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Studies on somaclonal variation in *Phalaenopsis*

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Abstract The morphological and genetic variations in somaclones of *Phalaenopsis* True Lady "B79-19" derived from tissue culture were evaluated. In 1360 flowering somaclones, no apparent difference was found in the shape of the leaves, whereas flowers in some somaclones were deformed. We have demonstrated that 38 selected random primers can be used to generate amplified segments of genomic DNA and to differentiate polymorphisms of somaclonal variations in *Phalaenopsis*. The random amplified polymorphic DNA (RAPD) data indicated that normal and variant somaclones are not genetically identical. We also studied the banding patterns of aspartate aminotransferase (AAT) and phosphoglucomutase (PGM) in young leaves of variant and normal somaclones of *Phalaenopsis*. With respect to AAT, three distinct banding patterns were found in normal somaclones and only two-banded phenotypes were detected in variant somaclones. In a comparison of the banding patterns of PGM isozymes, three to four bands were detected in normal somaclones and two to three bands in variant ones.

Key words Somaclonal variation · Random amplified polymorphic DNA · Isozyme · *Phalaenopsis*

Introduction

Tissue culture has been widely used for mass production in superior varieties of *Phalaenopsis* (Tokuhara and Mii 1993). However, the occurrence of somaclonal variation during culturing is a frequent and consistent event. Somaclonal variations not only can be distinguished by their morphological traits (Gamborg et al. 1977) but also by their biochemical, physiological and genetic characteristics. Several reports in the literature indicate that variations can be detected by identifying chromosome numbers (Bayliss 1973; Ogura 1990), isozyme patterns (Fernandez et al. 1996; Pereira et al. 1996) and random amplified polymorphic DNA (RAPD) (Rani et al. 1995; Roy et al. 1992; Taylor et al. 1995; Williams et al. 1990) in many crops. Avends (1970) reported that although the diploid karyotype in somaclonal variation of *Phalaenopsis* can be detected, the difficulty lay in the relatively small size of its mitotic chromosomes and that gross rearrangement (chromosome structure) of the karyotype in cultured cells is difficult to examine. This is probably the reason why the data on chromosome variability in cultured cells and regenerated plants in *Phalaenopsis* are rather scanty. It is important to detect somaclonal variations in *Phalaenopsis* during propagation by tissue culture. The narrow genetic diversity and the low reliability of phenotypic characters make the identification of somaclonal variation in *Phalaenopsis* especially difficult.

The aim of the experiments presented here was to examine plant characteristics, RAPD and isozyme patterns in normal and variant somaclones of *Phalaenopsis*.

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Materials and methods

Plant material

Induction of adventitious shoots

Dormant buds dissected from flower stalks of hybrid *Phalaenopsis (Phal.)* orchid (True Lady 'B79-19') were used. The buds were surface-sterilized by immersion in 70% (v/v) ethanol for 1 min, then in a 10% (v/v) commercial sodium hypochlorite solution for 15 min to surface-disinfect, followed by rinsing with sterile distilled water. The buds were placed vertically in a separate test tube (30×150 mm) containing 20 ml of agar-solidified medium. The composition of the

medium was half-strength (1/2) MS (Murashige and Skoog 1962), 2.6 μ*M* BAP (N^6-benzyladenine), 0.5 μ*M* NAA (α-naphthaleneacetic acid), 0.8% (w/v) agar (Difco, Detroit, Mich.), 10% (v/v) coconut milk. The cultures were kept at 27 °C under a 16-h photoperiod with the light intensity (cool-white fluorescent lamp) at 60 mol m^{-2} s^{-1} for 20 days. The adventitious shoots were then subcultured on the medium containing 1/2 MS, 0.9 μ*M* BAP, 0.1 μ*M* NAA, 0.8% agar (w/v), 10% (v/v) coconut milk to induce further development. Shoots were transferred to 0.2% (w/v) active charcoal medium for plantlet growth.

Induction of protocorm-like body from the leaf of in vitro plantlets

Young leaves cut from *in vitro* plantlets of *Phalaenopsis* were transferred to a liquid medium containing VW (Vacin and Went 1949) mineral salts, 0.4 m*M* $Ca_3(PO_4)_2$, 1.9 μ*M* adenine, 0.9 μ*M* BAP, 0.3 μ*M* NAA, 1.5% (w/v) sucrose, 2% (v/v) coconut milk. After 10 days, the leaf explants were transferred to a medium containing VW mineral salts, 0.4 m*M* $Ca_3(PO_4)_2$, 8.5 μ*M* adenine, 4.5 μ*M* BAP, 1.8 μ*M* NAA, 0.5% (w/v) sucrose, 10% (v/v) coconut milk and 0.8% (w/v) agar. The derivatives induced from the leaf explants were subcultured in KCpc medium (Tsai et al. 1992) for induction of protocorm-like body. Protocorm-like bodies were cut in half once a week, and then the halfs were cultured in the same medium for further development. Plantlets were obtained by further culturing in a L8 medium (Chen et al. 1994) for 3–4 months and then grown to maturity in a greenhouse (30 °C day/20 °C night). The variant somaclones R1-48, R1-84 and R1-85 were derived from the same cultivar of Phal. True Lady 'B79-19'.

Evaluation of flower morphology

Plant growth and flower morphology were recorded by photography 2 days after flower opening. The variation in flower phenotypes including color, flower form etc. was investigated.

RAPD analysis

DNA extraction

Somaclones exhibiting both the normal phenotype and variations with abnormal flower characteristics were selected for RAPD analysis using the methods of Fu et al. (1994). About 0.3 g of *Phalaenopsis* young leaves were crushed and used for total genomic DNA extraction. Leaf samples were extracted with pre-heated extraction buffer [1% (w/v) cetyltrimethylammonium bromide, 100 m*M* TRIS-HCl (pH 8.0), 1.4 *M* NaCl, 20 m*M* EDTA, 0.1% (v/v) mercaptoethanol] (65 °C) for 30 min, and then 7.5 ml of phenol : chloroform : isoamyl alcohol (24 : 23 : 1 v/v) was added. The solution was kept at 0 °C for 30 min with occasional shaking. After 15 min, the solution was centrifuged at 4000 *g* for 5 min at 4 °C. An equal volume of isopropanol was added to the supernatant and the mixture stored at –20 °C for 1 h. The mixture was further centrifuged at 10.000 *g* for 25 min at 4 °C, and the supernatant was then discarded. DNA was dissolved by adding 300 μl ice-cold TE buffer solution, and 1 μl of RNAase (2 U/μl) was added. The solution was then incubated at 37 °C for 1 h, and 30 μl of 3 *M* NaOAc and 1 ml of absolute alcohol were added. The DNA pellet was resuspended in 300 μl of ice-cold 70% (v/v) ethyl alcohol, and then recovered following a 25-min centrifugation at 4000 *g* at 4 °C. The DNA solution was dried in air and resuspended in 0.1 ml of TE buffer solution. The extraction procedure consistently yielded between 165 to 300 μg of DNA/g fresh tissue.

Amplification

The random amplified polymorphic DNA reaction mixture (10 μl) contained 1 μl of 10× polymerase chain reaction (PCR) buffer, 6 pmole of each primer, 20 m*M* each dNTP, 0.5 U *Taq* DNA polymerase, 4 μl of distilled water and 6 ng of *Phalaenopsis* DNA. Amplification was conducted in a thermal cycler (Air Thermo-Cycler, Idaho) programmed for 2 cycles of 94 °C for 60 s, 36 °C for 7 s, and 72 °C for 70 s, followed by a program run through 55 cycles of 94 °C for 6 s, 40 °C for 7 s, and 72 °C for 70 s. These reaction conditions produce consistently reproducible results for a range of *Phalaenopsis* DNA concentrations. Amplification products were separated in 2% (w/v) agarose gels in 1×TAE buffer and visualized by ethidium bromide staining.

Primer screening

One hundred RAPD primers (Operon Technologies, Alameda, Calif., kits OPA-1, OPD 1 & 3, OPG 1-12, OPJ 1-17 & 19-20, OPM 1-20, OPQ 1-20, OPS 19-20, OPT 1-4, OPY 1-20) were used. Primers that produced reproducible polymorphic bands were scored, followed by determination of the fragment size in base pairs. The RAPD analyses were repeated three times.

Isozyme analysis

Isozyme extraction

Leaf samples used for isozyme analysis was prepared in the same way (i.e. age, position etc.) as that of RAPD. Leaf tissues were crushed in an extraction buffer consisting of 1 *M* sucrose, 56 m*M* 2-mercaptoethanol, 0.01 m*M* antipan, 0.01 m*M* leupeptin and 0.2 *M* TRIS-HCl (pH 8.5). Plant extracts were centrifuged at 12.000 *g* for 20 min at 4 °C, and the supernatants were stored in –20 °C until isozyme analysis.

Electrophoresis

Isozyme separation was carried out by electrophoresis on 7.5% (w/v) polyacrylamide gels. The tray buffer was a mixture of 5 m*M* TRIS-HCl (pH 8.5) and 38 m*M* glycine. Electrophoresis was carried out for 11 cm at 200 V at 4 °C. The staining procedure was that from Shield et al. (1983).

Results and discussion

Plant characteristics

Interest in measuring the genetic variation derived from tissue culture regenerants has been reported in many plant species (Bajaj 1990, Larkin and Scowcroft 1981). Unfortunately, few investigations have been conducted at the levels of phenotypic and genetic variations in somaclones of *Phalaenopsis* (Tokuhara 1992). Morphological characterization is a traditional method for identifying phenotypic variation in many plants (Bailey 1983; Pereira et al. 1996). The plants used in the present study were obtained from tissue culture, and the abnormal somaclones were excluded during the flask transplantation stages. Therefore, the leaf appearance (i.e. shape, color etc.) of the variant somaclones during flowering was not discernible (data not shown). The results of this study demonstrate that flower morphology is a clear indication for investigating somaclonal variation (Figs. 1, 2). Of the 1360 mericlones of Phal. True Lady 'B 79-19' which were propagated from protocorm-like bodies for 4 years and then flowered for first time, 20 exhibited variation in flower morphology (1.5% of the total).

Fig. 1 a–d Normal and variant flowers from somaclones of Phal. True Lady 'B79-19'. The somaclones were transferred from in vitro to pot culture in 1993 (i.e. propagated by protocorm-like bodies for 4 years and then flowered for the first time). **a** Flower in normal somaclones (7.5×5.9 cm in flower dimensions), **b** wing petals had shrunk in variant somaclones; R1-48 was 6.0×5.6 cm in flower dimensions, **c**, **d** all flower parts in the variant somaclones showed significant abnormalities including color and shape, when compared with normal flower. R1-84 was 7.9×5.7 cm, and R1-85 8.5×5.7 cm in flower dimensions, respectively

The normal flower was found to be 7.5×5.9 cm in size, whereas the dimensions of those of the variants were 6.0×5.6 cm (R1-48) 7.9×5.7 cm (R1-84) and 8.5×5.7 cm (R1-85). All of the flowers of the variant somaclones were deformed (Fig. 1). Also, in a comparison of flower parts between normal and variant somaclones, R1-84 revealed significantly abnormal morphologies (Fig. 2). Variant somaclones R1-48, R1-84 and R1-85 were single individuals derived from the same cultivar, True Lady 'B79-19', a single genotype, by tissue culture. The altered flower morphology which we used as the basis to select abnormal variants was a stable trait.

RAPD polymorphisms

The RAPD method offers great potential for generating large numbers of markers representing a random sample of the genome and has efficiently been used to give reliable and reproducible results for estimating the genetic variation. In the present study on *Phalaenopsis*, 78 out of 100 primers gave 1 to 10 distinct bands per primer, resulting in a total of 1116 bands. Figure 3 shows four sets of RAPD profiles from this study that demonstrate the heterozygosity revealed in somaclonal variants of *Phalaenopsis*. Polymorphic and reproducible RAPD profiles were obtained from 38 primers. Table 1 shows the results of RAPD analysis for variant somaclones of True Lady 'B79-19'. Although about 38% of the primers related to the variant somaclones, no one single primer was suitable for separating the somaclones into normal versus variant ones. Therefore, it is likely that a group of variant somaclones might share the change in the same DNAs. The present work indicates that RAPDs will be a useful tool in identifying somaclonal variation in regenerants from tissue culture of *Phalaenopsos*. However, the same RAPD patterns in Fig. 3 b between the two variant strains (b-1: R1-84 and b-2: R1-85) were observed when primer OPG-11 was used, whereas primers OPQ-7 (a-1 for R1-84 in Fig. 3 a) and OPJ-4 (c-3 for R1-85 in Fig. 3 c) revealed significant polymorphic patterns for these two strains. As no apparent similarity in flower characters was found in R1-84 and R1-85,

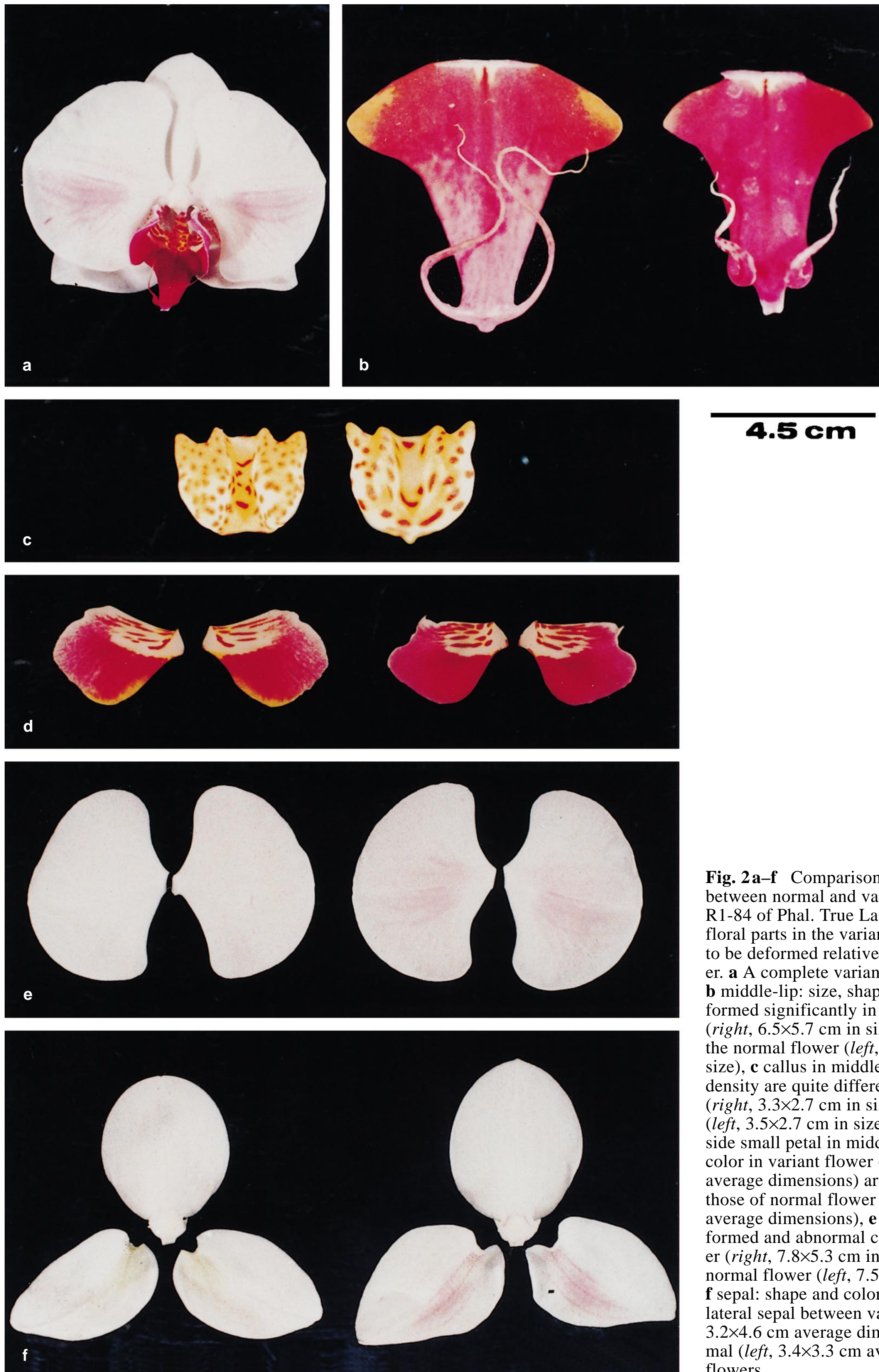

Fig. 2 a–f Comparison of each flower part between normal and variant somaclones in R1-84 of Phal. True Lady 'B79-19'. The floral parts in the variant somaclone appear to be deformed relative to the normal flower. **a** A complete variant flower of R1-84, **b** middle-lip: size, shape and tendril are deformed significantly in variant flower (*right*, 6.5×5.7 cm in size) as compared to the normal flower (*left*, 7.5×7.2 cm in size), **c** callus in middle-lip: shape and spot density are quite different between variant (*right*, 3.3×2.7 cm in size) and normal (*left*, 3.5×2.7 cm in size) somaclones, **d** side small petal in middle-lip: shape and color in variant flower (*right*, 3.5×2.6 cm average dimensions) are different from those of normal flower (*left*, 3.4×2.5 cm average dimensions), **e** wing-petal: deformed and abnormal color in variant flower (*right*, 7.8×5.3 cm in size) compared to normal flower (*left*, 7.5×5.1 cm in size), **f** sepal: shape and color are different in lateral sepal between variant (*right*, 3.2×4.6 cm average dimensions) and normal (*left*, 3.4×3.3 cm average dimensions) flowers

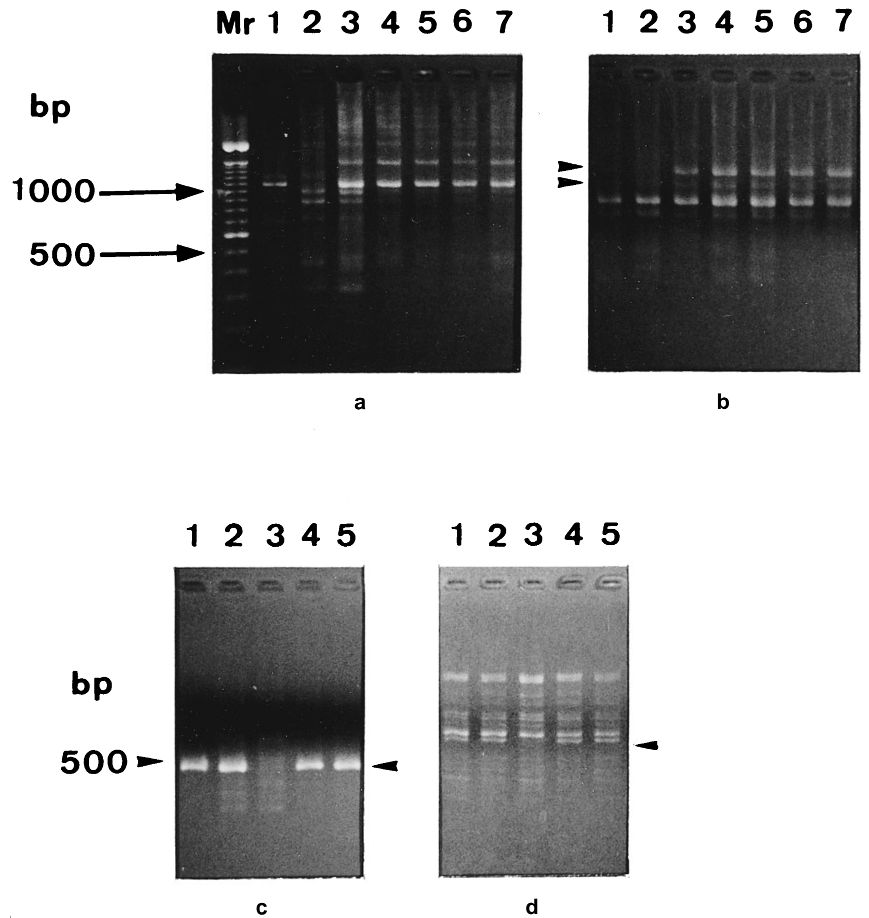

Fig. 3a–d RAPD analysis in the leaves of normal and variant somaclones of Phal. True Lady 'B79-19'. The primers were: **a** OPQ-7, **b** OPG-11, **c** OPJ-4, **d** OPY-13. The variant somaclones were: R1-48 (**d**-*3*), R1-84 (**a**-*1* and **b**-*1*), R1-85 (**a**-*2*, **b**-*2* and **c**-*3*).The *other numbers above* the figure represent normal somaclones

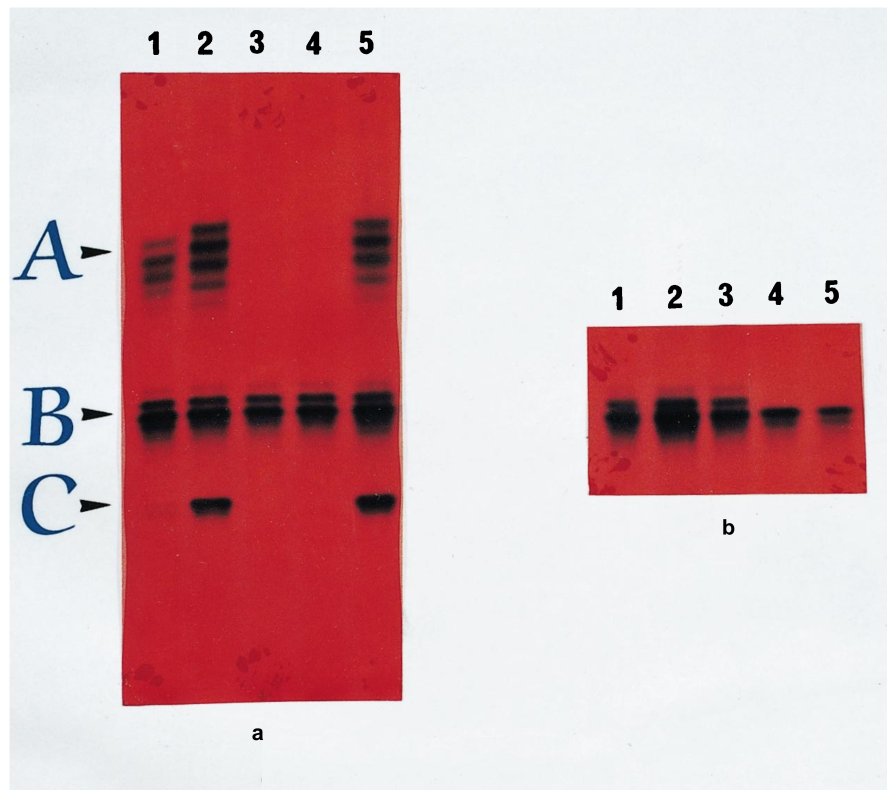

Fig. 4a, b Isozyme AAT patterns of normal and variant somaclones, respectively, in Phal. True Lady 'B79-19'. There are three zones in photograph **a** (containing *A*, *B* and *C* zones) of isozyme profiles. However, only zone *B* in photograph **b** exhibited stable patterns and has been used for identifying differences between normal and variant somaclones. *Lanes 1*, *2*, and *3* denote normal somaclones showing three-banded patterns, and *lanes 4* (R1-84) and *5* (R1-85) revealed variant somaclones where only two bands were found

Table 1 Distribution of RAPD in variant somaclones in Phal. True Lady 'B79-19'

Variant somaclones	Number of tested primers	Primers showing polymorphism (%)	Number of total bands	Number of polymorphic bands (%)	Primers showing DNA polymorphisms
R1-48	62	1 (1.6)	174	1 (0.6)	OPY-13
R1-84	100	18 (18.0)	307	33 (10.7)	OPY-1, -2 and -3, OPJ-1, -5 and -11, OPM-4, -11 and -14, OPQ-4, -7, -14, -15 and -20, OPT-1, OPG-10, -11 and -12
R1-85	100	19 (19.0)	316	35 (11.1)	OPY-1, -2 and -3, OPJ-1, -4, -5 and -11, OPM-4 and -14, OPQ-4, -7, -8, -14, -15 and -20, OPT-1, OPG-5, -10 and 11

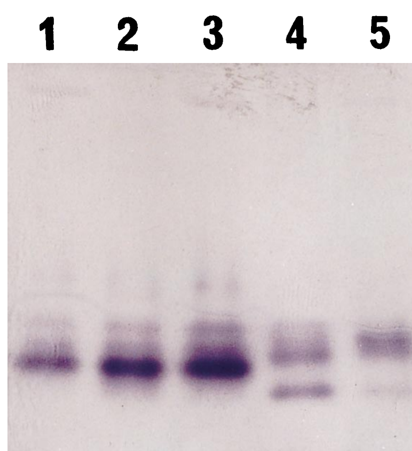

Fig. 5 Isozyme PGM patterns of normal somaclones (*lanes 1–3*) revealing three- to four-banded patterns, whereas in variant somaclones [*lanes 4* (R1-84) and *5* (R1-85)] only two to three bands were detected in the relatively lower position in photographs of Phal. True Lady 'B79-19'

as shown in Fig. 1, these differences in RAPD profiles should be considered to have no significant relationships to the phenotypic differences observed in these two variants.

Isozyme pattern

In our preliminary experiment, 2 out of 11 the enzymes tested, aspartate aminotransferase (AAT) and phosphoglucomutase (PGM), presented clear consistent isozyme patterns. Therefore, all subsequent studies were focused on these enzyme systems. For ATT, there were three zones of staining (A, B and C) in normal somaclones (Fig. 4 a). Each zone consisted of either one (C), three (B) or four (A) bands, but only zone B exhibited the stable and dimeric nature of the enzyme. Staining for ATT in zone B after polyacrylamide gel electrophoresis resulted in obvious differences between normal and variant somaclones, three distinct bands were found in normal somaclones, and only two banded patterns were detected in variant somaclones (Fig. 4 b). A similar difference in isozyme pattern was also found for PGM. Normal somaclones (1, 2 and 3) showed three- to four-banded patterns, whereas variant somaclones (4 and 5) exhibited only two to three zymograms (Fig. 5).

The fact that more AAT and PGM isozyme bands were expressed in normal somaclones than in variant, indicates that variant somaclones involved the repression of genes normally activated in the normal somaclones. Regular monitoring of somaclonal variation in *Phalaenopsis* using isoenzymes is feasible for propagators at an early stage.

The results of this study suggest that considerable somaclonal variations in flower morphology, including color and shape, occur in *Phalaenopsis* regenerants derived from tissue culture and that it should be possible to discern some of the somaclonal variations using genetically characterized RAPD markers. Also, biochemical traits such as isozymes could help in the identification of somaclonal variations as a complement to monitoring morphological traits.

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