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Histogenetic variation in flowers of *Angelonia* Humb. et Bonpl.

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Summary

Angelonia, a genus of the family *Scrophulariaceae*, provides new flowering pot plants. The flower colour of *Angelonia* hybrids ranges from blue to violet, white and pink. Bicoloured *Angelonia* hybrids are grown in horticultural production.

Here, the reasons for bicolourness in *Angelonia* flowers are discussed. It was found that the flower colour patterns of bicoloured *Angelonia* hybrids are chimeral patterns as a consequence of histogenetic variation. This result was proven by light microscopy, double marking of tissues and ploidy analysis as well as somatic and generative segregation. Furthermore, the constitution of periclinal chimeras of bicoloured *Angelonia* hybrids was verified. Three new bicoloured *Angelonia* types including two cytochimeras resulted from colchicine treatment of axillary buds. These new types were histogenetically analysed. Thus, the first demonstration of a complete system of ploidy marked histogenetic variation in flower colour patterns is given.

Introduction

The genus *Angelonia* consists of approximately 25 species which are native to tropical Latin America, especially Brazil (BAILEY, 1949). *Angelonia* was first used in European horticulture in the early nineteenth century (BONSTEDT, 1932; BHATTACHARYYA and JORI, 1999; SCHOELLHORN, 2002; STARMANN, 2004). New species are still being discovered. For example, in 1997 a new species was found in North East Brazil in the state of Tocantins, *Angelonia alternifolia* V. C. Souza (SOUZA, 1997). So far, the background of the species of current *Angelonia* cultivars is unclear. It is assumed that these cultivars are hybrids. According to the characteristics they show it is hypothesised that *Angelonia angustifolia* Benth. and *Angelonia salicariifolia* Humb. et Bonpl. are progenitors of current *Angelonia* cultivars.

The zygomorphic flowers of *Angelonia* have a high ornamental value and form racemose clusters. *Angelonia* flowers consist of five petals fused together at their base. The anthers and stigma are located adnate to the upper part of the corolla. The flower colour of *Angelonia* ranges from blue to violet, white and pink. Malvidin is the basic colour-giving pigment in petals of blue and violet coloured flowers just like pelargonidin in petals of pink coloured flowers. The flavonols kaempferol and quercetin as well as the flavone apigenin were found in petals of coloured and white flowering *Angelonia* (PLASCHIL et al., 2004).

One cultivar has bicoloured flowers, whose petals have a blue centre and a white margin (Fig. 1, Type 1). The two colours are clearly separated from each other. Such flower colour patterns occur also in flowers of other horticultural plants (CASSELLS and MINAS, 1983; POHLHEIM and RÖSSEL, 1989; OLBRICHT and POHLHEIM, 1996; PLASCHIL, 1997; OLBRICHT et al., 2006). Most such patterns are periclinal chimeras. Flower colour pattern was analysed using self-pollination and *in vitro* methods including the double marking of the different apex layers to discover the cause of bicolourness in this cultivar. Normally, angiosperms have three apex layers (SATINA and BLAKESLEE, 1941; NAPP-ZINN, 1984, 1988). Petal tissues usually originate from the two outer apex layers (POHLHEIM and RÖSSEL, 1989; POHLHEIM et al., 1998). Chimeral bicolourness is caused by genetic differences between the layers in respect to flower colour.

Regarding *Angelonia*, the following hypotheses are proposed:

1. The outer layer (L1) is anthocyanin-defective, whereas the second layer (L2) is anthocyanin-intact.
2. The L1 forms not only the epidermal tissue, but also participates in the formation of mesophyll at the petal margin. In the centre of the petals, the mesophyll is derived from the anthocyanin-intact L2.
3. Although the epidermis is anthocyanin-defective, the epidermal cells in the centre synthesise anthocyanin under the influence of the mesophyll below derived from L2.

This phenomenon is reported in literature as *partner induction* (BERGANN, 1961, 1962). Therefore, the following layer constitution is suggested for the described bicoloured *Angelonia*:

First apex layer (L1):	white	=	anthocyanin-defective
Second apex layer (L2):	blue	=	anthocyanin-intact
Third apex layer (L3):	blue	=	anthocyanin-intact

Materials and methods

Plant Material

This study is based on *Angelonia* 'Mandiana' Bicolor, a cultivar with blue petals and a white margin, classified as type1 (Fig. 1).

Microscopy

The upper and lower epidermis of fresh petals of all types were skinned, infiltrated in 10 % KNO_3 solution to localise the anthocyanin in the cells. In addition, cross sections of fresh petals were produced by hand and treated in the same way as the epidermis and analysed by light microscopy (Jenaval, Carl Zeiss).

Colchicine treatment and ploidy analysis

Axillary buds of the bicoloured *Angelonia* were treated with 0.2 % colchicine. To see whether or not the epidermis of the plant had become polyploid, stomata lengths were measured (SCHWANITZ, 1952). In order to analyse the ploidy level of the different layers in the apex longitudinal sections through shoot apices (sometimes with flower bud primordia) were produced. In such sections the size of cells were evaluated and chromosomes and nucleoli were counted (ADANIYA and ARDIAN, 1994). Apices were dehydrated and then embedded in hydroxyethylmethacrylate (Fa. Kulzer) (PLASCHIL, 1997; OLBRICHT, 1998). The sections were stained with haematoxylin (ROMEIS, 1989).

Somatic segregation

Somatic segregation of chimeras, which results in visible changes of flower colour, can occur spontaneously by L1-reduplication (re-

placement: the inner layer is lost by duplication of the outer layer), L1-perforation (displacement: the outer layer is lost by duplication of the inner layer) or adventitious shoots from roots (BATESON, 1916; TILNEY-BASSETT, 1986). Segregated shoots were selected from the *Angelonia* types and vegetatively propagated by cuttings.
Callus induction and subsequent *in vitro* regeneration of adventitious shoots is a method to induce the segregation into all different components of chimeral plants (CASSELLS and MINAS, 1983). Leaf explants of *Angelonia* were regenerated into flowering plants using modified MS-medium enriched with phytohormones (MURASHIGE and SKOOG, 1962).

Generative segregation

Segregation may also be induced by self-pollination. Male and female gametes are usually derived from L2 (TILNEY-BASSETT, 1986). Therefore the L2-component can be individualised by self-pollination, thus, chimeral determined flower patterns are eliminated. Bicoloured types of *Angelonia* were self-pollinated.

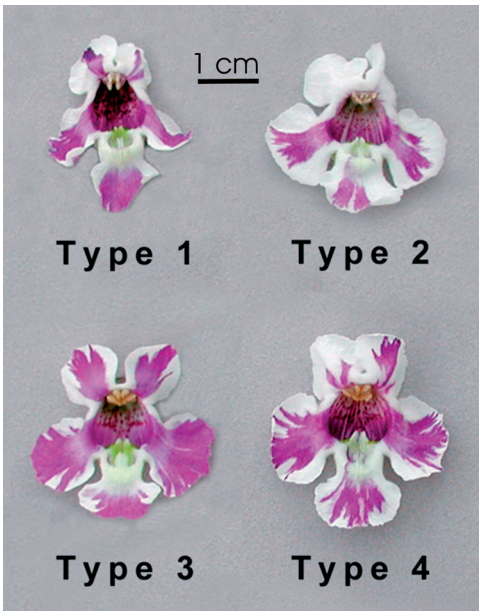

Fig. 1: Bicoloured *Angelonia* type 1 (DDD), 2 (TDD), 3 (DTT) and 4 (TTT); (D=diploid, T=tetraploid).

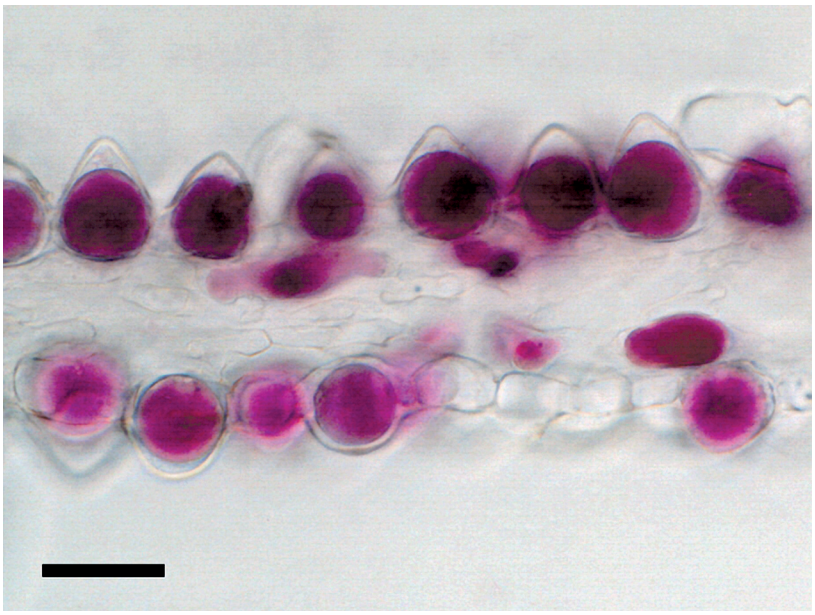

Fig. 2: Cross section of a petal of *Angelonia*: location of anthocyanin in both epidermal layers and in the mesophyll; scale bar = 50µm.

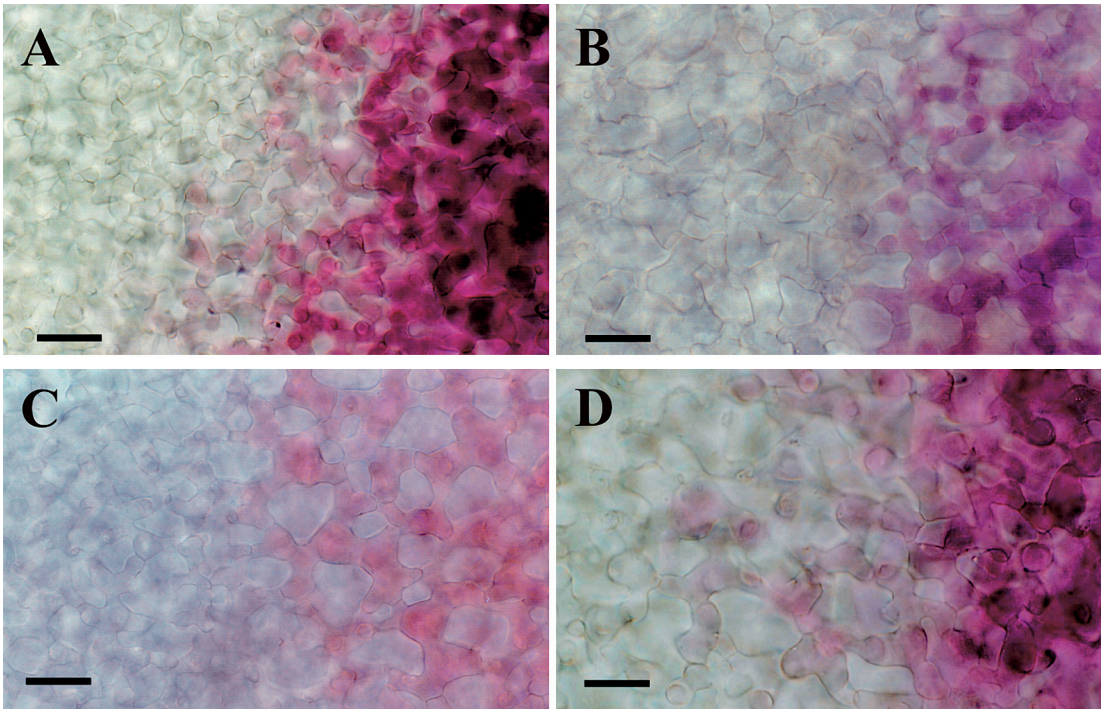

Fig. 3: Mesophyll of petals of *Angelonia*, border from white to blue, **A:** type 1 (DDD); **B:** type 2 (TDD); **C:** type 3 (DTT); **D:** type 4 (TTT); (D=diploid, T=tetraploid); scale bar = 50µm.

Results

Description of the new phenotypes

Three new phenotypic patterns resulted from colchicine treatment of *Angelonia* type 1 (Fig. 1). These types were separated from the treated plant and propagated as a clone by cuttings (types 2, 3 and 4). Flowers were characterised as follows:

Angelonia type 2: larger flower and a wider white margin of petals than type 1, blue centre

Angelonia type 3: flower in the same size as type 1, a smaller white margin than type 1, blue centre

Angelonia type 4: larger flower than type 1, a white margin and a blue centre in the same proportions as type 1

Microscopy

Fresh sections of petals of all bicoloured *Angelonia* types revealed that in the central stripes anthocyanin synthesis takes place in all cell layers (epidermis and mesophyll, Fig. 2), whereas in the margins anthocyanin was not synthesised. In type 2 and 3, differences in the size of the mesophyll cells correlate with differences in the colour of the mesophyll tissue (Fig. 3 B, C). Type 1 had uniform and small mesophyll cells, type 4 uniform and large mesophyll cells independent of the anthocyanin synthesis in the cells (Fig. 3 A, D).

Ploidy analysis

Stomata length measurement, chromosome and nucleoli counting in longitudinal sections through shoot apices allowed the characterisation of the ploidy level of all apex layers of the four different *Angelonia* types. Twenty chromosomes were counted in diploid cells (Fig. 4) as confirmed for all other available original accessions (RAGHAVAN and SRINIVASAN, 1940), 40 chromosomes were counted in polyploid cells (Fig. 5). Therefore, polyploid cells of colchicine treated types are tetraploid. The ploidy level of the four types can be described as follows:

Angelonia type 1: homohistic diploid

Angelonia type 2: tetraploid L1, diploid L2 and L3, cytochimera

Angelonia type 3: diploid L1, tetraploid L2 and L3, cytochimera

Angelonia type 4: homohistic tetraploid

Somatic and generative segregation

Angelonia type 1

Uniform white flowers or white sectors in the bicoloured flowers occurred spontaneously. The size of the white flowers was the same as in the bicoloured flowers of type 1. Most of the regenerated plants from *in vitro* callus culture had blue flowers in size of type 1. Sporadically, plants with white flowers with the same size as type 1 appeared (Fig. 6). Very rarely bicolour flowered plants developed. After self-pollination the entire progeny consisted of monochromatic flowering seedlings with different shades of blue. All of them were diploid. White or bicoloured types were not observed.

Angelonia type 2

Spontaneously white flowering sports with larger flowers than type 2 occurred. Type 2 dissociated into its different components after callus culture – mostly plants with blue flowers smaller than in type 2 and occasional plants with white flowers larger than those of type 2 (Fig. 6). Very rarely the same bicoloured type 2 regenerated. Seedlings from self-pollination segregated in the same way as type 1.

Angelonia type 3

Plants of this type formed sports with white flowers in the same size as type 3 and rarely sports with blue flowers larger flowers than type 3. Plants originating from callus culture flowered mostly blue with larger flowers than type 3 or seldom white in the same size as type 3 (Fig. 6). Plants with bicoloured flowers like type 3 very rarely occurred. All seedlings from self-pollination were tetraploid and flowered in different shades of blue.

Angelonia type 4

Sports arose with white flowers of the same size as type 4. Most regenerated plants after *in vitro* callus culture had blue flowers and some white flowering variants also occuring. All flowers had the same size as the flowers of type 4 (Fig. 6). The self-pollinated progeny flowered in different shades of blue and was tetraploid.

Tab. 1 summarises the results of the callus regeneration and the self pollination of the four types of *Angelonia.* The periclinal chimeral constitution of the four *Angelonia* types is described in Tab. 2 and in Fig. 7.

Discussion

Three general characteristics denote chimeral plants:

1. Somatic segregation: spontaneous as sports or induced by *in vitro* callus culture or adventitious shoot formation; the plants can separate into their different genetic components.
2. Chimeral patterns are not maintained through sexual reproduction (self-pollination or crossing). Generally pollen and ovules only arise from L2.

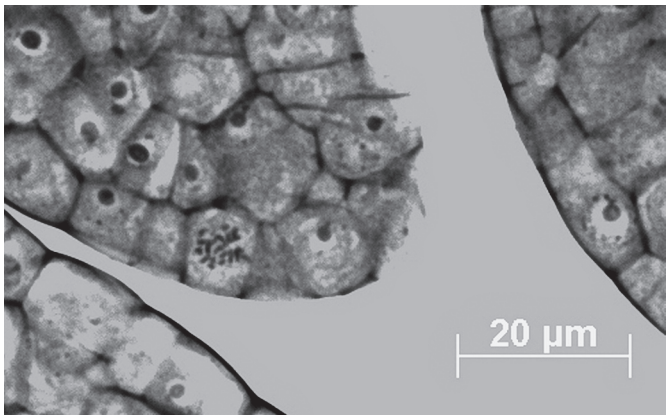

Fig. 4: Cell with diploid chromosome set, 2n = 2x = 20.

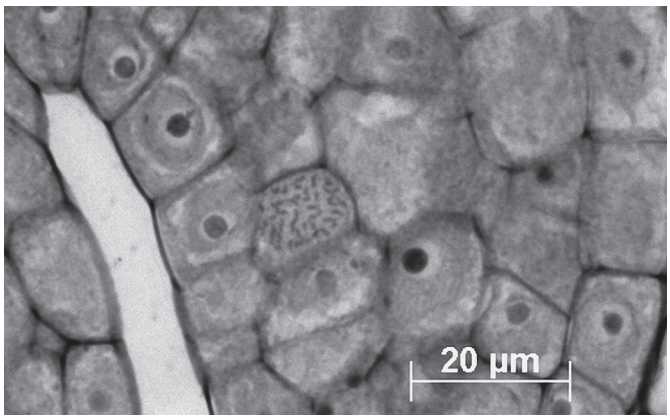

Fig. 5: Cell with tetraploid chromosome set, 2n = 4x = 40.

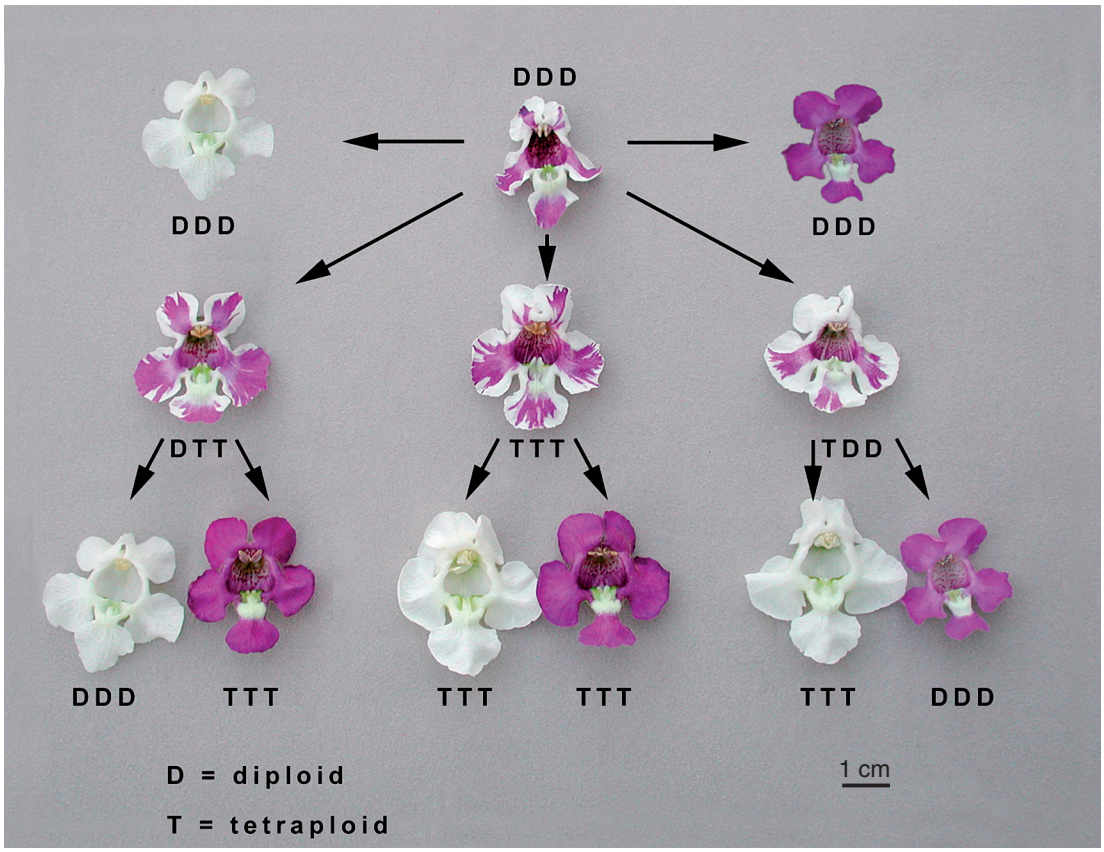

Fig. 6: Mutation pedigree and somatic segregation of the bicoloured *Angelonia* types after *in vitro* callus regeneration: type 1 (DDD), type 3 (DTT), type 4 (TTT) and type 2 (TDD); (D=diploid, T=tetraploid).

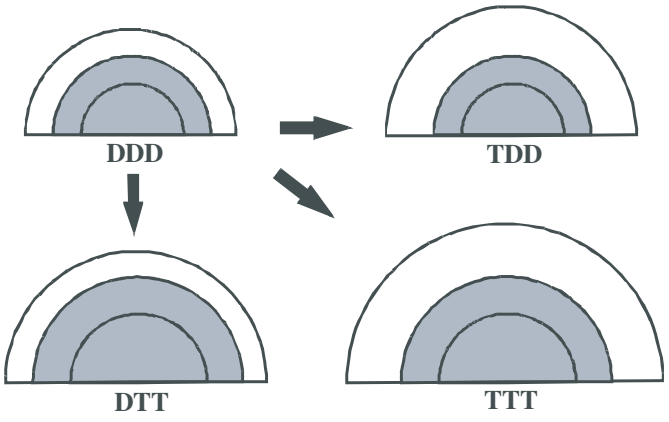

Fig. 7: Schematic graph of the apices (L1, L2, L3) of the four *Angelonia* types (the arrows symbolise the mutation steps) (D=diploid, T= tetraploid).

3. Single layers can be double-marked (e.g. by differences in anthocyanin synthesis in linkage with different ploidy levels), which makes it possible to categorise tissues to the appropriate apex layers over the whole ontogenesis of the plant (POHLHEIM and RÖSSEL, 1989; POHLHEIM and PLASCHIL, 2001).

Colchicine treatment is a common method to induce polyploid plants (SATINA and BLAKESLEE, 1940; GAO et al., 2002). By various methods of ploidy determination it was possible to classify the different ploidy levels of the three apex layers. *Angelonia* type 2 and 3 flowers are double-marked with differences in anthocyanin synthesis and ploidy level. L1 of type 3 is not only diploid but also is anthocyanin-defective. In contrast, L2 is tetraploid and produces tissues containing anthocyanin. In the mesophyll, at the border between colourless cells and cells containing anthocyanin an abrupt change of cell size is observed (ploidy level). The opposite situation is found in *Angelonia* type 2, where in the mesophyll the white cells are large (tetraploid) and the blue cells are small and diploid (Fig. 3 B, C). In the centre of the bicoloured petals, vacuoles of epidermal cells contain colour pigments although the epidermis is derived from L1, which is characterised as anthocyanin-defective. Adjacent anthocyanin-intact cells of the mesophyll (origin L2) influence the epidermal anthocyanin-defective cells (origin L1), whereas the synthesis of colour pigments is continued. This phenomenon is reported for various bicoloured flowering ornamentals (LINEBERGER and DRUCKENBROD, 1985; PLASCHIL, 1997; POHLHEIM et al., 1998; PLASCHIL et al., 2005) and known as *partner induction*. *Partner induction* was first reported by Bergann (BERGANN, 1961, 1962) for *Euphorbia pulcherrima* Willd. 'Eckes Rosa'. However, the way *partner induction* acts in leaves and bracts (in the case of *Euphorbia*) is comparable to petals, although in petals only L1 and L2 take part in their formation.

Partner induction is recognised as a cell-to-cell interaction where diffusion of substances is assumed (POHLHEIM and RÖSSEL, 1989). It has been demonstrated in experimental studies on plasmodesmata (LUCAS, 1999; LEE et al., 2005) that the plasmodesmal cytoplasmic annulus can establish a pathway for the diffusion of substances such as metabolites, ions and proteins. Thus far, *partner induction* has only been mentioned by phenotypical observation. It is still unknown which substances are transferred from cell to cell in this physiological process (BRABEC, 1965; PLASCHIL et al., 2003). However, the continuation of blocked anthocyanidin pathways in the mutated cells is possible by the transmitted substances.

Tab. 1: Description of the four different bicoloured *Angelonia*-types and their somatic and generative segregation

Type	Origin and description of the flower phenotype	Flower of sports *in vivo*	Flower variation of plants after *in vitro* callus regeneration	Segregation after self-pollination including ploidy level of seedlings (measurement of stomata length)
1	hybrid with blue petals and a white margin the same size as type 1 diploid	white, the same size as type 1	white or blue, different shades of blue,	seedlings flower in
2	appearance after colchicine treatment of type 1, larger flowers and a wider white margin of the petals than type 1, blue centre	white, larger flowers than type 2	white or blue, white flowers larger than type 2, blue flowers smaller than type 2	seedlings flower in different shades of blue, diploid
3	appearance after colchicine treatment of type 1, flowers in same size as type 1, a smaller white margin than type 1, blue centre larger than type 3	white or very seldom blue, white flowers in same size as type 3, blue flowers	white or blue, white flowers in same size as type 3, blue flowers larger than type 3	seedlings flower in different shades of blue, tetraploid
4	appearance after colchicine treatment of type 1, larger flowers than type 1, a white margin and a blue centre in the same proportion of type 1	white, the same size as type 4	white or blue both with the same size as type 4	seedlings flower in different shades of blue, tetraploid

Tab. 2: Chimeral constitution of the four bicoloured *Angelonia* types

Type	Constitution L1-L2-L3, colour	Constitution L1-L2-L3, ploidy level
1	white - blue – likely blue*	diploid – diploid – diploid
2	white - blue – likely blue*	tetraploid –diploid – diploid
3	white - blue – likely blue*	diploid – tetraploid – tetraploid
4	white - blue – likely blue*	tetraploid – tetraploid – tetraploid

* L3 was not tested.

In the bicoloured *Angelonia*, *partner induction* causes anthocyanin-defective L1-derived epidermis cells in the centre of the petals to synthesise pigments because of the influence from the anthocyanin-intact, L2-derived mesophyll cells. In contrast, in the margin there is no anthocyanin synthesis in the epidermis cells as well as in the mesophyll cells because both are derived from the first apex layer (L1) which is anthocyanin-defective. The formation of the margin of the petals by L1-derived tissue and the *partner induction* between genetically different cells within the petals leads to such bicoloured patterns (PLASCHIL, 1997).

Chimeral flower colour patterns could be used for physiological studies in cell-to-cell interactions. Both cytochimeras (type 2 and 3) are monectochimeras. There is no evidence about the genotype of the third apex layer regarding the flower colour, because petal tissues usually originate only from the two outer apex layers L1 and L2 (POHLHEIM and RÖSSEL, 1989; POHLHEIM et al., 1998; TILNEY-BASSETT, 1986). Only in cases of perforation of L2 and/ or re-duplication of L3, the third apex layer might become important for the colouration of the petals. Spontaneously *in vivo*, and more commonly after *in vitro* callus regeneration, chimeras segregate into their chimeral components. For example, the double-marked *Angelonia* type 2 segregates into white flowering, tetraploid plants which originate from L1-derived tissue and into blue flowering, diploid plants which originate from subepidermal tissue. *Angelonia* mainly regenerated *in vitro* from subepidermal tissues, seldom from epidermal tissue. Moreover, the four bicoloured types of *Angelonia* could not be maintained through sexual propagation as is usual for chimeras, and segregated into a blue flowering progeny, which represented the genotype of the L2.

The four chimeral *Angelonia* types are the first complete demonstration of chimeral flower colour patterns where flower colour mutation in one apex layer coincides with all possible ploidy constitutions in different layers. This allows not only to demonstrate the chimeral constitution of the apex but also to evaluate the contribution of single apex layers to the formation of petal tissue. Since the ratio of blue and white areas in the flower is equal for the homohistic diploid or tetraploid types (type 1 and 4), the white proportion is increased for a tetraploid L1 (type 2) and decreased in case of a diploid L1 with a tetraploid subepidermal layer (type 3). Additionally to the suggested studies in *partner induction* of bicoloured chimeral flowers, the four bicoloured types of *Angelonia* are appropriate objects for histological studies in regard to the layer competition in floral development. Stability of chimeral plants is important for horticultural use. It was observed, that *Angelonia* type 2 is the most stable type among the four described types (only 3 of 141 vegetatively propagated plants flowered white). Plants of type 1, 3 and 4 more often segregated into white flowering plants. The reasons of these differences in stability are unknown and require further investigation. Tetraploid plants which developed from vegetative and generative segregation were used to breed successfully new *Angelonia* cultivars with a compact habit and large flowers (Angelface ™ Blue, White and Sky).

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