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CONSTRUCTION OF PLANT TRANSFORMATION VECTORS CARRYING BEET NECROTIC YELLOW VEIN VIRUS COAT PROTEIN GENE (II)- PLANT TRANSFORMATION

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ABSTRACT

Fragments containing the coat protein gene of beet necrotic yellow vein virus were cloned in two plant transformation vectors: pCAMBIA3301M with the *bar* gene as selectable marker, and pCAMBIA1304M, with resistance to hygromycin. Three constructs were made of each vector: CPL, containing coat protein gene with leader sequence; CPS with coat protein gene, and CPSas with coat protein gene in antisense orientation. Vectors pC3301MCPL, pC3301MCPS, and pC3301MCPSas were used in *Agrobacterium*-mediated transformation of *Nicotiana tabacum* (tobacco), *Nicotiana excelsior* and *Nicotiana benthamiana*. Regenerants that developed roots on selective media were tested for the presence of CP fragments and the *bar* gene, but most regenerants were nontransformed (50-83% escapes). After all rooted plants had been selfed, and T₁ seed germinated on selective media, only plants descending from one *N. excelsior* regenerant transformed with pC3301MCPS were positive for presence of *bar* gene and CPS fragment. Tobacco and *Nicotiana benthamiana* were transformed with constructs pC1304MCPS and pC1304MCPSas. Transformation efficiency was much higher and approximately 50% of regenerants that rooted on media with 20 mg l⁻¹ hygromycin were positive for the presence of CP fragments. All T₁ plants were positive for presence of CP fragments.

Introduction

Rhizomania is the most important disease of sugar beet (*Beta vulgaris* L.) worldwide, caused by beet necrotic yellow vein virus (BNYVV) (32). Rhizomania is characterized by massive lateral proliferation of rootlets, constriction of the main taproot and a necrosis of vascular tissue, which leads to reduction of root yield up to 90%, while sugar content and processing quality can be badly affected as well. This virus is transmitted by soil-borne fungus *Polymyxa betae*, which is known to be able to survive in the soil for more than five years in the form of very robust resting spores (6).

Since agronomical and agrochemical management of rhizomania infested soil is inefficient, the most hopeful aspect of rhizomania control is breeding for resistance (1). Most of rhizomania tolerant genotypes, that perform very good in the conditions of mild infection, have one of the following types of resistance (11, 29): "Rhizor", "Holly", or resistance deriving from crosses with *Beta vulgaris* subsp. *maritima*. However, since genotypes with different degrees and types of resistance vary in their response to different BNYVV strains, climatic and soil conditions (14) many sugar beet breeding programs are now directed

towards combining known sources of resistance and introducing the new ones, including transgenic resistance.

There were few reports about inducing of transgenic resistance to rhizomania in sugar beet, by introducing the BNYVV coat protein gene (CP gene). Transformation of protoplasts with BNYVV CP gene was achieved by 17 but no transformed plant could be regenerated. The similar results were obtained with *A. rhizogenes* transformation, where many transformed hairy roots were formed, but none of them regenerated transgenic plant (9). Successful transformation was reported by 23 where transgenic sugar beet, plants were obtained after *Agrobacterium* transformation with constructs containing BNYVV coat protein gene and coat protein gene with leader sequence.

A possible reason for so few reports with successful sugar beet transformation is the fact that it has been very recalcitrant to regeneration after transformation with *A. tumefaciens*, although the bacteria is able to inoculate the plant tissue (20). No matter what type of explant was used (16, 21, 34), the induction of transgenic plants was very low and strongly dependant on genotype or binary vector. Particle bombardment of apical meristem (22), cell suspension (15) and embryogenic callus (31) did not give better results. The only transformation method that gave stable results regardless of genotype or vector is PEG transformation of stomatal guard cells protoplasts (12, 30), but this method is technically demanding and expensive.

In order to test ability of constructed plant transformation vectors to incorporate their T-DNAs in plant genome, we used them for transformation of some model plants. In the paper are presented results of tobacco, *Nicotiana excelsior* and *Nicotiana benthamiana* transformation with binary vectors containing BNYVV coat protein gene in sense or antisense orientation, with and without leader sequence.

Materials and Methods

Binary vectors

For plant transformation was used *Agrobacterium tumefaciens* strain LBA 4404 carrying binary vectors pCAMBIA3301M and pCAMBIA1304M. The coding sequences of BNYVV coat protein gene were isolated as 731 bp (CPL), and 587 bp (CPS) cDNAs, and cloned in plant transformation vectors pCAMBIA3301M and pCAMBIA1304M. Three constructs were obtained from each vector: CPL, CPS and CPSas, with the coat protein gene in antisense orientation.

In all constructs (**Fig. 1**), the coat protein gene was driven by the cauliflower mosaic virus promoter (35S) and followed by 3' nopaline synthase (nos) terminator. As selectable marker, constructs deriving from vector pCAMBIA3301M contained *bar* gene conferring resistance to herbicide phosphinotricin, while constructs deriving from pCAMBIA1304M had gene for resistance to hygromycin (*hyg*). Selectable markers were driven by the 35S promoter and followed by 35S polyA.

Plant transformation

Tobacco and *Nicotiana excelsior* were transformed with *A. tumefaciens* carrying pC3301MCPL, pC3301MCPS, and pC3301MCPSas, while tobacco and *Nicotiana benthamiana* were transformed by *A. tumefaciens* carrying pC1304MCPS and pC1304MCPSas. All transformations were done following slightly modified leaf disc transformation method (13): cells of *A. tumefaciens* were grown on a rotary shaker, at 28°C in 20 ml NB medium for 24 h. The medium contained 100 µg/ml of rifampicin and 50 µg/ml kanamycin, or 100 µg/ml rifampicin and 20 µg/ml hygromycin. The overnight cultures were centrifuged at 3000 rpm at 4°C and resuspended in 2ml of MS (26) medium. The leaves were cut into pieces of 1 cm² size, submerged in 10 ml MS medium with 2 mg l⁻¹ BAP and 0.2 mg l⁻¹ IAA, in which was dissolved 600 µl of *A. tumefaciens* suspension and left for 30

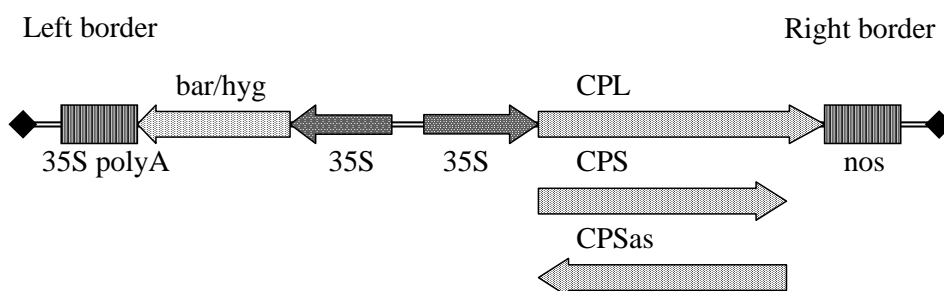


Fig. 1. T-DNA of plant transformation vectors pC3301MCP/pC1304MCP.

min. The explants were then transferred on solid MSD medium and left on cocultivation, at 21°C in the dark for three days. After cocultivation, the explants were washed, blotted dry and put on MSD medium with 500 mg l⁻¹ cefotaxime. Selective media for explants transformed with pC3301MCP vectors also contained 5 mg l⁻¹ phosphinotricin (DUCHEFA, Holland), while those for explants transformed with 1304MCP vectors had 20 mg l⁻¹ hygromycin. All regenerants were transferred on solid MS medium with 500 mg l⁻¹ cefotaxime and 5 mg l⁻¹ phosphinotricin, i.e. 20 mg l⁻¹ hygromycin.

Detection of transgenic plants

Regenerants of tobacco, *N. excelsior* and *N. benthamiana* that developed roots on selective media, were tested for the presence of *bar* gene and CP fragments.

Isolation of DNA was carried out as described in 27, and 30 - 50 ng was used in PCR reactions with specific primers. PCR was performed in 25 µl volume with 1x PCR buffer, 1.5 mM MgCl₂, 2 mM of dNTP, 2 units *Taq* polymerase (Amersham Pharmacia Biotech) and 100pM of each primer. For detection of *bar* gene specific primers were used: BARf (5'AGCCGCAG-GAACCGCAGGAGTG3') and BARr (5'ATGCCAGTTCCCGTGCTTGA AG3') giving 362 bp PCR product. Specific primers were made to complement the coat protein gene on BNYVV (3): two 5' primers P1 (5'CGAGATCTAAATTCTA-ACTATTATCTCC3') and P2 (5'GTAGA-

TCTATGTCGAGTGAAGGTAG3'), and one 3' primer P3 (5'CCGATA TCCAGC-TAATTGCTATTGT C3'). For detection of transgenic plants, primers specific for 35S promoter (5'AAACCTCCTCGGATT-CCATTG3') and *nos* terminator (CCATC-TCAT AAATAACGTCATGCAT) were used as well. Thermocycling was carried out as follows, 94°C, 5 min., then 35 cycles of 92°C for 30 s., 50.5, 56 or 57°C (depending of primer combination) for 1 min., 72°C for 1 min., followed by 72°C for 6 min.

Plants of tobacco, *N. excelsior* and *N. benthamiana* positive for presence of coat protein gene or *bar* gene were grown in the chamber, on 22±1°C and photoperiod 16/8h until flowering, when self pollination was done. Seeds of T1 generation were sterilized and germinated on selective MS media with 5 mg l⁻¹ phosphinotricin or 20 mg l⁻¹ hygromycin. T1 plants that developed on selective media were analyzed for presence of coat protein gene or *bar* gene following described PCR protocol.

Results and Discussion

Transformation of tobacco and

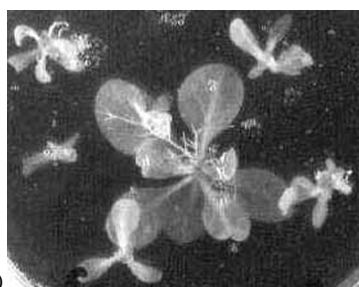
N. excelsior with pC3301MCP constructs

Seven days after inoculation with *Agrobacterium*, all explants showed tissue proliferation and after two weeks the first organogenic buds could be observed (Fig. 2). Well developed regenerants were transferred on selective MS medium for rhizogenesis. After 7-14 days some regene-



1)

Fig. 2. Organogenesis on transformed leaf explant.



2)

Fig 3. Rhizogenesis of regenerants on selective medium.

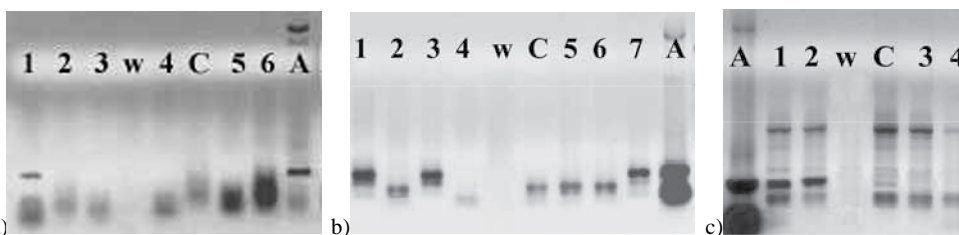


Fig. 4. Detection of *bar* gene in regenerants rooted in selective medium: **a)** *N. excelsior* after transformation with pC3301MCPL (1-6 - regenerants, w - water, C - control plant, A - pC3301MCPL), **b)** *N. excelsior* after transformation with pC3301MCPS (1-7 - regenerants, w - water, C - control plant, A - pC3301MCPS) **c)** tobacco after transformation with pC3301MCPSas (1-4 - regenerants, w - water, C - control plant, A - pC3301MCPS).

rants developed roots on selective media (**Fig. 3**): 21.4% of *N. excelsior* regenerants transformed with pC3301MCPL, and 23.6% after transformation with pC3301MCPS. After transformation with pC3301MCPSas, 26.9% of tobacco regenerants developed roots on selective medium.

PCR analysis of rooted regenerants for presence of CP fragments did not give consistent results, but PCR detection of *bar* gene showed that some *N. excelsior* regenerants transformed with pC3301MCPL (**Fig. 4a**) and pC3301MCPS (**Fig. 4b**) were *bar* positive, while only four out of six rooted tobacco regenerants developed enough to be micropropagated, and two of them were *bar* positive (**Fig. 4c**).

All regenerants that developed roots on selective medium were transferred to pots and grown until flowering and self pollination. Seed of T1 generation was put on the medium with phosphinotricin and only

those deriving from one *N. excelsior* plant, transformed with pC3301MCPS construct, had germination over 90%. They developed into the plants that, compared to non transformed, showed no morphological differences (**Fig. 5a**). All T1 plants were positive for presence of both, *bar* gene and CPS fragment (**Fig. 5b,5c**).

Transformation of tobacco and *N. benthamiana* with pC1304MCP constructs

After transformation with pC1304MCPS and pC1304MCPSas, explants of tobacco and *N. benthamiana* developed in the same way as those transformed with pC3301MCP constructs. After transformation with pC1304MCPS, 53% of *N. benthamiana* regenerants gave root on the selective medium, while after transformation with pC1304MCPSas rhizogenesis occurred in 70% of tobacco regenerants. One regenerant of *N. benthamiana* and four regenerants of tobacco that were positive

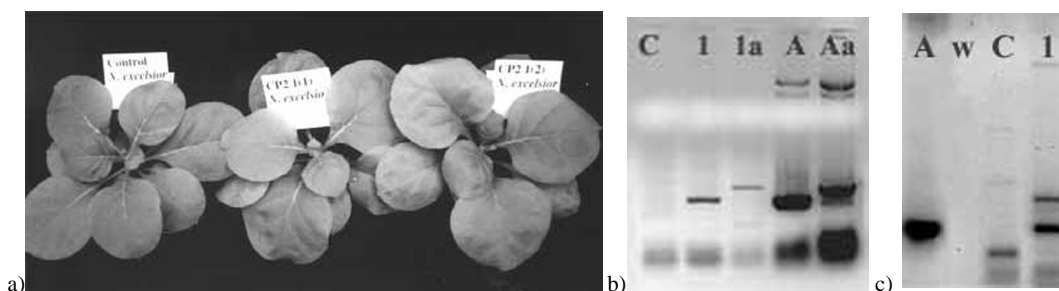


Fig. 5. T1 plants of *N. excelsior*: **a)** morphology (control is left), **b)** detection of CPS (C - control; 1 - transgenic plant/P2+P3; 1a - transgenic plant/P2+NOSrev; A - pC3301MCPS/P2+P3; Aa - pC3301MCPS/P2+NOSrev), **c)** detection of *bar* gene (A - pC3301MCPS; w - water; C - control; 1 - transgenic plant).

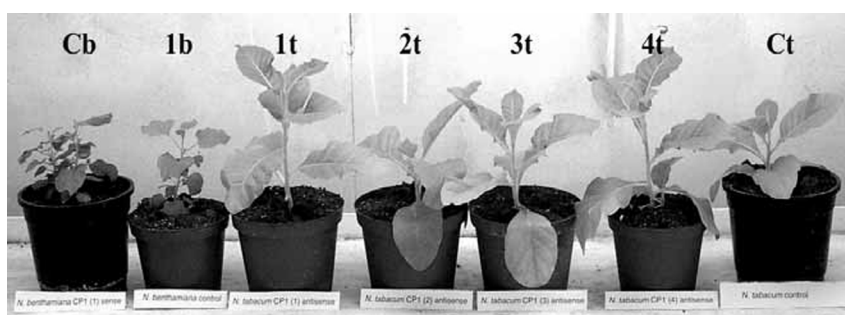


Fig. 6. T1 plants of *N. benthamiana* and tobacco, transformed with pC1304MCPS and pC1304MCPSas (Cb - *N. benthamiana* control plant, 1b - *N. benthamiana* transformed with CPS, 1t, 2t - tobacco transformed with CPS, 3t, 4t - tobacco transformed with CPSas, Ct - tobacco control plant).



Fig. 7. Detection of CPS and CPSas fragments in tobacco and *N. benthamiana* plants (1 - *N. benthamiana*/35Sfw+P3, 2 - *N. benthamiana*/P2+P3, 3 - tobacco/35Sfw+P3, 4 - tobacco/ P2+P3, w - water, 5, 7, 9 - tobacco/P2+P3, 6, 8, 10 - tobacco/P3+NOSrev, A - pC1304MCPS/ P2+P3, B - pC1304MCPSas/P2+P3, C - pC1304MCPS/35Sfw+P3, pC1304MCPSas/P3+ NOSrev, C - control plant/P2+P3).

for presence of coat protein gene, were transferred to the pots and grown until flowering, when the self pollination was done. Seed deriving from all CP positive tobacco and *N. benthamiana* plants had over 90% germination on medium with

hygromycin and developed into the plants normal morphological characteristics (**Fig. 6**). All plants were positive for the presence of CPS fragment in sense or antisense orientation (**Fig. 7**).

After inoculation of tobacco and *N. ex-*

celsior with *A. tumefaciens* carrying pC3301MCP constructs, resistance to glufosinate ammonium seemed to be the most stringent selectable marker, allowing growth and rhizogenesis of 14%, 21.5% and 19.4% after transformation with CPL, CPS and CPSas constructs respectively. Nevertheless, the amount of escapes was rather high (83% for CPL, 57% for CPS and 50% for CPSas), confirming the fact that efficiency of this marker differs from case to case (18, 28, 33). Percent of transformation for all three constructs was under 1%, which indicates that incorporation of T-DNA in plant genome was difficult. At first it was presumed that vector was somehow damaged during modification or cloning of CP fragment, but transformation efficiency of original pCAMBIA3301 on model systems was also extremely low (unpublished data). Probably this vector demands further modifications of transformation protocols, which would be in agreement with results of 5 who determined that transformation efficiency with pC3301 strongly depends on transformation protocol.

Over 60% of regenerants that developed after transformation with pC1304MCPS and pC1304MCPSas gave roots on medium with hygromycin and the number of escapes was under 50% for both vector. This results, as well as the fact that that all T1 plants deriving from CP positive regenerants were transformed, indicates that hygromycin is highly reliable selectable marker for *Nicotiana* species (7, 28). Its efficiency could be improved by use of high amounts of hygromycin in first subcultivations (2, 24), or by gradually increasing its concentration during micropropagation (19).

One of possibilities to improve transformation efficiency could also be construction of transformation vector with both *bar* and *hyg* gene (4) which would further improve hygromycin resistance with additional *bar* selection. The other option is to

use some of the methods for removal of an unfavorable *hyg* marker and combination with positive *bar* marker (8, 10, 25)

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