Production of transgenic carnation with antisense ACS *(1-aminocyclopropane-1-carboxylate synthase)* gene

INTERNATIONAL JOURNAL OF HORTICULTURAL SCIENCE

AGROINFORM
Publishing House, Hungary

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Key words: ACS, *Agrobacterium tumefaciens, Dianthus caryophyllus, Dianthus chinensis*, ethylene biosynthesis, *npt II*

Abbreviations: ACS: 1-aminocyclopropane-1-carboxylate synthase, BA: benzyladenine: NAA: naphtalene acetic acid, *npt II:* neomycin phosphotransferase

Summary: *Dianthus chinensis* and *Dianthus caryophyllus* varieties were tested for shoot regeneration from leaf and petal explants and transformed with *Agrobacterium tumefaciens* strains (EHA 105 and LBA 4404) harbouring an apple derived ACS cDNA in antisense orientation in order to reduce ethylene production and influence the ethylene dependant traits in carnation. After transformation regenerating shoots were selected on MS medium containing 50-75-100-125-150 mg/l kanamycin and supplemented with 1 mg/l BA, 0.2 mg/l NAA. Transgene integration was proved by PCR analysis with *npt II* spcific primers followed by Southern hybridisation of DNA isolated from green shoots on medium containing 150 mg/l kanamycin. Several putative transformants were subjected to RT-PCR in order to examine the *npt II* expression at mRNA level. Both the transformant and the non-transformant plants were potted into glasshouse to observe the effect of changed ethylene production on flowering time, petal senescence and vase life.

Introduction

Carnation is one of the leading species in the global flower trade, therefore genetic improvement of carnation in respect of several ethylene dependant traits such as flowering time, petal senescence characteristics or vase life can have beneficial economic effect in addition to scientific significance.

ACS (1-aminocyclopropane-carboxylate synthase) is the key enzyme of ethylene biosynthesis. Ethylene – this "small hormone with many functions" *(Van der Straeten* et al. 1995) – influences different physiological and biochemical processes in plant from germination till maturation, including floral senescence and fruit ripening. Down-regulation of ethylene production *via* antisense transformation proved to be an effective tool in delaying fruit ripening in tomato *(Oeller* et al. 1991) and petal senescence of carnation *(Savin* et al. 1995). ACS gene – a member of a multigene family of relatively high DNA sequence homology – has already been isolated and sequenced from many different plant species *(Theologis* et al. 1993) e.g. tomato, apple, rice, avocado, winter squash, zucchini, wheat, carnation *(Park* et al. 1992, *Have & Woltering,* 1997). In our transformation experiments with carnation an apple-derived cDNA clone was used in antisense orientation *(Kiss* et al. 1995, *Rosenfield* et al. 1996) to down-regulate ethylene biosynthesis *(Blokland* et al. 1993), in order to study how the reduced ethylene

production influences flowering characteristics of carnation varieties. Heterologous expression of the *Arabidopsis etr1-1* allele has already been shown to inhibit senescence of carnation flowers *(Bovy* et al. 1999).

Material and methods

Carnations varieties tested in regeneration experiments were: *Dianthus chinensis* 'Simon' (Hungary) and *Dianthus caryophyllus:* 'Nice white', (Vetőmag Hungary), 'Chabaud pink', (Denmark), 'Chabaud mixed colours' (Royal Sluis), 'Marie Chabaud yellow' (Denmark), 'Bíbor'/Purple (Óbuda Horticultural Laboratory Budapest, Hungary), 'Improved White Sim' (Óbuda Horticultural Laboratory Budapest, Hungary).

For shoot regeneration MS *(Murashige & Skoog,* 1962) basal medium (pH: 5.8) supplemented with 1 mg/l BA and 0.2 mg /l NAA, 3% sucrose and 0.4 % Phytagel *(Sigma P* 8169) was used both in case of leaf and petal explants. Leaves were harvested from *in vitro* shoot culture or from plantlets, germinated on hormone free MS medium after desinfection of the seeds (1 min in 70% ethanol, 20 min shaking in 1:3 diluted commercial sodium hypochlorite solution, 3x rinse in autoclaved distilled water). Petals were collected from buds and desinfected in the same way as the seeds after using a first washing step with steril water containing 10% TWEEN 20. After removal of sepals and external petals the internal petals were put onto shoot regeneration medium.

The scheme of transformation from gene isolation *via* plasmid construction, *Agrobacterium* transformation to regenerated plant can be seen in *Figure 1. A and 1.B.* The 1.1 kb fragment of ACS cDNA was isolated from McIntosh apple cultivar and inserted *via* subcloning steps into pBI 121 binary vector (Clontech) in reverse orientation *(Kiss* et al. 1995). This plasmid was introduced into two different *Agrobacterium tumefaciens* strains, LBA 4404 and EHA 105. Only the well-regenerating 'Simon' (*D. chinensis*, Chabaud Pink, Improved White Sim, Bíbor (*D. caryophyllus*) were transformed using leaf *(Horsch* et al. 1988, *Altvorst* et al. 1995) and petal explants *(Altvorst* et al. 1995, *Nakano* et al. 1994). Regenerating shoots were selected on kanamycin containing MS medium in five steps. Green shoots developing on medium supplemented with 50 mg/l kanamycin were transferred to medium of higher kanamycin concentration (75-100-125-150 mg/l).

Plants surviving on 150 mg/l kanamycin concentration were analysed at molecular level, for this purpose genomic DNA was isolated according to *Dellaporta* et al. (1983) analysed by PCR using neomycin-phosphotransferase (*npt II*) specific primers (5'-CTG AAT GAA CTG CAG GAC GAG GAGG-3' and 5'-GCC AAC GCT ATG TCC TGA TAG C, *Maas* et al. 1997). PCR was carried out in Perkin-Elmer GeneAmp 9700 thermocycler with the following reaction conditions: Precycle: 94 C° 2 min, 35 cycles: 94 C° 10 sec, 48 C° 30 sec, 72 C° 1 min, postcycle: 72 C° 2 min.

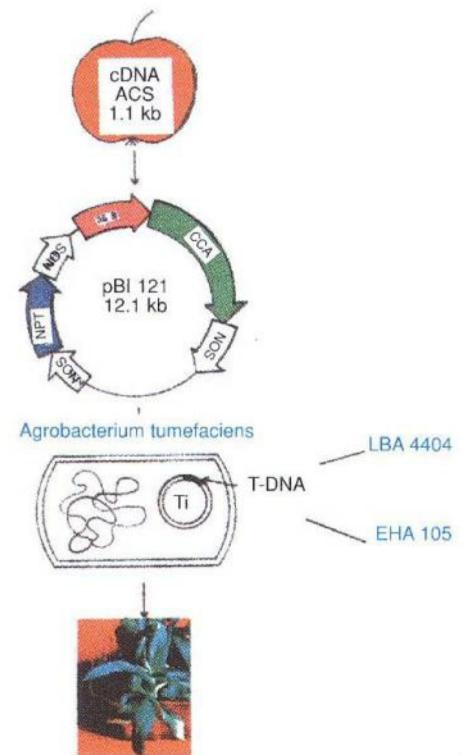

Figure 1.A. Scheme of transformation from gene isolation *via* plasmid construction, *Agrobacterium*-mediated transformation to regenerated transgenic plant

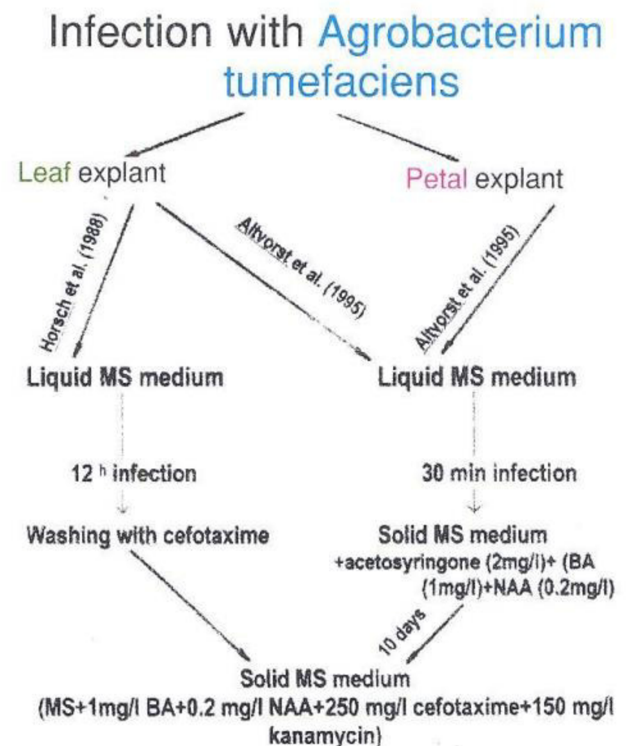

Figure 1.B. Steps of *Agrobacterium* transformation of leaf and petal explants. Petals and leaves were infected according to van Altvorst et al. method (1995), Horsch et al. (1988) protocol was tried only in case of leaves.

Southern hybridisation was carried out with digoxigenin (DIG) labelled probes according to the manufaturer's protocol (Boehringer-Mannheim) after blotting the DNA onto Nylon membrane (Boehringer- Mannheim 120972). The probe was the Pst I fragment of the pBI 121 plasmid (Clontech). Detection was done by chemiluminescent CSPD substrate of Boehringer-Mannheim and followed by autoradiography. For further proving of transgene integration mRNA was isolated from the putative transformants of variety 'Bíbor' (*D. caryophyllus*) according to Dynal Dynabeads mRNA DIRECT Micro kit protocol, followed by immediate RT-PCR with *npt II* specific primers after the recommendation of Superscript One-Step RT-PCR System (GIBCO BRL Life Technologies). cDNA was synthetised at 50 C° for 15 min and amplified under the same conditions as in case of genomic DNA amplification.

Transgenic regenerants were potted in glasshouse in order to make observations of phenotypic effect of the heterologous antisense ACS transformation on the intact plants e.g. flowering time, flower senescence and vase life.

Results and discussion

Leaves of responsive varieties harvested from three weeks old *in vitro* seedlings put on shoot regeneration medium (*van Altvorst* et al. 1995) produced the first shoots after two weeks on the basal part of the leaves. Petal explants regenerated shoots also within three weeks.

The four varieties selected for transformation 'Simon' (*D. chinensis)*, Bíbor, Improved White Sim and Chabaud pink with two *Agrobacterium tumefaciens* strains EHA 105, LBA 4404 regenerated shoots with various efficiency. Table 1. shows the results of shoot regeneration from transformed leaf explants comparing *van Altvorst* et al.' (1995) and *Hoersch* et al.'s (1988) infection method *(Figure 1.B)*. As the results demonstrate the infection method influenced shoot regeneration to a great extent, at the same time remarkable differences due to the *Agrobacterium* strains could not be observed, therefore their effect was not evaluated (data not shown).

Shoot regeneration from *Agrobacterium* infected leaves according to van *Altvorst* et al.' (1995) protocol proved to be more efficient in case of each variety, therefore further transformations are being carried out according to this method. There are data in literature on using microprojectile

Table 1 Comparison of shoot regeneration from leaf explants on MS medium supplemented with 1 mg/l BA+0.2 mg/l NAA+250 mg/l cefotaxime+50 mg/l kanamycin after transformation with van Altvorst et al.' (1995) and Hoersch et al.' (1988) procedure.

Variety	Shoot regeneration (%)	
	van Altvorst et al. (1995)	Hoersch et al. (1988)
'Simon' (*D. chinensis*)	40	8
'Chabaud pink' (*D. caryophyllus*)	17	7
'Improved white Sim' (*D. caryophyllus*)	25	7
'Bíbor' (*D.* caryophyllus)	48	13

bombardment *(Zuker* et al. 1995) and combination of wounding by bombardment with *Agrobacterium* transformation for production transgeneic carnation *(Zuker* et al. 1999), but in our transformation experiments these methods have not been tried.

In the five-step selection of transformants the green shoots developed on 50 mg/l kanamycin were transferred to 75-100-125-150 mg/l kanamycin containing regeneration media resulting in greening shoots even at 150 mg/l kanamycin concentration *(Figure 2)*. This result suggested to replace the five steps for two steps: 50 mg/l 150 mg/l kanamycin.

Figure 2 Green and white shoots of variety 'Simon' (*D. chinensis)* developing on regeneration medium containing 50 mg/l kanamycin.

PCR and Southern hybridisation analysis of these green shoots served further proofs of transformation. The PCR amplification results of 'Simon' (*D. chinensis)* and 'Bíbor' (*D. caryophyllus*) with *npt II* specific primers can be seen in *Figure 3*. The expected a 503 bp fragment was amplified in several samples both in case of transformation with EHA 105 and LBA 4404 *Agrobacterium* strains.

The integration of *npt II* gene was confirmed by blotting of PCR amplification products onto Nylon membrane and hybridised with DIG-labelled Pst I fragment of pBI 121 *(Figure 4)*.

Figure 5. shows the positive result of RT-PCR with *npt II* specific primers for one of the transformants of variety 'Bíbor' (*D. caryophyllus*). Appearance of the expected 503 bp fragment confirmed not only the integration but the expression of *npt II* gene responsible for kanamycin resistance at mRNA level, too.

After the molecular analysis the plants were analysed for ethylene production, and despite of high individual differences both in the control and the transformant group, the average ethylene production of the antisense ACS transformant 'Bíbor' plants was about 40% lower compared to the control plants (data not shown).

In vitro shoot regeneration capacity of leaf explants of transformants were checked also, since there are data in the literature (Eng-Chong Lee 1994, Purnhauser et al. 1987), that decreased ethylene concentration enhances shoot regeneration in plant tissue cultures. According to the first results the shoot regeneration of variety 'Bíbor' plants increased by 10 % even on *hormone free* MS medium

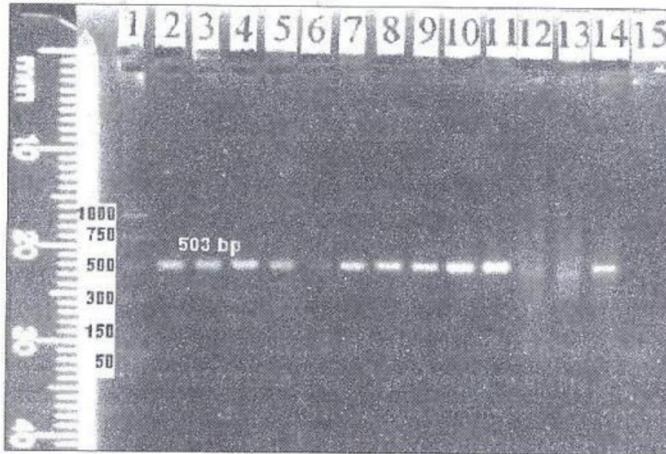

Figure 3 Results of PCR with *npt II* specific primers. The expected band of 503 bp can clearly be observed in lanes 2–5 (variety 'Bíbor', *D. caryophyllus)* 7–11 and 14 'variety 'Simon', *D. chinensis)* they are faint in lanes 6, 12–13 corrresponding the putative transformants of 'Simon' *(D. chinensis),* but it is missing from lane 15, representing the control non-transformed plant DNA. Lane 1: DNA molecular weight marker, Promega PCR marker.

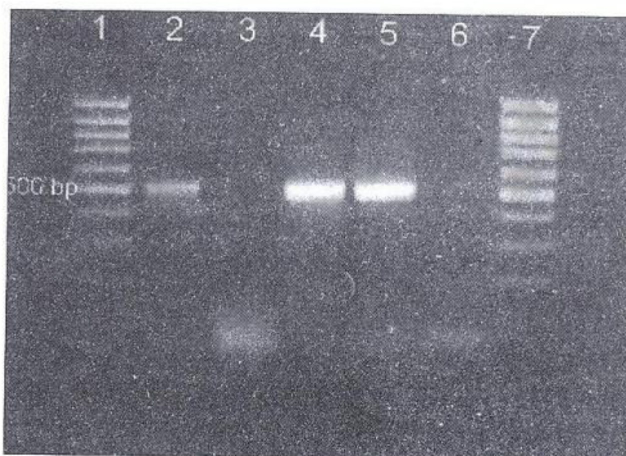

Figure 5 Result of RT-PCR obtained for variety 'Bíbor' *(D. caryophyllus).* mRNA was isolated by Dynal Dynabeads® mRNA DIRECT Micro kit protocol, followed by immediate RT-PCR with *npt II* specific primers. Superscript™ One-Step™ RT-PCR System (GIBCO BRL® Life Technologies™). cDNA was used for cDNA synthesis. *npt II* specific primers were applied for amplification. 1: DNA molecular weight marker (Fermentas 100-1000 bp ladder), 2: RT-PCR product of one of the transformants 'Bíbor' plant mRNA, the expected 503 bp DNA fragment can be seen, 3: non-transformed 'Bíbor' plant sample, 4–5: PCR product of pBI 121 plasmid DNA , 6: other transformant of 'Bíbor' variety, showing no amplification product, DNA molecular weight marker (Fermentas 100–1000 bp ladder).

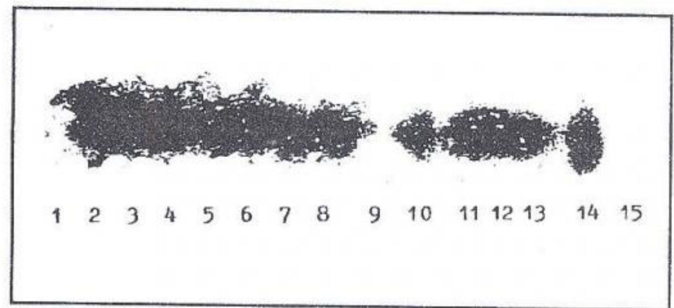

Figure 4 Southern hybridisation with digoxigenin labeled *npt II* probe. 1: DNA molecular weight marker, 2–5 putative transgenic plant DNA samples of variety 'Bíbor' *(D. caryophyllus)* 6–14: DNA samples of putative transformants of variety 'Simon' *(D. chinensis),* 15: non-transformed control plant DNA.

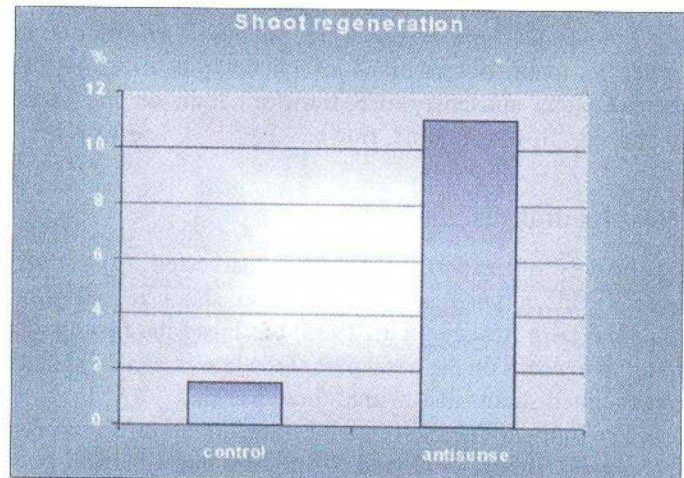

Figure 6 Comparison of shoot regeneration from transformed (antisense) and non-transformed (control) leaf explants of Bíbor carnations on hormone free MS medium.

Figure 7 Growing transgenic 'Bíbor' *(D. caryophyllus)* plants (120) in glasshouse of Óbuda Horticultural Laboratory (Budapest) before flowering.

(Figure 6.). Further experiments have already been started to test the effect of ethylene production on shoot regeneration in the different transformation procedures.

Transformant and non-transformant plantlets were potted *(Figure 7)* in glasshouse in Óbuda Horticultural Laboratory (Budapest) and are being observed to determine what phenotypic changes can be the consequences of integration of antisense ACS cDNA. Evaluation of the effect of changed ethylene production on commercial traits, e. g. flowering time, petal senescence, vase life are in progress.

Acknowledgement

The research is supported by National Science Foundation of Hungary (OTKA TO 26502) and by the Hungarian Ministry of Education (FKFP 0494).

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