and genotypes were determined under an illuminator. To ascertain the accuracy of the determination of variation percentages by multiplex PCR, leaf samples were randomly collected from in vitro-regenerated shoots of cultured ‘Thamires’ leaf explants, and these plants were potted in vermiculite, acclimatized under intermittent misting, and grown in a greenhouse; during flowering, their phenotypes were assessed. To avoid overestimating mutation percentages, the numerical value of the accuracy rate of PCR determination compared with the phenotype was used to correct the mutation percentages, as shown in “Development of multiplex PCR evaluation of somaclonal variation” in Results.

Evaluation of the percentages of somaclonal variations under different conditions

We evaluated four candidate factors reported to be involved in the induction of somaclonal variation. First, to assess whether the somaclonal variations are derived from pre-existing mutated cells or newly mutated cells during shoot regeneration, the percentages of somaclonal variation were assessed using regenerants from leaves and stamens. After the percentages of pre-existing mutated cells in both organs were assessed by real-time PCR as described above, ex vitro-harvested explants were sterilized with 0.5% sodium hypochlorite for 10 min and leaf discs (5 mm in diameter) and stamens cut at the joined base with blossom ends were placed on modified MS medium supplemented with 0.5 ppm 6-benzylaminopurine (BA) and 0.1 ppm α-naphthaleneacetic acid (NAA) for leaf discs and 1 ppm BA and 1 ppm NAA for stamens. A leaf collected from each regenerated plant was used for multiplex PCR and the number of mutated regenerants by multiplex PCR was statistically compared with each number of mutated regenerants expected from the percentages of pre-existing mutated cells obtained by real-time PCR. Second, for comparison of the growing conditions of

S. Matsuda, M. Sato, S. Ohno, S.-J. Yang, M. Doi and M. Hosokawa
mother plants, percentages of somaclonal variation using ex vitro- and in vitro-cultured mother plants were compared. Leaves harvested from in vitro and ex vitro mother plants were inserted into vermiculite in a container (35 × 45 × 8 cm) located in a greenhouse and 10 g of slow-release fertilizer (IB Kasei; JCAM Agri, Tokyo, Japan) was added in each container. Third, for the comparison of regeneration conditions, the percentages of occurrence of somaclonal variation were compared between in vitro and ex vitro regeneration conditions. Leaves from in vitro mother plants were cultured on modified MS medium supplemented with 3% (w/v) sucrose and 0.3% (w/v) gellan gum (Wako). A leaf collected from each regenerated plant was used for DNA extraction and multiplex PCR was performed.

For statistical evaluation of the effect of in vitro conditions on the occurrence of somaclonal variation, the numbers of somaclonal variations of leaves from in vitro-cultured plants were compared with those of ex vitro regenerants by the chi-square test. In addition, to assess the effect of in vitro shoot regeneration conditions on the occurrence of somaclonal variation, the numbers of somaclonal variations of in vitro regenerants were compared with those of ex vitro regenerants by the chi-square test.

To test the effect of cutting explants on the percentage of somaclonal variations, the variation percentages were compared between regenerants from cut leaves and uncut whole leaves. For cutting treatment, a 10 × 5-mm disc at the center of the leaf blade of a young leaf (2-cm length) was cut and cultured on modified MS medium supplemented with 3% (w/v) sucrose and 0.3% (w/v) gellan gum without PGRs. The data for in vitro shoot regeneration from in vitro mother plants in the above-mentioned experiment were used for comparison. To test the effect of PGRs on the percentages of somaclonal variations, cut or whole leaves harvested from in vitro plants were also cultured on media with 0.5 ppm BA and 0.1 ppm NAA. A leaf collected from each regenerated plant was used for DNA extraction and multiplex PCR was performed. The chi-square test was conducted to assess the effect of cutting stress or PGRs using the numbers of somaclonal variations in the regenerants with or without cutting stress or in those with or without PGRs.
Statistical analyses

Data collected from multiplex PCR were inserted into a Microsoft Excel spreadsheet for chi-square tests calculated using Excel 2012 add-on software.

Results

Development of multiplex PCR for evaluation of somaclonal variation

Single or double bands on agarose gels were visible (Fig. 1b); the signal intensity of each band represented the contents of the mutated and wild-type cells. Samples showing an intense upper band and a weak lower band were assigned as wild-type plants that had wild-type petals with some blue splotches. Samples for which only the lower band was detected were designated as solid blue mutants. Samples for which similar signal intensities were observed in the upper and lower bands were designated as chimeric plants (Fig. 1b-b). Eighteen of 20 samples showed an intense upper band with a trace lower band and had wild-type flowers (Fig. 1b-a) when they flowered (data not shown). Two of them were chimeric plants, one having blue flowers comprising mutated layer1 (L1) and wild-type internal layer and the other having striped flowers comprising wild-type L1 and blue mutated internal layer when they flowered. All eight plants showing only the single lower band (Fig. 1b-c) had blue flowers (data not shown). The accuracy rate of samples with both bands of almost the same intensity was relatively low; 6 of 9 plants (67%) were chimeric. Three plants were not so-

Table 1. The number of somaclonal variations of the shoots regenerated from leaf segment and stamens.

| Cultured organs | Percentage of pre-existing mutated cells (%) | The number of tested plants | Expected variation number | The number of mutated plants determined by multiplex PCR | Estimated variation number | The percentages of variations (%) | Chi-square (P-value) |
|-----------------|--------------------------------------------|-----------------------------|--------------------------|---------------------------------------------------------|----------------------------|----------------------------------|----------------------|
| Leaf segment    | 3.6                                        | 187                         | 6.7                      | 109 (43, 66)                                            | 87.2                       | 46.6                             | 997.8 (0.000)         |
| Stamen          | 1.4                                        | 46                          | 0.6                      | 26 (25, 1)                                              | 25.7                       | 56.5                             | 1012 (0.000)         |

* This value is calculated by multiplication of “Percentage of pre-existing mutated cells (%)” and “The number of tested plants”.

The number of chimeric plants is corrected by multiplying by 0.67 to avoid overestimation of somaclonal variations. This number is the sum of solid blue mutants and chimeric plants.

The percentages of somaclonal variations among the total regenerated shoots

Although the percentages of mutated cells on leaf laminas and stamens were low at 3.6% and 1.4%, the percentages of somaclonal variations among total shoots were high at 46.6% and 56.5%, respectively (Table 1). There were significant differences at the 1% level between the estimated values calculated from the real-time PCR results of the pre-existing mutated cells and the estimated number of variations after downward correction by multiplex PCR fidelity (0.67).

When leaves from in vitro and ex vitro mother plants were used for ex vitro shoot regeneration, the percentages of somaclonal variation did not depend on the growth conditions of mother plants; the mutation percentages in ex vitro and in vitro mother plants were 9.2% and 8.5%, respectively (Table 2). No significant difference was observed in the estimated variation numbers between different growth conditions of mother plants ($P = 0.8602$) at the 5% level. When leaf laminas harvested from in vitro shoots were used for shoot regeneration in vitro for comparison of the effects of regeneration conditions on somaclonal variation, the percentage of somaclonal variations was 4.9%, a value not significantly different from that for ex vitro regener-

Table 2. The effect of the sources of mother plants and shooting conditions for the number of somaclonal variations.

| The source of mother plants | Condition for shoot regeneration | The number of tested plants | The number of mutated plants determined by multiplex PCR | Estimated variation number | The percentages of variations (%) | Chi-square (P-value) |
|-----------------------------|----------------------------------|-----------------------------|---------------------------------------------------------|----------------------------|----------------------------------|----------------------|
| (1) ex vitro                | ex vitro                         | 94                          | 11 (4, 7)                                               | 8.7                        | 9.2                              | 0.03103 (0.8602)     |
| (2) in vitro                | ex vitro                         | 47                          | 6 (0, 6)                                                | 4.0                        | 8.5                              | 0.8526 (0.3558)      |
| (3) in vitro                | in vitro                         | 48                          | 3 (1, 2)                                                | 2.3                        | 4.9                              |                     |

* The number of chimeric plants is corrected by multiplying by 0.67 to avoid overestimation of somaclonal variations. This number is the sum of solid blue mutants and chimeric plants.

The figures in the parentheses indicate the numbers of solid blue mutant and chimeric plants, respectively.
ants from *in vitro* leaf laminas (*P* = 0.3558) at the 5% level (Table 2).

When 10 × 5-mm cut-leaf laminas were cultured on the PGR-free medium, the somaclonal variation percentage significantly increased, reaching 26.4% after correction for multiplex PCR fidelity (Table 3). The difference in estimated variation numbers between uncut and cut leaves was significant at the 1% level by chi-square test (*P* < 0.001).

When PGRs were added to the medium, the occurrences of somaclonal variations significantly increased (Table 3). When uncut leaves were used, the mutation percentage was 39.9%, a value of the estimated variation number significantly different from that for uncut leaves regenerated on the PGR-free medium (*P* < 0.001) (Table 3). When cut-leaf laminas were used, the estimated variation number in PGR-containing medium was also significantly higher than that in the PGR-free medium (*P* < 0.001) (Table 3).

**Discussion**

**Multiplex PCR as a bioassay tool**

The triggers of somaclonal variation in tissue culture remain controversial (Joyce et al., 2003). Numerous factors have been suggested, including PGRs, lighting conditions (George, 1993), aseptic conditions, imbalances of medium components, the relationship between high humidity and transpiration (Cassells and Roche, 1994; Cassells and Walsh, 1994), saline stress, oxidative stress, and nutrient deficiencies (Joyce et al., 2003).

For development of a bioassay method, there are two difficulties to be overcome: model plant selection and the development of a convenient detection method such as DNA markers. *Saintpaulia* has the ability to regenerate via tissue culture from any part of the plant, including the leaves, petioles, stalks, sepals, petals, and anthers, and has accordingly often been used as a model for research on adventitious shoot regeneration (Bilkey et al., 1978; Cassells and Plunkett, 1984; Cooke, 1977; Mølgaard et al., 1991; Smith and Norris, 1983; Start and Cumming, 1976; Weatherhead et al., 1982). In addition, adventitious shoot formation of *Saintpaulia* occurs not only *in vitro* but also *ex vitro*. The method developed in the present study, which involves multiplex PCR for mutation determination, is suitable for evaluating several samples because of its simplicity and rapidity. The time from the start of culture to shoot regeneration is 2–3 months, and multiplex PCR allows the evaluation of more than 50 plants per day. Thus, using this bioassay system, the experimental period was shortened with respect to the conventional method, which requires a very long time, 6–8 months, until flowering.

We were able to identify wild-type plants with blue-splotched flowers, solid blue-flowered mutants, and periclinal chimeric plants comprising different genetic histogenic layers of flower color. Periclinal chimeras variations have a blue mutant layer in their L1 or L2. A blue stripe in the center of flowers appears in L2-mutated periclinal chimeric plants covered by a wild-type L1, which is a genotype that can be identified by color phenotype. In contrast, variations with a mutated L1 covering a wild-type L2 are difficult to discriminate from solid blue mutants by phenotype. Although the determination rate for chimeric plants was low, a rough count of chimeric plants was possible. Two bands were observed by multiplex PCR in chimeric and wild-type plants (Fig. 1b) and assignment of phenotype was performed according to the difference in intensities of the bands. Because the smaller product is prone to be multiplied by PCR, the lower band intensity was sometimes strong even in wild-type plants, leading to incorrect identification as a chimeric plant. Through the experiment, 67% of total chimeric plants judged by multiplex PCR were evaluated as the estimated number of chimeric plants in order to avoid overestimation of this.

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**Table 3. The effect of cutting explants and adding plant growth regulators (PGRs) to regeneration medium on the number of somaclonal variations.**

| Cutting treatment | PGRs | The number of tested plants | The number of mutated plants determined by multiplex PCR | Estimated variation number | The percentages of variations (%) |
|------------------|------|-----------------------------|--------------------------------------------------------|---------------------------|----------------------------------|
| (1) Non-cut      | –    | 48                          | 3 (1, 2)*†                                              | 2.3                       | 4.9                              |
| (2) Non-cut      | +    | 41                          | 19 (11, 8)                                              | 16.4                      | 39.9                             |
| (3) Cut          | –    | 142                         | 48 (16, 32)                                             | 37.4                      | 26.4                             |
| (4) Cut          | +    | 187                         | 109 (43, 66)                                            | 87.2                      | 46.6                             |

Chi-square (P-value)
(1) vs. (3) 144.5 (0.000)
(1) vs. (2) 111.4 (0.000)
(3) vs. (4) 39.70 (0.000)

*0.5 ppm 6-benzylaminopurine (BA) and 0.1 ppm α-naphthaleneacetic acid (NAA) were used for adding PGR treatment.
† The number of chimerical plants is corrected by multiplying by 0.67 to avoid overestimation of somaclonal variations. This number is the sum of solid blue mutants and chimeric plants.
‡ In these treatments, the numbers are the same as in Table 2.
§ The figures in parentheses indicate the numbers of solid blue mutant and chimerical plants, respectively.
number. However, this correction does not change the statistical significance of the results in the experiments. One of the solutions for this incorrect identification of the wild type is to reduce the number of PCR cycles or the amount of template.

On the other hand, among the in vitro regenerants from cultured explants of ‘Thamires’, blue-flower mutants as well as a few pink-pigmented or purple-pigmented flower mutants were obtained in our previous experiments (Sato et al., 2011b). These rare pink- and purple-flowered mutants occur as a result of the loss of transposition ability of VGs1 and incomplete transposon deletion, respectively. If these variations occurred, they were counted as non-mutated plants in the multiplex PCR described below. In our previous study, these mutants were not observed often, so they may not have had a marked effect on the mutation percentage in the experiments.

**Endogenous factors involved in somaclonal variation**

Determination of whether somaclonal variations arise from pre-existing mutated cells or newly developed mutated cells is difficult. In previous studies, repeated culture (Broertjes, 1969) was used to answer this question. However, this method is time-consuming and difficult to use for evaluating several treatments. In our previous report, mutated cells in ‘Thamires’ leaves contributed to the number of somaclonal variations, but the percentage was low (Sato et al., 2011a). The main source of somaclonal variation developed after shoot regeneration in vitro. In the present study, our data presented in Table 1 are consistent with this inference. Pre-existing mutated cells on stamens constituted only 1.4%, but the percentage of somaclonal variations was over 50%, indicating that somaclonal variations associated with VGs1 transposition occurred during the shoot regeneration step. Considering that shoots were regenerated from single cells, the observation that multiple chimeric plants regenerated indicates the occurrence of mutation during shoot regeneration. It may be stated conclusively that some exogenous factors activate VGs1, resulting in somaclonal variation.

**Which exogenous stimulus activates VGs1?**

Activation of TEs by tissue culture has been previously reported (Peschke and Phillips, 1991; Peschke et al., 1987). In maize, the TE Activator (Ac) could be detected in 2–3% of regenerated plants, but not in materials before tissue culture (Peschke et al., 1987). Our experiment using leaf laminas of ex vitro and in vitro plants revealed that in vitro conditions, including the addition of sucrose, rich nutrient contents, and high humidity, did not activate VGs1 (Table 2). In addition, the growth conditions of mother plants were not considered to activate VGs1 (Table 2); VGs1 is not prone to transpose in in vitro-grown mother plants. Aside from in vitro conditions themselves, one of the exogenous factors inducing somaclonal variation has been considered to be cutting stress. In our results, the percentage of somaclonal variation was higher with the culture of cut leaves on the medium than that with uncut leaves (Table 3). However, further experiments increasing number of plants will be needed. *Saintpaulia* has been reported to be one of the genera most sensitive to wounding stress such as leaf-cutting (Yang et al., 2006). Cutting leaves or local wounding of *Saintpaulia* plants induces a “hypersensitive state” in the whole plant, which lasts for at least 20 min (Yang et al., 2006). Thus, it is an interesting question whether the hypersensitive state induced by cutting stress is conducive to the occurrence of somaclonal variation via the transposition of TEs.

Adding PGRs to the medium also increased the percentage of somaclonal variation (Table 3). The highest percentage was obtained from cultured cut explants on medium with PGRs (Table 3). PGRs, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and BA, have been implicated in the induction of somaclonal variation (Evans, 1988; Griesbach et al., 1988; Shoemaker et al., 1991). There are several reports of the influence of PGRs on somaclonal variation; some on auxin (Ahmed et al., 2004; Hitomi et al., 1998) and others on cytokinin (Jain, 1997; Venkatachalam et al., 2007). Cytokinin induces somaclonal variation via cell-cycle disturbance (Peschke and Phillips, 1991). It has been reported that activation of the retrotransposon Tnt1 during protoplast isolation resulted from hypersensitive responses to cell-wall digestion enzymes from fungi (Pouteau et al., 1991). There are other examples of physical or hormonal effects on the transposition of TEs; methyl jasmonate and fungal elicitors were triggers for transposition of the retrotransposon Tos1 (Takeda et al., 1999) and salicylic acid activated the Tnt1 promoter (Grandbastien, 1997). PGRs are assumed to induce the transposition of TEs in two different ways: one is by activating cell division and the other is by functioning as a mutagen. Future studies should first determine which PGR, cytokinin or auxin, is associated with mutation induction in *Saintpaulia* and then determine whether or not the identified PGR functions as a mutagen. In addition, an epigenetic study could reveal how the exogenous triggers induce the transposition of VGs1 and shed further light on the relationship between the exogenous factors and transposition. In tissue culture, epigenetic changes, such as altered methylation states, are associated with the activation of TEs (Kaeppler et al., 2000). For example, Hirochika et al. (1996) showed that the retrotransposons Tos10, Tos17, and Tos19 in rice (*Oryza sativa*) are activated by tissue culture, and Liu et al. (2004) showed that activation of retrotransposons is closely associated with a decrease in the methylation rate of the genome. Brettell and Dennis (1991) showed that the Ac elements in maize, which belong to the hAT superfamily, are activated in tissue culture with a re-
duced DNA methylation rate. To develop a new culture method, decreasing the occurrence of somaclonal variations or screening anti-mutagens available for in vitro culture and DNA markers for assessing epigenetic states in plants should be developed in addition to the DNA marker developed in this study.

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