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

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ABSTRACT

Coat protein gene of beet necrotic yellow vein virus (BNYVV) was isolated from inoculated sugar beet roots and leaves of Chenopodium quinoa and Tetragonia expansa, by RT-PCR and imuno capture RT-PCR. Specific primers were made to complement coat protein gene and untranslated leader sequence, so that two fragments were obtained: long (731 bp), which contained coat protein gene and leader sequence, and short (587 bp), with coat protein gene. Fragments were cloned in two plant transformation vectors: pCAMBIA 3301M and pCAMBIA 1304M, which were modified by removing multicloning site and NcoI restriction site at the 5' end of the reporter genes. Vector pC3301M had bar gene which confers resistance against the herbicide gluphosinate ammonium as selectable marker, and pC1304M had gene for resistance to antibiotic hygromycin. Three constructs were made from each vector: CPL, containing coat protein gene with leader sequence; CPS with gene for coat protein, and CPSas with coat protein gene in antisense orientation. All constructs were transfered to Agrobacterium tumefaciens strain LBA4404.

Introduction

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, 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 ribosomes on viral RNA (2, 22). Presence of coat protein gene leader sequence can sometimes enhance translation and expression of transgene leading to higher level of resistance (8, 13). RNA mediated virus resistance is based on homology between sequence of transgene and viral RNA and is analogous to process known as gene silencing (7). 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 (27, 28).

Beet necrotic yellow vein virus (BNYVV) is the causal agent of severe disease called "rhizomania" in sugar and fodder beet plants (26), which is characterized by massive lateral proliferation of rootlets. BNYVV is positive sense, single-

stranded RNA virus, with multipartite genome of four to five RNAs, which belongs to *Benyvirus* genus (4, 15). It is a soil-borne virus, transmitted by fungus *Polymyxa betae* Keskin, (11). The viral particles are 20 nm wide, while their length varies from 44 to 769 nm (5). RNA2 contains coat protein gene, located at the 5' end, from nt 145 to 708 which codes synthesis of virus coat protein P21 (3, 23).

Fungus *Polymyxa betae* remains in the soil in the form of cystosory, very resistant resting spores which are capable of preserving viability more than five years (6, 17). Since no chemical treatment is available for eliminating the fungus, the only way to grow sugar beet in infected areas is to use genetically resistant genotypes. Most of rhizomania tolerant genotypes, that perform very good in the conditions of moderate soil infection, have one of the following types of resistance (9, 25):"Rhizor", "Holly", or resistance deriving from crosses with *Beta vulgaris* subsp. *maritima.* However, on the soils heavily infected with BNYVV those types of protection are 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.

The aim of this research was to clone beet necrotic yellow vein virus (BNYVV) coat protein gene and make functional plant transformation vectors.

Materials and Methods

Inoculation of plant material

Sugar beet hybrid variety DANA was sown in pots with mixture of 40% sand and 60% rhizomania infected soil from fields of Institute of field and vegetable crops, Novi Sad. Plants were germinated and grown in the greenhouse, and after ten weeks root was tested for presence of BNYVV by DAS ELISA test, following the standard protocol of company BIOREBA AG (Switzerland). Half of every sample positive for presence of BNYVV, was sent to Center for fruit growing and viticulture in Cacak. There, in order to obtain plant material with high concentration of viruses, was done infection of indicator plants *Chenopodium quinoa* and *Tetragonia expansa*. Leaves of indicator plants with symptoms of local infection were analyzed with ELISA test for BNYVV. The positive samples were used as starting material in further investigation.

Cloning of BNYVV coat protein gene in pUC18

Primers specific for BNYVV coat protein gene (CP gene) were created according to RNA 2 sequence (3). The two 5' primers had incorporated restriction site for enzyme *Bgl*II, P1: CGAGATCTAAATTCTAACTATTATC TCC, covering leader sequence, and P2: GTAGATCTATGTCGAGTGAAGGTAG , covering the sequence around start codon of BNYVV CP gene. One 3' primer, had incorporated restriction site for *Eco*RV, P3: CCGATATCCAGCTAATTGCTATTGT C. For isolation of CP gene two approaches have been taken: (i) isolation of RNA from infected leaves of indicator plants and sugar beet roots positive for BNYVV, following protocol 18; and (ii) fixing virus particles on the walls of centrifugation tubes coated with BNYVV antibodies, i.e. "imuno capture" (14), and use of viral RNA in RT-PCR reaction.

For reverse transcription and polymerase chain reaction (RT-PCR) was used »*Ready To Go* RT-PCR Beads« kit (Amersham Pharmacia Biotech). In reaction mixture was put 15 pmol of both primers in combinations P1/P3 and P2/P3, isolated RNA and filled up to 50 µl with DEPC treated water. Synthesis of first cDNA chain was done at 42°C, for 25 min., and then PCR reaction with denaturation at 95°C, 5 min., and 35 cycles : 95°C 1 min., 50°C 1 min., 72°C 1 min., and terminal elongation at 72°C for 10 min. PCR products were cloned in phosphorylated plasmid vector

pUC18/*Sma*I, which was then transferred in *E. coli* strain JM109 (10). Plasmids from bacterial colonies that remained white on selective medium and gave PCR product of expected size, were sent for sequencing. The fragment sequences were identified with search engine BLAST (1), version BLASTN 2.2.3, while translation product of CPS sequence was analyzed with same program, version BLASTX 2.2.3.

Plant transformation vectors

For plant transformation were selected vectors pCAMBIA3301 and pCAMBIA1304 (CAMBIA - Center for Application of Molecular Biology to International Agriculture, Australia). Vector pC3301 had *gus* as reporter gene, while pC1304 had *gus* and *mgfp* genes, with 35S as promoter, and *nos* as terminator sequences.. As selectable marker pC3301M had *bar* gene which confers resistance against the herbicide gluphosinate ammonium, and pC1304M had gene for resistance to antibiotic hygromycin. Selectable markers were under control of 35S promoter and followed by 35S polyA. In both vectors restriction site for *Nco*I (C↓CATGG), which contains start codon of reporter gene was destroyed and multicloning sites were extracted.

Cloning of CP fragments in modified plant transformation vectors

CP fragments were cloned in modified plant transformation vectors on the place of reporter genes - *gusA* in pC3301M, and *mgfp5* and *gusA* in pC1304M.

CPS and CPL fragments were multiplied from pUC18CPS and pUC18CPL plasmids with combinations of specific primers. PCR products were extracted from the gel, and after their polyA ends were removed they were cut with *Eco*RV and *Bgl*II. In order to create CPS antisense construct (CPSas), CPS was multiplied with M13/pW sequencing primers (Amersham Pharmacia Biotech): IF5 (GTAAAACGACGGCCAGT) and IR6 (CAGGA AACAGCTATGAC). PCR product was extracted from gel, blunted and digested with *Bam*HI (G↓GATCC, Amersham Pharmacia Biotech).

In order to extract *gusA*, i.e. *mgfp5* and *gusA* genes, vectors were digested with *Pma*CI (CAC↓GTC, Amersham Pharmacia Biotech) and *Bgl*II (Gibco BRL). After ligation recombinant vectors were transferred in *E. coli*. Colonies were checked for CPL, CPS and CPSas by PCR with following combinations of primers: a) For CPL - P1/P3 (annealing on 56°C), and 35Sfw (AAACCTCCTCGGATTCCATTG) / NOSrev (CCATCTCATAAATAA CGTCATGCAT) (annealing on 57°C); b) for CPS - 35Sfw/P3, and 35Sfw/NOSrev with annealing 57°C; c) for CPSas - P2/35Sfw (annealing on 50.5°C) and P3/NOSrev (annealing on 57°C)

Transformation of *Agrobacterium tumefaciens*

Vectors pCAMBIAMCPL, pCAMBIAMCPS and pCAMBIAMCPSas were transferred to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating, with pRK2013 as helper. Colonies were checked for CPL by PCR with two primer combinations: 35Sfw/P3 and P1/NOSrev with annealing at 57°C. Primer combinations for CPS were as for colony checks, and for detection of CPSas was used P3/NOSrev primer combination.

Results and Discussion

Inoculation of plant material

In ELISA test of sugar beet roots for BNYVV average absorbance of negative control was A_{405}=0.597 and positive A_{405}=1.175. Samples differed in amount of virus particles in root sap, but for further analysis were taken those with absorbance A_{405}=0.912 - 1.201.

Ten days after inoculation of *Chenopodium quinoa* and *Tetragonia expansa* the symptoms of local infection developed in the form of chlorosis (**Fig. 1**). Average absorbance of healthy leaves was A_{405}=0.170

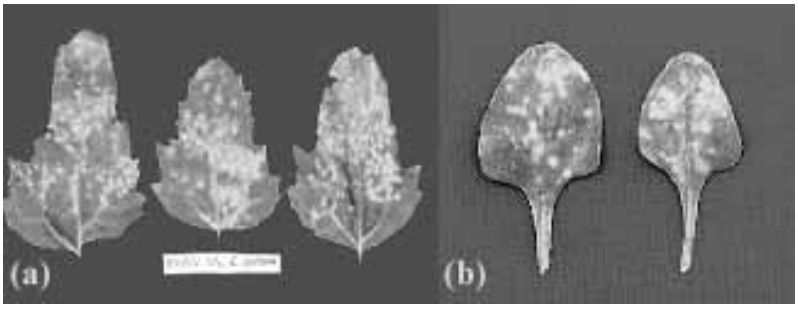

Fig. 1. Local symptoms of BNYVV infection on *Chenopodium quinoa* **(a)** and *Tetragonia expansa* **(b)** leaves

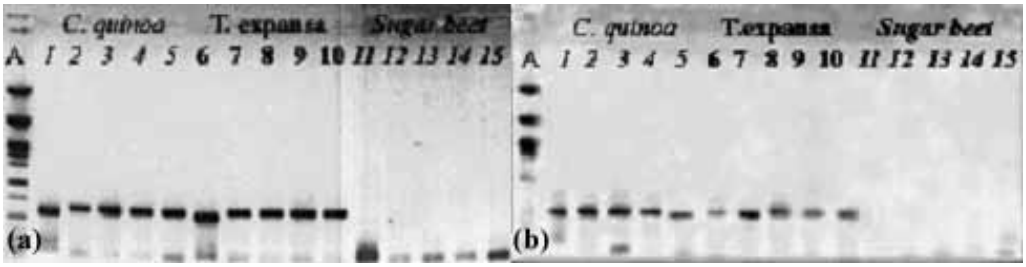

Fig. 2. RT-PCR and IC RT-PCR of indicator plants and sugar beet with primers P1/P3 **(a)** and P2/P3 **(b)** (1, 2 - RT-PCR; 3, 4, 5, - IC RT-PCR; 6, 7 - RT-PCR; 8, 9, 10 - IC RT-PCR; 11, 12 - RT-PCR, 13, 14, 15 - IC RT-PCR, A - λ/PstI).

and in infected leaves A_{405}=2.950. That way we obtained plant material with increased concentration of virus, which could enable easier isolation of BNYVV coat protein gene.

Cloning of BNYVV coat protein gene in pUC18

As a result of RT-PCR and imuno capture RT-PCR (ICRT-PCR) with primer combination P1/P3, samples of indicator plants gave fragments with expected length of coat protein gene with leader sequence (CPL) of 731 bp (**Fig. 2a**). RT-PCR and IC RT-PCR of indicator plants with primer combination P2/P3, resulted in fragment of expected size for BNYVV coat protein gene of 587 bp (CPS) (**Fig. 2b**). There was no product in reactions with sugar beet samples with both primer combinations.

After both CP fragments have been cloned in pUC18 plasmid and transferred in *E. coli*, colonies were checked for CPL and CPS. Colonies transformed with plasmid containing longer CP fragment that stayed white on selective medium. White colonies that developed after CPS transformation were not positive for presence of the fragment. The only positive ones were two colonies blue in the center and white at the edges. Plasmids from colonies positive for CPL and CPS were sent for sequencing (GenBank accession nubmers AY920456 and AY920466 respectively). Fragment sequences and sequence of protein made by their translation were compared with other in sequence data bases. The sense orientation of CPL fragment could be determined according to position of start and stop codon, and *Bgl*II and *Eco*RV restriction sites. Comparing the fragment sequence with sequences in data bases, the best match was obtained with genomic RNA of different BNYVV isolates and BNYVV coat protein gene with homology of 96 - 99%. According to the position of start and stop codon, and restriction sites for *Bgl*II

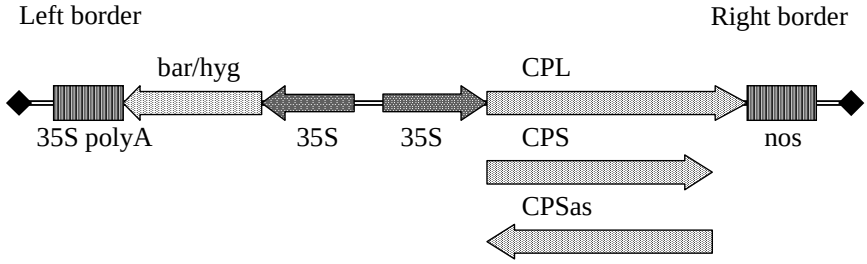

Fig. 3. T-DNA of plant transformation vectors pC3301MCP/pC1304MCP.

and *Eco*RV, it was determined that CPS fragment was cloned in antisense orientation. This was confirmed after compairing fragment with sequence data bases, where the highest level of homology (97 - 98%), was obtained for BNYVV coat protein gene in antisense orientation.The constructed protein sequence from CPS fragment, had highest homology (96 - 99%) with BNYVV coat protein.

Cloning of BNYVV coat protein gene in plant transformation vectors

Fragments CPL,CPS and CPSas were cloned in modified plant transformation vectors in the place of reporter genes. As the result, six transformation vectors were obtained: pC3301M and pC1304M with longer fragment (CPL), shorter fragment (CPS) and shorter fragment cloned in antisense orientation (CPSas) (**Fig. 3**).

Transformation of *Agrobacterium tumefaciens*

Transkonjugation colonies that developed on selective medium after transformation with vector pC3301MCPL, were tested for presence of construct with primer combinations 35Sfw/P3 and P1/NOSrev (**Fig. 4a**). Colonies 2 and 3 gave PCR product of expected size with both primer combinations. After transformation of *Agrobacterium* with pC3301MCPS two colonies developed on selective medium. They were tested for the presence of construct with primer combinations 35Sfw/P3 and 35Sfw/NOSrev (**Fig. 4b**) and both proved to be positive. *Agrobacterium* transformed

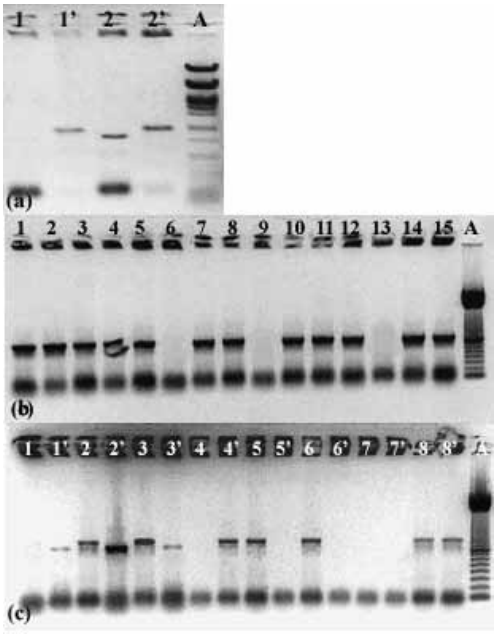

Fig. 4. Check of *A. tumefaciens* colonies for **(a)** pC3301MCPS (1, 2 - primers 35Sfw/P3; 1', 2' - primers 35Sfw/NOSrev, A - λ/*Pst*I); and **(b)** pC3301MCPSas (primers 35Sfw/P2, A - 100 bp ladder) **(c)** pC3301MCPL (1-8 - primers 35Sfw/P3; 1'-8' - primers P1/NOSrev, A - 100 bp ladder);

with pC3301MCPSas developed 15 colonies on selective medium, who were tested with primer combination 35Sfw/P2 (**Fig. 4c**). Only three colonies were not positive for presence of CPs fragment in antisense orientation.

Mechanical inoculation of indicator plant leaves with root extract of sugar beet in-

fected with rhizomania virus, showed to be successful method for obtaining plant material with high virus concentration. Isolation of BNYVV coat protein gene was easily achieved, since all samples gave PCR products after RT-PCR with total RNAs as well as with IC RT-PCR. These results are not unexpected because in previous research isolation of BNYVV CP gene from sugar beet tissue was possible only by two-step PCR (12), or RT-PCR with "nested" primers (20). Even when BNYVV RNA fragments were multiplied with single PCR their amount was so low that they could be analyzed only with polyacrilamide gels and stained with silver (14), which is more sensitive than ethidium bromide. Therefore isolation of higher amounts of BNYVV RNA fragments with one step PCR should be done from indicator plants which have very high concentration of viral particles in infected tissue.

For isolation of beet necrotic yellow vein virus coat protein gene, two approaches were taken: (i) isolation of total RNA from samples positive to BNYVV, and its use as template in RT-PCR, and (ii) attachment of viral particle to walls of tubes coated with BNYVV-specific antibodies, i.e. imuno capture, and use of viral genomic RNA as template in RT-PCR. The amount of the PCR product was roughly the same for both methods, which leads to suggestion that for further work with BNYVV or other viruses for which there are commercial antibodies IC RT-PCR should be used in order to avoid sensitive and sometimes troublesome RNA isolation step.

The obtained fragments, CPL and CPS, were cloned, sequenced and compared with each other and with sequences in data bases. CPL and CPS sequences had very high homology level with BNYVV coat protein gene sequences in data bases, which confirms the fact that differences in BNYVV coat protein gene between different isolates are very small (24), and confirms the fact that sequence of this gene can remain unchanged for long period of time (16). Although sequences of our fragments had high homology with other sequences of BNYVV coat protein gene, it differed from all of them in two nucleotides 273 and 519, which could be considered as specific feature of this isolate. The differences in leader sequence between isolates were minimal confirming the hypothesis that stability of loop which it forms is essential to BNYVV (19). Differences in nucleotide sequence did not affect coat protein sequence which agrees with (5), where was determined that point mutations in BNYVV coat protein sequence do not affect protein sequence. This high level of gene and protein sequence preservation indicates that eventual selection pressure on population of this pathogen which is made when rhizomania resistant genotypes are grown might not induce changes in protein and gene sequence. Detection and spreading of P strain (14, 16) shows that there is much higher probability for virus to try overcoming existing resistance by developing strains with more RNAs, i.e. more virulent strains.

Since transformation efficiency is much higher in constructs with shorter T-DNA (21), reporter genes were removed and T-DNAs were significantly reduced, since *gusA* and *mgfp5* and *gusA* genes, were 2050 bp, i.e. 2400 bp long, while CPL and CPS had 720 bp, and 567 bp. Chances for higher transformation efficiency were increased by the fact that at the start of reporter genes there are consensus sequence for translation initiation, which is considered to significantly increase gene expression. Six plant transformation vectors were obtained: pC3301MCPL, pC1304MCPL, pC3301MCPS and pC1304MCPS with BNYVV coat protein gene in sense orientation, and vectors with antisense construct pC3301MCPSas and pC1304MCPSas.

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