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Growth habit and photosynthetic activity of shoot cultures of *Medicago sativa* L. transformed with the *oryzacystatin II* gene

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Abbreviations:
C-3 Control non-transformed shoot culture
OC-II-1 Transformed shoot culture, clone 1
OC-II-16 Transformed shoot culture, clone 16

Key words: alfalfa, chlorophyll fluorescence, CO_2 influx, genetic transformation, *Medicago sativa*, morphology, *oryzacystatin II*

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Abstract

Background and Purpose: *Introduction of a foreign gene into plant genome may induce morphological and physiological alterations in transgenic plants. The purpose of this study was to assess the impact of gene introduction on morphology and photosynthetic activity of two transgenic alfalfa shoot cultures.*

Material and Methods: *In vitro maintained shoot cultures of alfalfa (Medicago sativa L. cv. Zaječarska 83) that were transformed with the oryzacystatin II (OC-II) gene and propagated on growth regulator-free medium were subjected to analysis of morphological characteristics and photosynthetic activity. For analysis of morphological characteristics, the length of the main axis, the number of axillary and adventitious shoots, total number of leaves and the number of senesced leaves as well as plant dry mass were determined. Net photosynthetic rate was determined as CO_2 influx using a LI-6200 closed photosynthesis system. Photosynthetic function was assessed as the rate of basic chlorophyll fluorescence and determined with a Plant Stress Meter by method of induced fluorometry. Chlorophyll content in leaf samples was determined spectrophotometrically.*

Results: *The most striking feature of transformed cultures was reduced apical dominance and the absence of adventitious roots. In comparison with control, main axis length was also reduced. In addition, a general decrease in photosynthesis in transgenic shoots was also observed.*

Conclusion: *Both morphology and the key processes in photosynthesis were modified in transgenic shoots. However, the fact that transgenic plants carry a gene that confers pest resistance gives potential agronomic value to at least some of the clones whose morphological characteristics and photosynthetic acivity were minimally disturbed.*

INTRODUCTION

The production of transgenic plants involves the delivery of a foreign gene of interest and a selectable marker gene that usually encodes either herbicide tolerance or, more often, resistance to antibiotics. However, introduction of such single-gene traits may produce more complex agronomic traits such as altered growth habit and photosynthetic activity *(1)*. Therefore, a thorough evaluation of whole plant physiology and performance should always be considered to exclude possible negative interactions with endogenous plant components or the occurrence of unusual

phenotypes created either by introduction of the transgene into plant genome (position effects) or its expression.

Alfalfa (*Medicago sativa* L.) is an important forage legume that is widely grown in a number of regions throughout the world because of its ability to fix atmospheric nitrogen and its high protein content. Molecular breeding of alfalfa, as well as other *Medicago* species, can be enhanced through the application of transgenesis. Oryzacystatin II (OC-II) is a cysteine proteinase inhibitor from rice in the phytocystatin family of proteinase inhibitors. Phytocystatins are found in a number of plants where they may play a role in defense against pathogens and pests. *OC-II* gene under the regulation of a wound-inducible promoter was introduced into alfalfa in order to increase its resistance to *Phytodecta fornicata* Brüggemann, the field pest that causes serious crop/economical losses in the Balkan peninsula of Europe *(2)*.

Given that photosynthetic pigments have the primary role in the process of photosynthesis, their qualitative and quantitative analyses are frequently carried out in physiological research. Total amount of chlorophyll is a commonly used parameter when comparing plants that differ in their metabolic activities. The relative ratio of Chl *a/b* changes with leaf stress and growth conditions and reflects the relative contents of photosystem I. As criteria for the state of photosynthetic apparatus, the rate of CO_2 assimilation and prompt chlorophyll fluorescence parameters were used. Chlorophyll fluorescence from intact leaves proved to be a reliable, non-invasive method for monitoring photosynthetic events and for judging the physiological status of the plant *(3)*. The ratio of variable (F_v) to maximum (F_m) fluorescence is an important parameter used to assess the physiological state of photosynthetic apparatus. It represents the maximum quantum yield of primary photochemical reaction of photosystem II (PSII). Environmental stresses that affect PSII efficiency are known to provoke a characteristic decrease in the F_v/F_m ratio *(4)*. In plants grown *in vitro* where environmental conditions are generally not considered stressful, alterations in ecophysiological performance are assumed to be a consequence of metabolic changes caused by the insertion of a foreign gene(s) into plant genome.

Demand for producing increased yields by the use of transgenic crops is determined by ever-increasing world human population. Therefore, analysis of plant growth parameters and net photosynthesis in transgenics while still in *in vitro* conditions can serve as a useful tool for an early selection of transformed clones with improved agronomic characteristics. The present study was undertaken in order to assess the impact of gene introduction on morphology and photosynthetic activity of two independently transformed alfalfa shoot cultures.

MATERIAL AND METHODS

Shoot cultures of alfalfa (*Medicago sativa* L. cv. Zaječarska 83) designated as OC-II-1, OC-II-16 and C-3 were used in this study. C-3 was a nontransformed control shoot culture. OC-II-1 and OC-II-16 were transformed with *Agrobacterium tumefacines* strain EHA101 carrying the binary vector pGV-OC-II that contains the *OC-II* (oryzacystatin II) gene under the control of pin2p promoter *(5)*. Single somatic embryos at the cotyledonary stage, initially obtained from immature zygotic embryos that were propagated by recurrent embryogenesis in culture for 3 years were used for transformation. Inoculation methods and establishment of axenic transformed embryogenic cultures were as described by Ninković *et al*. *(6)* and Uzelac *et al. (7)*. Plantlets that regenerated from the control and transformed single somatic embryos were used to establish shoot cultures. Shoot cultures were maintained on Murashige and Skoog *(8)* medium supplemented with 3% sucrose and solidified with 0.6% agar (*Torlak*, Belgrade, Serbia). The cultures were grown in a temperature controlled chamber at 25 ± 2 °C under a 16-h photoperiod. Photosynthetic photon flux density of 31 µmol m^{-2} s^{-1} was provided by white fluorescent tubes (*Tesla*, Pančevo, Serbia, 65 W, 4500 K). Shoot cultures were subcultured to fresh media every 60 days.

For analysis of morphological characteristics, the length of the main axis as well as the number of both axillary and adventitious shoots, total number of leaves and the number of senesced leaves were determined 60 days after transfer to fresh media. Subsequently, plant material was oven-dried at 75 °C until reaching the constant mass and then weighed.

Net photosynthetic rate was determined as CO_2 influx using a LI-6200 closed photosynthesis system (*Li-Cor Inc*. Lincoln, Nebraska, USA). Measurements were conducted with leaf chamber CO_2 concentration of 350 ppm, at the chamber temperature of 20 °C, relative humidity of 55%, and photosynthetic photon flux density above 850 µmol photons m^{-2} s^{-1}. Eight shoots of each culture were subjected to CO_2 exchange measurements. Averaged value of five measurements per plant made evenly over 5 min was taken as a final value for leaf CO_2 exchange per shoot. Net photosynthetic rate is expressed on the leaf area basis that was detected using the »Areameter« software *(9)*.

Photosynthetic function was assessed as the rate of basic chlorophyll (Chl) fluorescence, i.e. the ratio of variable to maximum fluorescence (F_v/F_m) from a dark-adapted leaf (covered with non-transparent plastic bag for 20 min). Steady state fluorescence was determined with a Plant Stress Meter (*Polartech*, Umea, Sweden), by the method of induced fluorometry *(10)*. Leaves were illuminated with low saturating light (100 µmol m^{-2} s^{-1}) for 2 s, after having been in darkness for at least 20 min.

The same leaf samples which were subjected to fluorescence measurements were incubated with dimethyl sulfoxide at 65 °C for at least 2 h until the complete discoloring of the leaf tissue *(11)*. Chl content in the extract was determined using spectrophotometer *Shimadzu UV-160* (Shimadzu, Japan), by the method of Arnon *(12)*.

Break-down one way ANOVA (Statistica for Windows) was used to compare differences among analyzed clones for all investigated parameters at the 0.05 level of significance.

TABLE 1

Plant growth parameters of control, non-transformed (C-3) and transformed (OC-II-1 and OC-II-16) alfalfa shoots after 8 weeks in culture. Results are given as means ± SD of six replicates.

	Main axis length (cm)	No. of axillary shoots	No. of adventitious shoots	Leaf number	Senesced leaves (%)	Plant dry mass (g)
Control	8.76 ± 1.09 a*	2.1 ± 0.2 a	4.4 ± 0.3 a	30.1 ± 2.1 a	11	0.22 ± 0.02 a
OC-II-1	7.05 ± 1.90 c	3.3 ± 0.4 c	2.4 ± 0.5 b	42.4 ± 5.0 b	39	0.21 ± 0.02 a
OC-II-16	2.91 ± 0.29 b	4.3 ± 0.4 b	4.3 ± 0.3 a	35.4 ± 3.7 a	15	0.19 ± 0.02 a

*Statistically significant differences ($P < 0.05$) are marked by different letters

TABLE 2

Leaf photosynthethic parameters of control non-transformed (C-3) and transformed (OC-II-1 and OC-II-16) alfalfa shoots cultured for 8 weeks. Net CO_2 assimilation rate (A), stomatal conductance to CO_2 (g), variable to maximum chlorophyll fluorescence ratio (F_v/F_m), leaf chlorophyll content (Chl total) and chlorophyll a to b ratio (Chl a/b). Results are given as means ± SD of eight replicates.

	A (μmol CO_2 m^{-2} s^{-1})	g (mol m^{-2} s^{-1})	F_v/F_m (relative units)	Chl total (mg g^{-1} dry mass)	Chl a/b (relative units)
Control	9.65 ± 1.04 a*	0.76 ± 0.04 a	0.56 ± 0.02 a	4.1 ± 0.55 a	1.9 ± 0.20 a
OC-II-1	1.38 ± 0.41 c	0.72 ± 0.07 a	0.52 ± 0.03 c	2.5 ± 0.26 c	3.7 ± 0.33 b
OC-II-16	7.47 ± 1.74 b	0.99 ± 0.05 b	0.61 ± 0.02 b	3.0 ± 0.28 b	3.2 ± 0.27 b

*Statistically significant differences ($P < 0.05$) are marked by different letters.

RESULTS AND DISCUSSION

Significant differences in morphological characteristics among the analyzed shoot cultures were observed (Table 1). In control non-transformed cultures (C-3), the main shoot axis, with elongated internodes, developed axillary shoots that were also mostly elongated. Adventitious shoots of variable length and mostly with short internodes were induced from the compact whitish callus formed at the base of the main axis. While non-transformed shoots had a growth habit similar to the field grown individuals, transformed shoots OC-II-1 and OC-II-16 had somewhat altered growth habit. Transgenic cultures had bushy phenotype, with reduced shoot height (Table 1) and internode length (data not shown) when compared with control non-transformed clone. OC-II-1 shoots were similar to control, only somewhat shorter, while the length of OC-II-16 shoots was conspicuously smaller (Table 1). Taking into account that the total plant biomass did not differ significantly among the cultures, such short stem phenotype with higher leaf area, particularly in clone OC-II-16, may be advantageous since much of the increase in yields can be achieved by decreased stem elongation. Van der Vyver *et al. (13)* observed similar decrease in stem elongation in transgenic tobacco plants expressing *OC-I* gene. When grown at a relatively low light intensity, *OC-I* expressing lines were smaller with fewer expanded leaves when compared not only to non-transformed control plants, but also to empty vector controls. This indicated that phenotypic differences were linked to *OC-I* expression and were not due to somaclonal variation consecutive to the transformation or tissue culture processes. On the other hand, Gutierrez-Campos *et al. (14)* demonstrated that transgenic tobacco plants overexpressing *OC-I* gene showed increased height, growth rate and dry weight.

Apart from the length and branching of the main axis, other morphological characteristics such as the total number of leaves and the number of senesced leaves also varied significantly between transformed and control cultures, as well as between two transformed clones (Table 1). In comparison to control and OC-II-16 shoots, which had intensively green leaves, OC-II-1 had considerably greater number of leaves, but many of which were senescent.

Control shoots developed numerous adventitious roots, the average length of the longest among them being 11.8 cm. The most striking feature of analyzed transformed shoots was the absence of adventitious roots, which is a very unfavorable trait from the viewpoint of plant mineral nutrition. In addition, good rooting efficiency, which improves water uptake during early acclimatization leading to rapid plantlet growth, is an important factor that governs the efficiency of transgenic plant recovery *(15)*.

In non-transformed cultures, net CO_2 assimilation rate (A) was lowered, while participation of Chl *b* in total Chl amount was increased when compared to available data regarding greenhouse or the field-grown plants *(16, 17)*. Photosynthetic rate and consequent biomass pro-

duction in plants grown *in vitro* are often limited by low irradiance and low CO_2 concentration during the light period *(18, 19)*. In leaves grown at low light, the relative proportion of chlorophyll *a* decreases due to increased size of photosystem antennae complexes. As chlorophyll *b* is prominent in outer regions of the antenna, the observed decrease in the chlorophyll *a/b* ratio can be accounted for by a relative increase in chlorophyll *b*-containing antennae pigments, as shown by Anderson *et al. (20)*.

In comparison with non-transformed cultures, both transformed cultures had reduced rate of CO_2 uptake and decreased total Chl (Table 2). However, their photosynthetic capacities did not correspond to their morphological features (the main axis length and total leaf number, Table 1). Plants belonging to OC-II-16 had moderately high net CO_2 assimilation rate, A (77% of control plants, Table 2), high stomatal conductance (g) values and the highest F_v/F_m ratio of all examined cultures. Plants belonging to OC-II-1, however, had the lowest A (14% of control plants) and the lowest F_v/F_m ratio, as well as the lowest total Chl content in active (i.e. green) leaves and relatively high percentage of senesced leaves (39%, Table 1). Decrease in fluorescence parameters from optimal values, recorded both in control and in transformed plants, implies decreased photochemical conversion efficiency of PS II, which could be considered as an indicator of stress conditions. Interestingly, very close values of F_v/F_m ratio in non-transformed and OC-II-1 transformed plants were recorded despite remarkable differences in their Chl content, suggesting that the quantum efficiency of photosystem II in plants belonging to clone OC-II-1 was not affected by transformation.

Chl *a/b* ratio in both transformed cultures was significantly greater in comparison to control. However, since the values of variable to maximum Chl *a* fluorescence ratio of transformants were similar to those of control plants, increase in Chl *a/b* ratio is likely to be the consequence of a relative decrease in chlorophyll *b* content. In addition, increase in Chl *a/b* ratio could be caused by disturbed nitrogen assimilation and/or allocation in transformed shoots since changes in nitrogen allocation to different components of photosynthetic system lead to changes in photosynthetic performance *(21)*.

Van der Vyver *et al*. *(13)* showed that maximal CO_2 assimilation rates were decreased in all transgenic tobacco lines as a result of *OC-I* gene expression. However, since the absolute amounts of chlorophyll were increased, there was no photoinhibition in *OC-I* expressing plants. On the other hand, Georgieva *et al. (18)* found that photosynthetic incorporation of CO_2 in the leaves of transformed tobacco plants, although limited due to restricted gas exchange in the vials, showed no difference when compared to control plants. Consequently, the authors presumed that genetic transformation itself had not influenced the photosynthetic characteristics of tobacco plants and that *in vitro* conditions were much more unfavorable for photosynthesis.

The abovementioned alterations of photosynthesis and growth characteristics in both transformed cultures could result from gene insertion rather than *OC-II* expression. Actually, in order to conserve metabolic energy of plants and minimize physiological effects of the transgene on plant metabolism, the *OC-II* gene was fused to a promoter that is inducible by wounding and insect feeding (pinII) *(5)*. However, certain level of *OC-II* gene expression due to *pinII* promoter leakage cannot be excluded since it was clearly shown that growth characteristics as well as key processes of photosynthesis and respiration are modified by constitutive expression of *OC-I* gene *(13, 14)*.

The data presented indicate that observed morphological alterations and lowered photosynthetic capacity in transgenic shoots cannot be accounted for only by unfavorable *in vitro* conditions. Although certain level of *OC-II* gene expression cannot be excluded, it seems more likely that changes in their growth characteristics and photosynthetic activity could result from genetic transformation itself.

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