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Full Length Research Paper

Agrobacterium-mediated transformation of two Serbian potato cultivars (*Solanum tuberosum* L. cv. Dragačevka and cv. Jelica)

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An efficient protocol for *Agrobacterium*-mediated transformation of Serbian potato cultivars Dragačevka and Jelica, enabling the introduction of oryzacystatin genes *OCI* and *OCII*, was established. Starting with leaf explants, a two-stage transformation protocol combining procedures of Webb and Wenzler provided high shoot regeneration efficiency: 84 - 89% for Dragačevka cultivar and 60 - 68% for Jelica cultivar as compared to 76 - 86% for Desiree, the most frequently used cultivar in transformation experiments. PCR analysis of a small sample of putative transformants showed a *nptII* integration frequency of 90.9, 76.9 and 86.4% for Dragačevka, Jelica and Desiree, respectively. Regeneration and transformation efficiency was strongly genotype-dependent.

Key words: *Agrobacterium tumefaciens,* oryzacystatin, *Solanum tuberosum* L.

INTRODUCTION

Following the first trials performed by Ooms et al. (1986), a number of transformation protocols have been proposed and well elaborated for all important potato cultivars. Some of them like those using leaf (De Block, 1988; Visser et al., 1989), stem (Visser et al., 1989; Newell et al., 1991; Beaujean et al., 1998), or tuber discs explants (Sheerman and Bevan, 1988; Hoekema et al., 1989), are still in use as the basic, starting transformation protocols.

Most of the transformation studies were conducted with the intention to transfer genes/traits expected to increase the resistance of potato against predators and pathogens (Wierenga et al., 1996; Hefferon et al., 1997; Lyapkova et al., 2001; Urwin et al., 2001; Naimov et al., 2001; Chue et al., 2004) or to modify common metabolic pathways, such as starch and sucrose synthesis (Wolters et al., 1998; Edwards et al., 1999).

Although for all major potato cultivars, transformation is considered as routine, there are still some less amenable genotypes (Banerjee et al., 2006; Gustafson et al., 2006), that require further improvement of transformation methods. Transformation efficacy in potato is actually highly genotype-dependent, which is the main reason for the existence of many different protocols (Vinterhalter et al., 2008b).

For our popular cultivars Jelica and Dragačevka, we used the existing transformation protocols of Webb et al. (1983) and Wenzler et al. (1989) which were combined and slightly modified. Dragačevka and Jelica are well known for their high and regular yields, universal cooking features and good nutritional quality. However, they are higly susceptible to insect herbivores.

The transgenes used in this study, rice cystatins cDNAs: OCI (Abe et al., 1987) and OCII (Kondo et al., 1990), showed potential in controlling pests relying on cysteine proteinases for digestive protein hydrolysis (Leple et al., 1995; Samac and Smigocki, 2003; Ribeiro et al., 2006;

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Abbreviations: **MS**, Murashige and Skoog; **OCI,** oryzacystatin I; **OCII,** oryzacystatin II; **CIM**, callus-induction medium; SIM, shoot-induction medium; **BA,** benzylaminopurine; **NAA,** naphthalene acetic acid; **GA3**, gibberellic acid; **2,4-D**, 2,4-dichlorophenoxyacetic acid**; kinetin**, N6-furfuryladenine.

Ninković et al., 2007). Thus, introduction of oryzacystatin genes into Dragačevka and Jelica genome could potentially enhance their resistance to predators (Colorado potato beetles, etc.) or pathogens (*Erwinia carotovora*, etc).

MATERIALS AND METHODS

Plant materials

Dragačevka and Jelica were obtained from Potato Research Center, Guča; Serbia, and Desiree, used here as a control cultivar, were obtained from PKB INI Agroeconomic institute, Belgrade. Shoot cultures were established from sprouts and propagated *in vitro* by monthly subculture of single-node stem explants on basal MS medium containing Murashige and Skoog (1962) mineral salts, Linsmaier and Skoog (1965) vitamins, 3% sucrose and 100 mg/l myoinositol solidified with 6 g/l agar. Cultures were grown under controlled conditions in a growth room with a 16/8 h light/dark photoperiod, 47 µmol m^{-2} s^{-1} irradiance at the culturing surface provided by 58 W fluorescent tubes and temperature 25 ± 2°C.

Bacterial strains and transformation vector

Three *Agrobacterium tumefaciens* strains EHA101 carrying pGV-GFP-OCII-4.2A7, pGV-GFP-OCI-3.8(19) or pGV-GFP-OCI-3.1D-16 plasmids were used for genetic transformation (Samac and Smigocki, 2003; Ninković et al., 2007). Plasmids were carrying the rice OC-II, OC-I sense or OC-I antisense cDNAs, respectively, fused to the pin2 promoter, as well as 35S-*GFP* reporter gene and nos-*nptII* selectable gene.

Transformation and plant regeneration

Leaves excised from 4-week old *in vitro* maintained shoot cultures were used as explants for transformation. Explants (~10mm^2 lamina) were incubated 5-10 min in an overnight bacterial suspension (~10^8 bacterial cells/ml), blotted dry on a filter paper and cultured on CIM (callus induction medium) according to Webb et al. (1983): MS supplemented with 3% sucrose, 2 mg/l BA and 0.2 mg/l NAA. After 3 days of co-cultivation, explants were washed with sterile water containing cefotaxime (1000 mg/l), dried on filter paper and transferred onto CIM supplemented with 50 mg/l kanamycin and 300 mg/l cefotaxime. After 4 weeks, explants were transferred on SIM (shoot induction medium) according to Visser et al. (1989): MS supplemented with 1.5% sucrose, 2 mg/l BA and 5 mg/l GA_3 with 300 mg/l cefotaxime and 50 mg/l kanamycin. Explants were regularly subcultured to fresh SIM medium in two-week intervals until shoots were regenerated.

Individual shoots reaching 10 - 20 mm in length (only one shoot per explant) were excised and transferred to plant growth regulator-free MS medium supplemented with 300 mg/l cefotaxime and 50 mg/l kanamycin for rooting. Plantlets with well developed roots were multiplied and used further for histological and molecular analyses.

PCR analysis

Genomic DNA was isolated from putative transformants after 7 - 9 subcultures on Km-containing medium according to Zhou et al. (1994). The presence of the transferred *nptII* gene was confirmed by PCR analysis using specific primers (5'- ATGAT-TGAACAAGATGGATTGCACGCAGG-3' and 5'-GAAGAACTCGT-CAAGAAGGCGATA-3'), which delimit an 800-bp fragment from the *nptII* coding region. The conditions employed for its amplification were 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. PCR and DNA gel analysis followed standard procedures (Sambrook et al., 1989).

Light microscopy analysis

For light microscopy, the material was fixed in formalin: acetic acid: ethanol (10:5:85) and embedded in paraffin. Sections (10 - 15 µm thick) were stained with haematoxylin.

Glasshouse cultivation

Thirty replicates of two randomly chosen PCR positive lines for all three constructs X three cultivars plus appropriate non-transformed controls were planted out in a compost mix containing peat: perlite: sand (1:1:1) and grown under glasshouse conditions: 25/18°C day/night temperature and 16 h day-light regime.

RESULTS AND DISCUSSION

Shoot regeneration capacity

Our main interest was to evaluate a need for separate callus (CIM) and shoot regeneration (SIM) media, as well as effects of BA, Kin, 2,4-D and GA_3 on shoot regeneration of Dragačevka and Jelica cultivars. Few simple shoot regeneration procedures employed for potato leaf explants by different authors were investigated first (Table 1). It is apparent that treatments B and C (Table 1) supported only abundant and fast proliferation of undifferentiated callus as a consequence of high 2,4-D concentration. In the BA + NAA treatments, moderate callus proliferation was accompanied by shoot regeneration that was highest in treatment D. The two-stage regeneration treatments D and E supported higher callus proliferation and shoot regeneration than the single-stage treatment A. This difference was most pronounced when comparing treatments A and D, containing basically the same plant growth regulators, only combined in a different way and sequence of application. Anyhow, the first medium used for cultivation of leaf explants seems to have a crucial effect on the sub-sequent morphogenesis. Thus the 2, 4-D containing CIM medium in treatment B prevented the otherwise stimulatory effect of SIM medium on shoot regeneration (Treatment B versus D).Treatment D, consisting of CIM medium according to Webb et al. (1983) and SIM medium of Wenzler et al. (1989), was selected for shoot regeneration in all further transformation studies, since it provided high shoot regeneration in both Serbian cultivars and Desiree used here as a control.

Transformation studies

Within 7 - 10 days, after bacterial infection, leaf explants cultivated on CIM-D medium supplemented with 50mg/l kanamycin and 300 g/l cefotaxime manifested callus

Table 1. Callus proliferation and shoot regeneration of potato leaf explants. Subculture duration: Treatment A, 6 weeks; treatments B, C, D and E, 4 weeks. *Number of explants: A , 100; B, C, D, E, 50.

Plant growth regulators (mg /l)		Treatments	Parameters	Desiree	Dragačevka	Jelica
CIM	SIM					
BA 2.0 + NAA 0.2 + GA_3 10.0	same as CIM	A	Callusing (%)	41.0	46.0	28.0
			shoots per explant* ± SE	2.2 ± 0.4	2.6 ± 0.5	1.2 ± 0.2
Kin 0.25 + 2,4-D 5.0	BA 2.0 + GA_3 5.0	B	Callusing (%)	100	100	100
			shoots per explant* ± SE	0	0	0
	Zea 1.0 + NAA 0.1 + GA_3 0.1	C	Callusing (%)	100	100	100
			shoots per explant* ± SE	0	0	0
BA 2.0 + NAA 0.2	BA 2.0 + GA_3 5.0	D	Callusing (%)	89.0	92.0	57.0
			shoots per explant* ± SE	8.8 ± 0.6	9.1 ± 0.8	5.1 ± 0.4
	Zea 1.0 + NAA 0.1 + GA_3 0.1	E	Callusing (%)	58.0	62.0	50.0
			shoots per explant* ± SE	4.2 ± 0.6	5.4 ± 0.6	3.8 ± 0.4

CIM A, CIM of Wenzler et al. (1989); CIM B,C, media for intensive callus proliferation, Anstis and Northcott (1973) and Bajaj and Dione (1967); CIM D,E, CIM of Webb et al. (1983). SIM A, same as CIM A; SIM B, D, SIM of Visser et al. (1989), similar to SIM of Wenzler et al. (1989) containing 2x less GA_3; SIM C, E, CIM of de Block (1988) supplemented with GA_3 0.1 mg/l.

Table 2. Transformation frequency and shoot bud regeneration efficiency of leaf explants.

Control	Number of explants that developed calli (%)*	Number of explants that developed buds (%) **	Number of buds/explants
Desiree	97.0 ± 0.3 e	90.0 ± 0.9 fg	8.7 ± 0.6 d
Dragačevka	98.0 ± 0.4 e	92.0 ± 0.4 g	9.9 ± 0.7 d
Jelica	82.0 ± 0.4 b	58.0 ± 0.9 a	4.9 ± 0.4 ab
OCII			
Desiree	86.0 ± 0.4 bc	82.0 ± 0.9 de	6.3 ± 0.5 bc
Dragačevka	90.0 ± 0.8 cd	84.0 ± 0.4 def	7.0 ± 0.4 c
Jelica	74.0 ± 0.4 a	60.0 ± 0.4 ab	4.1 ± 0.7 a
OCI			
Desiree	90.0 ± 0.3 cd	86.0 ± 0.4 fg	9.0 ± 0.7 d
Dragačevka	94.0 ± 0.4 de	86.6 ± 0.5 fg	9.2 ± 0.6 d
***OCI* (*antisense*)**			
Desiree	86.0 ± 0.4 bc	76.0 ± 1.2 cd	6.4 ± 0.4 bc
Dragačevka	92.0 ± 0.4 d	89.3 ± 0.9 fg	8.5 ± 0.4 d
Jelica	72.0 ± 0.4 a	68.0 ± 0.4 bc	5.9 ± 0.7 abc

Number of explants = 100 for control while 200 for each separate transformation experiment. Results are expressed as mean ± SE. Within columns, means with different letters are significantly different according to Duncan's multiple range test ($P < 0.05$). *After 4 weeks on CIM medium, mean No. of explants per petri dish with calli/No. of explants was calculated. ** After 4 weeks on SIM medium, mean No. of explants per petri dish with developed buds/No. of explants was calculated.

proliferation along the cut edge. Callus proliferation depended on potato genotype and bacterial strains and it significantly differed in comparison to control, non-transformed explants (Table 2). Overall callus induction efficiency of Dragačevka, Jelica and Desiree in transformation studies was 92.0, 73.0 and 87.3%, respectively.

Transferring of the explants to SIM-D medium and callus proliferation was suppressed and shoot regeneration was observed after 10 days in Dragačevka and Desiree and after 14 days in Jelica (Figure 1A, B and C). It is noticeable that inoculated explants regenerated shoots 5 days earlier than non-inoculated controls (data

Table 3. Shoot length distribution after 6 weeks on SIM media.

SIM media	Number of shoots (%)									
	<2 mm	2-4 mm	4-6 mm	8-10 mm	10-12 mm	12-15 mm	15-20 mm	20-25 mm	25-30 mm	30 mm >
Desiree										
control	21.52	25.46	19.16	11.03	7.35	4.98	4.20	3.41	1.84	0.70
OC II	19.91	22.36	17.80	9.02	7.03	3.86	7.38	4.33	3.86	4.45
OC I (as)	27.63	31.20	15.51	9.80	3.92	2.85	3.57	1.78	2.14	1.60
OC I	18.38	22.91	18.62	12.17	8.83	5.97	4.29	3.82	2.86	2.15
Dragačevka										
control	18.18	23.78	24.72	14.22	4.43	6.06	3.96	2.09	1.86	0.70
OC II	16.44	25.92	19.22	7.64	4.86	3.01	5.09	8.56	4.17	5.09
OC I (as)	15.28	16.85	19.37	16.49	11.19	7.10	4.45	3.73	3.37	2.19
OC I	17.53	24.84	21.30	10.01	3.55	10.86	7.39	1.87	1.25	0.86
Jelica										
control	59.71	35.97	4.32	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OC II	28.57	19.05	9.52	9.52	7.34	3.18	0.0	0.0	0.0	0.0
OC I (as)	29.82	31.29	18.71	7.60	4.68	3.51	0.0	0.0	0.0	0.0

not shown), which could be attributed to physiological stresses or stimulatory effects of antibiotic cefotaxime. Antibiotics are known to have phytohormone-like effects on cultured plant tissues and could affect morphogenesis in many plant species (Bhau and Wakhlu, 2001). Histological analysis confirmed that shoot regeneration from leaf explants was achieved via indirect organogenesis, as was expected.

There were no apparent differences between shoot buds regenerating from control and transformed explants, as well as among different cultivars. However, organogenesis was not synchronized, and different shoot regeneration stages from meristem initiation to well differentiated buds with leaf primordia, could be observed on the same explant (Figures 1D, E and F).

Shoot regeneration rate varied widely among investigated cultivars and bacterial strains (Table 2). After 4 weeks of cultivation on SIM-D medium, the highest shoot proliferation rate was 89.3% for Dragačevka, 68.0% for Jelica and 86.0% for Desiree. The average number of shoots reg-enerated per explant was 8.3 in Dragačevka, 5.0 in Jelica and 7.2 in Desiree. Until now, higher efficiency of 7-9 shoots/explant was reported only by Beaujean et al. (1998).

Regeneration efficiency was found to be strongly dependent on the genotype, thus confirming previously published results (Wenzler et al., 1989; Conner et al., 1991). Generally, among three cultivars, Jelica had the lowest shoot regeneration response in all transformation studies. The total number of regenerated shoots per 100 leaf explants was 900 for control and 600-800 for Dragačevka transformants. Desiree control regenerated 780 shoots, while transformants regeneration ranged from 490-760. It should be noted that Wenzler et al. (1989) reported 20 shoots per 100 Desiree leaf explants. Regeneration response of Jelica cultivar was more efficient with transformants (400) than with control (300), opposite to other two genotypes. This increase in morphogenetic potential could be related to a process occurring during transformation itself that stimulated conversion of differentiated cells into cells with meristematic features (Wang et al., 2005).

Shoot bud length distribution measured after 6 weeks on SIM medium showed apparent differences among genotypes (Table 3). Here again, Jelica responded specifically, regenerating mostly short shoots. More than 93% of the shoots of all three cultivars initiated roots 7 days after transfer to the rooting medium (Figures 1 G, H and I). In general, complete rooted plants were obtained as early as in 4 - 5 weeks after the initial *Agrobacterium* infection, adding this protocol to the most efficient potato transformation protocols (Beaujean et al., 1998; Banerjee et al., 2006).

Due to high total number of regenerated shoots, we could not accurately determine the percent of transgenic plants. Using PCR analysis, the sample of fifty six independent lines was tested for the presence of the *npt II* gene. Transformation frequency was highly variable and probably connected with the shoot regeneration ability. The average transformation efficiency according to PCR was 90.9% for Dragačevka, 76.9% for Jelica and 86.4% for Desiree.

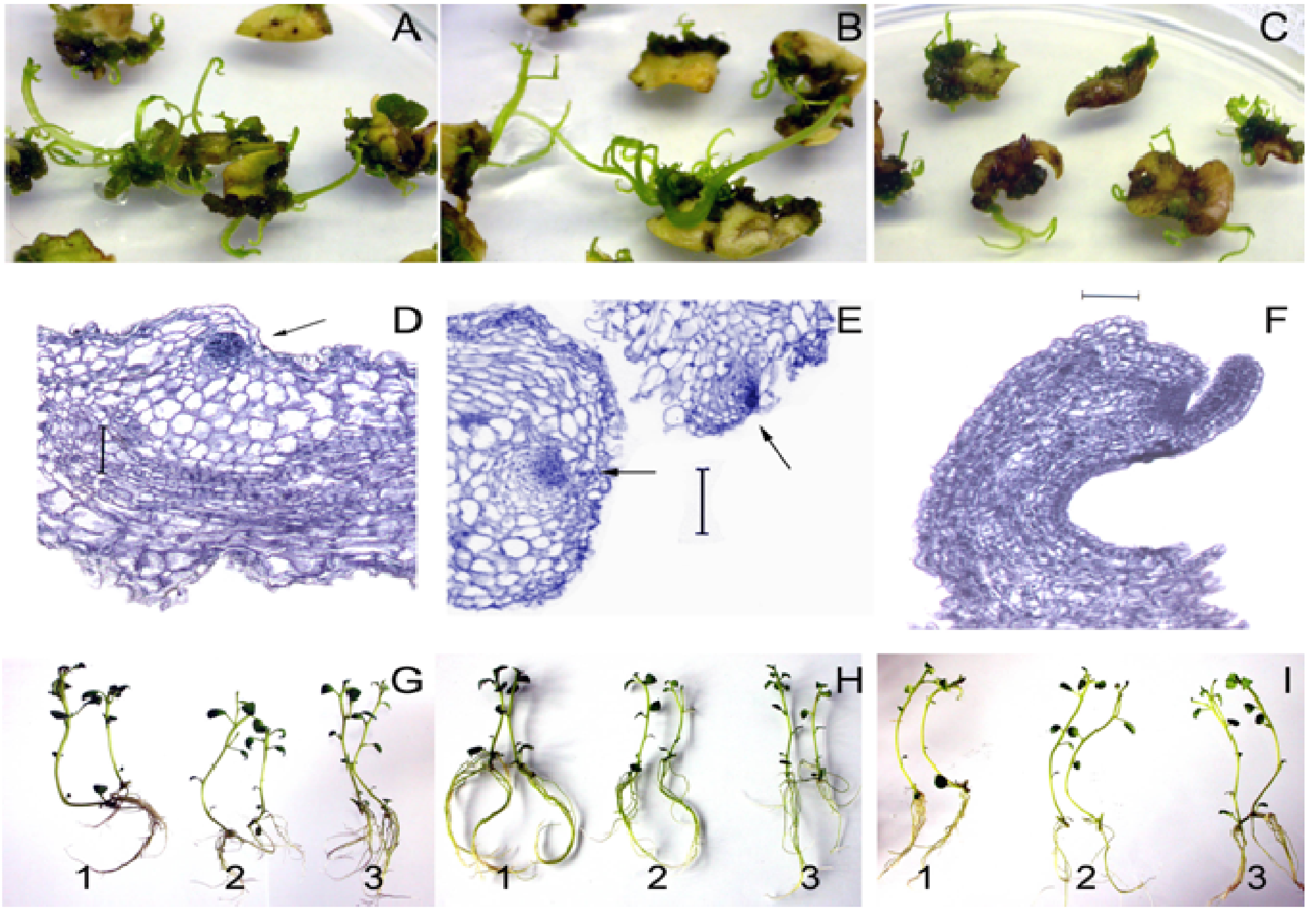

Figure 1. In vitro regeneration of transgenic potato plants: morphological and histological analyses. (A, B, C): Bud formation from the leaf explants cultured on the SIM-D medium: (A) Desiree transformed with pGV-GFP-OCI-3.8(19); (B) Dragačevka transformed with pGV-GFP-OCI-3.1D-16; (C) Jelica transformed with pGV-GFP-OCII-4.2A7. (D, E, F): Meristem shoot bud initiation (arrows) and differentiation in the control (E) and pGV-GFP-OCI-3.8 (19) transformed Dragačevka explants (D, F). (G, H, I): Control (1) and two transformed clones (2, 3), after 3 weeks on hormone-free MS medium. (G) OCI Desiree; (H) OCII Jelica; and (I) OCI Dragačevka.

An average of 87.5% of all tested transformed plants displayed an 800-bp amplification product that was missing from non-transformed control plants (Figure 2). Several escapes were recorded (Figure 2B, lanes 4 and 6), especially for Jelica (up to 37.5%). This indicates that kanamycin at 50 mg/l, adequate to control development of nontransformed cells and efficient to support early shoot bud regeneration, allowed appearance of some escapes (Wenzler et al., 1989). Thus, as it is suggested by Banerjee et al. (2006), for efficient screening of non-transformed shoots, level of kanamycin in the rooting medium should be slightly increased. It is important that the selection intensity is not too high, since it could lead to production of false negatives, resulting from the failure to recover transformed plants.

Problem of somaclonal variation was emphasized in many potato transformation protocols (Ooms et al., 1987; Imai et al., 1993; Badr et al., 2008). Visual inspection of selected transformed clones of our three cultivars cultured *in vitro* indicated statistical differences (data not shown) in morphological parameters such as number of nodes, number of axillary buds and shoot length. However, the overall phenotypes of the transformants were normal (Figures 1G, H and I). Also, visual inspection of 60 plants for each of 15 selected transformed clones in the green-house did not reveal morphological abnormalities indicating low frequency of somaclonal variation.

In summary, the protocol for transformation of two Serbian potato cultivars described here is simple, efficient and produces high percentage of transformed shoots which can be further used in biotests with predators and pathogens. In addition, current protocol eliminates pre-incubation, dark incubation of explants, as well as delaying addition of the selection agent to the culture medium after co-cultivation, commonly used in potato transformation protocols.

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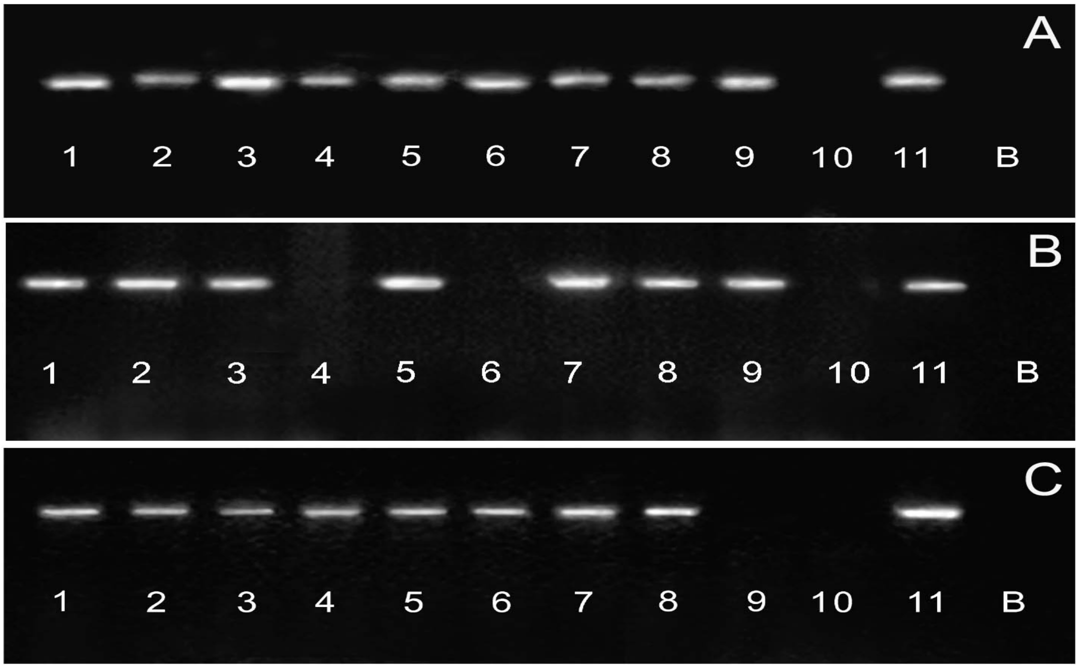

Figure 2. PCR analysis using specific *nptII* primers. (A) Plants transformed with pGV-GFP-OCII-4.2A7: lanes 1 - 3: Jelica OCII clones, lanes 4 - 6: Dragačevka OCII clones, lanes 7 - 9: Desiree OCII clones, lane 10: nontransformed control Jelica, lane 11: positive control pGV-GFP-OCII-4.2A7, lane B: blank. (B) Plants transformed with pGV-GFP-OCI-3.1D-16: lanes 1 - 3: Dragačevka OCI(as) clones, lanes 4 - 6: Jelica OCI(as) clones, lanes 7 - 9: Desiree OCI(as) clones, lane 10: nontransformed control Jelica, lane 11: positive control pGV-GFP-OCI-3.1D-16, lane B: blank. (C) Plants transformed with pGV-GFP-OCI-3.8(19): lanes 1 - 4: Dragačevka OCI clones, lanes 5 - 8: Desiree OCI clones, lanes 9 and 10: nontransformed control, Desiree and Dragačevka, lane 11: positive control pGV-GFP-OCI-3.8 (19), and lane B: blank.

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