PORTULACA GRANDIFLORA: A MODEL SYSTEM FOR THE STUDY OF THE BIOCHEMISTRY AND GENETICS OF BETALAIN SYNTHESIS

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Abstract

The segregation pattern of petal and stem colouration, resulting from crosses between betalain producing and non-producing clones of Porculaca grandlflora (Hook.), was consistent with a biosynthetic regulatory mechanism involving 3 genes. Calli derived from both betalain producing and non-producing P. grandlflora clones produced betalain pigments. The betalain contents of whole plant tissues and callus were determined.

1. . Introductiog

Betalains are alkaloid-type pigments characteristic of the order Centrospermae. In this group of plants the betalains replace anthocyanins as the major pigments. Betalain pigments i.e. betacyanins, have been used as natural food additives. Despite their importance, the biochemistry and genetics of betalain synthesis remain relatively undetermined. Portulaca grandiflora **(x-9)** is an ideal system for such a study; its flowers exhibit numerous phenotypes ranging from yellow through orange and red to violet; it has a short life cycle (ca. 3 months) and produces in excess of 300 seeds per ovary and **so is** ideal for genetic studies. In addition, P. grandiflora is readily amenable to tissue culture.

All betalain pigments contain betalamic acid as the chromophore. Depending upon the nature of the betalamic acid addition residue the betalains can be classified as either betacyanins or betaxanthins. Betacyanins contain a cyclo-DOPA (usually glycosylated) residue and exhibit a red/violet pigmentation, while the betaxanthins contain various amino acid or amine side chains and exhibit a yellow/orange pigmentation.

2. Materials and methods

2.1. Plant material

From commercially obtained seeds, two plants exhibiting two different phenotypes were selected for study: PCV (violet coloured flowers and stems) and PCB (white coloured flowers and green stems). After sterilisation with hypochloride, plants were maintained by vegetative propagation on basal 1/2 strength M&S (Murashige and Skoog, 1962) media solidified with 0.6% agar.

2.2. Derivation of tissue cultures

Callus tissue was initiated from stem explants of both the PGV and PGB phenotypes on 1/2 strength M&S media supplemented with the vitamins of Morel and Wetmore (1951). Growth regulators were included as described in the results.

2.3 Analysis of betalain pigments

Tissues were extracted, on ice, in 20 volumes of solution A (methanol : HCl 0.29; 80:20) and centrifuged at 20,000 g for 15 min at 4°C. The pellet was resuspended in a further 20 volumes of solution A, centrifuged at 20,000 g for 15 min. The pooled supernatants were evaporated to dryness under reduced pressure using a rotary evaporator. The residue was resuspended in deionised water (1 ml) and centrifuged prior to analysis by HPLC. Separation was performed using an ODS RP C-18 column (250 x 4.6 mm ID) packed with 3 μm Hypersil (Shandon). The system was operated using a stepped gradient over a period of 25 min, from 100% solution A (50 mM NaH_2PO_4 + 2.5 mM triethylamine, adjusted to pH 4.2) to 80% solution B (40% acetonitrile). Detection was performed using a Hewlett Packard 79994A HPLC ChemStation in conjunction with a Hewlett Packard 1040A HPLC Detection System.

2.4. Genetics

PGV and PGB clones were self-crossed *in vitro*, but all other crosses were performed under greenhouse conditions. Seeds were collected and dried for 1 month. Prior to germination on sterile, basal M&S media, seeds were soaked in 1 M HCl for 1 h and subsequently in water for 24 h. Ten-day-old plants were potted individually and transferred to the greenhouse for flowering.

3. Results

3.1 Tissue culture of P. *grandiflora*

3.1.1 Establishment of cultures

Callus initiated from the violet genotype was pigmented violet, while callus initiated from the white genotype was pigmented white. Upon lowering the medium 2,4-D concentration, from the maintenance level of 0.1 mg/l to 0.02 mg/l, red cell clusters appeared, unexpectedly, on the white callus, while white cell clusters appeared on the red callus. After prolonged maintenance on media containing the reduced level of 2,4-D, the two callus lines exhibited almost identical 'mosaic' phenotypes. Individual coloured cell clusters could be isolated and maintained as stable, uniform cell lines.

In a second experiment, callus tissue was initiated from stem explants of the violet P. grandiflora variety using media containing both BA (1 mg/l) and 2,4-D (0.25 mg/l). The primary callus consisted of both violet and white (non-pigmented) cell clusters. In subsequent passages callus was transferred to basal media containing no hormones either by transferring directly to zero-hormone media or by progressively reducing the medium hormone concentration to zero over a period

of 4 subcultures. Upon reducing the media hormone levels to zero, either directly or progressively, all cells exhibited the white phenotype. Restoration of auxin rich (1 mg/l NAA + 0.1 mg/l 2,4-D) medium induced production of violet pigmented cells, selection of which resulted in a uniform violet cell line.

3.1.2 Regeneration of P. grandiflora plants

P. grandiflora plants were readily regenerated from meristem tissue in the presence of 1 - 5 mg/l kinetin. Regenerants were transferred to basal media for root induction. This method facilitates the rapid vegetative propagation of specific genotypes. Attempts at inducing plantlet regeneration from secondary callus tissue proved unsuccessful.

3.2 Preparation of P. grandiflora protoplasts

Protoplasts were prepared from P. *grandiflora* leaf and petal tissues. Tissue sectors were incubated in KS medium (Muller et al., 1983) supplemented with Macerozyme RL (4 g/l), Cellulase Onozuku R-10 (6 g/l), Cellulysin Onozuku R-10 (6 g/l) and the osmolarity adjusted to 520 mOsm/Kg with glucose. After digestion (4.5 h), protoplasts were passed through a 64 μm filter, collected by flotation on a 20 % sucrose solution and subsequently washed, twice, with W06 media (Meyer and Abel, 1975) containing 0.02% tween 80. The yield of protoplasts by this technique was reduced by the high polysaccharide (mucilage) content of the starting tissue.

Protoplasts from petal tissues remained viable (>90% by the fluorescein diacetate method) and retained their vacuolar content of pigment even after 24 h incubation at 4°C. Protoplasts derived from either violet or yellow petals gave rise to only violet and yellow cell populations, respectively. Protoplasts derived from red petals, however, exhibited mixed phenotypes. The population consisted predominantly of the red phenotype, but a limited number of cells exhibited the violet or the yellow phenotype.

Within two days protoplasts derived from leaf tissue had begun cell wall regeneration and within seven days had completed the first cell division. The experiment was discontinued after 15 days at the microcallus stage of development, but it was expected that cell division in microcalli could be maintained.

3.3 The genetics of betalain biosynthesis

The pathway of betalain synthesis is shown in Fig. 1. P. *grandiflora* plants exhibiting different petal colours (phenotypes) were crossed as described in Table 2. The resultant segregation patterns indicated that a minimum of three genes, C, R and I, were involved in petal pigmentation. In our genetic model, locus C, a dominant allele, is responsible for the conversion of DOPA (dihydroxyphenylalanine) to betalamic acid, locus R for the transformation of DOPA to cyclo-DOPA (-glycosylated) and locus I (inhibitor) prevents conjugation of amino acid residues with betalamic acid. It was observed, however, that a yellow pigment was always produced in flower buds when locus C was active. This compound was identified as the condensation product of

betalamic acid and tyrosine and was named Portulacaxanthin II (Trezzini and Zryd [a], in preparation). The conjugation of R and C gene products, resulting in the production of betanin (Fig. 1), was not apparently under genetic control since no flowers containing only cyclo-DOPA and betalamic acid (results not shown) have been obtained. According to the above genetic model, the PCV (violet) clone was of the genotype CCRrII. The F1 generation of the self-crossed PGV clone, however, exhibited a segregation pattern typical of linked genes (Table 2). Plants displaying red flowers, the result of both betacyanin and betaxanthin synthesis, were of the genotype CCRRII. Plants displaying pale yellow flowers were of the genotype CCrrII. Clone PGB (white flowers) was of the genotype ccrrII. An explanation of the segregation pattern exhibited by the self-crossed PGB (white) genotype (Table 2) is given elsewhere (Trezzini and Zryd, unpublished).

3.4 Biochemical analysis of PGV and PGB tissues

The betalain and betalain precursor content of PGV and PGB tissues are given in Table 1. PCV stem tissue contained 4 betacyanins, one of which was betanin (<20 %) and the remainder were unidentified. The violet and variegated PGV callus lines, maintained on 0.1 and 0.02 mg/l 2,4-D respectively, exhibited a pigment composition similar to that of PGV stem (Table 1). The pigment compositions of variegated callus lines from both PGV and PGB were similar. The only betacyanin present in PCV petals was betanin. Betacyanins were not present in PGB petals, but high levels of DOPA and dopamine were detected. Betalain precursors were not detected in either PCV or PCB callus tissues.

4. References

Meyer, Y., Abel, W.O., 1975. Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. Planta 123:33-40.

Morel, G., Wetmore, R.H., 1951. Fern callus tissue culture. Am. J. Bot. 38:138-140.

Muller, J.F., Missionier, C., Caboche, M., 1983. Low density growth of cells derived from Nicotiana and Petunia protoplasts: influence of the source of protoplasts and comparition of the growth-promoting activity of various auxins. Plant Physiol. 57:35-41.

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

Trezzini, G.F., Zryd, J.-P., 1989a. HPLC analysis of betaxanthins from *Portulaca grandiflora*, in preparation.

Table 1 - Content of betalains and precursors in cell lines.

Cell line or tissue	Content (mg/g DW) Total cyanins	Total xanthins	Betalamic acid	Tyr	DOPA	Dopamine
PGV 0.1 2,4-D	*0.8*	0	0	*0*	*0*	*0*
PGV 0.02 2,4-D	0.2	0	0	0	0	0
PGB 0.1 2.4-D	0	0	0	0	0	0
PGB 0.02 2,4-D	0.2	0	0	0	0	0
PGV 1 NAA +0.1 2,4-D	1.0	0	0	0	0	0
PGV petals	9.0	0.7	(+)	(+)	0	0
PGB petals	0	0	0	0	*4.9*	10.2
PGV stem	0.1	0	0	0	0	0
PGB stam	0	0	0	0	0	0

Table 2 - Segregation genetics of flowers of P. grandiflora clones used for the derivation of tissue cultures. V- violet, pY- pale yellow, R- red, W- white, Y- yellow, Ym- predominantly yellow, but limited violet pigmentation also apparent.

Clone	Cross	Phenotype (parental)	Segregation	Genotype (parental)
PGV .	selfed	violet	2V:1pY:1R	CC Rr Ii
PGB	selfed	white	**97W+1Y+2Ym**	cc rr Ii
	PGV x PGB	violet x white	100% coloured	
(PGV x PCB)	selfed	coloured	3 coloured : 1W	Cc .. ..

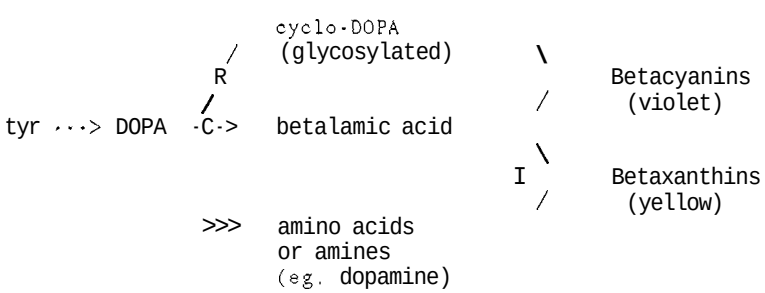

Figure 1 - The pathways of betalain biosynthesis. Locus C is responsible for the production of the chromophore batalamic acid; locus R for the formation of cyclo-DOPA and may also be involved in its glycosylation. Both betalamic acid and cyclo-DOPA are formed from tyrosine derived DOPA. Locus I inhibits betaxanthin formation by preventing the conjugation of betalamic acid with amino acids/amines.