Arch. Biol. Sci., Belgrade, 60 (1), 9P-10P, 2008 DOI:10.2298/ABS080109PN

IN VITRO MULTIPLICATION OF ORYZACYSTATIN II TRANSFORMED ALFALFA ON GA3-CONTAINING MEDIUM.

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Keywords: Alfalfa, transgenic shoots, gibberellic acid, adventitious root induction

UDC 633.31:577.2:581.3

Transformation of plant genomes with reconstructed proteinase inhibitor (PI) genes has been shown to enhance plant resistance to insects (L e p l e et al., 1995). Several coleopteran pests that commonly use cysteine proteases for protein digestion, as well as nematodes, were inhibited by cysteine PI genes of the oryzacystatin I (*OCI*) and II (*OCII*) PI gene family (A b e and A r a i, 1985; S a m a c and S m i g o c k i, 2003).

Here we described attempt to obtained large-scale propagation of transformed plants of a local alfalfa variety carrying oryzacystatin II (OCII) gene, which will be further used in insect biotest.

Embryogenic cultures of alfalfa (*Medicago sativa* L. cv. Zaječarska 83), subsequently propagated by recurrent embryogenesis for 3 years on the MSB_5 medium (MS salts + B_5 vitamins + 3% sucrose), were inoculated with *Agrobacterium tumefaciens* strain EHA101, carrying the binary vector that contains the rice *OCII* cDNA (K o n d o et al., 1990) fused to the *pinII* gene promoter (S a m a c and S m i g o c k i, 2003), CaMV35S: *GFP* reporter gene and a selectable marker gene nos:*nptII* (U z e l a c et al., 2007; N i n k o v i ć et al., 2007). Transformed embryogenic clones were selected on MSB_5 medium containing 50 mgl^{-1} kanamycin (Km). To induce germination of selected somatic embryos sucrose concentration was increased up to 40 g. Multiplication of regenerated control and transformed plantlets were tested on MS medium supplemented with different plant growth regulators: BA (6-benzyladenin), NAA (naphthaleneacetic acid) and GA_3 (gibberellic acid), using shoot tips as explants. Five explants were subcultured per flask. Shoot multiplication was calculated on the basis of production of new lateral shoots suitable to be used as explants for subculturing. For rooting ability single shoots were transferred to MS medium supplemented with indole-3-butyric acid (IBA) in the following concentrations: 0.1, 0.25, 0.5 and 1.0 mg; an auxin-free MS medium was used as control. Rooting percentage was determined after seven weeks. Cultures were grown at 25±2˚C under 16 h / 8 h photoperiods. Photosynthetic photon flux density of 31 µmol m^{-2} s^{-1} was provided by white fluorescent tubes (Tesla, Pančevo, Serbia, 65 W, 4500 K). Basal internodes of transformed and untransformed shoots were fixed in FAA (formalin: acetic acid : ethanol, 10:5:85). Sections (10 µm thick) were stained with haematoxylin and photographed using a Leitz MRB photomicroscope (Leica, Germany). LSD test was used for statistical analyses.

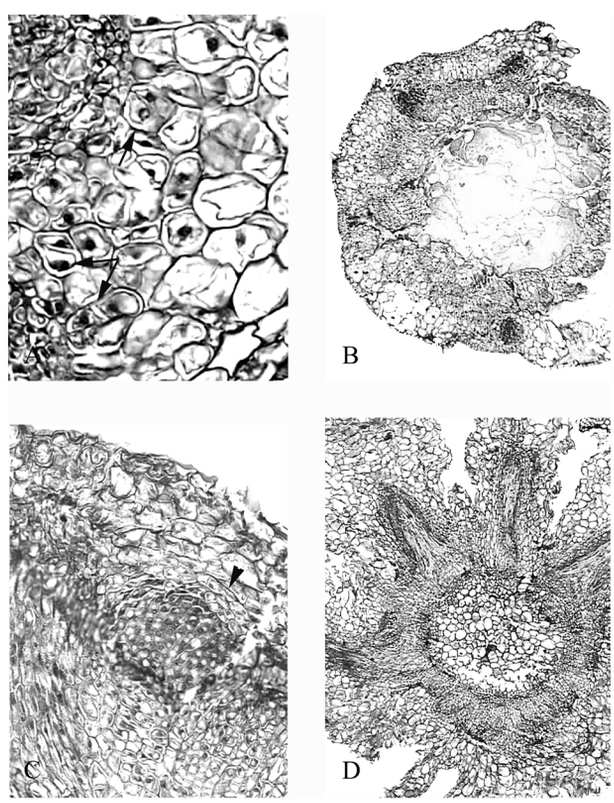

Fig. 1. Histological analysis of adventitious root initiation. (A) Transverse section of the basal internode of transgenic OCII-16 shoot cuttings. Note division of cortical parenchyma cells adjacent to the vascular cylinder (arrows). (B) Transverse section of the basal internode of control shoot cuttings, with several meristematic centers. (C) Transverse section of the basal internode of transgenic OCII-16 shoot cuttings. The meristematic center is composed of densely stained meristematic cells with prominent nuclei. Note the organized root cap directed towards the stem periphery. (D) Transverse section of the basal internode of transgenic shoot cuttings. Emergence and elongation of adventitious roots which have vascular connections with the central cylinder of the stem.

Table 1. Number of lateral shoots/explant in control and OCII transformed lines after 30 days on multiplication medium (MS medium supplemented with 0.2 mgl-1 BA, 0.5 mgl-1 BA, 4.0 mgl-1 BA+ 0.1 mgl-1 NAA, or 7.0 mgl-1 GA_3). Means±SE, n=20. Means followed by the same letter were not significantly different according to the LSD multiple range test at $p<0.05$.

Clone	MS0	BA 0.2	BA 0.5	BA 4.0 NAA 0.1	GA_3 7.0
Control	0.70 ± 0.33a	0.75 ± 0.25a	1.25 ± 0.48a	1.00 ± 0.00a	**2.50 ± 0.15b**
OCII-1	1.25 ± 0.30	0.80 ± 0.37	2.20 ± 0.80	1.80 ± 0.68	**2.35 ± 0.46**
OCII-2	1.40 ± 0.44	1.80 ± 0.25	1.20 ± 0.13	1.40 ± 0.37	**2.10 ± 0.41**
OCII-3	1.50 ± 0.34	1.60 ± 0.54	1.40 ± 0.45	1.60 ± 0.45	**3.50 ± 0.42**
OCII-4	0.60 ± 0.40a	0.45 ± 0.22a	0.45 ± 0.22a	0.00a	**2.00 ± 0.36b**
OCII-5	1.50 ± 0.42	1.40 ± 0.34	1.60 ± 0.16	1.80 ± 0.13	**1.83 ± 0.40**
OCII-15	1.70 ± 0.24	1.80 ± 0.21	2.00 ± 0.21	2.60 ± 0.45	**3.50 ± 0.49**
OCII-16	1.40 ± 0.29	1.40 ± 0.34	0.40 ± 0.26	1.60 ± 0.37	**1.30 ± 0.28**

From the two hundred embryos that were infected 28 embryogenic clones (lines) survived the Km selection pressure conferred by the *npt*II gene. Transformed embryogenic lines differed in color, size and numbers of germinated embryos (2 - 8 germinated embryos/g tissue). Plantlets were regenerated from 11 of the 28 lines. Presence of the inserted genes in transformed lines was confirmed by PCR analysis (data not shown). In an attempt to obtained large scale propagation of the regenerated plants, different concentration of growth regulators, applied to MS medium, were tested (Table 1). In many reports shoot multiplication was found to depend mostly on the concentration of BA (Vinterhalter and Vinterhalter, 1992, 1998). In our case the presence of BA in two different concentration, as well as in combination with auxin, did not influence the number of lateral shoots produced per explant what is in accordance with privious report obtained on *Medicago sativa* (Pupilli et al., 1992). Unexpectedly, MS medium containing 7.0 mgl^{-1} GA_3 had been shown as a more stimulated medium for shoot multiplication in control (Table 1). Proliferation rate of transformed plants was not statistically different from cytokinin containing medium, except in line OCII-4, but shoots regenerated on GA_3 were looking healthier (Table 1, note line OCII-16). That was a reason of using GA_3 containing medium for further work. The number of shoot produced per explant was related to the clone used as explant source. The best proliferation rate was achieved in OCII-15 line (Table 1). GA_3 also seems to positively affect the rooting of this line. Exogenous applied GA_3 promotes both initiation and differentiation of somatic embryos of *Medicago sativa* (Rudus et al., 2000) but, according to our knowliege, the promotive effect of singular supplement of GA_3 on shoot multiplication was not reported earlier.

In further studies rooting response to indole-3-butyric acid concentrations ranging from 0.1 to 1.0 mg was determined in control and two low responsive transformed lines (OCII-4 and OCII-16). In comparison with control, where rooting of about 100% was induced over the entire range of IBA concentrations tested, rooting of transformed shoots was low (4.5% – 21%) and with quite irregular frequency. Cytological examination revealed that the adventitious roots were of endogenous origin in both nontransformed and transformed shoots (Fig. 1A-C). Neither the place of origin nor further development (Fig. 1D) of adventitious roots differed among the clones, but their emergence on the internode surface was observed earlier in nontransformed shoots. That is in a accordance with preliminary results which showed that most efficient protocol for rooting of OCII shoots is prolong exposure to GA_3 containing medium. It was shown that medium supplemented with GA_3, facilitated internodes and shoot elongation, latter being an important step in successful micropropagation because elongated shoots could be rooted easier (De Klerk, 2002).

In conclusion, GA_3-containing MS medium can be successfully used for multiplication of OCII transformed, as well as non-transformed alfalfa plants.

Acknowledgment – This work was supported by the Serbian Ministry of Science Grant No. 143026.

References: Abe, K., and S. Arai (1985). *Agric. Biol. Chem*. **49,** 3349-3350. - De Klerk, G. J. (2002). *In Vitro Cell. Dev. Biol*. **38**, 415-422. - Kondo, H., Abe, K., Emori, Y., and S. Arai (1990). *FEBS Lett.* **278**, 87-90. - Leple, J. C., Bonade-Bottino, M., Augustin, S., Pilate, G., Le Tan, V. D., Delplanque, A., Cornu, D., and L. Jouanin (1995). *Mol. Breed.* **1,** 319-328. - Murashige, T. and F. Skoog (1962). *Physiol. Plant.* **15**, 473-497. - Ninković, S., Miljuš-Đukić, J., Radović S., Maksimović, V., Lazarević, J., Vinterhalter, B., Nešković, M., and A. Smigocki (2007). *Plant Cell Tiss. Org. Cult*. **91**, 289-294. - Samac, D. A. and A. C. Smigocki (2003). *Phytopathol.* **93**, 799-804. - Uzelac, B., Ninković, S., Smigocki, A., and S. Budimir (2007). *Biol. Plant.* **51,** 1-6. – Vinterhalter, D., and B. Vinterhalter (1992). *Arch. Biol. Sci*. Belgrade **44**, 177-186. - Vinterhalter, B., and D. Vinterhalter (1998). *Arch. Biol. Sci*. Belgrade **50**, 177-182.