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Interspecific crosses between *Pelargonium* × *hortorum* and *P. quinquelobatum* using embryo rescue and molecular characterization of hybrids by an endogenous *chs* probe

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Abstract

Interspecific hybridizations were performed between *Pelargonium* × *hortorum* (subgenus *Ciconium*) and a yellow-flowered species, *Pelargonium quinquelobatum* (subgenus *Eumorpha*). Hybrids, using embryo rescue, were obtained only when *P. quinquelobatum* was used as the female parent. A polymerase chain reaction (PCR) cloned *Pelargonium chs* gene fragment was used as probe to characterize hybrids by restriction fragment length polymorphism (RFLP) analysis. Chromatographic flavonoid analysis showed that hybrids could synthesize both flavonol glycosides and anthocyanin in the petals which were pale yellow to pale pink from the proximal to the distal part.

Key words: *Pelargonium* × *hortorum* — *P. quinquelobatum* — chalcone synthase — embryo rescue — flavonoids — interspecific crosses

The genus *Pelargonium* contains more than 250 species in 13 sections (van der Walt and Vorster 1988). The 'so-called' geraniums (*P. × hortorum* Bailey, *Ciconium* section), and the ivy-leaved geraniums (*P. × hederacifolium* Bailey, *Dibrachya* section) are the most economically important. Since the beginning of the nineteenth century more than 4000 varieties have been created by hybridization or mutation and it is now difficult to extend the flower colour palette. Thus, there is some interest in using newer techniques to create greater varietal diversity. Attempts have been made to produce somatic hybrids, however, plant regeneration has been reported only for parent protoplasts (Yarrow et al. 1987).

Recently, *Agrobacterium*-mediated transformation of *P. × hortorum* has been reported (Robichon et al. 1995). Modification of *Pelargonium* pigmentation by this method is, up to now, limited to the antisense-*chs* strategy (van der Krol et al. 1988). Interspecific hybridization is another field of investigation because of the broad genetic variability in the genus *Pelargonium*. The main limitation comes from the variation in chromosome numbers ($x = 8, 9, 10$ or 11 ; Darlington and Wylie 1955), and ploidy level. While intra-subgeneric crosses are generally successful, most inter-subgeneric crosses (i.e. between different sections) fail to produce seeds and hybrids from species with different chromosome numbers are an exception (Horn 1994; Yu and Horn 1984). Thus, embryo rescue has been used to recover hybrids consistently. In this respect, Kakihara et al. (1990) obtained *P. × hortorum* × *P. peltatum* hybrids; Bentvelsen et al. (1990) succeeded in recovering hybrids between *P. peltatum* and several species of the *Ciconium* section. The pre-

sent report describes for the first time the successful hybridization between a cultivar of *P. × hortorum* (*Ciconium* section) and the pale yellow-flowered wild species *P. quinquelobatum* (*Eumorpha* section). Restriction fragment length polymorphism (RFLP) analysis with an endogenous *chs* probe provided evidence of the biparental origin of the hybrids and for that purpose a chalcone-synthase gene fragment was polymerase chain reaction (PCR) cloned. The polymorphism of this multigene family (Niesbach-Klössgen et al. 1987) is observed in these two *Pelargonium* species. This result clearly indicates that molecular tools can be used at an early stage for the detection of hybrids in *Pelargonium* breeding. Flavonoid analysis of hybrids and parents gives a preliminary understanding of the yellow-coloured flower inheritance within the genus.

Materials and Methods

Plant materials: *Pelargonium × hortorum* cvs 'Happy Thought', 'High-field Promise', 'Hamelin' and 'Westphalen' (*Ciconium* section), *P. × hederacifolium* cv. 'Ville de Paris' (*Dibrachya* section) and yellow-flowered wild species *P. quinquelobatum* (*Eumorpha* section) and *P. aridum* (*Ligularia* section) were used as parents in controlled cross-pollinations. All forms were diploid ($2n = 2x = 18$). Flower buds (1 cm long) were emasculated and bagged, and pollen was applied to the stigmas 48 h later.

For embryo rescue (method adapted from Scemama and Raquin 1990) whole fruits were harvested 19 days after pollination and surface-sterilized in 70% ethanol, 15 s, calcium hypochlorite 20 g/l, 1 min, then washed twice with sterile water. Zygotic immature embryos were explanted aseptically on a sterile 0.5 MS medium (Murashige and Skoog 1962) supplemented with 20 g/l saccharose and 6 g/l agarose, pH 5.7. Cultures were maintained *in vitro* at 24°C, with a 16 h photoperiod. The plantlets were then transplanted into perlite-peat moss (1:1) and acclimatized in the greenhouse.

Molecular analysis of hybrids: Total DNA was isolated from young leaves (1-g aliquots) according to Doyle and Doyle (1990). Genomic *Pelargonium* DNA was used as template for amplification of the *chs* fragment, as described by Sambrook et al. (1989). PCR reaction was performed using Goldstar DNA polymerase (EUROGENTEC, Seraing, Belgium) and two partially degenerated primers flanking a 553-bp region and including an *EcoRI* and a *XbaI* site, respectively (Lurin and Jouanin 1995):

O15 *EcoRI*: 5'-CG GAA TTC ATG ATG TA(CT) CA(AG) CA(AG) GG(GCT) TGC TT(CT) GC-3';

O16 *XbaI*: 5'-GC TCT AGA C(AG)C AIG C(AG)C T(GCT)G ACA T(AG)T TIC C-3'.

Table 1: Results of crosses between *Pelargonium quinquelobatum* and *P. × hortorum* or *P. × hederaefolium*

Cross	Pollinations	Early developing fruits	Harvested fruits	Seeds	Plants	True hybrids
<i>P. quinquelobatum</i> × <i>P. × hortorum</i>						
'Westphalen'	5	2	2	2	0	0
'Highfield Promise'	11	6	6	5	2	2
'Hamelin'	27	7	7	8	8	0
'Happy Thought'	2	2	2	2	1	1
Total	45	17	17	17	11	3
<i>P. × hortorum</i> × <i>P. quinquelobatum</i>						
'Westphalen'	12	3	0	0	0	0
'Highfield Promise'	11	2	0	0	0	0
'Hamelin'	11	0	0	0	0	0
'Happy Thought'	6	2	0	0	0	0
<i>P. × hederaefolium</i> × <i>P. quinquelobatum</i>						
'Ville de Paris'	72	0	0	0	0	0
Total	112	7	0	0	0	0

The PCR programme was: denaturation 93 °C (5 min), 35 cycles of 93 °C (1 min), 50 °C (1 min), 72 °C (2 min), followed by a final elongation step at 72 °C (20 min). The PCR product was digested by *EcoRI* and *XbaI* and subcloned into pUC19 according to standard protocols (Sambrook et al. 1989) and partially sequenced by Euro Séquences Gènes Services (ESGS) (Montigny le Bretonneux, France).

Genomic DNA (10-µg samples) was digested overnight by *EcoRI* and *HindIII*, run on a 0.8% agarose gel at 5V/cm, denatured and transferred to a Hybond N (Amersham, Slough, UK) nylon membrane according to standard protocols (Sambrook et al. 1989). The *chs* fragment was labelled with ³²P by the random prime method. Hybridization was performed overnight at 62 °C. The membrane was then washed twice in 2 × SSPE/0.1% sodium dodecyl sulphate (SDS) at room temperature for 10 min, in 1 × SSPE 0.1% SDS at 62 °C for 15 min and in 0.1 × SSPE/0.1% SDS at 62 °C for 10 min. Finally, the blot was exposed to Kodak X-OMAT film with two intensifying screens at -80 °C for 48 h.

Flavonoid pigment extraction and chromatography: Fresh petal tissue was extracted in MeOH-HCl 0.1% (2 g/ml) at room temperature according to standard procedures (Harborne 1967). Extracts were filtered through glass wool and concentrated under vacuum. One-microlitre aliquots were loaded for the mono-dimensional high-performance thin layer chromatography (TLC) (cellulose plates, Merck, Nogent Sur Marne, France) of pigments in BAW (*n*-butanol:acetic acid:water, 4:1:5). The remaining volume was diluted in 2N HCl (2 ml) and digested for 30 min at 100 °C. Aglycones were extracted with 300–500 µl of isoamyl alcohol. 5–10 µl were then used for the two-dimensional TLC of aglycones (cellulose plates, Merck) using formic acid:HCl:water (100:2.5:37.5) and *n*-amyl alcohol:acetic acid:water (2:1:1). The chromatograms were analysed under visible light (anthocyanins) and ultraviolet light (flavonols).

Results

Hybrids

From 157 pollinations between *P. quinquelobatum* and cultivated species, fruits could be harvested only when *P. quinquelobatum* was used as the female parent (Table 1). When *P. quinquelobatum* was the male parent some fruits began to grow after pollination, but they died after 8–10 days.

Eleven plants were greenhouse-acclimatized after embryo rescue from crosses between *P. quinquelobatum* (female) and *P. × hortorum* (male). However, eight showed a female phenotype, suggesting that they were self-pollination products. *P. quinquelobatum* is highly self-fertile and there is a high risk of self-fertilization even if emasculation is performed early.

Nevertheless, three plants looked like hybrids with some intermediate characters—lobate leaves with zonation and less elongated than the female parent (Fig. 1a). The two hybrids from 'Highfield Promise' did not grow quickly and failed to flower. Conversely, the hybrid *P. quinquelobatum* × 'Happy Thought' grew normally and bloomed quickly (Fig. 1b). Floret peduncles showed an intermediate 10 cm average length which would be at least 20 cm for those of *P. quinquelobatum*. There were eight to nine flowers per floret, the petals were pale yellow at the proximal part and pale pink at the distal part (Fig. 1c).

Twelve crosses between *P. aridum* × *P. × hortorum* were performed, and fruits were obtained only when *P. aridum* was used as the female parent. Three plants were greenhouse acclimatized. However, all three plants showed a female phenotype suggesting they were products of self-pollinations (confirmed by RFLP analysis).

PCR cloning of the *chs* probe

A 553-bp PCR *chs* fragment was amplified from *P. × hederaefolium* genomic DNA and checked first by hybridization with a *Petunia chsA* probe. The fragment was subsequently cloned in the *XbaI* and *EcoRI* sites of pUC 19 and partially sequenced (single strand monodirectional sequencing). A deduced amino acid sequence of 156 residues corresponding to the 468 nucleotides analysed was then compared with the *chs* consensus sequence of Niesbach-Klößgen et al. (1987), revealing a high degree of homology (80.9%). The sequence will appear in the EMBL nucleotide sequence database under the accession number X 95801.

RFLP analysis

The characterization of hybrids was achieved by Southern blot analysis using the 553-bp *chs* fragment as probe. Figure 2 shows the *chs* hybridization pattern of the hybrid *P. quinquelobatum* × *P. × hortorum* compared with its parents. For both restriction patterns, the parents showed two major bands (that probably correspond to largely homologous sequences) and several faint bands (that probably correspond to related sequences in the *chs* multigene family). The hybrid showed in both cases bands of biparental origin. The *EcoRI* restriction pattern indicates two major bands at 5 and 10 kbp for *P. quinquelobatum* and two closed bands at ≈ 7 kbp for *P. × hortorum*.



Fig. 1: a. Leaves from *Pelargonium quinquelobatum* (left), hybrids (middle) and *P. × hortorum* (right); b. hybrid *P. quinquelobatum* × *P. × hortorum* ('Happy Thought'); c. hybrid flowers pale yellow (Y) to pale pink (P)

The four bands are present in the hybrid. The *Hind*III pattern revealed two major bands at 1.9 and 3.2 kbp for *P. quinquelobatum* and two bands at 1.8 and \approx 12 kbp for *P. × hortorum*, whereas the hybrid contains the two maternal bands and only the largest paternal band. Currently, no data are available for the heterozygosity level of the parental genotypes. However, the *Hind*III restriction pattern shows that *P. quinquelobatum* bands were both transmitted to the hybrid, in this case the maternal plant would be homozygous for both (one locus containing a *Hind*III site or two loci). Only one band of *P. × hortorum* was transmitted to the hybrid, thus the male parent might be heterozygous at this locus. In both restriction patterns the hybrids present bands from the two parents. This result clearly indicates that the plant analysed is really a hybrid between *P. quinquelobatum* and *P. × hortorum*. The other two hybrids gave the same hybridization pattern with bands of both parents

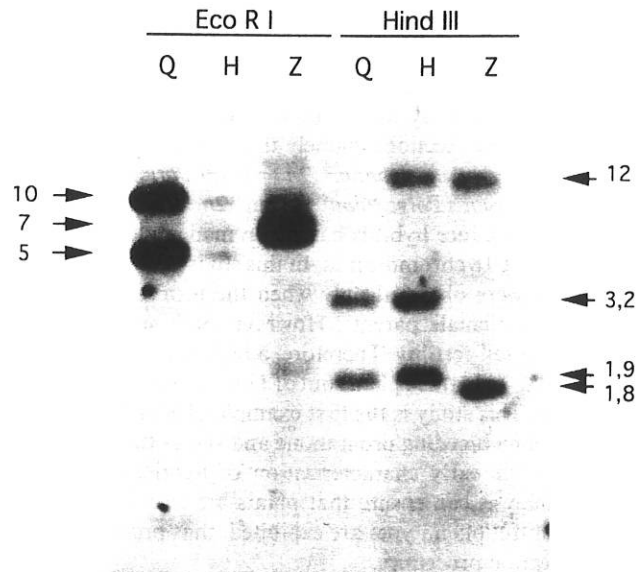


Fig. 2: Southern-blot analysis of *Pelargonium quinquelobatum* (Q) *P. × hortorum* cv. 'Happy Thought' (Z) and hybrid (H) digested by *Eco*RI and *Hind*III. Probe: *chs*. Fragment size is expressed in kbp

(data not shown). Conversely, RFLP analysis of the presumed hybrids '*P. quinquelobatum* × *P. × hortorum*' exhibiting a female phenotype and of the presumed '*P. aridum* × *P. × hortorum*' hybrids showed exactly the same hybridization pattern for hybrids and their female parent, supported the hypothesis that they were products of self-pollination (data not shown).

Flavonoid analysis

Flavonoid chromatograms showed both the presence of flavonol glycosides and low amounts of anthocyanins in the *P. quinquelobatum* × 'Happy Thought' hybrid whereas large amounts of flavonol glycosides are present only in *P. quinquelobatum* flowers and anthocyanins are only present in *P. × hortorum* flowers. Aglycone analysis by two-dimensional TLC confirmed that the hybrid contains low amounts of paeonidin and myricetin and large amounts of kaempferol and quercetin (Table 2).

Discussion

The objective of this study was to evaluate the possibility of extending the flower colour palette in the cultivated species of *Pelargonium* by interspecific hybridization. This is the first report of the production of hybrids between *P. quinquelobatum* and *P. × hortorum*. Only one case of hybridization between sections *Eumorpha* and *Dibrachya* has previously been reported (Gibby and Westfold 1986). However, the authors described a wild plant containing 26 chromosomes and of a presumed origin *P. alchemilloides* × *P. peltatum* L.

Table 2: Flavonoid analysis of parents and hybrid. M = myricetin. Q = quercetin. K = kaempferol. P = paeonidin. Pe = pelargonidin. ++ main component, + comp. present, ○ traces, - absent

	M	Q	K	P	Pe
<i>Pelargonium quinquelobatum</i>	+	++	++	-	-
<i>P. quinquelobatum</i> × <i>P. × hortorum</i>	+	++	++	+	-
<i>P. × hortorum</i> cv. 'Happy Thought'	-	-	○	++	++

Embryo rescue by *in vitro* culture has become a useful tool in interspecific hybridization, already reported by Bentvelsen et al. (1990) between the two related sections *Ciconium* and *Dibrachya*. This study shows that such a technique can be extended to other sections, namely the three pale yellow-flowered species *P. quinquelobatum* ($2n = 18$), *P. aridum* ($2n = 18$) and *P. gibbosum* (*Polyactium* section, $2n = 22$). Until now, attempts to produce hybrids have been made only by crossing species having 18 chromosomes. In this study, developing fruits and plants were obtained only when the forms with $2n = 18$ were used as female parents. However, both species show a high level of self-fertility. Therefore, a high self-pollination risk exists, and so only three plants out of 11 have been characterized as hybrids. This study is the first example of RFLP analysis in a *Pelargonium* breeding programme and shows that it could be of use for the early characterization of hybrids. Restriction pattern analysis can ensure that plants are true hybrids, even when doubtful phenotypes are exhibited, thus providing a tool in the selection procedure.

Flavonoid analysis had shown that the pale yellow colour of *P. quinquelobatum* petals results from the large amounts of flavonol glycosides, whereas the *P. × hortorum* parent contains only anthocyanins. Previous analyses have shown that flavonol glycosides are not detectable in mature flowers of *P. × hortorum* and *P. × hederifolium* cultivars — even in white ones (data not shown). This suggests that neither species, in the petal tissue, can oxidize dihydroflavonols which are transformed into anthocyanidins by the dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS) (for review, see Heller and Forkmann 1993). Lack of significant flavonol synthase (FLS) activity might occur at the regulatory level. However, the hybrid synthesizes both anthocyanins and flavonols in mature petals, indicating both FLS and DFR activity on dihydroflavonols. This result suggests that production of a yellow-coloured flower would involve crosses with a white *P. × hortorum* genotype lacking DFR activity.

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