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Light-Dependent Betanin Production by Transformed Cells of Sugar Beet

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Summary

The objective of this work is to transform sugar beet (*Beta vulgaris* L. var. *altissima*) cells using *Agrobacterium tumefaciens*, a wild octopine strain B6S3, and to study metabolic changes associated with the transformation. From the primary tumours on leaf fragments, two tumour lines were established: one pale green and the other red-violet in colour. The red-violet pigment was identified by means of high-performance liquid chromatography as betanin. Betanin production is strongly light-dependent. To enhance the betanin yield, nutrient media with different carbohydrates like sucrose, a combination of glucose and fructose, or only glucose or only fructose were tested. The selection of carbohydrate affected betanin production and yield. It was observed that the production of betanin per g of dry mass was 20–40 % higher on simple carbohydrates, especially fructose, than on sucrose or the combination of glucose and fructose. However, due to higher biomass production on the medium with sucrose, the highest total yield of betanin was obtained in the presence of sucrose. The most suitable time for possible betanin extraction was between days 7 and 14 for tissue grown on sucrose. The tumour line seems to be promising as an alternative source of betanin as well as a model to study sugar-regulated genes involved in tissue morphology control.

Key words: *Agrobacterium tumefaciens,* betacyanins, carbohydrates, *in vitro* tissue culture, pigment, secondary metabolites

Introduction

Sugar beet and red beet (*Beta vulgaris* L.), as important industrial crops, have been studied to improve their agronomical traits (*1,2*). *In vitro* cultures of normal and habituated sugar beet cell lines have been established as a model to study habituation and cell differentiation (*3,4*). A way to modify the state of differentiation of a plant cell may be to change the ratio of plant hormones auxin and cytokinin by adding them directly into nutrient media. Alternatively, cell transformation by means of *Agrobacterium tumefaciens* can be performed (*5*). It offers the advantage that no exogenous hormones are required and any gene of interest can be inserted into the plant genome. However, a visual marker of transformation and cell differentiation would be beneficial. Red beets produce pigments, betalains. These pigments are commercially important as natural dye instead of artificial colorants. Red betacyanins and yellow betaxanthines are iminium derivatives of betalamic acid with 5,6-dihydroxyindoline-2-carboxylic acid (cyclo-DOPA) and amino acids or amines (*6*). All betacyanins are com-

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posed of aglycone (betanidin) and glycosyl moieties, and some betacyanins are modifed with acyl groups, whereas betaxanthins are not. In the betacyanin biosynthetic pathway, both cyclo-DOPA and betalamic acid are thought to be synthesized from DOPA, and then an aglycone, betanidin, is formed from the conjugation of these two moieties (*6*). Betanin, a member of the betacyanin pigments subfamily, is a powerful antioxidant and anticancer compound (*7,8*). The growing knowledge of their therapeutic and preventive properties is likely to further increase the research interest in their *in vitro* biosynthesis (*9*). Although the biosynthetic pathway is known, little is known about the enzymes involved in biosynthesis of these compounds (*6*). Red beet cell cultures (*10–13*) and hairy root cell lines (*14*) capable of betalain production have been described. Sugar beet can be easily transformed with a T-DNA of tumour inducing (Ti) plasmid from *Agrobacterium tumefaciens* (*3,15*). So far there has been no report on *A. tumefaciens*-transformed sugar beet cells with enhanced capacity of betalain production.

The aim of this work is to establish *in vitro* culture of sugar beet tumour tissue and to study metabolic changes caused by transformation of leaf cells into tumour cells. In this study sugar beet was transformed by the wild strain B6S3 of *Agrobacterium tumefaciens*. One tumour line with high capacity of betalain production was established. The pigment was analyzed by high-performance liquid chromatography (HPLC), quantified and identified as betanin. The influence of different sugars on betanin production was evaluated.

Materials and Methods

Tumour induction and tissue cultivation

Tumours were induced by infecting leaf fragments of axenically germinated sugar beet (*Beta vulgaris* L.) plantlets with a wild octopine strain B6S3 of *A. tumefaciens* following the procedure of Horsch *et al.* (*5*). Bacteria were eliminated by culturing tumour tissue in the liquid PG0 medium (*15*) containing the antibiotic Pyopen (Pliva, Croatia) in the concentration of 1 mg/mL during the first week and 0.5 mg/mL during the second and third weeks of subculture. Bacteria-free tumour tissue was subcultivated on hormone-free PG0 medium solidified by 0.9 % (by mass per volume) agar (*16*). The light/dark photoperiod (42 μmol/(m^2·s)) was 16/8 hours. For experimental purposes, sucrose, glucose, fructose and the combination of glucose and fructose were added to the nutrient medium of tumour red in light cell line (Trl), at the final concentration of 88 mM. Cells were harvested at days 4, 7, 14 and 21, lyophilized, weighed and stored at –20 °C until analysis.

PCR amplification

PCR amplification was carried out in a 25-μL reaction mixture containing 5–10 ng of sugar beet DNA, 4 μM primers, 200 μM dNTP, 10 mM Tris-HCl (pH=8.3), 50 mM KCl, 2 mM $MgCl_2$ and 1 U Taq DNA Polymerase (Fermentas, Germany). After an initial denaturing step at 95 °C for 5 min, 35 amplification cycles were performed on a Gene Amp PCR System 2400 (Applied Biosystems, CA, USA) thermal cycler, each consisting of a denaturation step at 95 °C for 45 s, an annealing step at 60 °C for 45 s, an extension step at 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Primer pair 6a1 and 6a2, which amplified part of the T-DNA region with expected fragment length of 336 pb of *A. tumefaciens*, was used to verify the transformed character of sugar beet cells. In addition, primer pair vir1 and vir2, which amplified part of *virB10* region present only in *A. tumefaciens*, was a negative control (Table 1). Amplified fragments were separated on 1 % (by mass per volume) agarose gels.

Extraction of pigments

For HPLC and spectrophotometric analyses, lyophilized sugar beet tumour tissue was homogenized in ice-cold 90 % ethanol (by volume). Tissue to solvent ratio was 1:20 (by mass per volume). In a parallel experiment freshly cut root flesh of the red beet was homogenized. Root to solvent ratio was 1:2 (by mass per volume). Prior to the analysis, homogenates were filtered through a 0.2-μm nylon filter. For betanin quantification, 20 mg of the lyophilized sugar beet tumour tissue were homogenized and resuspended in 1 mL of 80 % methanol (by volume) supplemented with 50 mM ascorbic acid. Homogenates were centrifuged for 20 min at 15000×*g*. The pigment was reextracted twice with additional 2 mL of the same buffer. Extracts were joined and the absorbance was measured at 534 nm (ATI/Unicam UV4-100, Cambridge, UK).

Spectral and HPLC analysis

Qualitative chromatographic analyses were performed on the HPLC system (Agilent 1100 Series, CA, USA) equipped with a quaternary pump, multiwave UV/VIS detector, autosampler and fraction collector. The column used was a 5-μm Zorbax RX-C18 (250×4.6 mm,

Table 1. Primer pairs, primer sequences and expected PCR fragment length used for verification of the transformation of sugar beet tumour lines Trl and Tg with *A. tumefaciens* B6S3 wild strain

Amplified region and primers	Primer sequences	Fragment length
T-DNA		
6a1	5′-TGCTTCAGATGGATTGCTTGCC-3′	336 pb
6a2	5′-GATAGCACCATCTAACTCCACG-3′	
virB10		
vir1	5′-CAATCCCGATCAAGTCGTGCGC-3′	644 pb
vir2	5′-AGACGCCAACCTCGTGAAACCG-3′	

Agilent Technologies, CA, USA). The elution profile consisted of a 30-minute linear gradient from solvent A (1 % (by volume) acetic acid in water) to 12 % (by volume) solvent B (1 % (by volume) acetic acid in acetonitrile). The injection volume was 20 μL and the constant flow rate 1.0 mL/min. HPLC grade solvents (Merck, Germany) were used in all experiments. Betanin, a major pigment component of red beet root, and the investigated compound from sugar beet tumour tissue were isolated from the extracts semipreparatively under the same conditions (injection volume of 1.8 mL). The spectrum (350 to 650 nm) of ethanol extracts of betanin and the investigated compound were recorded on a spectrophotometer (Beckmann, Germany). Finally, equal volume of ethanolic solution of betanin and the investigated compound were mixed (1:1, by volume) and this mixture was submitted to qualitative HPLC analysis.

Results

The infection of sugar beet leaf fragments with a wild strain *A. tumefaciens* B6S3 resulted in the growth of primary tumours (Fig. 1A). Tumours were greenish with a snowy surface and some of them had red-violet patches. After antibiotic treatment and elimination of bacteria, an axenic culture of tumour tissue was established (Fig. 1B). In the course of subculture, two tumour lines were separated. They were labelled according to their colour as Tg (tumour green line), green and compact tissue (Fig. 1C), and Trl, red and loose tissue (Fig. 1D). When placed in darkness, the Trl tumour stopped producing the red-violet pigment and became more friable. This colourless tissue was labelled Trd (tumour red in darkness cell line) (Fig. 1E). A switch from the red-violet to white phenotype was reversible and strongly light-dependent. It took several weeks for the colourless tissue to become coloured again, under the influence of light. The same tumour morphology was obtained in two repeated transformation experiments. Greenish and red-violet tumour tissue stably maintained their phenotypes during more than 10 years of subculturing. Light microscopy showed that tumour cells were mostly spherical with few elongated cells in the size range from 50 μm (Trl and Trd cells) to 150 μm (Tg cells) (Figs. 1F, G and H). Only Trl cells accumulated the pigment. Plastids of Tg cells were chloroamyloplasts, and those of Trl cells were smaller amyloplasts.

The PCR amplification of a 336-bp fragment of T-DNA region with primer pair 6a1 and 6a2 (Table 1) confirmed that both lines, Tg and Trl, had been transformed (Fig. 2A). The T-DNA fragment present in tumour-inducing bacteria (a positive control) was not detected in an untransformed sugar beet callus. Additional PCR with primer pair vir1 and vir2 (Table 1) excluded the presence of bacteria in tumour tissues (Fig. 2B). The 644-bp fragment, corresponding to *vir* region of Ti plasmid, was detected only in *A. tumefacines*.

The pigment of Trl tissue was compared with the pigment of red beet roots. The ethanol extracts of both

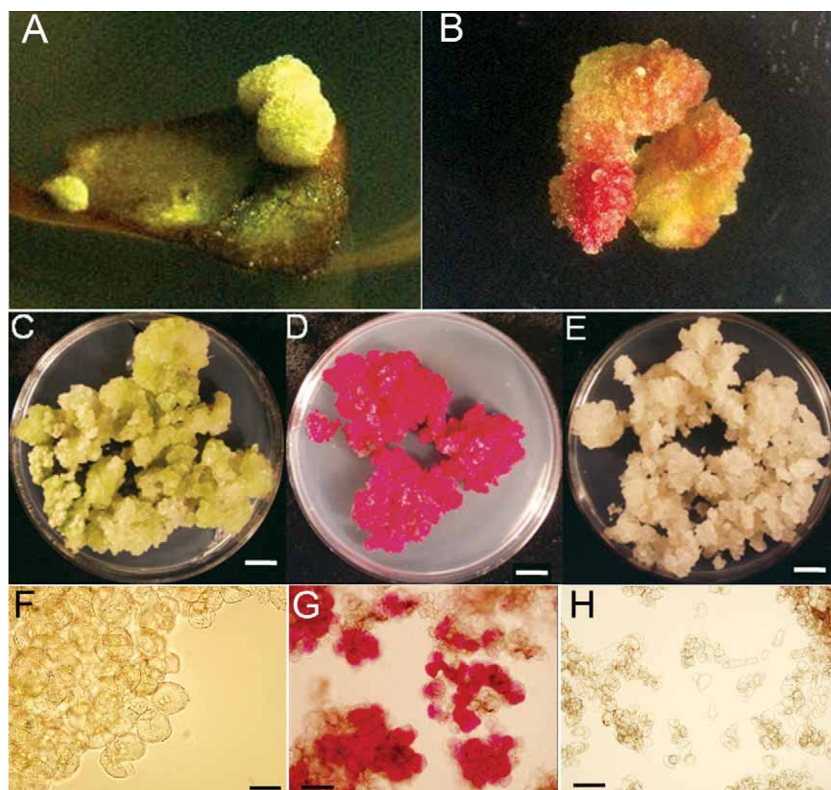

Fig. 1. Sugar beet tumour cultures. A – primary tumours (white arrow) on the leaf fragment. B – tumour tissue grown on hormone-free PG0 medium after bacteria elimination. White arrow shows betanin-producing tumour parts. Macroscopic (C-E) and microscopic (F-H) view of sugar beet tumour tissues 14 days after subculture on PG0 medium. C, F – green tumour line (Tg); D, G – red tumour line (Trl); E, H – white tissue of Trl line grown in the dark (Trd). White bar=1 cm, black bar=200 μm

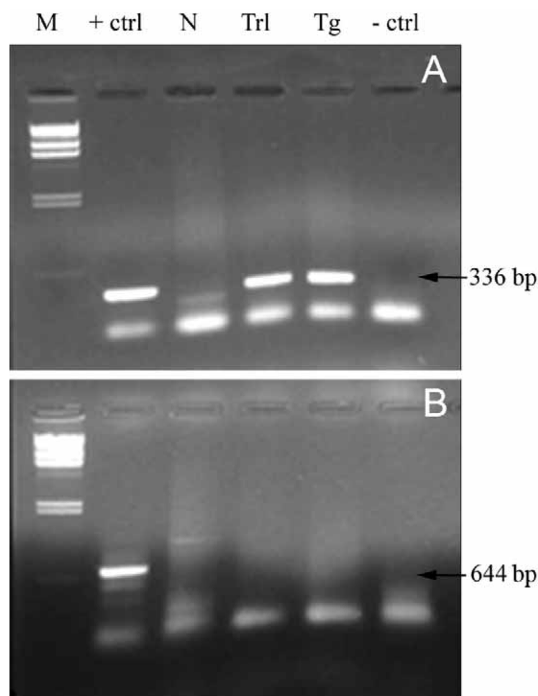

Fig. 2. Verification of transformation of sugar beet tissue lines by PCR amplification with two primer pairs. A) PCR amplification of 336 bp fragment of T-DNA region with primers 6a1 and 6a2 in *A. tumefaciens* strain B6S3 Ti-plasmid and sugar beet tissue lines. B) PCR amplification of 644 bp fragment as part of *virB10* region achieved with vir1 and vir2 primers only in *A. tumefaciens* strain B6S3 excluded the presence of bacteria in sugar beet tissues. M – marker, λ-DNA digested with HindIII; + ctrl – DNA from *A. tumefaciens*; N – normal sugar beet cell line; Trl – Tumour red in light cell line; Tg – Tumour green line; – ctrl – DNA without the template

tissues showed one identical absorbance maximum at 534 nm, corresponding to betacyanins. Betanin, which belongs to betacyanins, is known as the major pigment component in red beet root (*17*). The HPLC analyses of extracts of Trl and red beet root tissues showed only one major peak, eluting at around 17.09 min. This major compound with a retention time of around 17.09 min was isolated semipreparatively from both extracts. Ethanolic solutions of these isolated compounds were mixed together and the mixture was submitted to HPLC analysis. Only one peak with the same retention time of 17.09 min was recorded (Fig. 3). Based on this result and the data reported by Fernández–López and Almela (*17*) about the retention time and absorption spectra investigated, the compound was identified as betanin.

With the intention of optimizing biomass production and betanin yield, the Trl tissue was cultured on the solid PG0 media, supplemented with different sugars: monosaccharides glucose (Glc) or fructose (Fru), disaccharide sucrose (Suc) and with a combination of monosaccharides, Glc with Fru. Growth was measured up to 21 days for Suc and Glc with Fru, but only up to 14 days for Glc and Fru. After 14 days, phenolic compounds accumulated in the media and necrosis of the tissues started. To compensate for possible differences in water content, the tissue was lyophilized, and dry mass

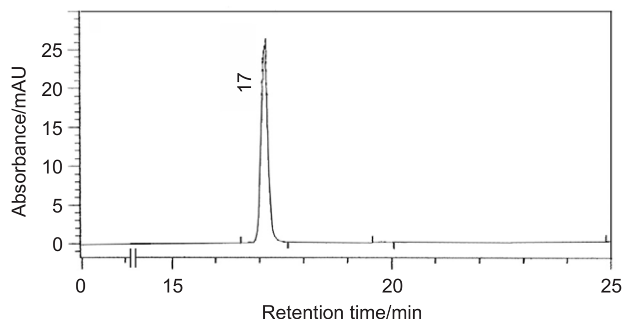

Fig. 3. HPLC chromatogram of the mixture of ethanolic solutions of betanin and Trl pigment. (1:1, by volume). Detection was carried out at 534 nm (maximum absorbance of both compounds)

was measured and related to betanine accumulation (Fig. 4). The betanin accumulation was positively correlated with the time course of tissue growth on the Glc or Fru, but it was negatively correlated with the growth on Suc or Glc with Fru. If expressed per dry mass, tissue grown on Glc or Fru synthesized more betanin than tissue grown on Suc or on Glc with Fru. If expressed per Petri dish, the highest yield of betanin was measured in tissue which grew on the medium containing Glc with Fru at day 21 (4.36 mg of betanin per Petri dish), followed closely by the tissue grown on Suc, but at day 14 (4.15 mg of betanin per Petri dish). Time of cultivation and biomass accumulation are important factors in the production of phytochemicals, and Suc was the most effective carbohydrate due to the highest growth rate of the Trl. The betanin synthesis in Trl grown on sucrose remained almost constant until day 14, so the optimal extraction time of the pigment would be on day 14.

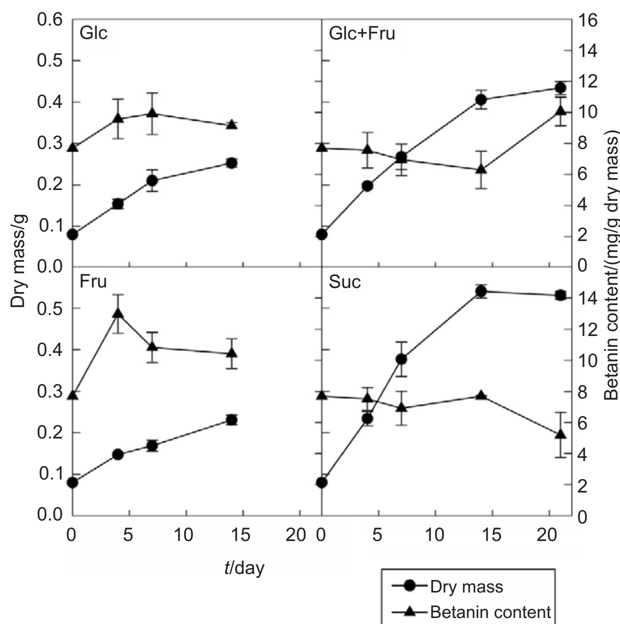

Fig. 4. Comparison of dry mass accumulation and betanin content in Trl tumour subcultured on PG0 medium supplied with different carbohydrates. Glc – glucose, Fru – fructose, Glc+Fru – glucose with fructose, Suc – sucrose (error bars represent the standard deviation, *N*=3)

Discussion

Transformation of sugar beet leaf cells with the wild type *A. tumefaciens* B6S3 of Ti plasmid resulted, as expected, in the growth of unorganized tumours. Primary tumours on sugar beet leaf fragments contained red-violet tissue patches, which also appeared in the initial tumour cultures (Figs. 1A and B). During subculture two tumour lines were separated, one greenish and the other entirely red-violet (Figs. 1C and D). Tumour morphology depends on the ratio of auxin to cytokinin, so if both loci, *aux* and *cyt*, of T-DNA are active, an unorganized tumour should develop. However, in horseradish, the same type of transformation resulted not only in unorganized tumours, but also in teratomas with malformed shoots (*18*,*19*), showing that tumour morphology also depends on the host plant. Polymerase chain reaction confirmed the transformed state of both tissue lines, Tg and Trl, but it did not answer the question why the Trl line produces betanin. It can only be supposed that T-DNA insertion, by positional effect, activated the light-induced betanin production in Trl cells. According to some literature data, the integration of T-DNA into plant genome occurs randomly at different places and in different copy numbers, which can explain tumour tissue morphology (*20*–*22*). Other reports suggest that T-DNA integration under selective pressure may not be completely random (*23*). Analysis of T-DNA insertion site distribution in *Arabidopsis* revealed that transcription initiation and polyadenilation site regions of genes are favourite targets for T-DNA integration (*23*). Light microscopy revealed that Trl contained clustered, spherical cells that accumulated pigment in vacuolar space (Fig. 1G). Similar cellular shape and pigment production is obtained when red beet calli are cultured under specific auxin to cytokinine ratio (*10*). The auxin to cytokinin ratio determines cellular shape, from spherical to elongated, and the production of pigments, from betacyanins to betaxanthines (*9*). The HPLC spectra (Fig. 3) confirmed that the Trl line contained betacyanins, specifically betanin, while no betaxanthins were detected. The light-dependency of pigment production was demonstrated as a dark-grown tumour becoming depigmented after few weeks (Figs. 1E and H). Light is also a prerequisite for pigment production in cell cultures of red beet and *Portulaca grandiflora* that produce betacyanin, but the mode of action of light is not yet resolved (*11*,*24*). Results for time course of betanine production and its quantity in the tumour line (Fig. 4) were comparable to those published for red beet cultivated *in vitro* (*11*,*25*,*26*). We observed that the production of betanin per g of dry mass was 20–40 % higher on simple carbohydrates, especially fructose, than on the sucrose or the combination of glucose and fructose. However, due to higher biomass production on the medium with sucrose, the highest total yield of betanin was obtained in the presence of sucrose. Regarding energy, sucrose has been established as the most suitable carbon source for the cultivation of *in vitro* plant cultures, particularly for biosynthesis of secondary metabolites (*26*,*27*). On the other hand, betanin production could be increased. The possible improvements of betanin production could be tested by using modified Linsmaier-Skoog medium in which inorganic nitrogen concentration is reduced to 30 mM, the ratio of ammonium to nitrate is set to 1:14, Zn concentration is reduced to 0.3 μM, and Cu and Co are removed from the medium (*12*,*13*). With such modifications, the authors gained a 300 % increase in yield. The biosynthesis of betanin in Trl could be enhanced additionally in liquid medium using metabolic precursors of betanin or elicitors such as jasmonic acid, salicylic acid or yeast extract (*9*). Transformed cultures of red beet can produce quantities of betanin at a comparable rate to the original plants (*6*). Most of *in vitro* systems that produce betanin are based on hairy root transformants of red beet (*9*,*27*). Our novel system is based on *A. tumefaciens*. It might offer enhaced synthesis of betanin interesting for commercial purposes.

Conclusion

Agrobacterium tumefaciens-mediated transformation of plants represents one of the most convenient methods for inserting genes of interest in the plant DNA for further studying. In this study, using the above mentioned method, two transformed sugar beet tumour lines were established with commercially interesting traits. One of them is characterised by betanin overproduction, which is light-dependent. The production is also affected by the carbohydrate type supplied in the nutrient medium. The tumour lines are promising for studying proteins involved in betanin production and sugar-regulated genes involved in morphogenetic processes in transformed sugar beet tissues.

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