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Production of transgenic carnation with a heterologous 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase bifunctional enzyme cDNA

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Summary: Transgenic carnations were produced with a modified mammalian bifunctional enzyme cDNA coding 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. Relative activity of this enzyme determines the fructose 2,6-bisphosphate (fru 2,6-P2) cytosolic concentration. This metabolite – as a signal molecule – is one of the carbohydrate metabolism regulators. The regenerated *Dianthus chinensis* and *Dianthus caryophyllus* shoots were selected on MS basal medium containing 150 mg/l kanamycin. Transgene integration was proven by PCR analysis with cDNA specific primers followed by Southern hybridization of DNA isolated from selected green shoots, which survived on kanamycin containing medium, so 3 *D. chinensis* and 20 *D. caryophyllus* transgenic plants were produced. Transgene expression were examined by RT-PCR. Transformed and control plants were potted in glasshouse to evaluate the effect of modified fru 2,6-P2 on development, growth and carbohydrate metabolism.

Key words: 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase, *Agrobacterium tumefaciens*, fructose 2,6-bisphosphate, RT-PCR, transgenic carnation, *Dianthus chinensis, Dianthus caryophyllus*

Introduction

Transgenic approach is a powerful tool to understand the function of isolated genes, metabolic pathways and could be extensively used in plant breeding to produce new varieties. This technique makes the improvement of agronomic and economic traits of plants possible.

Carnation is one of the most important floricultural crops in the world. Many kinds of gene transfer systems have been established to introduce new traits into commercial cultivars. *Agrobacterium tumefaciens*-mediated transformation systems have been developed for leaves, petals and stems (*Lu* et al., 1991; *Van Altvorst* et al., 1995, 1996; *Nontaswatsri* et al., 2004). In addition to indirect gene transfer, the microprojectile bombardment alone or combined with *Agrobacterium* was also successfully applied (*Zuker* et al., 1995, 1999).

Recently, different transgenic carnations have been produced with novel traits. Long vase life is a very important characteristic of the cut flowers. Down-regulation of ethylene production by antisense ACC-synthase (*Kiss* et al., 2000; *Veres* et al., 2005) and antisense ACC-oxidase (*Savin* et al., 1995) extended the vase life by 6–8 days. Florigene Ltd. (1995) produced violet flower carnation varieties Moondust™ and Moonshadow™ by means of anthocyanin biosynthesis modification (delphinidin accumulation). Introduction of *rolC* gene from *Agrobacterium rhizogenes* improved the rooting ability of cuttings and as a result the transgenic plants produced three times more flowering shoots (*Zuker* et al., 2001a). For generating *Fusarium oxysporum f. sp. dianthi* resistant transgenic carnation osmotin, PR-1 and chitinase genes (in various combinations) were introduced into highly susceptible variety. The disease symptoms in these lines were markedly less severe (*Zuker* et a., 2001b). Antisense suppression of flavonone 3-hydroxylase gene modified simultaneously the flower colour and fragrance. Depending on the rate of inhibition the colour varied and the volatile methylbenzoate content was 10 to 100 times higher, so transgenics flowers were more fragrant than controls (*Zuker* et al., 2002).

Photosynthesis and carbohydrate metabolism are fundamental physiological processes in plants, providing energy for normal growth and development. Fructose 2,6-bisphosphate (fru 2,6-P2) as a signal metabolite is an important regulator of carbohydrate metabolism in plants (*Stitt*, 1990). In order to clarify the physiological role of fru 2,6-P2 in carnation, transgenic plants were produced with *Agrobacterium tumefaciens* harbouring modified mammalian bifunctional enzyme (6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase) cDNA encoding a› a polypeptide with the capacity to decrease the fru 2,6-P2 concentration (F-2,6-BPase) and aa› a polypeptide to increase the fru 2,6-P2 (6PF2K).

Materials and methods

Plant materials

Carnation species and varieties of good regenerating capacity were selected for transformation (*Kiss* et al., 2000):

Dianthus chinensis 'Simon' (Hungary), *Dianthus caryophyllus* variety 'Improved White Sim' (IWS) and 'Bibor' (Óbuda Horticultural Laboratory Budapest, Hungary).

Culture media, conditions and transformation

Carnation leaves were transformed with LBA4404 *Agrobacterium tumefaciens* strain harbouring the constructs shown in *Figure 1* as described by Van Altvorst et al. (1995). Shoots were regenerated on the following medium (RMS): MS salts and vitamins supplemented 3% sucrose, 1 mg/l benzyladenine, 0.1 mg/l naphthaleneacetic acid and 0.8% Oxoid agar or Phytagel (Sigma). For selection of transgenic plants, RMS medium was supplemented with 150 mg/l kanamycin and 200 mg/l cefotaxime.

Shoots were regenerated in climatic chamber under 16 h light/8 h dark photoperiode, 200 W/m^2 light intensity at 18 °C.

Gene construct

The modified coding region of the rat liver 6-phospho-fructo-2-kinase/fructose 2,6-bisphosphatase contains point mutations (*Tauler* et al., 1991; *Kurland* et al., 1992; *Li* et al., 1992). This sequence was inserted *via* subcloning steps into pBIN 19 under the control of CaMV 35S promoter and introduced into *Agrobacterium tumefaciens* strain LBA 4404. Binary vector pBIN 19 carries neomycin phospho-transferase (*nptII*) gene as a selectable marker (*Figure 1*).

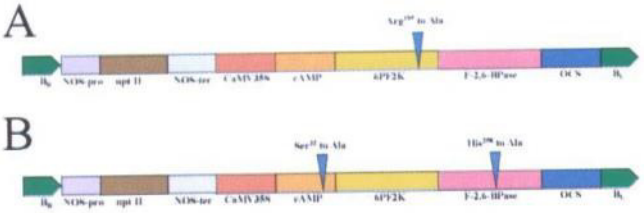

Figure 1. The applied vector construction. **A:** cDNA coding bifunctional enzyme, which possesses only fructose 2,6-bisphosphatase activity (F-2,6-BPase). **B:** cDNA coding bifunctional enzyme, which exhibits only 6-phosphofructo-2-kinase activity (6PF2K). Abbreviations: BR: right border; NOS-pro: nopaline synthase promoter; npt II: neomycin phosphotransferase; NOS-ter: nopaline synthase terminator; CaMV35S: cauliflower mosaic virus promoter; cAMP: cyclic adenosine monophosphate binding site; 6PF2K: 6-phosphofructo-2-kinase; F-2,6-BPase: fructose 2,6 bisphosphatase; OCS: octopin synthase; BL: left border

PCR, Southern hybridization, RT-PCR

From plants that survived on selective medium genomic DNA was isolated with QIAGEN® DNeasy Plant Mini Kit and analysed by PCR using cDNA specific primers (5'-ATG TCT CGA GAG ATG GGA GAA CTC ACT CAA-3' and 5'-TCA GTA ATG GGC AGG TAC AGT GTC CAA GGA-3'). PCR was carried out in BioRad iCycler with the following reaction conditions: Precycle: 94 °C 2 min, 40 cycles: 94 °C 10 sec, 56 °C 30 sec, 72 °C 1 min, postcycle: 72 °C 2 min.

Southern hybridization was carried out with digoxigenin (DIG) labelled bifunctional enzyme cDNA probe. Genomic DNA (30 µg) was digested with *Eco*RI and blotted onto nylon membrane and hybridized at 54 °C according to the manufacturer's protocol (Boehringer-Mannheim). Hybridization signal was obtained by colorimetric detection (NBT/BCIP: nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate).

Total RNA were isolated with QIAGEN® RNeasy Plant Mini Kit. RT-PCR with cDNA specific primers were performed with Invitrogen™ SuperScript III One-Step Kit according to the manufacturer's protocol.

Qualitative determination of starch

Qualitative analysis of starch content was carried out by Lugol dying. To remove the chlorophyll, 1 g leaf tissue was washed with 10 ml acetone. The colourless tissues were ground in liquid N_2. The starch was extracted with 3 ml 8 M HCl and neutralized with 5 M NaOH, 15 µl Lugol solution/1 ml extract was added (Lugol: 1 g KI, 1 g I_2 in 100 ml distilled water).

Results

After inoculation and cocultivation, the leaves were placed on regeneration medium containing 50 mg/l kanamycin (*Figure 2*). When the shoots reached 0.5–1 cm length, they were transferred to medium supplemented with 150 mg/l kanamycin. Non-transformant plantlets whitened and died. The number of the regenerated transgenic shoots of *D. chinensis* and *D. caryophyllus* varieties is presented in *Table 1*.

Figure 2. Shoot regeneration after transformation on medium containing 50 mg/l kanamycin. Green shoots are the putative transformants. Non-transformant plantlets whitened and died

In order to test the effect of agar type on shoot quality the regeneration was carried out on two types of agar (Oxoid, Phytagel). On Oxoid agar the regenerating shoots had normal phenotype, while on Phytagel the shoots became hyperhydric. They were saturated with water, translucent and glassy (*Figure 3*). These hyperhydric tissues could be reverted to normal growth and morphology, when they were transplanted onto Oxoid agar.

Table 1. Effect of the genotype and the vector type on regeneration capacity and transformation efficiency

	Regeneration and transformation efficiency			
Genotype/vector type	**Total number of transformed leaves**	**Regenerating explants number/%**	**Total number of regenerated shoots**	**Number/% of transgenic shoots**
D. chinensis				
6PF2K	150	10/6.7	16	1/6.3
F-2,6-BPase	150	16/10.7	22	2/9.1
D. caryophyllus variety IWS				
6PF2K	150	27/18	31	5/16.1
F-2,6-BPase	150	33/22	45	8/17.7
D. caryophyllus variety Bibor				
6PF2K	150	22/14.7	26	4/15.4
F-2,6-BPase	150	19/12.7	23	3/13.0

Figure 3. Effect of agar type on shoot development. Left: a normally growing shoot on culture media solidified Oxoid agar, right: a hyperhydric shoots on Phytagel containing medium

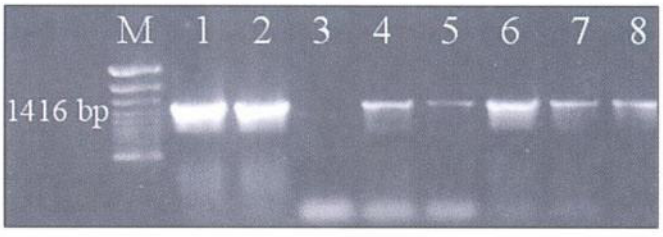

Figure 4. Results of PCR with gene specific primers. Putative transgenic IWS plants containing 6PF2K amplified the 1416 bp DNA fragment (4–8). M: molecular weight marker (Fermentas GeneRuler 100 bp ladder / 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80 bp), 1–2: positive controls, 3: negative control

Transgene integration was proven with PCR. The PCR amplification resulted in the expected 1416 bp fragment in transgenic plants which was absent in controls (*Figure 4*). DNA fragment of the same size amplifies both in the kinase and the phosphatase transformed plants with the applied primers. The integration of the foreign gene was confirmed by Southern hybridization, too. Genomic DNA was digested with *Eco*RI, blotted onto nylon membrane and hybridized with DIG-labelled cDNA PCR product. DNA from control carnation did not show any fragment hybridizing with the probe, while putative transgenic plants produced coloured fragments with the hybridization probe (2–6 samples in *Figure 5*).

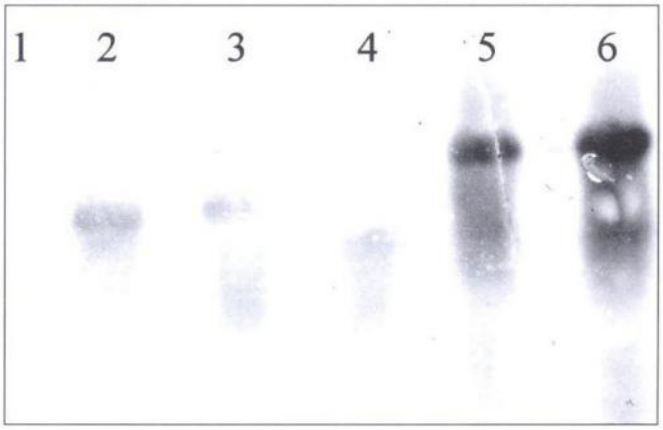

Figure 5. Southern hybridization with DIG labeled probe. 1: negative control, 2–6: putative transgenic IWS plants containing 6PF2K

Transgene expression was analysed by RT-PCR. Appearance of the expected 1416 bp fragment in both types of transformants indicated the mRNA production in both 6PF2K and F-2,6-BPase plants (*Figure 6*).

The rooted, transgenic plants were potted into glasshouse to examine the effect of modified fru 2,6-P2 levels on carbohydrate metabolism, growth and development. At an early stage of the development and growth 6PF2K plants grew more slowly than controls but after 6 months this distinction was insignificant. There were differences in the length of internodes, which can be a characteristic trait for carnation. Internodes of F-2,6-BPase lines were 4 cm long compared to the 2 cm length of the wild type and 1 cm of 6PF2K transgenic plant (*Figure 7*).

As a first step in carbohydrate content analysis, a qualitative test (Lugol dying) was applied to determine the effect of the carbohydrate modification for starch content (*Figure 8*). As it can be seen, the intensive blue colour of 6PF2K leaf tissue extract markedly differs from that of the pale blue shade of control and colourless tone of F-2,6-BPase lines. The detailed evaluation of transgenic plants is going on and will be published in a separate article.

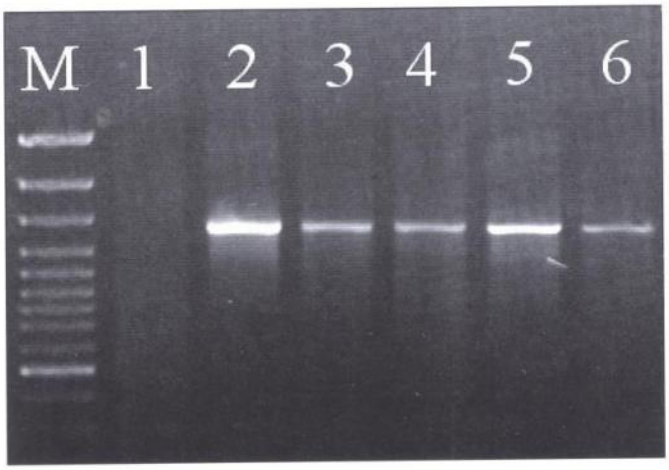

Figure 6. Results of RT-PCR obtained for PFK IWS transgenic plants. 1. non-transformant IWS, 2–6: IWS transformants with the expected 1416 bp amplification product, M: DNA molecular weight marker (Fermentas GeneRuler 100 bp ladder / 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80 bp)

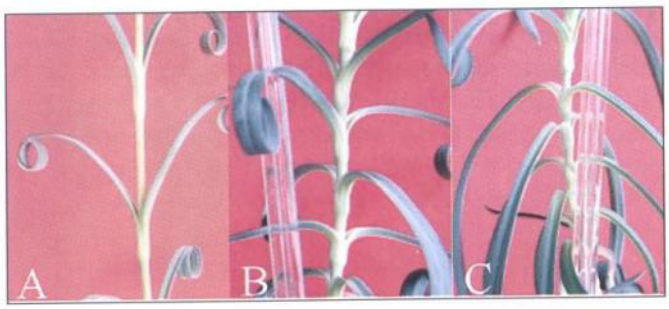

Figure 7. Differences in internode length between control and transgenic plants. **A:** plant transformed with phosphatase (F-2,6-BPase) construct, **B:** control plant, **C:** plant transformed with kinase (6PF2K) construct

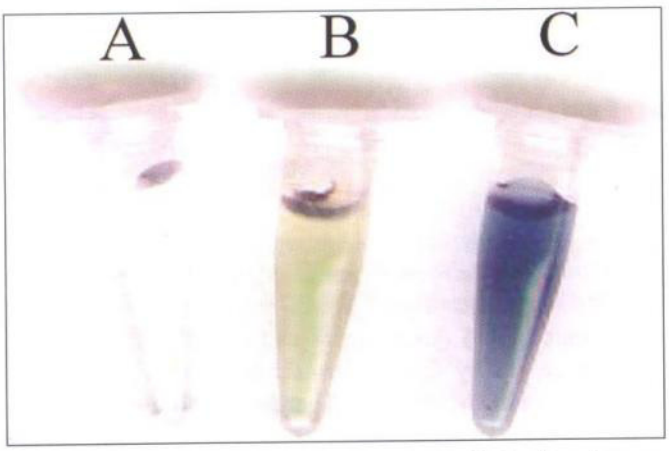

Figure 8. Qualitative analysis of starch content. (A) No discolouration can be observed in F-2,6-BPase plants, where starch synthesis is repressed, (B) Lugol reaction in control plant tissue extract, (C) blue colour indicates the starch overproduction in 6PF2K sample

Discussion

Transgenic carnation was obtained by *Agrobacterium*-mediated transformation. Binary vector carried a modified mammalian bifunctional enzyme gene, responsible for fru 2,6-P2 synthesis and breakdown. For transformation, leaves of *in vitro* grown shoots were used, because their transformation frequency is higher than those of *in vivo* shoots (*Miroshnichenko & Dolgov*, 2000). Transformation was performed according to the protocol described by *Van Altvorst* et al. (1995). Frequency of transgenic plants varied depending on species between 6.3–9.1% (*D. chinensis*) and 13.0–17.7% (*D. caryophyllus*). In *D. caryophyllus* this was higher than in the original protocol (1.5–12.0%), but it did not reach the effectiveness of Nontaswatsri et al. (2004) method, which produced 20.0-93.0% depending on varieties.

Number of regenerating explants was lower in *D. chinensis* (26) than in *D. caryophyllus* (101). In *D. caryophyllus* the higher regeneration occured in variety IWS (60) than in variety Bibor (41), but significant differences in transformation efficiency between vector types and genotypes respectively could not be observed (Table 1). The differences between species can be explained by the size of the leaf explants base (*Van Altvorst* et al., 1994), which is smaller in *D. chinensis*.

During preselection, the regeneration medium contained lower concentration of kanamycin (50 mg/l), than in the selection phase. As antibiotics penetration is inhibited through leaf tissues (*Van Altvorst* et al., 1995), the regenerating 0.5–1 cm long shoots were cut off from leaf surface and placed onto 150 mg/l kanamycin containing medium.

The effect of two different agar types on the developing shoots were tested, too. The most plantlets regenerated in Phytagel were hyperhydric. Regarding the cause of hyperhydricity numerous hypotheses have been set up to explain the phenomenon (*George*, 1996). Selection in Phytagel resulted in false positive shoots, too, therefore Oxoid agar was used for regenerating transformant shoots.

PCR and Southern blot techniques confirmed the transgenic nature of survived plants. RT-PCR revealed, that the transgene not only integrated but expressed, too.

Differences observed in the morphology and the ontogeny between the greenhouse grown transgenic and control plants mean further confirmation of transgene expression. In other preceding studies the modification of sucrose export capacity caused also phenotypical and morphological alterations in tobacco (*Bürkle* et al., 1998).

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