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High Frequency of Polyploidization in Regenerated Plants of *Kalanchoe blossfeldiana* Cultivar 'Tetra Vulcan'

Ryutaro AIDA* and Michio SHIBATA

National Institute of Floricultural Science, National Agricultural Research Organization, Ano, Mie 514-2392, Japan
**Corresponding author* E-mail address: ryu@affrc.go.jp

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Abstract

We used flow cytometry to analyze chromosomal changes in regenerants of the *Kalanchoe blossfeldiana* cultivar 'Tetra Vulcan' (4x). About 80% of regenerants showed increased ploidy levels. Twenty-four (20.7%), 87 (75.0%), 1 (0.9%), and 4 (3.4%) regenerants had ploidy levels of 4x, 8x, 12x, and 16x, respectively. 4x and 8x regenerants grew normally and similarly to wild-type plants, but 12x and 16x plants showed remarkable delays in growth. Plant height and leaf size among the 4x and 8x plants were the same as in wild-type plants, but those of the 12x and 16x plants were dramatically reduced. Leaves of plants with ploidy levels of 8x or more were thicker than those of 4x plants, and this character increased with increasing ploidy level. Our results confirmed that regeneration from leaf segments is an efficient method of polyploidization in *Kalanchoe*.

Key words: flow cytometry, *Kalanchoe blossfeldiana*, polyploidization, regeneration, tissue culture

Introduction

Chromosomal changes have been observed in many plant species among plants regenerated through tissue culture. The most common chromosomal changes are increases in ploidy levels, by chromosomal doubling (Skirvin, 1978). Polyploidization of regenerants has been reported in many species, including ornamental plants such as *Eustoma grandiflorum* (Lindsay *et al.*, 1994), *Saintpaulia ionantha* (Winkelmann and Grunewaldt, 1995), and *Petunia hybrida* (Oh *et al.*, 1995).

The genus *Kalanchoe* (Crassulaceae) comprises 125 species (Huxley *et al.*, 1992), and *K. blossfeldiana* (2n = 34) is the most important species for ornamental use. The *K. blossfeldiana* cultivar 'Vulcan' has 2n = 2x = 34 chromosomes (van Voorst and Arends, 1982), and the cultivar chosen for our study, 'Tetra Vulcan', is its tetraploid version (2n = 4x = 68).

Schneider-Moldrickx and Horn (1985) reported that callus-derived *Kalanchoe* regenerants showed a high percentage of changes, such as dwarf type (24%). Schwaiger and Horn (1988) reported that 23% of *Kalanchoe* plants regenerated from leaves showed phenotypic changes; the typical characters of the regenerants were dwarf with thick, round leaves and large, round flowers. They suggested that the changes were caused by polyploidization, which occurs through cell differentiation, but they did not examine the ploidy level of the regenerants.

We developed an *Agrobacterium*-mediated transformation system of *K. blossfeldiana* with cultivar 'Tetra Vulcan', and we analyzed the expression pattern of the β-glucuronidase gene in transformants (Aida and Shibata, 1996, 1998). Some of the *Kalanchoe* transformants showed phenotypic changes such as dwarf. We seemed that the changes were caused by polyploidization occurred through tissue culture. Flow cytometry is a convenient method for estimating the DNA amount in a nucleus (Bennett *et al.*, 2000). In this study, we confirm the existence of high frequency polyploidization among these transgenic *Kalanchoe* plants regenerated from leaf segments by flow cytometry. We also demonstrated high frequency polyploidization among regenerants without the transformation procedure. These plants had altered morphological characters.

Materials and Methods

Regenerated Kalanchoe plants

We used regenerated plants of the *K. blossfeldiana* cultivar 'Tetra Vulcan', derived from leaf segments, obtained in former 4 experiments that

were conducted to introduce foreign genes (Aida and Shibata, 1996, 1998).

In this study, in a fifth experiment, we also obtained regenerants without the transformation procedure, as follows: 1) Leaves of plants grown *in vitro* were cut into 5-mm squares and cultured on MS (Murashige and Skoog, 1962) solid medium (0.2% gellan gum) containing 0.5 mg l^{-1} benzyladenine and 2.0 mg l^{-1} indoleacetic acid in a 16-h photoperiod regime under fluorescent light (about 5000 lux) at 25(C for about 2 months; 2) A single regenerated shoot was excised from each explant and planted on half-strength minerals of MS medium. This regeneration protocol is almost same with that of the transformation experiments.

Cuttings of 'Tetra Vulcan' and some of the regenerants were potted in soil and grown in a greenhouse. Plant height and size of mature leaves were measured about 6 months after potting.

Flow cytometric analysis of nuclear DNA content

For the isolation of nuclei, pieces of leaf about 5 mm square were chopped up with a razor blade in a few drops of buffer A of a Partec High Resolution Staining Kit for Plant DNA Analysis (Partec GmbH, Germany). After we had added 0.2 ml of buffer A, the suspension with nuclei was filtered through a 30 - μm mesh filter. About 1.5 ml of buffer B from the kit was added, and the intensity of fluorescence of the nuclei was immediately measured with a Partec PA flow cytometer. The fluorescence gain was modified to make the first main peak (4x nuclei) appear at the 50 point (see **Fig. 1**) in wild-type 'Tetra Vulcan'.

Results and Discussion

Flow cytometric analysis of nuclear DNA content

The patterns of the flow cytometry histograms could be classified into 4 types (**Fig. 1**). The peak that appeared near zero resulted from the presence of debris of nuclear DNA. Wild-type 'Tetra Vulcan' plants (4x) showed a type A pattern, in which the point of the first main peak was at about 50. The first peaks were located at 100, 150, and 200 for patterns B, C, and D, respectively. We did not count the actual chromosome numbers, but patterns B, C, and D were presumed to represent 8x, 12x, and 16x

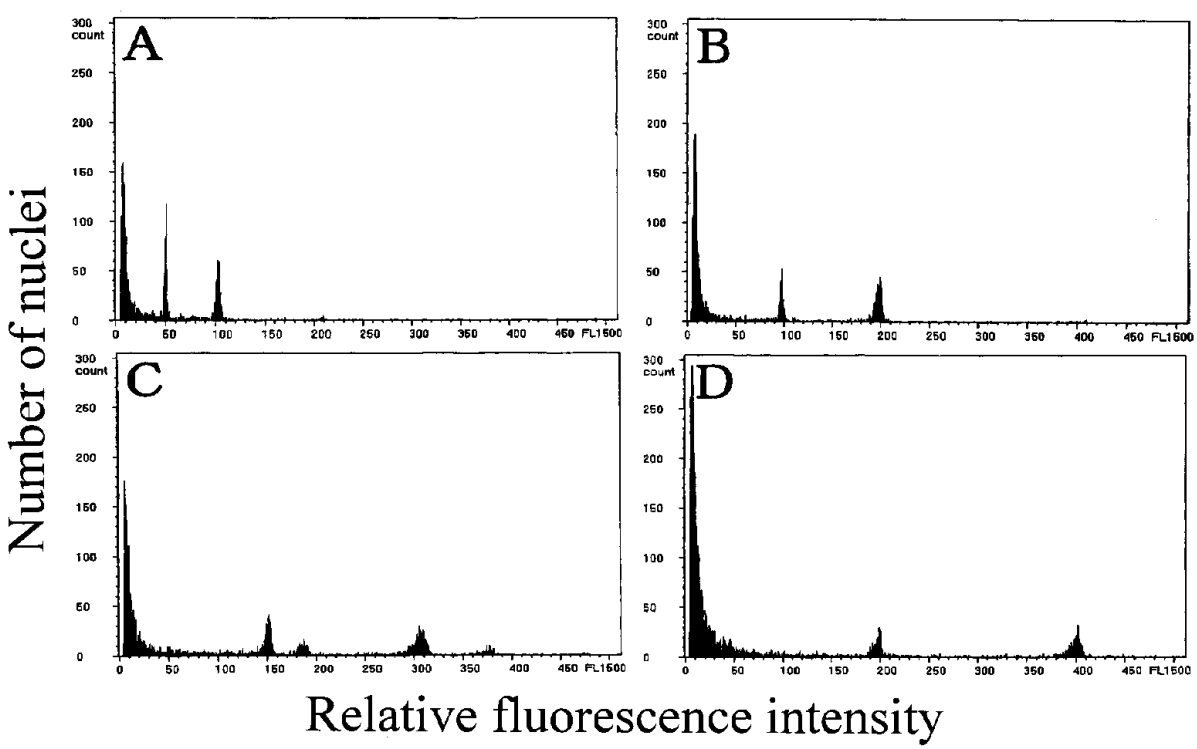

Fig. 1 Flow cytometric analysis among *Kalanchoe* regenerants.
Patterns could be classified into 4 types. The peaks that appeared near zero resulted from debris of nuclear DNA. Wild-type 'Tetra Vulcan' plants (4x) showed pattern A, in which the point of the first main peak was about 50. The first peaks were located at 100, 150, and 200 for patterns B, C, and D, respectively. Patterns B, C, and D represented 8x, 12x, and 16x, respectively. The second peak, located at about double the distance from the first peak, might be ascribed to the G2 period in the cell cycle. Pattern C also showed disorder peaks located at about 190 and 380 that were lower than the main peaks. These disorder peaks could signify chromosomal chimeric structures.

plants, respectively, because the first peaks was located in size order among the 4 types and the intensity of fluorescence should be in proportion to DNA amount. The second peak was located at about double the distance from the first peak in each pattern, and this second peak could be ascribed to the G2 period in the cell cycle.

Pattern C also showed disorder peaks located at about 190 and 380 that were lower than the main peaks. These disorder peaks might result from the presence of chromosomal chimeric structures, as reported in *Hosta* cultivars (Zonneveld and van Iren, 2000).

Table 1. Variation in ploidy level among regenerated *Kalanchoe* plants

Experiment no.[1)]	Ploidy level[2)] (number of plants (%))			
	4x	8x	12x	16x
1	1 (3.4)	26 (89.7)	0 (0.0)	2 (6.9)
2	10 (35.7)	17 (60.7)	1 (3.6)	0 (0.0)
3	3 (20.0)	12 (80.0)	0 (0.0)	0 (0.0)
4	5 (35.7)	9 (64.3)	0 (0.0)	0 (0.0)
5	5 (16.7)	23 (76.7)	0 (0.0)	2 (6.7)
Total	24 (20.7)	87 (75.0)	1 (0.9)	4 (3.4)

[1)]The regenerated plants were obtained in 5 independent experiments. Experiments 1 to 4 were conducted to introduce foreign genes with the vectors pIG121Hm, pBE2113G, pBI121 (Aida and Shibata, 1996), and pTRA415 (Aida and Shibata, 1998), respectively. Experiment 5 was conducted to obtain regenerants without the transformation procedure.

[2)]Ploidy levels were defined by flow cytometric analysis (see **Fig. 1**).

Table 1 shows the variations in ploidy level among regenerated plants in each of the 5 experiments. Experiments 1 to 4 were conducted to introduce foreign genes. Experiment 5 was conducted to obtain regenerants without the transformation procedure. More than half of the regenerants (64% to 97%) showed greater ploidy levels than that of the wild-type plant in each experiment. Regenerants showed polyploidization regardless of the transformation procedure, indicating that the regeneration step was enough and a transformation procedure was not required for polyploidization in *Kalanchoe*. In total, 24 (20.7%), 87 (75.0%), 1 (0.9%) and 4 (3.4%) of regenerants were determined as having 4x, 8x, 12x, and 16x ploidy levels, respectively. This result showed that high frequency chromosome doubling occurred through the regeneration step in the *K. blossfeldiana* cultivar 'Tetra Vulcan'.

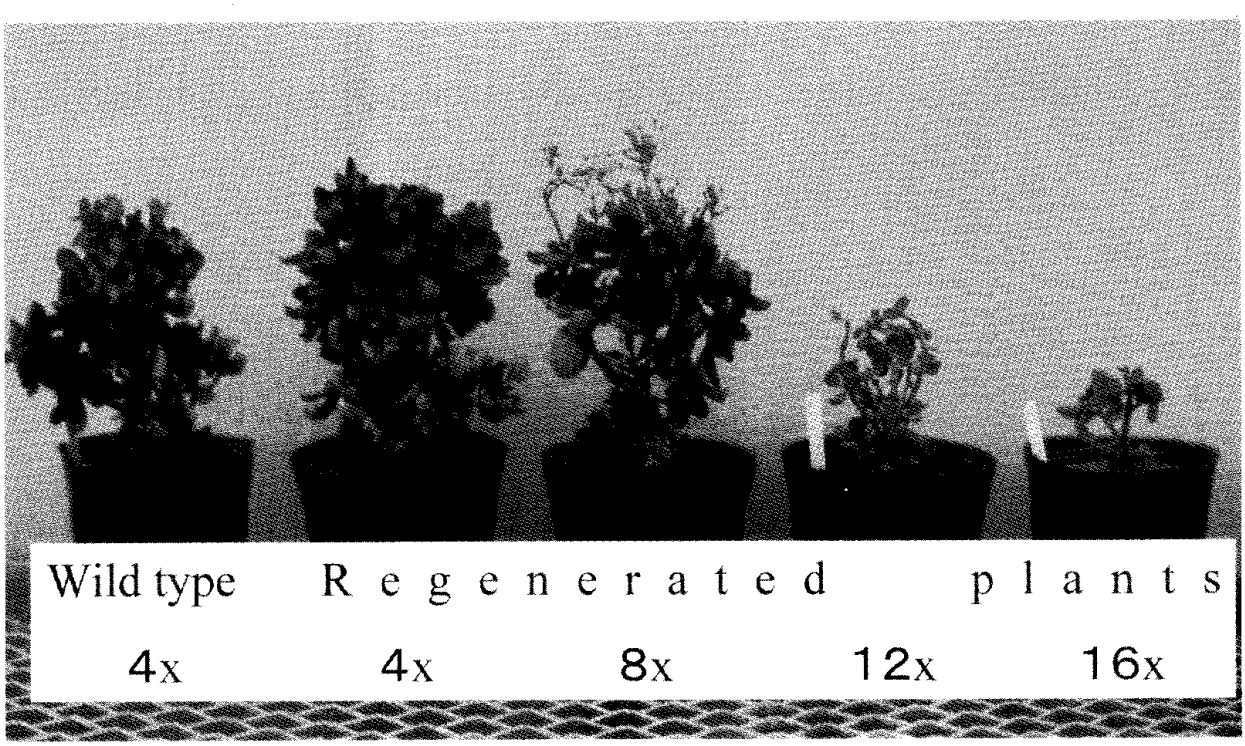

Fig. 2 Growing *Kalanchoe* regenerants with different ploidy levels.
4x and 8x regenerants grew normally at almost the same rate as wild-type plants, but 12x and 16x plants showed remarkably delayed growth. Mature 4x and 8x plants were similar in height to wild-type plants at about 180 mm. 12x plants were about 120 mm high, and the 16x plants were only 70 mm.

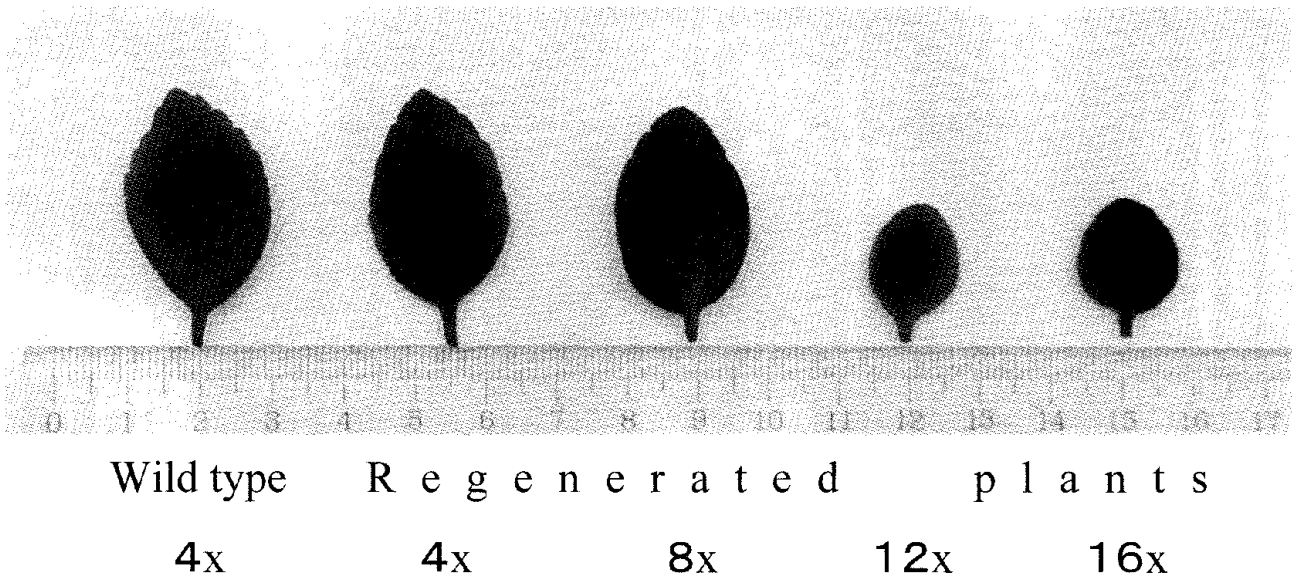

Fig. 3 Phenotypic differences in mature leaves of regenerants with different ploidy levels. Leaves of 4x and 8x plants were about the same size as those of the wild type, but leaves of 12x and 16x plants were dramatically smaller. The leaves of plants with ploidy levels of 8x or more were thicker than those of the 4x plants, and thickness increased with ploidy level.

Phenotypic changes with ploidy level

Regenerants with 4x and 8x ploidy levels grew normally and in almost the same manner as the wild-type plants. Regenerants with 12x and 16x ploidy showed remarkably delayed growth (**Fig. 2**). Plant height in mature 4x and 8x plants was about 180 mm – the same as in the wild type. The 12x plants were about 120 mm high and the 16x plants were only 70 mm high. In other studies of *Kalanchoe*, 24% of callus-derived regenerants showed a dwarf phenotype (Schneider-Moldrickx and Horn, 1985) and 23% of leaf-derived regenerants also showed dwarf changes, with thick, round leaves and large, round flowers (Schwaiger and Horn, 1988). We also observed dwarf change in the 12x and 16x plants, but the 8x plants had almost the same form as the 4x regenerants and the wild-type plants (**Fig. 2**). **Fig. 3** shows the phenotypic changes in mature leaves with increasing ploidy level. Leaf size in the 4x and 8x plants (length 29 to 32 mm / breadth 18 to 20 mm) was almost the same as in the wild type, but that of the 12x and 16x plants (length 16 to 17 mm / breadth 12 to 14 mm) was dramatically reduced (significant at 1% level). The thickness of the leaves was 0.8 to 0.9 mm in wild-type and 4x plants, 0.9 to 1.0 mm in 8x plants, and 1.2 to 1.8 mm in 12x and 16x plants. Thus plants with ploidy levels of 8x or more had thicker leaves than 4x plants, and thickness increased with ploidy level. Leaves of the 12x and 16x plants were rounder and had shorter sinuses than leaves of the 4x/8x plants. Flower shape in the 4x and 8x plants appeared almost the same as in the wild type, and the 12x and 16x plants did not flower.

These results suggested that the phenotypic change in *Kalanchoe* regenerants were caused by polyploidization. Comparisons between changes of cell conditions such as size or number and the phenotypic change among the *Kalanchoe* regenerants would serve to clarify the causes of such phenotypic variations.

In general, the most vigorous plants are obtained with 4x level, and plants with higher ploidy levels are far less vigorous than the 4x (Sharp, 1943). We also demonstrated that the most vigorous *Kalanchoe* plants seemed to be obtained at 4x level, and 12x and 16x plants showed less vigor than the 4x ones.

High frequency polyploidization in Kalanchoe

Chromosomal stability throughout the steps of regeneration is dependent on many factors, such as plant species, culture period, culture media, and regeneration pathway (Skirvin, 1978). The source of the explant also affects chromosomal stability. A high level (68%) of chromosomal variation was observed among regenerants from the basal end of tomato hypocotyl pieces, but only 15% of apical-end-derived regenerants showed chromosomal changes (Asakura *et al.*, 1995). In contrast, no polyploid regenerants were derived from hypocotyls in *Eucalyptus globulus* (Azmi *et al.*, 1997). Jacobs

and Yoder (1989) reported a polyploidy rate of 22% among transformed tomato plants regenerated from leaf explants.

In *Kalanchoe*, 23% of leaf-derived regenerants showed dwarf changes (Schwaiger and Horn, 1988), which might have been caused by an increase in ploidy levels, but the ploidy levels of the regenerants were not clarified. In contrast, in our study 79% of the *Kalanchoe* regenerants derived from leaf explants had increased ploidy levels. Only about 4% of regenerants (12x and 16x) showed a dwarf phenotype, and the 8x plants had almost the same shape as the wild-type plants (4x). We used a different cultivar and modified regeneration steps, however, it is possible that some regenerants with slightly increased ploidy levels were regarded as phenotypically normal in the above-mentioned study.

Seventy-nine per cent of the *Kalanchoe* regenerants had increased ploidy levels - a relatively high rate of polyploidization. In *Kalanchoe* - or at least in the cultivar 'Tetra Vulcan' - it might be easy to cause polyploidization during the steps in the regeneration process. Hybrid plants of *K. blossfeldiana* and *K. flammea* (both species have 2n =34) generally have 2n = 4x = 68 chromosomes instead of 2n = 2x = 34. Although the mechanism of tetraploidization in the hybrid is still unknown, van Voorst and Arends (1982) hypothesized that unreduced (2n) gamete production in the hybrid may lead to the production of tetraploid progeny after cross- or self-fertilization. This phenomenon also suggests that *Kalanchoe* possesses chromosomal instability.

Conclusion

Chromosomal doubling has been widely used as a breeding method, especially in ornamental plants. Polyploidization to a proper degree produces larger and more heavily textured flowers, and more vigorous plants with thicker leaves. In *Kalanchoe*, most modern cultivars have higher ploidy levels than the wild species (van Voorst and Arends, 1982). In hybrids of *K. blossfeldiana* and *K. flammea* it seems to be easy to cause polyploidization through fertilization steps. Our results confirmed that regeneration from leaf segments is an efficient method of polyploidization in *Kalanchoe* without fertilization. This method could be used for breeding in *Kalanchoe*. We also point out that careful attention should be paid to chromosomal changes in the transfer of foreign genes in *Kalanchoe*. The phenotypic changes that occur after transformation may be affected by chromosomal changes as well as the effect of transgenes.

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References

Aida, R., Shibata, M., 1996. Transformation of *Kalanchoe blossfeldiana* mediated by *Agrobacterium tumefaciens* and transgene silencing. Plant Sci., **121**: 175-185.

Aida, R., Shibata, M., 1998. Constitutive expression of β-glucuronidase gene fused with stress-inducible promoter of the pathogenesis-related 1a protein gene of tobacco in transgenic *Kalanchoe blossfeldiana*. Acta Hort., **454**: 373-376.

Asakura, N., Misoo, S., Kamijima, O., Sawano, M., 1995. High frequency regeneration of diploids from apical end of cultured hypocotyl tissue in tomato. Breed. Sci., **45**: 455-459.

Azmi, A., Noin, M., Lande, P., Prouteau, M., Boudet, A. M., Chriqui, D., 1997. High frequency plant regeneration from *Eucalyptus globulus* Labill. hypocotyls: ontogenesis and ploidy level of the regenerants. Plant Cell, Tissue Organ Cult., **51**: 9-16.

Bennett, M. D., Bhandol, P., Leitch, I. J., 2000. Nuclear DNA amounts in angiosperms and their modern uses - 807 new estimates. Ann. Bot., **86**: 859-909.

Huxley, A. J., Griffiths, M., Levy, M., 1992. The New Royal Horticultural Society Dictionary of Gardening, pp. 728-731. The Macmillan Press Limited, London and Basingstoke.

Jacobs, J. P., Yoder, J. I., 1989. Ploidy levels in transgenic tomato plants determined by chloroplast number. Plant Cell Rep., **7**: 662-664.

Lindsay, G. C., Hopping, M. E., O'Brien, I. E. W., 1994. Detection of protoplast-derived DNA tetraploid lisianthus (*Eustoma grandiflorum*) plants by leaf and flower characteristics and by flow cytometry. Plant Cell, Tissue Organ Cult., **38**: 53-55.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15**: 473-497.

Oh, M.-H., Lee, H. S., Song, J. Y., Choi, D.-W., Kwon, Y. M., Lee, J. S., Kim, S.-G., 1995. Origin of tetraploidization in protoplast cultures of petunia (*Petunia hybrida*). J. Heredity, **86**: 461-466

Schneider-Moldrickx, R., Horn, W., 1985. *In vitro* propagation of *Kalanchoe blossfeldiana* hybrids II. Callus and suspension cultures. Gartenbauwissenschaft, **50**: 9-13

Schwaiger, G., Horn, W., 1988. Somaclonal variations in micropropagated *Kalanchoe* hybrids. Acta Hort., **226**: 695-698

Sharp, L. W., 1943. Fundamentals of cytology. McGraw-Hill, New York.

Skirvin, R. M., 1978. Natural and induced variation in tissue culture. Euphytica, **27**: 241-266

van Voorst, A., Arends, J. C., 1982. The origin and chromosome numbers of cultivars of *Kalanchoe blossfeldiana* Von Poelln.: their history and evolution.

Euphytica, **31**: 573–584

Winkelmann, T., Grunewaldt, J., 1995. Analysis of protoplast-derived plants of *Saintpaulia ionantha* H. Wendl. Plant Breed., **114**: 346–350

Zonneveld, B. J. M., van Iren, F., 2000. Flow cytometric analysis of DNA content in *Hosta* reveals ploidy chimeras. Euphytica, **111**: 105–110