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ADVANCES IN DEVELOPMENT OF TRANSGENIC RESISTANCE TO BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) IN SUGAR BEET

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Fragments of viral cDNA containing the coat protein gene of beet necrotic yellow vein virus were cloned in plant transformation vector pCAMBIA3301M with the *bar* gene as selectable marker. Vector pC3301MCPL carrying coat protein gene with leader sequence, and pC3301MCPS with coat protein gene, were used in *Agrobacterium* - mediated transformation of sugar beet. The transformation method used was based on the fact that sugar beet develops axillary shoots in *in vitro* conditions, when placed on media with citokinins. Since this ability is not genotype or ploidy dependant it is widely used for sugar beet vegetative multiplication. Sterile seedlings, with removed cotyledons and lower half of hypocotyl, were used as starting material. After transformation explants were put on micropropagation medium with cephotaxime and phosphinotricyn (ppt), where axillary shoots started to develop. Since concentration of ppt was not selective enough, after two subcultivations it was increased twofold. Only one sample, transformed with pC3301MCPS

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preserved morphogenetic potential for micropropagatio, and it was tested for presence of CPS fragment and *bar* gene by PCR with specific primers.

Key words: rhizomania, coat protein, gluphosinate amonium, *Beta vulgaris* L.

Abbreviations: CAMBIA - Center for application of molecular biology in agriculture, Australia

INTRODUCTION

Rhizomania is sugar beet disease caused by beet necrotic yellow vein virus (BNYVV) (TAMADA, 1975), characterized by massive lateral proliferation of rootlets, constriction of the main taproot and a necrosis of vascular tissue, which results in severe reduction of root and sugar yield. BNYVV, the type species of the *Benyvirus* genus, is positive sense, single-stranded RNA virus. Its genome consists of four or five RNA components (BOUZOUBAA *et al.*, 1988; KOENIG *et al.*, 1997), with the coat protein gene located on RNA 2 and extending from nt 145 to 708nt (BOUZOUBAA, 1986).

Virus is transmitted by soil-inhabiting obligate parasite of sugar beet, *Polymyxa betae*, which is known to be able to survive in the soil for more than five years in the form of resting spores (DACHM and BUCHENAUER, 1993). Since no chemical control is available for eliminating the fungus, the only way to grow sugar beet on area infected with BNYVV, is to use genetically resistant genotypes. Most of rhizomania tolerant genotypes, that perform very good in the conditions of mild infection, have one of the following types of resistance (GEYL *et al.*, 1995; SCHOLTEN and LANGE, 2000): ”Rhizor”, “Holly”, or resistance deriving from crosses with *Beta vulgaris* subsp. *maritima.* However, on the soil heavily infected with BNYVV that type of protection is not efficient enough, so many sugar beet breeding programs are now directed towards combining known sources of resistance and introducing the new ones, including transgenic resistance.

Virus resistance can be engineered by transforming the plant with genes or sequences derived from viral genomes, and is known as pathogen-derived resistance (PDR). It can be result of expression of a viral protein (movement or coat protein), or an RNA-mediated mechanism. Protection from virus infection mediated by expression of viral coat protein (CP) in transgenic plant has been demonstrated for number of viruses, and although the underlying mechanism of resistance is still not fully known, it is suggested that presence of coat protein in transgenic plant cells inhibits decapsidation and attachment of ribozomes on viral RNA (BEACHY *et al.*, 1990; REIMANN-PHILIPP and BEACHY, 1993). The presence of the coat protein gene leader sequence can sometimes enhance translation and expression of transgene leading to higher level of resistance (KAWAGUCHI and BAILEY-SERRES, 2002; GALLIE, 1998). RNA mediated virus resistance is based on homology between sequence of transgene and viral RNA and is analogous to process known as gene silencing (DEMPSEY *et al.*, 1998). Transgenic resistance to viruses is based on post-transcriptional gene silencing (PTGS), that can be defined as degradation of both the transgene mRNA and the viral RNA, which contains either the

same or complementary nucleotide sequence (WATERHOUSE *et al.*, 2001; VAZGUEZ *et al.*, 2002).

There were few reports about inducing of transgenic resistance to rhizomania in sugar beet, by introducing the coat protein gene (CP gene) of BNYVV. Transformation of protoplasts with BNYVV CP gene was achieved by KALLERHOFF *et al*. (1990) but no transformed plant could be regenerated. The similar results were obtained with *A. rhizogenes* transformation, where many transformed hairy root were formed but none of them regenerated transgenic plant (EHLERS *et al*., 1991). Successful transformation was reported by MANNERLOEF *et al.* (1996) 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 sugar beet tissue (KRENS *et al.*, 1988). No matter what type of explant was used: cotyledons and hypocotyl (JACQ *et al.*, 1993), transition zone (KRENS *et al.*, 1996) or organogenic and embryogenic callus (ZHANG, 1998), every time the induction of transgenic plants was very low and strongly dependant on genotype or binary vector. No better results were obtained with particle bombardment of apical meristem (MAHN *et al.*, 1995) or cell suspension (INGERSOLL *et al.*, 1996), and only SNYDER *et al.* (1999) reported higher regeneration frequency after bombardment of embryogenic callus than after *Agrobacterium* transformation. The only transformation method that gave stable results regardless of genotype or vector is PEG transformation of stomatal guard cells protoplasts (HALL *et al.*, 1996; SEVENIER *et al*., 1998), but this method is technically very demanding and can not be preformed in many laboratories.

In the paper are presented results of sugar beet transformation with binary vectors containing BNYVV coat protein gene with and without leader sequence, as well as gluphosinate amonium resistance gene as selectable marker. The selection of potential regenerants as well as their molecular analysis are presented as well.

MATERIAL AND METHODS

Binary vectors - For transformation was used *Agrobacterium tumefacien*s strain LBA 4404 carrying binary vector pCAMBIA3301M. The coding sequences of BNYVV coat protein gene were isolated as 731 bp (CPL), and 587 bp (CPS) cDNAs, and cloned in plant transformation vector (NAGL *et al.*, 2005)

In all constructs, pC3301MCPL and pC3301MCPS, the coat protein gene was driven by the cauliflower mosaic virus promoter (35S) and followed by 3' nopalin synthase (nos) terminator. As selectable marker, constructs contained *bar* gene conferring resistance to herbicide phosphinotricin. Selectable markers were driven by the 35S promoter and followed by 35S polyA (Fig. 1).

Plant transformation - One hundred sterile seedlings of three genotypes, in stage of four leaves, were used as explants for sugar beet transformation with *A.*

tumefaciens carrying pC3301MCPL and pC3301MCPS. *A. tumefaciens* was 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. The overnight cultures were centrifuged at 3000 rpm at 4°C and resuspended in 2ml of MS medium (MURASHIGE and SKOOG 1962).as descibed before and dissolved in 300 ml MS medium.

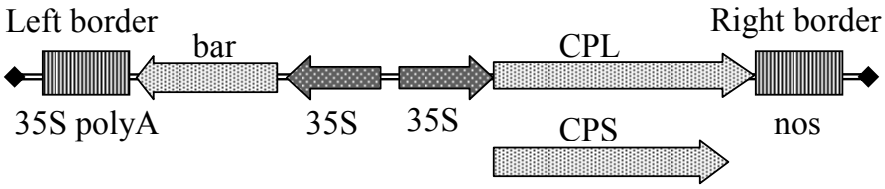


Fig 1. T-DNA of plant transformation vectors pC3301MCP

Sugar beet explants were prepaired in the following way: the root and most of the hypocotyl (except 0.5 –1 cm below the cotyledons) was removed. The cotyledons and the rest of the explant were held with two pair of forceps and pulled in opposite directions until cotyledons were peeled of, and zone between hypocotyl and leaf petiole was severed and exposed. Approximately twenty explants were put in 50 ml of *Agrobacterium* suspension and left on a rotary shaker in the dark for 1 h. The explants were transferred on solid MS 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 selective MS medium for micropropagation with 0.3 mg l^{-1} BAP, 0.01 mg l^{-1} GA_3, 500 mg l^{-1} cefotaxime and 5 mg l^{-1} phosphinotricin (DUCHEFA, Holland) under 16 h light/8 h dark cycle. Subcultivation was done every three weeks, and after two subcultivations phosphinotricin concetration was doubled.

Detection of transgenic plants - From sugar beet explants that preserved morphogenetic potential for micropropagation, i.e. were able to develope axillary buds on medium with herbicide, DNA was isolated following the protocol of SHURE *et al.* (1983). In order to prove the presence of the coat protein and *bar* gene 30 ng were used for the PCR reactions with specific primers.

PCR was performed in 25 μl volume with 1x PCR buffer, 1.5 mM $MgCl_2$, 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'AGCCGCAGGAACCGCAGGAGTG3') and BARr (5'ATGCCAGTTCCCGTGCT TGAAG3') giving 362 bp PCR product. Specific primers were made to complement the coat protein gene on BNYVV (3): two 5' primers P1 (5'CGAGATCTAAATTCTAACT ATTATCTCC3') specific for longer (CPL) fragment and P2 (5'GTAGATCTATGTC GAGTGAAGGTAG3') specific for shorter (CPS) fragment, and one 3' primer P3 (5'CCGATATCCAGCTAATTGCTATTGTC3'). For detection of transgenic plants, primers specific for 35S promotor - 35Sfw

(5'AAACCTCCTCGGATTCCATTG3') and *nos* terminator – NOSrev (CCATCT-CATAAATAACGTCATGCAT) 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 prmer combination) for 1 min., 72°C for 1 min., followed by 72°C for 6 min.

RESULTS AND DISCUSSION

Fourteen days after transformation of seedlings with pC3301MCPL and pC3301MCPS, in 25% of explants was observed the formation of axillary buds

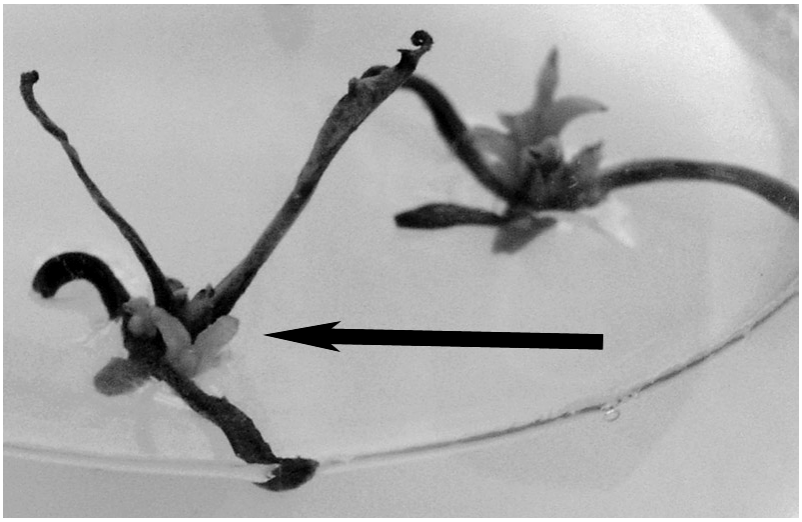


Fig.2. Formation of axillary buds in sugar beet explants after transformation with pC3301MCPS and pC3301MCPL on 5mg/l ppt

(Fig. 2) that gave rise to axillary shoots. Morphology of most shoots indicated that they did not derive from transformed cells or tissue - their leaves were pale green or yellow, they were formed outside the medium and multiplied at the very slow rate. Therefore, after second subculture, all explants with axillary shoots were transferred on medium with doubled phosphinotricin concentration (10 mg/l). This amount proved to be more selectable, because after two subcultivation there was only one sample, transformed with CPS fragment, that preserved morphogenetic potential for multiplication (Fig. 3).

During micropropagation leaves and leaf stalks were taken for DNA isolation and analysis. PCR analysis with specific primers showed that shoots were positive for presence of CP fragments and *bar* gene (Fig. 4). Although PCR gave products of appropriate size, this is still no final proof that tested plants were genetically transformed, because the positive signal can be result of residual *Agrobacterium*. Since the tested plant showed resistance to high concentrations of gluphosinate ammonium, it could be considered transient transformant, since the

full confirmation of its transgenic nature could be obtained by Southern blot analysis or PCR detection in next, T_1 generation.

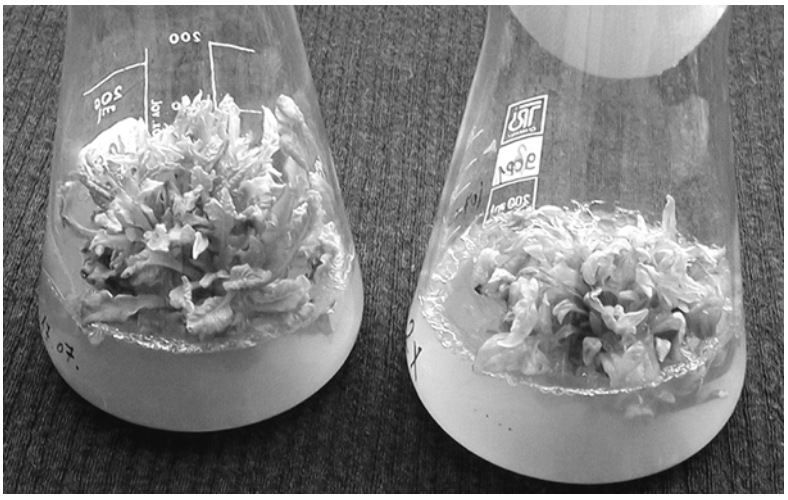
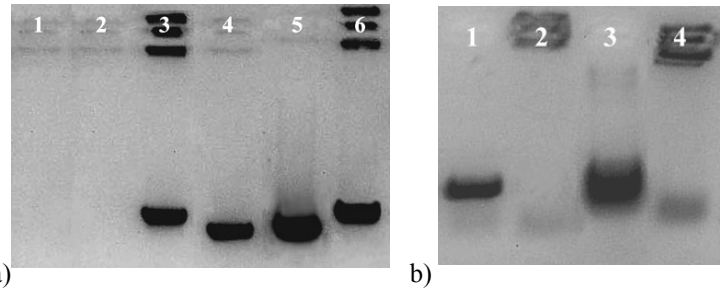


Fig.3. Micropropagation of sugar beet transformed with pC3301MCPS on medium with 10 mg/l ppt (control right)

The sugar beet transformation method presented in this paper is based on its ability to form a great number of axillary buds on media with cytokinin (ATANASSOV, 1980; MIEDEMA *et al.*, 1982). Since induction of axillary meristems, unlike regeneration ability, is not genotype dependant and can be stimulated from different explants (SAUNDERS, 1982; MEZEI, 1988) it could be considered as promising system for regeneration of transgenic sugar beet plants. Presented results are not extensive enough to offer better estimation of this transformation method but presence of even one putative transformant indicates that, with further optimization and use of other binary vectors, it could be used on wide range of sugar beet genotypes.



a) b)

Fig.4. Detection of a) pC3301MCPS (1-water, 2-control, 3-candidate plant with 35Sfw/P3, 4-candidate plant with P2/NOSrev, 5-pC3301mCPS with 35Sfw/P3, 6-pC3301MCPS with P2/NOSrev), b) bar gene (1-candidate plant, 2-control, 3-pC3301MCPS, 4-water)

Results presented in the paper is still does not offer enough information for determination of efficiency of the presented method, but if even one transient transformant is obtained it indicates to eventual potential that this method might have. There are many possibilities to increase transformation efficiency: use of vacuum, vectors with higromycin or other selectable markers, or use of binary vectors with enhanced *vir* genes.

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RAZVOJ TRANSGENE OTPORNOSTI ŠEĆERNE REPE NA VIRUS NEKROTIČNOG ŽUTILA NERAVA REPE (BNYVV)

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I z v o d

Fragmenti virusne cDNK sa genom za protein omotača virusa nekrotičnog žutila nerava repe su klonirani u vektor za transformaciju biljaka pCAMBIA3301M koji je sadržao *bar* gen kao selektivni marker. Vektori pC3301MCPL, sa genom za protein omotača virusa i njegovom lider sekvencom, i pC3301MCPS, sa genom za protein omotača, su korišćeni u tramsformaciji repe pomoću *Agrobacterium*-a. Metod transformacije se zasniva na sposobnosti repe da u uslovima *in vitro* razvije aksilarne pupoljke na podlozi sa citokininima. Pošto ova sposobnost ne zavisi od genotipa ili od nivoa ploidnosti, postala je standardni metod za vegetativno umnožavanje repe. Kao početni materijal su korišćeni sterilni ponici kojima su odstranjeni kotiledoni i donja polovina hipokotila. Nakon transformacije eksplantati su postavljeni na selektivnu podlogu za mikropropagaciju sa cefotaksimom i fosfinotricinom (ppt) gde je došlo do razvoja bočnih pupoljaka. Pošto koncentracija fosfinotricina nije bila dovoljno selektivna, ona je nakon dve subkultivacije dvostruko povećana. Samo je jedan uzorak, transformisan vektorom pC3301MCPS, nakon dve subkultivacije sačuvao mofrogenetski potencijal za mikropropagaciju, i bio testiran na prisustvo CPS fragmenta i *bar* gena PCR rakcijom sa specifičnim prajmerima.

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