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Somaclonal Variations in Flower and Inflorescence Axis in Micropropagated Plants through Flower Stalk Bud Culture of *Phalaenopsis* and *Doritaenopsis*

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Abstract

Somaclonal variations in flower and inflorescence axis were investigated among the plants micropropagated through protocorm-like bodies induced by flower stalk bud culture of various cultivars of *Phalaenopsis* and *Doritaenopsis*. Eighteen hundred to 14,750 micropropagated plants were grown for each of 11 genotypes in greenhouses for 1-1.5 years till flowering. Somaclonal variations appeared in flower and inflorescence were classified into 9 categories irrespective of cultivars. The frequencies of these somaclonal variations in each genotype ranged from 0 to 100%, but most of the cultivars showed variations less than 10%. Changes in leaf shapes and ploidy level were not detected in these variants. Possible mechanisms involved in these variations and the problems of the micropropagation method used in this study were discussed.

1. Introduction

Recently, the production of pot plants and cut flowers of orchids has greatly increased and 7.4 million pot plants and 88 million cut flowers of orchids in volume were produced in 1992 in Japan [1]. In orchid production, 22% of pot plants and 3% of cut flowers consisted of *Phalaenopsis* and its intergeneric hybrid with *Doritis* (*Doritaenopsis*). In most of the commercially important orchids such as *Cattleya*, *Dendrobium* and *Cymbidium*, cultivars propagated vegetatively through tissue culture are now predominantly used for production. In *Phalaenopsis*, however, production using seedlings is still dominant despite the variabilities of the products. Although there have been several reports on successful micropropagation of *Phalaenopsis* using different explant sources [2-4], clonal micropropagation is still not popular in this orchid because of the difficulties such as low multiplication rate and occurrence of somaclonal variations in applying these methods to large scale production of plantlets.

For commercial micropropagation of plants, the occurrence of somaclonal variation is one of the most serious problems [5, 6]. In some orchids such as *Cymbidium* and *Dendrobium*, there are a few reports on somaclonal variation of micropropagated plants, however, no numerical data such as the number of plants produced and the frequency of variants were shown [7, 8]. In *Phalaenopsis*, a low frequency of somaclonal variation was reported previously by Tanaka *et al.* [9] using a relatively small number of plants. However, it is necessary to use a large number of plants for appropriate evaluation of the frequency of variations which might occur in commercial scale micorpropagation. In our previous studies, we established an efficient method of micropropagation of *Phalaenopsis* [4] and showed the low frequencies of somaclonal variations in the micropropagated plants [10]. In the present report, we show the details of somaclonal variations in flowers and inflorescence axis that appeared in various genotypes of micropropagated *Phalaenopsis*.

2. Materials and Methods

Flower and inflorescence axis were investigated at flowering time in the plants obtained through culture of shoot tips from lateral buds of flower stalk [4] in 11 genotypes which included five genotypes of *Phalaenopsis* Wedding Promenade (PM70, PM78, PM79, PM122, and PM166), 2 genotypes of *Phal.* Crystal Veil (PM68 and PM107), 2 genotypes of *Phal.* Magic Girl (PM69 and PM190), 1 genotype of *Doritaenopsis* Tsuei Hoa Truth (DTM43) and 1 genotype of *Dtps.* Wedding Ring (DTM73). In each genotype, protocorm-like bodies (PLBs) were induced from 2-3 lateral buds on the basal part of a single inflorescence axis, subcultured 5-10 times at one month-intervals for PLB micropropagation, and grown into plantlets by the methods previously reported [3]. Finally, 1,800

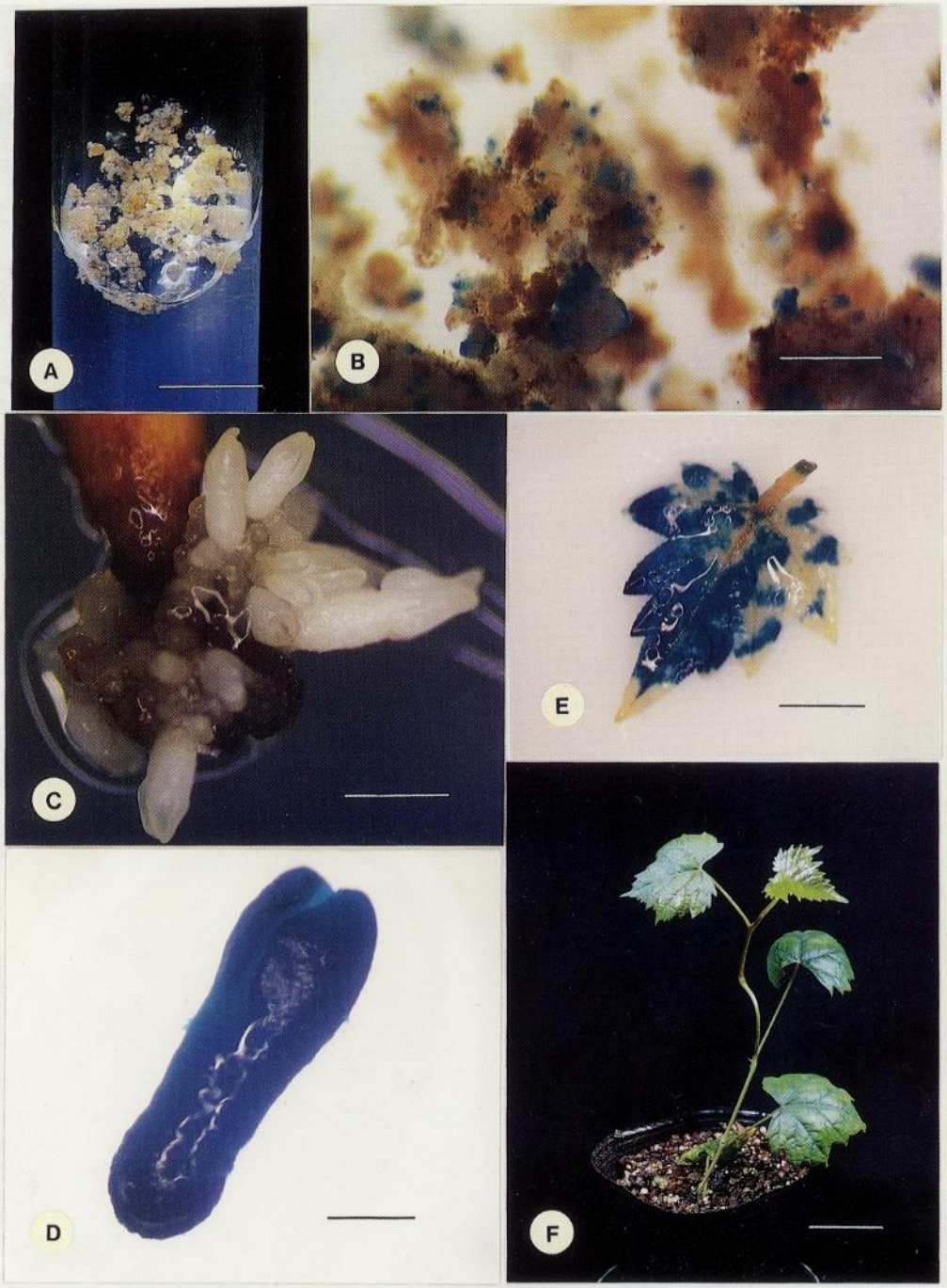

Fig. 1 Regeneration of transgenic plants via *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli.

(A) Embryogenic callus used for co-cultivation with *A. tumefaciens*. Bar = 10 mm

(B) Embryogenic calli showing GUS activities 7 days after co-cultivation. Bar = 5 mm

(C) Secondary embryos formed at the base of a somatic embryo derived from embryogenic callus after 3 months of culture. The original somatic embryo turned dark brown on 50 mg/*l* kanamycin-containing medium while secondary embryos could grow on the same medium. Bar = 4 mm

(D) Secondary embryo developed 4 months after bacterial inoculation showing GUS activity. Bar = 1 mm

(E) Leaf segment showing GUS activity excised from a regenerated plant derived from secondary embryo. Bar = 2.5 mm

(F) Regenerated plant having GUS activity established in a pot after transfer to the growth chamber. Bar = 30 mm

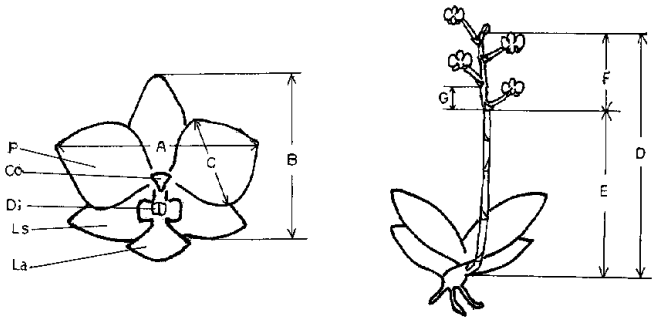

Fig. 1 Terminology on flower and plant of *Phalaenopsis* used in this study.
A: flower width, B: flower height, C: petal width, D: whole length of inflorescence axis, E: length of basal inflorescence axis, F: length of flower-bearing inflorescence axis, G: internode length between first and second flowers, P: petal, Co: column, Di: disc, La: labellum, Ls: lateral sepal.

to 14,750 plantlets with 3-4 leaves in each genotype were successfully transferred to pots within 2 years after starting the multiplication of PLBs. These plantlets were further grown in a greenhouse for 1-1.5 years till flowering. Abnormality in flower and inflorescence characters were investigated during the full bloom period in the first year with randomly selected 222-1,173 plants for each genotype. After observation of flower characters, inflorescence axes were removed and plants were grown in greenhouses until the next flowering to confirm the stability of the variations.

In the 3 variants of PM70, several characteristics in flower and inflorescence axis (**Fig. 1**) were investigated at flowering time using 3-10 plants for each variant. The flower morphology terms for *Phalaenopsis* adopted in this study were those used by Hodgson *et al.*[11].

The ploidy level of variants was confirmed using flow cytometry. About 1 cm^2 tissue segments were excised from petals of normal and variated plants in 2 genotypes of *Phal.* Wedding Promenade (PM70, PM79) and 1 genotype of *Dtps.* Wedding Ring (DTM73), respectively. These petal segments were chopped into small pieces with surgical knives in 4,6-diamidino-2-phenylindole (DAPI) solution (10 mM Tris-HCl buffer at pH 7.5, 0.1% Triton X-100, 2 mM $MgCl_2$ and 2 mg l^{-1} DAPI) for releasing and staining the nuclei. The DAPI solutions containing free nuclei were filtered with 20 μm nylon mesh to remove the large cell debris, and used as samples for assessing DNA contents by flow cytometry (CA II, Partec Ltd. Münster, Germany).

3. Results

The variations in flower characters observed in the present study were classified into 7 types as shown in **Fig. 2**. Compared with the other monocots, orchids have unique flower shape, in which one of the three petals forms a labellum, a landing platform for vector insects [12] (**Fig. 2-A**). One of the variants had petals with incomplete labellum-like color and a shape. The petal showed a yellow tongue-like small disk (Type 1, **Fig. 2-B**). Another variant also showed abnormal petals similar to a labellum in shape and in color (Type 2, **Fig. 2-C**). Consequently, the flower looked as though it possessed 3 labellums. The morphological character of the labellum was also transferred onto sepals in the other variant, in which the lower half of the lateral sepals turned into a labellum-like structure and color (Type 3, **Fig. 2-D**). A variant with irregular shape in the perianths was also obtained (Type 4, **Fig. 2-E**). All of the perianths of this variant showed an irregular shape with some notched edges and blurred coloration. The variants with faded flower color (Type 5, **Fig. 2-F**) and dwarfed flowers (Type 6, **Fig. 2-G**) were also observed. In Type 6, all of the parameters examined on the flower size showed smaller values (**Table 1**). The remaining flower variant possessed an abnormal column with petal-like wings at the abaxial side (Type 7, **Fig. 2-H**). A combination of two variations was also observed in a variant (**Fig. 2-I**) which had a dwarfed flower (Type 6) and a petaloid column (Type 7).

On the inflorescence axis, 2 types of variations were observed. One of the variants had a shorter internode between each flower without any change in inflorescence axis length from the base to the node with the first flower, which resulted in an aggregation of flowers (Type 8, **Fig. 3** and **Table 2**). A variant which had both aggregated flowers (Type 8) and faded color flowers (Type 5) was also observed (**Fig. 3**). The other type of variant was characterized by the shortening of the inflorescence axis from the base to the node with the first flower (Type 9, **Fig. 4** and **Table 1**). In this variant, however, the internode length between each flower did not show the shortening which was observed in the Type 8 variant.

All of the variants investigated in the present study showed normal leaves during *in vitro* culture as well as under cultivation in a greenhouse.

The frequencies of the somaclonal variants observed in all the genotypes examined are summarized in **Table 3**. More than 90% had normal flowers and normal inflorescences in most of the genotypes of the 3 hybrids (*Phal.* Wedding Promenade, *Phal.* Magic

Table 1. Comparison of several characteristics between normal plant and 2 different variants, Type 6 (dwarfed flower) and Type 9 (dwarfed basal inflorescence axis) in *Phal.* Wedding Promenade (PM70).

Character	Normal	Variants	
		Type 6 (dwarfed flower)	Type 9 (dwarfed basal inflorescence axis)
Whole length of inflorescence axis (mm)	383.6±15.4	337.5±19.0	211.0±16.5
Flower width (mm)	59.4± 1.3	42.3± 2.6	56.7± 1.7
Flower height (mm)	51.4± 0.8	38.0± 3.0	52.8± 1.8
Petal width (mm)	28.3± 0.3	19.9± 1.3	28.7± 0.6
Internode length (mm)	27.5± 0.8	23.1± 1.2	26.9± 1.3

Table 2. Comparison of several characteristics between normal plant of *Phal.* Wedding Promenade (PM70) and its Type 8 variant which has aggregated flowers.

Character	Normal	Type 8 variant
Whole length of inflorescence axis (mm)	482.3± 5.9*3	442.2±13.9
Length of basal inflorescence axis*1(mm)	315.0± 7.1	320.4±10.8
Length of axis with flower*2(mm)	167.3± 1.2	121.8±14.8
Internode length (mm)	28.3± 1.2	23.6± 0.7

*1The length from base of inflorescence axis to the node with first flower.

*2The length from the node with the first flower to the apex was measured when 6-7th flowers were blooming.

*3The value considerably differs from that shown in **Table 1** due to the difference in age of the plants.

Fig. 3 Flowering of normal plant (left) and variants with aggregated flowers (Type 8, middle and right) in genotype PM70 of *Phal.* Wedding Promenade.
The variants had shorter internodes at flower-bearing part of the axis than the normal plant, resulted in shorter flower-bearing inflorescence axis. The plant in the right also had a variation with faded flower color (Type 5+8).

Fig. 4 Plant (genotype DTM73 of *Dtps.* Wedding Ring) with normal flower (left) and the Type 9 variant (right) with shorter inflorescence axis than normal plant.

Girl and *Phal.* Crystal Veil). All the regenerants were normal in two genotypes of *Phal.* Crystal Veil. However, in *Dtps.* Tsuei Hoa Truth all regenerants showed variation of dwarfed flowers and in *Dtps.* Wedding Ring 91% of the regenerants showed dwarfed inflorescence axis. No such single types of variations were observed in most of the other genotypes.

The variants observed in the first flowering season was stable and showed the same types of variations in next flowering season.

Flow cytometric analysis of all the types of variants showed that they had the same DNA contents as that of normal plants, suggesting that no polyploids were included in the variants observed in this study.

Table 3. Frequencies of somaclonal variations in the plants micropropagated from shoot tip culture of flower stalk buds.

Genotype		*Phal.* Wedding Promenade					*Phal.* Magic Girl		*Phal.* Crystal Veil		*Dtps.* Tsuei Hoa Truth	*Dtps.* Wedding Ring
		PM70	PM78	PM79	PM122	PM166	PM69	PM190	PM68	PM107	DTM43	DTM73
No. of plants propagated		9,150	7,600	8,750	5,050	7,700	5,500	14,750	8,800	10,100	1,800	5,250
No. of plants investigated		982	586	1,173	892	1,017	918	369	643	1,046	350	222
Total No. of variants(%)		78(7.9)	44(7.5)	137(11.7)	16(1.8)	15(1.5)	61(6.6)	1(0.3)	0	0	350(100)	202(91.0)
Type of variation*2	1	3(0.3)*1	0	2(0.2)	0	1(0.1)	36(3.9)	1(0.3)	0	0	0	0
	2	2(0.1)	0	0	0	0	25(2.7)	0	0	0	0	0
	3	1(0.1)	0	1(0.1)	0	1(0.1)	0	0	0	0	0	0
	4	4(0.4)	2(0.3)	1(0.1)	1(0.1)	1(0.1)	0	0	0	0	0	0
	5	12(1.2)	12(2.0)	13(1.1)	1(0.1)	7(0.7)	0	0	0	0	0	0
	6	8(0.8)	1(0.2)	17(1.4)	2(0.2)	1(0.1)	0	0	0	0	350(100)	0
	7	1(0.1)	0	7(0.6)	12(1.3)	1(0.1)	0	0	0	0	0	0
	6+7	0	0	2(0.2)	0	0	0	0	0	0	0	0
	8	8(0.8)	29(4.9)	93(7.9)	0	3(0.3)	0	0	0	0	0	0
	5+8	1(0.1)	0	1(0.1)	0	0	0	0	0	0	0	0
	9	38(3.9)	0	0	0	0	0	0	0	0	0	202(91.0)

*1 Numbers in the parentheses show the percentages.

*2 The variations observed are classified into 11 categories; 7 variants in flower characters (Type 1-7), 2 variants in inflorescence axis length (Type 8 and 9) and 2 doubled variants (6+7 and 5+8). The details of these variants are described in the text.

4. Discussion

The occurrence of somaclonal variations in micropropagated plants has been reported in various species such as *Begonia* [13], *Chrysanthemum* [14], *Cucumis* [15], *Musa* [16], and *Saintpaulia* [13]. In commercially cultivated orchids such as *Cattleya*, *Cymbidium*, *Dendrobium*, *Miltonia* and *Oncidium*, most of the cultivars are propagated using shoot tip culture and the occurrence of somaclonal variations has been recognized as one of the most serious problems for commercial growers of orchids. However, there have been few studies on the somaclonal variations in orchids propagated by tissue culture [7, 8]. These studies reported variations in color, size and shape of flower, and plantlet morphology in micropropagated *Dendrobium* and *Cymbidium*. Furthermore, polyploids including tetraploid, hexaploid and octaploid which showed thicker leaves and slower growth than the original plants were observed. In the present study, we showed various variants among the plants of *Phalaenopsis* and *Doritaenopsis* micropropagated at commercial scale levels examing totally 8,198 plants obtained through shoot tip culture of lateral buds on flower stalk nodes. The characteristics of the variants observed in the present study were different from those previously reported. Generally in commercial laboratories, plantlets with some variations in leaves are eliminated during subculture, because they usually show abnormal morphologies in flower characteristics as well. However, all of the variants examined in this study showed normal leaves and grew normally during *in vitro* propagation as well as during cultivation in a greenhouse. Moreover, polyploids were not included in these plants as assessed by flow cytometry. Therefore, it is possible that changes in DNA sequence and/or their expression might be involved in these variants.

To avoid or reduce somaclonal variations, it has been recommended to limit the period of subculture [5, 6]. In commercial micropagation of orchids, however, it is required to subculture periodically for more than 1-1.5 years to propagate sufficient numbers of plants. Therefore, it is possible that the frequency of somaclonal variation would increase during the subculture. In the present study, less than 10% of somaclonal variation was obtained in most of the cultivars except for 2 genotypes (DTM 43 and DTM 73) in which almost all the micropropagated plants showed a single type of somaclonal variation, types 6 and 9 respectively. In DTM43 and DTM73, it is considered that genetic changes would have occurred at the stage of PLB induction from shoot tips of lateral buds on the flower stalk. Also, it is possible that these 2 genotypes were genetically unstable in tissue culture condition or were periclinal chimeras because the data of somaclonal variations in **Table 3** were obtained from the culture of 2-3 shoot tips. To avoid this kind of variation, it is necessary to check flower and inflorescence axis characteristics in advance using PLBs which developed in early stage of multiplication.

Although the somaclonal variations observed in the present study might be undesirable characteristics, some of the plants with abnormal flower morphology have been utilized as novel cultivars, such as *Cattleya intermedia* var. *aquinii* 'Dragon Stone', *Laeliocattleya* Mari's Song 'Daniella'[17] and *Phal. equestris* 'Cats' [18] which have Type 1 variation, and *Dtps.* Grebe 'Poker Chips'[17] which is classified as a Type 2 variation in the present study, respectively. Although the genetic mechanisms involved in the formation of these variants are still unclear, it is possible that they could be induced by the mutation of some homeotic genes which control the flower morphogenesis [19-21].

Recently, DNA-metylation has been recognized as a cause of somaclonal variations observed during tissue culture [5]. The variations observed in the present study are also possibly associated directly or indirectly with the DNA-metylation. Further studies on the genetic analysis of the variants will be needed to clarify these phenomena through progeny test with the exception of one variant (Type 2) which has no pollen fertility.

Somaclonal variations are generally considered to be induced by plant growth regulators (PGRs) or culture conditions used for *in vitro* culture [5, 6]. In the present study, however, we only used the same PGRs and culture conditions throughout the micropropagation. Therefore, it is difficult to identify the causal agents of the somaclonal variations observed in this study.

Although further studies will be required for analyzing the genetic mechanisms involved in the variations observed in the present study, a search for molecular markers associated with each variation will enable us to predict and remove the variations at an early stage of micropropagation before flowering.

References

[1] Asada, H., 1994. In "Flower Data 94", p. 237-243, Kaki-Engei Shinbunsya Inc., Nagoya.

[2] Arditti, J., Ernst, R., 1993. In "Micropropagation of Orchids", p. 373-375, 467-520, Jhon Wiley and Sons, Inc., New York.

[3] Ichihashi, S., 1992. Lindleyana, **7**: 208-215.

[4] Tokuhara, K., Mii, M., 1993. Plant Cell Rep., **13** : 7-11.

[5] George, E. F., 1993. In "Plant Propagation by Tissue Culture Part 1 The Technology", p. 67-94, Exegetics, Edington.
[6] Karp, A., 1989. IAPTC News Letter, **58**: 2-11.
[7] Vajrabhaya, M., Vajrabhaya, T., 1974. "Proc. 7th World Orchid Conference" (ed. by Ospind, M. H.), p. 231-242, Medellin.
[8] Vajrabhaya, T., 1977. In "Orchid Biology 1" (ed. by Arditti, J.), p. 179-201, Cornell Univ Press, New York.
[9] Tanaka, M., Muraguchi, H., Goi M., 1987. Abstr. J. Japan Soc. Hort. Sci. Autumn Meet: 560-561.
[10] Tokuhara, K., 1992. In "Proc. Nagoya International Orchid Show '92" (eds. by Ichihashi, S., Nagata, H.), p. 317-319, NIOS '92, Kariya.
[11] Hodgson, M., Paine, R., Anderson, N., 1991. In "A guide to orchids of the world", p. 6, Harper Collins Publishers, North Ridge.
[12] Van der Pijl, L., Dodson, C. H., 1966. In " Orchid flowers, their pollination and evolution", p. 1-6, University of Miami Press, Florida.
[13] Jain, S. M., 1993. Scientia Hort., **54**: 221-231.
[14] Khalid, N., Davey, M. R., Power, J. B., 1989. Scientia Hort., **38**: 287-294.
[15] Ezura, H., Amagai, H., Yoshida, K., Oosawa, K., 1992. Plant Science, **85**: 209-213.
[16] Vuylsteke, D., Swenssen, R., Wilson, G. S., De Langhe, E., 1988. Scientia Hort., **36**: 79-88.
[17] Maguire, A., 1993. Awards Quarterly, **24**: 29, 119, 271, Amer. Orchid Soc, Florida.
[18] Maguire, A., 1994. Awards Quarterly, **25**: 34, Amer. Orchid Soc, Florida.
[19] Bowman, J. L., Smith, D. R., Meyerowitz, E. M., 1991. Development, **112**: 1-20.
[20] Coen, E. S., Meyerowitz, E. M., 1991. Nature, **353** : 31-37.
[21] Griesbach, R. J., 1995. Amer. Orchid Soc. Bull., **64**: 364-370.