Observation of High-frequency Occurrence of Chimeral Adventitious Shoots in Tissue Culture from the Chimeral Tissues of *Pelargonium zonale*

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Abstract. In *Pelargonium*, the plastid mutation in three independent cell layers L1, L2, and L3, can produce plastid chimeras with visible shoot colour difference such as GWG (green-white-green) and GGW (green-green-white). Chimera can be used to trace the relationship between the cell layers of different genotypes during shoot development and the effect of the mutated genes on shoot development. In this study, we have obtained different adventitious shoots with GGG, GWG, GGW, and WWW combinations of cell layers through tissue culture of petioles and internodes from GGW and GWG chimeras of *Pelargonium zonale* 'Mrs Pollock'. Much higher percentage (14.9%) of chimeral adventitious shoots was obtained from GGW tissues than from GWG tissues (4.2%). Of the 10.8% chimeral adventitious shoots regenerated in this experiment, 8.6% are different from the original type of explants. This result indicated that cells at least in both L2 and L3 of the explants were involved in the regeneration of the adventitious shoots. The number of shoot types regenerated is likely dependent on the number and the type of cells that were in direct contact with the culture medium. It is suggested that the mixed cells can be used to produce the chimera by tissue culture. Three possible ways to form the chimeras *in vitro* culture were discussed. Chemical names used: TDZ = 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (Thidiazuron); IAA = Indole-3-acetic acid; PVP = polyvinylpyrrolidone.

Pelargonium is among the most popular ornamental plants over the world. The meristem in shoot apex is consisted of three independent cell layers (Baur, 1909). The cells in the two outer layers (L1 and L2) of the meristem divide anticlinally (i.e. the new cell walls are perpendicular to the meristem surface), resulting in the daughter cells remaining in the same layer. This ensures that the cell lineages of L1 and L2 are kept separate from each other. The cells in inner layer (L3) divide periclinally in any plane; occasionally divisions can result in daughter cells invading a different layer (Carpenter and Coen, 1995). The L1 gives rise to the epidermis, the L2 gives rise to the subepidermal mesophyll and the germ cells of the reproductive organs, and the L3 forms most of the internal and vascular tissues of the plant (Hantke et al., 1995).

Leaves always arise in association with a shoot meristem. The leaf epidermis is derived solely from the L1. The L2 gives rise to first palisade parenchyma and the under layer of the spongy parenchyma on the leaf margins. The L3 gives rise to the upper and middle layers of the spongy parenchyma, but makes no contribution to the blade margins (Burk et al., 1964).

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Mutations often occur in cells in one or two but not all three layers in shoot apex, resulting in chimera (Baur, 1909). A plant chimera is a specific type containing at least two different genotypes in a shoot apical meristem, where they continue to give rise to the cells that form the body of the plant. Chimeras due to genotypical difference in layers are called periclinal chimeras, while those from sector mutation are called sectorial chimeras. It was called mericlinal chimeras, when a fraction of one or more cell layers (but not the entire layer) of a shoot apical meristem is genetically distinct from the remainder of the layer. Periclinal chimeras are the most stable form, and can be multiplied by vegetative propagation to produce distinctive and valuable plant phenotypes for horticulturalists. Two different genotypes in the three apical layers can produce 2³ different periclinal arrangements with six periclinal chimeras.

The plastid mutation in three independent cell layers L1, L2, and L3, can produce plastid chimeras with visible color difference. The plastid chimera contains the wild green plastids and mutated white plastids, such as GWG (green-white-green) and GGW (green-green-white). GWG type was a sandwiched form with white subepidermal layer between epidermis and the inner layer. GGW type had a white inner layer with green epidermis and green subepidermal tissue. In the mutated white appearance, the tissue also produces a range of visible color changes. The change of the plastid genotype is easily detectable without

expensive apparatus. So it is very useful for the observation of signal transition between different genotypes. In our greenhouse, the plastid mutant chimeras in types of GWG, GGW, and GWW were observed in *Pelargonium zonale*.

Bergann and Bergann (1959) observed that genotypes of adventitious buds by leaf cutting of *Pelargonium zonale* 'Mrs Pollock' are identical with the cell types of inner component (L3) of a leaf. Kameya (1975) separated the components of a white-over-green *Pelargonium* leaf in a different way and regenerated *in vitro* only white shoots from white protoplast callus and green shoots from green protoplast callus, and there were no variegated ones. Cassells et al. (1980) obtained no chimeral plants from petiole derived callus culture of *Pelargonium zonale* 'Madame Salleron'. This is usually explained on the basis of that the ultimate origin of the apex of an adventitious shoot is from one (original) cell in explant / callus culture (Broertjes and Keen, 1980).

Bergann and Bergann (1959), however, observed only a few parental chimeral adventitious shoots by leaf cutting in *Pelargonium zonale* 'Madame Salleron'. Skirvin and Janick (1976) have observed 0.6% chimeral plants from tissue culture of the explants of *Pelargonium graveolens*. In this current experiment, high percentage chimeral adventitious shoots were obtained from *in vitro* culture of plastid chimeral explants of *Pelargonium zonale* 'Mrs Pollock'.

Materials and Methods

The plant used in this experiment was a plastid-mutated chimera *Pelargonium zonale* 'Mrs Pollock' with a GWG pattern that has genetically green L1, white L2, and green L3, and its variant in type GGW that has green L1, green L2 and white L3. The position and the size of the L2-derived and L3-derived tissue were variable. Some mixed cells (cells that contain both wild green plastids and the mutated white ones) were found in leaf section of variegated plants. In this experiment, 20 petioles with the leaf attached and 20 segments (about 1 cm) of internodes of the 2 typical chimeras, GGW and GWG, were used as explants for tissue culture. The explants were rinsed in distilled water and sterilised in 70% ethanol for 1 min, then shaken continuously in a sterilizing solution containing 0.25% Wofasteril E 400 (Kesla, Germany) and Tween 20 (2 drops/100 mL solution) for 5 min. The explants were then rinsed 3 times in sterile distilled water, placed upright in a 50-mL glass jar containing 10 mL liquid medium with Perlite, and incubated in dark for a week. Each glass jar contained one (petiole with the leaf attached) or two (segments of internodes) explants. The culture medium contained ½ macro elements and whole concentration of other elements of MS (Murashige and Skoog, 1962) with B5 (Gamborg et al., 1968) vitamins, 30 g·L⁻¹ sugar, 100 mg·L⁻¹ myo-inositol, and 22 µM TDZ. After induction of callus, the explants were transferred on to the surface of 20 mL solid medium containing 0.4% agar and 500 mg·L⁻¹ PVP in a 100 mL glass at 22 to 24 °C

in a culture room providing 12 h·d⁻¹ of cool white fluorescent light. The types of adventitious shoots and numbers of each type were recorded at the end of shoot induction. For the regeneration of roots, the adventitious shoots were isolated individually and transferred to a basal medium with 0.3 mg IAA/L and 50 µg·L⁻¹ Adenine instead of TDZ.

Results and Discussion

In this experiment, the different types of adventitious shoots were regenerated through the callus from explants of chimeral types *in vitro* culture (Table 1), of which most adventitious shoots were homogeneous green (GGG) and white (WWW). The white shoots died during isolation culture. This indicated that plant chimeral tissue could be dissected into their component genotypes by production of adventitious shoots. In addition, three chimeral histological combinations of the adventitious shoot and variegates were regenerated through the callus from explants of chimeral types GWG and GGW (Fig. 1). These results revealed that the various chimeras could be produced from calluses of the chimeral tissue of *Pelargonium zonale* 'Mrs Pollock'. The chimeras from chimeral explants, however, did not necessarily possess the same histogenic arrangement as the original explant, disagreeing with the investigation of *Pelargonium zonale* 'Madame Salleron' by Bergann and Bergann (1959).

Competence of the cell layers for regeneration. From the GWG explants, 85.9% green

shoots and 9.9% white shoots were observed, indicating that the white cells in L2 were involved in the production of the adventitious shoots. While the GGW explants also generated 42.1% white shoots besides 43.0% of green shoots. This suggested that the white cells in L3 participated in the production of the adventitious shoots as well. This result indicated that cells at least in both L2 and L3 of the explants were involved in the regeneration of the adventitious shoots. Furthermore, the L3 cells were the main source of adventitious shoot production.

In this study, the genotypes of the adventitious shoots were not all identical with the genotype of the cells in any one layer. The cells in at least two layers were involved in the regeneration of the adventitious shoots, but the two layers did not always went into the regenerated shoots together. Such a phenomenon was not only disagreed with the observation of the same plants 'Mrs Pollock' by Bergann and Bergann (1959), in which all adventitious shoots were regenerated from the cell in L3 by leaf cutting, but also was different from the investigation of leaf cutting by Bergann and Bergann (1959) in *Pelargonium* 'Salleron', in which the cells in all three layers together contributed to the regeneration of the adventitious shoots and only reproduced the parent chimeral types.

The reason that more adventitious shoots were derived from the cells in the L3 may be that more cells in L3 were directly contacted with the medium compared to cells in the L2 and L1 of the explants during tissue culture.

This result suggests that the competence of cells for regeneration from the explants depends on cell genotype and cellular location.

Cell origin of the chimeral shoot formation in tissue culture. Of the six possible periclinal arrangements of the chimeral genotypes with G and W markers, the two periclinal chimeras (GWG, GGW) and sectorial chimera in adventitious shoots were observed in this study by tissue culture of the 'Mrs. Pollock'. The average percentage of the chimeral shoots in this experiment is 10.8% (there were 8.6% new chimeral adventitious shoots), in which 4.2% chimeral shoots were from GWG explants and 14.9% from GGW explants (Table 1).

To explain the formation of the chimeras in *in vitro* culture, Geier (1989) suggested that the mutations during the development of the adventitious shoots could produce the chimeral shoot. Because the cell mutation often appeared during the shoot developing process, both the mutated and the wild type cells can maintain as one entity and grow together to produce a chimeral shoot that has at least two genotypes. Plastid mutation, leading to chimera formation, has been chemically induced in *Saintpaulia* (Pohlheim, 1974). In this way, a chimeral shoot was not developed from a single origin cell.

Several studies based on the investigation of chimeral plants suggested that chimeral adventitious shoots had multicellular origin of cells with different genetic makeup (Dulieu, 1967). Multicellular theory was also supported by histological investigation of Bergann and Bergann (1959) by *Pelargonium zonale* 'Madame Salleron' and by experiments of chimeral synthesis with artificially mixed calluses in *in vitro* culture (Marcotrigiano and Gouin, 1984), in which interspecific chimeral calluses of *Nicotiana tabacum* L. were used and 0.3% chimeral plants were obtained.

The results of this study were similar with the observation by Vaughn (1983), in which tissue culture of variegated leaf with periclinal chimeras gave a high percentage of variegated plantlets. In this study, some new types of chimera arose with a constitution different from the original chimeral explants, agreeing with Dulieu's experiment (Dulieu et al., 1967). This further substantiated the idea that cells from several tissue layers must be involved in apex formation. Therefore, it supports the notion that at least some chimeral adventitious shoots had a multicellular origin of cells with different genetic makeup.

The single cell origin theory precludes the formation of large numbers of multiple cell types and chimeras in adventitiously regenerated plants (Preil, 1986). However, as each plant cell contains many of plastids, it is possible to produce a mixed cell when some of these plastids were mutated while the others remained normal. Tilney-Bassett (1963) found that a chimeral shoot could be produced from a mixed cell with various genotypes if the normal and mutant plastids are similar in number and survival rate in a mixed cell. Hagemann and Scholze (1974) obtained chimeral shoots from mixed zygotes of a cross between a plastid chimeral 'Mrs. Pollock' and a green 'Trautlieb'

Fig. 1. Adventitious shoots from the chimeral tissues of *Pelargonium zonale* 'Mrs Pollock' *in vitro* culture. (A) Callus induction; (B) somatic embryo-like structure induction; (C) white shoot in WWW type; (D) chimeral shoot in GGW type; (E) chimeral shoot in sandwiched GWG type; (F) sectorial variegate.

Table 1. Regenerated shoots from the different chimeral explants *in vitro*; G = green, W = white.

Explants	Total shoots	GGG (%)	WWW (%)	GWG (%)	GGW (%)	Sectorial (%)
GWG	71	61 (85.9)	7 (9.9)	1 (1.4)	1 (1.4)	1 (1.4)
GGW	114	49 (43.0)	48 (42.1)	5 (4.4)	3 (2.6)	9 (7.9)
Total	185	110 (59.5)	55 (29.7)	6 (3.2)	4 (2.2)	10 (5.4)

in *Pelargonium zonale*. They concluded that the green and white plastids in *Pelargonium zonale* 'Mrs Pollock' had similar growth chance in practice. Seeni and Gnanam (1981) observed about 10% to 15% regenerated chimera shoots from the callus of hypocotyl and cotyledonary explants of tomato seedling heterozygous for the semidominant $\times$ anthophyll-2 mutant, but no chimeras occurred in shoots derived from the callus of homozygous plants. In addition, Pohlheim (1974) observed mixed cells in variegated leaves of variegated shoots produced by applying *N*-Nitroso-*N*-Methyurea in *Saintpaulia*. Therefore, it is possible that the plastid chimera plants could be regenerated from a mixed cell, in which some plastids are mutated and others are normal. The production of chimeras from mixed cells is similar to cell fusion by Philip (1990), in which the chimera adventitious shoots could be produced via fusion of two or three protoplasts of different genotypes. However, as a mixed cell is a single cell, the mixed cells may have a better chance to regenerate chimeras than fused cells in tissue culture. The mixed cell origin is a specific single cell origin, in which the mutant and normal plastids exist in one cell. The formation of the green, white, sectorial, mericlinal and periclinal adventitious shoots from the mixed cells as described by Tilney-Bassett (1986) depends on mass, distribution and the propagation ability of the white and the green chloroplasts in the mixed cells.

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