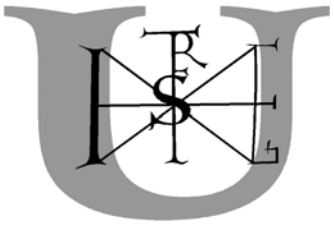

SZENT ISTVÁN UNIVERSITY

GÖDÖLLŐ

Inhibition of the ethylene-biosynthesis in carnation by apple derived antisense ACC-synthase gene

Ph.D. thesis

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1. INTRODUCTION AND AIMS

1. 1. TOPICALITY OF THE THEME

Ethylene is responsible for several plant physiological processes and has a regulatory role almost in all stages of the plant development from germination to senescing. It accelerates the development and maturation of fruits and flowers, causing wilting of the petals. It is of great practical importance in the induction of simultaneous flowering and fruit ripering. The clarification of the steps of the ethylene biosynthesis as well as the inhibition of the gene-expression by antisense RNA enabled the modification of the senescing and the fruit and crop maturation using molecular methods in plant kingdom.

The antisense RNA was discovered in the *Escherichia coli* during the investigation of the replication of the colE1 plasmid. The antisense RNA hybridizes with the sense RNA strand and because this duplex is unable to fulfil its primer function, the DNA replication is inhibited. In addition, the antisense RNA molecules also regulate the transcription and not only in prokaryotes, also in eukaryotes: theoretically the transcription of any kind of genes can be prohibited by antisense RNA molecules. However, the knowledge of the biochemical synthesis ways and the isolation of the appropriate structure genes are a prerequisite. Wide examinations showed also that genes which are isolated from different species and have the same function (heterolog genes) can generate the antisense inhibition in different plant species.

Flowers play an important role in our life. There is always a particular occasion (birthday, name-day, family-celebrations: wedding-day or marriage) where flowers are important accessories. Carnation is one of the most sold cut flowers both on the world and on the Hungarian market.

As a result of the technical improvement of the transportation and the cold-storage (cooled camion, aeroplane), cultivation of the carnation has been changed in the ’70 in the U.S., the previous growing areas swung to the south. Nowadays 95% of the cut carnation is imported from South-America. Similar tendency can be observed in Europe with approximately 20 year’s delay. The inland cultivation decreased dramatically in the industrialized, developed countries (Germany, England, and France), the most part of the necessary amount is imported from South-Europe, South-America and Africa. The situation is similar in Hungary. Moreover, after fully extinction of the importation customs duty in 2002, the cultivation of greenhouse cut flowers has increasingly been deemphasized. Therefore it has great importance to produce such

new varieties which have an particular character and so can compete with cheaper imported products.

Like in the case of all cut flowers, also in the case of the carnation, which is a typically ethylene-sensitive plant, one of the most important quality value characteristics is the length of the vaselife, which is significantly determined by the ethylene.

In order to increase the length of the vaselife, several methods have been elaborated: storage at low temperatures (between -0,5 and 0°C) and at pressure of 90-120 Pa or soaking the flowers in warm water bath after cutting. Among the heavy metal ions, the cobalt and the nickel is able to hinder the ACC (1-aminocyclopropane-1-carboxylic acid) ⇒ ethylene transformation. The silver ion, the carbon dioxide and the potassium permanganate bind the ethylene receptors. The 1-MCP (1-methylcyclopropene), the 3,3-DMCP (3,3 dimethyl cyclopropene), the AOA (amino acetic acid), the AVG (aminoethoxyvinilglycin) and the CHX (cycloheximyde) also hinder the ethylene biosynthesis. During in vitro vulture these enhance the short regulation as it was observed with several plant species.

Another important quality determining characteristic of the carnation is the colour of the petals. The Company Florigene produced a transgenic carnation with blue flowers according to customer demand. Customers looks for fragrant flowers; however, there is a negative correlation between the scent of the flowers and the length of the vaselife. Therefore, it is of great importance to produce such lines in which negative correlation between scent and vaselife does not exist and they are both excellent in characteristics.

Production of transgenic plants can mean a new plant breeding way for the ornamental plant cultivation. Using this method, economically important quality characteristics can be improved like the length of the vaselife and the colour. Researchers already produced carnation and rose transformed with antisense and sense ACC oxidase and synthase genes, prolonging the vase life petunia, carnation and gerbera were transformed with antisense chalcone synthase, and carnations are already commercialized both in the U.S. and the EC.

1.2. OBJECTIVES

Our experiments aimed at the following:

- to produce transgenic carnation, in which genome one of the genes responsible for ethylene production – the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase deriving from the apple – is introduced in “antisense” orientation;

- to reduce of the amount of the ethylene produced by the cut flower as a result of the transformation with the antisense gene;
- to increase the length of the vaselife and the shoot regeneration ability as a result of the reduced ethylene production.

2. MATERIAL AND METHODS

The ACS used for the transformation was isolated from McIntosh apple (MdACS2 – gene bank accession number: U73815). The insert was introduced into the pBI121 vector in antisense orientation using subcloning steps in order to produce a vector suitable for the transformation.

In our transformation experiments – where we compared two methods –LBA4404 *Agrobacterium tumefaciens* strain was used in which the modified pBI121 vector was introduced by using the 'triparental mating' method.

After preliminary regeneration experiments on regeneration from based on the leaf the economically valuable, sweet-smelling 'Bíbor' variety was selected for the transformation. In addition to the apple derived carnation were with genes indifferent in terms of the ethylene synthesis, such as the GUS gene, the cowpea inhibitor gene (pCpTi) and genes which play a significant role in the carbohydrate biosynthesis (6-PF-2-K, Fru-2,6-P_2-ase). These lines scored as transgenic controls regenerants obtained after all the besides transformation steps except these the *Agrobacterium* infection were appled as control as well.

Transgene integration was proven with molecular analysis (PCR, Southern hybridisation, RT-PCR). DNA from green plants regenerated on selective medium containing kanamycin (150 mg/l).

Transformant and control plants were planted in the greenhouse of the Óbuda Horticultural Laboratory. For sake of comperison carnation grown in the Óbuda Horticulture werealso potted at the same time as transgenic lines. Drop watering was applied normal greenhouse circumstances. No morphological differences could be observed between the transformant and control flowers.

The length of the vaselife from June 1999 to March 2004 on 22°C was estamited. In order to compare the length of the vaselife we took samples which were at the same flowering stage (the petal made an angle of 90º with the sepal) and had the same stem length (50 cm). Transgenic and control flowers werw always compared with horticultural control plants (KK) at the same flowering stage. Because of the different environmental conditions of the diverse seasons of the year, the length of the flowering period was different in the flowering cycles.

Therefore we determined the length of the vaselife in a relative value; this means that we took the difference between the transgenic and the corresponding horticultural control plant into consideration. In calculation of the length of the vaselife, the first day meant the sampling while the last, when 50% of the petals wilted.

Rate of ethylene production was mesaured at the Faculty of Horticultural Sciences, Department of Chemistry of the Szent István University (present Corvinus University). Flowers with a stem of 8 cm were cut at the same flowering stage as for measuring of the length of the vaselife. Since the site of ethylene analysis the sampling and the detection were different.first we had to exclude the production of the stress ethylene evoked by the sampling and the transportation from Óbuda to the laboratory of Department of Chemistry. For this purpose plants were kept in water 24 hours, after they were put moved them into a special measuring-jar. The jars contained stop rubber septum, through this 1% exogenous ethylene was added and gas sample were taken. After a 24 hours treatment we took out the flowers, ventilated them for 24 hours and put them into the measuring-jar again. After 24 hours we took gas samples. The amount of the produced ethylene was detected with a GC 6000 gas chromatograph using aluminium colon. The data measured in different time points depended on environmental impacts (temperature, light conditions) due to the different flowering time. Therefore, the ethylene results were presented within a treatment as the percentage compared to control, non transgenic carnation.

To be able to express objectively the observations, that transgenic Bíbor plants are less fragile, than the controls. In the course of the stem braking experiments the flowers were cut at the 8. node at flowering described for vase life and ethylene measuremed. For the modified tensile strength measuring-machine (type FM-250 produced in the former NDK). The perpendicular force in Newton neede for the breaking of the stem supported in 5 cm span at every node.

3. RESULTS

In the experiments where we compared the shoot regeneration, 24% of the leaves of the control plants regenerated shoot on a medium containing hormone, while this value was 49% in case of plants modified in ethylene biosynthesis. To prove that the increased shoot regeneration is a consequence of the modified ethylene biosynthesis, carnation plants harbouring other transgenes (GUS, pCpTi, 6-PF-2-K, Fru-2,6-P_2-ase) – which are neutral regarding the ethylene biosynthesis were also involved– into the investigation. The results showed that the regeneration in case of the

ethylene natural transforments were the same as in case of the control plants, proving that the increase of the shoot regeneration was caused by the down regulation of the ethylene biosynthesis.

We examined the vaselife of the planted carnation plants for 5 years. Our results show that control plants flowered 1 or 2 days shorter than the horticultural control flowers, or as long as these plants, the length of the vaselife of the transgenic lines extended even by six days longer at 22ºC storage temperature. The length of the vaselife increased in case of 40% of the plants containing the antisense ACC synthase gene. We detected a strong correlation (R^2=0,79) between the increase of the length of the vaselife and the decreased ethylene production, a longer vaselife was accompanied by decreased ethylene production.

Carnation 'Bíbor' is a variety which is susceptible to stem breaking. During the care we observed that the stem of the transgenic individuals showed less inclination to break, therefore we measured the stem strength. As a result of the breaking tests we could prove that the stem of transgenic individuals are really more strong, they can be characterized with higher "breaking index" compared with control plants and at the same time there was no statistically detectable difference between'Improved White Sim' which is no fragile variety. Between the improvement of the stem breaking and the prolonged vaselife characteristics a positive, between the amount of the produced ethylene and the stem breaking a negative correlation could be proven.

As a result of our experiments we could improve the quality characteristics of the economically valuable, fragrant carnation variety in such a way, that its other important characteristics have not changed. On the bases of these, the transgenic modification of the ethylene biosynthesis can be a new plant breeding tool in the improvement of the disadvantageous traits (such as short vaselife and inclination to stem breaking) of the existing and valuable carnation varieties.

3.1 NEW SCIENTIFIC RESULTS

1) We achieved the molecular inhibition of the ethylene biosynthesis in carnation by transformation with the antisense ACC-synthase (CCA) gene derived from the apple.
2) We demonstrated that the inhibition of the ethylene production in the CCA transgenic flowers showed individual variability.
3) We proved that the shoot regeneration ability from leaf explants of transgenic plants is double as high than in the case of non transgenic plants and this effect is not caused by the transformation procedure, but by the decrease of the ethylene production.

4) We showed that the modification of the ethylene biosynthesis prolonged the length of the vaselife of the 'Bíbor' flowers even by six days and the increase of the length of the vaselife is in a close, negative correlation with the produced amount of the ethylene (R^2 =0,96).

5) We proved that the stem strength of the transgenic plants improved. The advance of the stem break correlated with the length of the vaselife (R^2 = 0,79) and is in negative correlation with the ethylene production (R^2 = 0,45).

4. CONCLUSION AND PROPOSALS

We achieved the inhibition of the ethylene synthesis of the carnation by antisense the ACC-synthase cDNA deriving from another species – from apple. ACC-synthase catalyses the SAM→ACC conversation in the ethylene biosynthesis. On the basis of the obtained results it can be stated that after the transformation with the antisense ACC synthase gene the length of the vaselife of carnations can be lengthened even by 6 days at room temperature. Therefore, one of the most important characteristics of the varieties in commercial production can be improved. In our experiments, we were able to improve the length of the vaselife of the economically valuable, fragrante 'Bíbor' variety. Between the scentl and the length of the vaselife there is a negative correlation. Using this method, we were able to produce individuals, in which this disadventegous correlation does not exist.

Oroginally variety Bíbor has an unfavourable trait, its stem are fragile. The inclination to breaking is another important quality characteristic which could be improved as a consequence of the transformation with antisense ACC synthase according to our results. This was an unpredictable new results notiable only during the phanotypic avalution of the transgenic and control plants. The transformation with antisense ACC synthase gene is a new breeding possibility, which can be used for the improvement of two quality characteristics (length of the vaselife and stem break) of a valuable variety. The transgenic carnation, modified in ethylene biosynthesis can be competitive in comparison with the imported carnation which floods Hungary and other European countries by satisfying the demands (such as fragrance, long lasting flowers, non breaking stem). Furthermore, the increase of the length of the vaselife could mean an advantage both for exporting and importing countries as a result of the decrease of transport and storage losses.

Increased effectiveness of the in vitro shoot regeneration is an other important result due to down-regulation of ethylene biosynthesis. Utilizing this can be beneficial for both for gene technological and micropropagation aspects speeding up the breeding process and marketing.

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