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Selection of anthocyanin-accumulating potato (*Solanum tuberosum* L.) cell lines from calli derived from seedlings produced by gamma-irradiated seeds

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Abstract. Callus cell lines of potato (*Solanum tuberosum* L. cv. Zarevo) were obtained from seedlings germinated from gamma-irradiated seeds (200 Gy). Some of these cell lines produce red-violet pigments which were identified as acylated anthocyanins. The major anthocyanin was determined to be peonidin 3-O-[6-O-(4-O-*E*-*p*-coumaroyl-rhamnosyl)-glucoside]-5-O-glucoside ("peonanin"). Single cell-derived protoclones from non-pigmented protoplasts sometimes also gave rise to pigmented cell clusters thus indicating that the changes in the expression of the anthocyanin pathway can also occur after the stage of initial callus induction.

Key words: Solanum tuberosum, anthocyanins, gamma-irradiation, protoplasts, protoclones.

Introduction

Callus or suspension cultures of about 30 plant species are known to accumulate anthocyanins (Seitz and Hinderer 1988), but only a few of them show stable expression of this biosynthetic pathway. Among other systems, cell suspensions of *Euphorbia millii* (Yamamoto et al. 1981) and carrot isolated from different sources in several laboratories are able to produce anthocyanins in high amounts over years (Noé et al. 1980; Dougall et al. 1980; Ozeki and Komamine 1981). For potato callus (*Solanum tuberosum*), anthocyanin accumulation has already been described by Harborne and Simmonds (1962).

Besides the spontaneous production of pigments, anthocyanin biosynthesis can be stimulated by purposive induction with different effectors. Starvation in nitrogen and phosphate enhances anthocyanin accumulation (Knobloch et al. 1982). Phytohormones also influence the anthocyanin content (for review see Seitz and Hinderer 1988). Light induces anthocyanin synthesis in cell cultures of *Haplopappus gracilis* (Fritsch and Grisebach 1975), *Daucus carota* (Takeda 1988; Gleitz and Seitz 1989), *Centaurea cyanus* (Kagegawa et al. 1991), and in several other systems. In this paper, we describe the stable production of anthocyanins by potato cell lines derived from seedlings germinated from gamma-irradiated seeds. The production of coloured secondary metabolites allowed easy selection of high producing cell lines.

Materials and methods

Gamma-irradiation of seeds. Air-dried seeds (150) of *Solanum tuberosum* L. cv Zarevo (a gift from Dr. G. Petjuch, Institute of Potato Breeding, Nemeshajevo, Ukraine) were irradiated with 200 Gy (10 Gy·min^{-1}) of gamma rays (^{60}Co-source, "Issljedovatjel", USSR).

Callus induction. Irradiated and non-irradiated seeds were germinated on the 1-3-medium containing the mineral constituents of the MS-medium (Murashige and Skoog 1962), 100 mg·l^{-1} myo-inositol, 1 mg·l^{-1} each of pyridoxin, nicotinic acid, Ca-pantothenate, kinetin and 2 mg·l^{-1} thiamine, 0.01 mg·l^{-1} biotin, 3 mg·l^{-1} 2,4-D and 2.5% sucrose (w/v) solidified with 0.6% Agar. After three weeks in dim light (600-800 lux) or in the dark at 26-28°C, seedlings were wounded with a razor blade and explanted on 1-3-medium (see above) for callus formation. Red-violet cell clusters and white cell lines were isolated from primary callus tissue and subcultivated on fresh 1-3-medium.

Isolation and cloning of protoplasts. Protoplasts were isolated from callus with an enzyme mixture containing 0.5% cellulase (Onozuka R10), 0.25% Macerozyme R10, and 0.1% Driselase (Sigma Chemie, Deisenhofen, Germany) dissolved in W5-medium (Menczel et al. 1981) and cultivated as previously described (Sidorov et al. 1987). Two weeks later protoplast suspension was diluted to a cell titer ensuring the separation of single cell-derived protoclones.

Growth measurements. About 300 mg callus was plated on 6 cm Petri dishes with solidified 1-3-medium. Every 5 d the fresh weight increase was calculated as $\log\left(\frac{m_c - m_o}{m_o} * 100\right)$ where m_0 is the initial fresh weight and m_c indicates the fresh weight after the cultivation period. Each value is the mean of three independent experiments.

Extraction of pigments. Pigments were extracted from fresh tissue in 1 ml ethanol (containing 0.1% HCl) per 1 g fresh weight. The concentration of the pigments was measured photometrically at 535 nm.

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For identification of pigments, the callus was frozen in liquid N_2 and lyophilized. This material was suspended in 10 ml MAW (methanol: acetic acid: water = 50:8:42) per g dry weight, homogenised in a mortar and filtered two times through paper filters and a cotton plug. The filtrate was extracted four times with ethyl acetate, evaporated to dryness in vacuo at 35°C and redissolved in a minimal volume of MAW. The major component from two cell lines was isolated according to Gläßgen et al. (1992a).

HPLC analyses. Crude extracts and isolated fractions were analysed by RP-HPLC on a Hypersil ODS column (5 µm; 250×4.6 mm i.d.; Grom Herrenberg, Germany) with a two-step linear gradient of 10% formic acid in H_2O (solvent A) and 10% formic acid in MeOH (solvent B): during 0-15 min solvent B increasing from 5-25%, during 15-27 min from 25-28% at a flow rate of 1 ml·min^{-1}. Injection volume was 10 µl. The absorbance was detected at 530 nm and additionally at 260 nm for purity checks of isolated anthocyanin fractions.

Structure elucidation

a) Acid hydrolysis and identification of aglycones and monosaccharides. For acid hydrolysis, 2 ml of purified anthocyanin in MAW were refluxed with 2 ml of 4M HCl for 90 min at 100°C. The liberated aglycone was extracted with 2 ml isoamyl alcohol and identified by UV/VIS spectroscopy in 0.01% HCl in methanol, by ion spray MS (see below), and by TLC on cellulose with BAW (n-butanol: acetic acid: water = 4:1:5, upper phase) and forestal (HCl: acetic acid: water = 3:30:10) as solvents. Commercially available anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin: Roth, Karlsruhe, Germany) were used as standards. TLC analysis was also carried out with the organic phase after acid hydrolysis of crude extracts.

The liberated monosaccharides in the aqueous phase were converted to their respective alditol acetates and identified by capillary GC according to Gläßgen et al. (1992a).

b) NMR spectroscopy. A ^{1}H (400 MHz) NMR spectrum of the isolated major compound (5) of cell lines A 2002 and A 2003 was recorded on a Bruker AMX 400 NMR spectrometer (Karlruhe, Germany) in CD_3OD containing a trace of DCl. Chemical shifts δ are reported in ppm relative to tetramethylsilane and coupling constants J in Hz: H-4 (δ=9.05, *s*); H-6 (δ=7.06, *s*); H-8 (δ=7.13, *s*); OCH_3 (δ=4.02, *s*); H-2' (δ=8.20, *d*, J=1.7 Hz); H-5' (δ=7.39, *d*, J=8.7 Hz); H-6' (δ=8.27, *dd*, $J_{2',6'}$=1.8 Hz, $J_{5',6'}$=8.7 Hz); H-1 [5-O-β-Glc] (δ=5.17, *d*, J=7.6 Hz); H-1 [3-O-β-Glc] (δ=5.68, *d*, J=7.8 Hz); H-1 [α-Rha] (δ=4.71, *s*, unresolved coupling); H-4 [Rha] (δ=4.89, *t*, J=9.7 Hz); H-6 [Rha] (δ=0.96, *d*, J=6.2 Hz); H-2'' and H-6'' (δ=7.08, *d*, J=8.7 Hz); H-3'' and H-5'' (δ=6.81, *d*, J=8.5 Hz); H-7'' (δ=7.54, *d*, J=15.9 Hz); H-8'' (δ=6.22, *d*, J=15.9 Hz).

c) Ion Spray MS and Ion Spray MS-MS. Mass spectra were recorded on a triple-quadrupole mass spectrometer API III with a mass range of m/z 10-2400 Da equipped with an ion spray interface (Sciex, Thornhill, Ontario, Canada) (Bruins et al. 1987). The samples were dissolved in methanol/HCl or in aqueous acetic acid and introduced directly into the ion spray source at a constant flow rate of 5 µl·min^{-1} with a microliter syringe using a medicinal infusion pump (Harvard Apparatus, Southnatick MA, USA). Daughter ion scans (MS-MS) were performed by collision-induced dissociation experiments using argon as a collision gas (Gläßgen et al. 1992b).

Results and Discussion

Induction of anthocyanin accumulation by gamma-irradiation.

Seeds of higher plants exposed to lethal doses of gamma rays are able to germinate up to the two cotyledon stage, but when sown in soil they stop their development and die (Grodzynsky 1989). However, under *in vitro* conditions these "gamma seedlings" can be rescued and form plants. In the case of wounding, the seedlings proliferate calli which

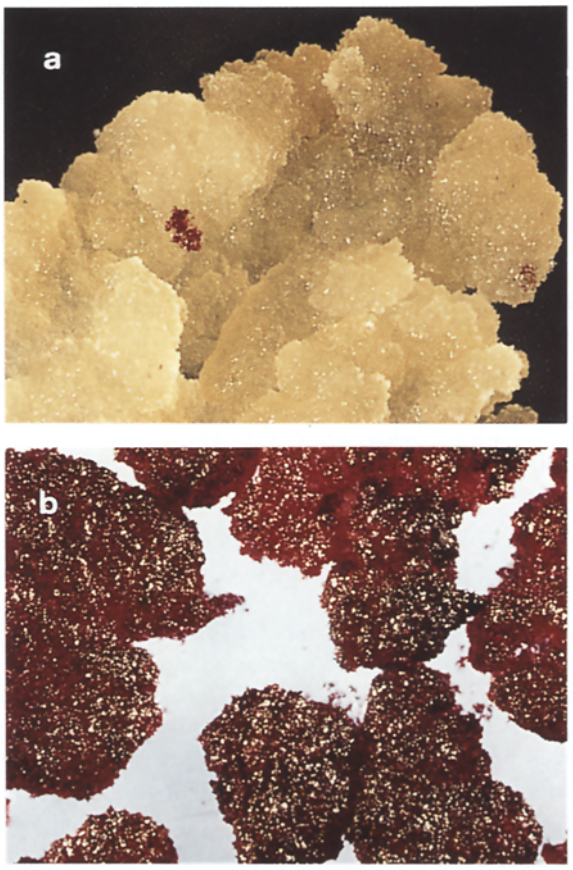

Fig. 1. Selection of anthocyanin-accumulating cell lines (A 2002) of *Solanum tuberosum* L. cv Zarevo. The calli were induced by wounding of seedlings germinated from seeds which were irradiated with gamma-rays. a) White callus with pigmented areas. Pigmented parts were selectively transferred to fresh medium (1-3). b) The stable pigmented cell line A2002 derived from the same "gamma seedling".

can be used to establish cell lines. This material can be studied with regard to changes of phenotypes on cytological and metabolic level (Zubko et al. 1990).

The potato calli emerging from the wound surface of seedlings after 2 or 3 weeks varied in colour from white to yellowish or light-green. In cell lines derived from gamma-irradiated seeds, red-violet callus material frequently appeared. Twenty calli were isolated and planted on fresh medium. Pigmented parts were transferred selectively and maintained by subculturing every 3 to 4 weeks. Three deeply pigmented cell lines derived from different "gamma-seedlings" were propagated successfully. Fig. 1 shows a pigment-free (2002) and a pigmented (A 2002) cell line, both derived from the same "gamma-seedling". The accumulation of red-violet pigment was preserved over many passages in the dark and light as well. The pigments were determined to be anthocyanins (see below).

The potato cell line described here, did not respond to high concentrations of 2,4-D (3 mg·l^{-1}), whereas in suspension-cultured carrot cells anthocyanin biosynthesis is inhibited by such concentrations (Sukuta and Komamine 1987).

Growth rates of anthocyanin-free and anthocyanin-

containing cell lines were nearly identical (Fig. 2). During the period of cultivation the fresh weight increased to six-fold in both cell lines.

Clonal propagation of cell lines.

During subcultivation, the anthocyanin-containing cell lines also produced compact, non-pigmented cell clusters on their surface. When these colourless parts were isolated, they retained their whitish colour during the following passages, but sometimes new pigmented areas were formed.

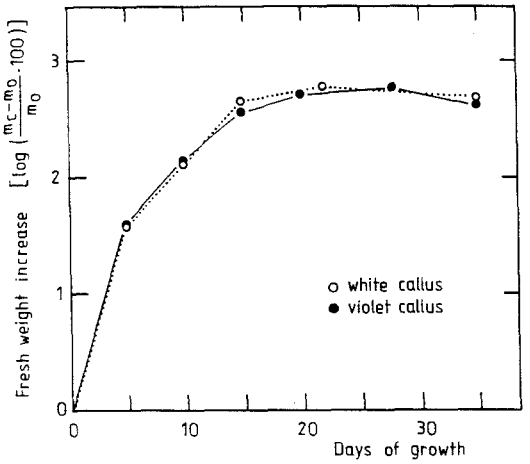

Fig. 2. Growth curves of white (2002) and violet callus (A2002) expressed as fresh weight increase (see Material and Methods).

These calli also retained their pigmentation during subcultivation. In order to reveal whether the pigmentation is irreversibly determined in each certain cell, protoplasts from colourless calli were prepared and plated to obtain single cell-derived subclones, which were then analysed for their capacity to form anthocyanins. These protoclones again segregated pigmented cell clusters which in turn retained their pigmentation during subculturing. The segregation of differently pigmented areas in the callus is therefore not related to initial heterogeneity of the material. The mechanism of this "switching" in anthocyanin production is still unknown. It must be emphasised that calli obtained from seedlings derived from non-irradiated seeds very seldom showed anthocyanin accumulation, and no pigmented subclones could be established.

Characterization of pigments.

Acidic (0.1% HCl) ethanol extracts of pigments showed maxima of absorbance at 535 nm and exhibited the typical features of anthocyanins when treated with alkali. Anthocyanin content reached values of up to 400 nmol per g fresh weight (7 μmoles per g dry weight) as estimated using an extinction coefficient of log ϵ = 4.48 (Strack and Wray 1989). After acid hydrolysis of crude extracts from all three pigmented cell lines, the major aglycones could be identified by TLC in two solvent systems. Peonidin was the major aglycone in the cell lines A 2002 and A 2003, whereas cyanidin was dominant in A 2001.

The anthocyanin composition of extracts from the different cell lines was compared by using reversed-phase HPLC (Fig. 3). Five anthocyanin peaks (**1**, **2**, **3**, **5**, **6**) were detected in varying portions in each of these cell lines. Cell line A 2001 contained an additional peak (**4**). A 2002 and A 2003 exhibited a very similar pattern with peak **5** (81% and 67% of total A_{530}, respectively) representing the major compound. Cell line A 2001 showed a somewhat different composition with peak **3** (59%) being the major constituent, followed by peak **5** (23%). The major component (**5**) of the cell lines A 2002 and A 2003 was isolated by repeated paper chromatography (Gläßgen et al. 1992a) and subjected to structure analysis.

After acid hydrolysis, the aglycone was determined to be peonidin by TLC in two solvent systems, by UV/VIS-spectroscopy (maxima at 279 and 534 nm, no spectral shift with $AlCl_3$), and by ion spray mass spectroscopy (m/z 301). The liberated sugars were converted to their corresponding alditol acetates and identified by GC (Gläßgen et al. 1992a) as the derivatives of glucose and rhamnose in a molar ratio of 2:1. After alkaline hydrolysis with 10% KOH, *p*-coumaric acid was identified as acyl group by HPLC of the ether phase (Gläßgen et al. 1992a).

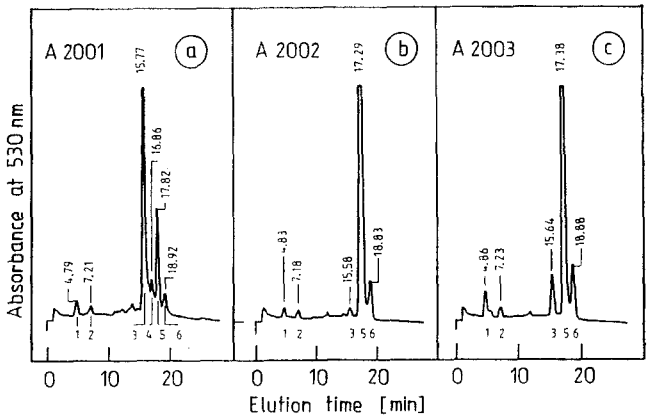

Fig. 3. HPLC-elution profiles of anthocyanin extracts from different callus cell lines of *Solanum tuberosum*. For chromatographic conditions see Materials and Methods. a) Cell line A2001, b) cell line A2002, c) cell line A2003.

The ion spray mass spectrum of **5** exhibited a molecular ion M^+ at m/z 917. A daughter ion spectrum of m/z 917 showed fragment ions at m/z 301 representing the aglycone peonidin, m/z 463 (peonidin + glc), m/z 625 (peonidin + 2 glc), and m/z 755 (peonidin + glc + rha + *p*-coumaric acid). The chemical shifts, coupling constants and the integration of signals in the 400 MHz ^{1}H-NMR spectrum of **5** were consistent with the data given by Andersen et al. (1991) for petanin from *Solanum tuberosum* tubers, with the exception of an additional doublet signal at $\delta = 7.39$ ppm for the proton H-5' in the B-ring of the aglycone peonidin (instead of a hydroxy group in petunidin), and an additional *ortho* coupling of H-6' ($J = 8.7$ Hz) making the signal for H-6' occur as a doublet of doublets (*dd*, $\delta = 8.27$ ppm). The major anthocyanin (**5**) from the cell lines A 2002 and A

2003 can thus be described as peonidin 3-O-[6-O-(4-O-*E*-*p*-coumaroyl-α-rhamnosyl)-β-glucoside]--5-O-β-glucoside ("peonanin", $C_{43}H_{49}O_{22}$, calculated mono-isotopic mass 917.37).

The molecular ions obtained from ion spray mass spectra of enriched fractions of minor components of these cell lines indicated the presence of additional acylated anthocyanins with the same glycoside part and the same acyl group, but with different aglycones (e.g. "cyananin", M^+ 903, $C_{42}H_{47}O_{22}$; "negretein", M^+ 947, $C_{44}H_{51}O_{23}$). In *Petunia hybrida*, another member of the Solanaceae family, analogous structures have been identified, which also differ only in their aglycones (Schram et al. 1983). The glycosylation of different aglycones with 3-rutinoside-5-glucosides is typical for all *Solanum* species and can be used as a taxonomic marker (Timberlake and Bridle 1982), thus the compounds synthesised by the potato cell line are typical Solanaceae anthocyanins.

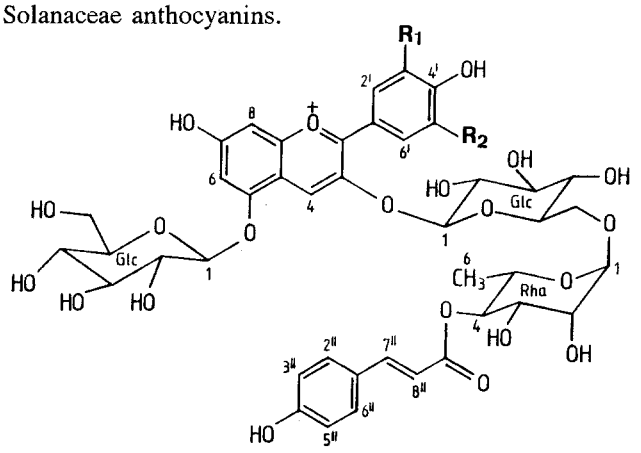

Fig. 4. Structural formula of the flavylium cations of anthocyanins found in callus cultures of *Solanum tuberosum*: R_1 = OCH_3, R_2 = H: peonanin (major component); R_1 = OH, R_2 = H: cyananin; R_1 = R_2 = OCH_3: negretein

Deduced from a molecular ion at m/z 903 and the identification of the major aglycone as cyanidin (see above), the major component (3) from cell line A2001 was tentatively concluded to be cyananin (calculated mono-isotopic mass 903.35). This component was not available in quantities sufficient to perform detailed structural analyses.

The present report shows that gamma-irradiated seedlings can be rescued by establishing a callus. The treatment with gamma-rays promotes regulatory changes in the expression of anthocyanin biosynthesis, and this constitutive over-expression is maintained over many generations in the majority of the cells. In addition to the light-induced and phytohormone-regulated anthocyanin biosynthesis (see Introduction) this is another example of a "derepression" of genes involved in this pathway. Further investigations will be necessary to elucidate the site of action of gamma-rays. Among other possibilities they may interfere with the respective promoters or lead for instance to a gene amplification.

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