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REGULAR ARTICLE

ISOLATION AND CHARACTERIZATION OF CHITINASE GENE FROM THE UNTRADITIONAL PLANT SPECIES

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ABSTRACT

Round-leaf sundew (*Drosera rotundifolia* L.) from *Droseraceae* family belongs among a few plant species with strong antifungal potential. It was previously shown that chitinases of carnivorous plant species may play role during the insect prey digestion, when hard chitin skeleton is being decomposed. As many phytopathogenic fungi contain chitin in their cell wall our attention in this work was focused on isolation and *in silico* characterization of genomic DNA sequence of sundew chitinase gene. Subsequently this gene was fused to strong constitutive CaMV35S promoter and cloned into the plant binary vector pBinPlus and tested in *A. tumefaciens* LBA 4404 for its stability. Next, when transgenic tobacco plants are obtained, increasing of their antifungal potential will be tested.

Keywords: carnivorous plant, chitinase, in silico analyses, sundew

INTRODUCTION

Currently, a large part of scientific community considers utilization of sophisticated breeding techniques and genetically modified (GM) technology as one of the most important factors for the growth of agricultural production efficiency (**Wang *et. al.*, 2011**). This

approach allows the selection of successful genotypes, isolation and cloning of genes for more useful features of plants and the development and introduction of transgenic cultivars in agricultural practice (**Moeller and Wang, 2008**). One of the important applications of molecular genetics and plant transformation is to increase the resistance of crops to pests, herbicides, phytopathogenic fungi and nematodes (**Collinge *et al.*, 2010**). It involves selection and isolation of suitable genes from any genetic resource, transfer into an economically important crop using methods of genetic engineering and subsequent testing of resistance to a certain type of pathogen. Current research showed that in the case of transfer of some chitinase genes *via* genetic transformation, the defense mechanism of these plants can be enhanced (**Brogue *et al.* 1991., Datta *et al.* 2001., Ganesan *et al.*, 2009**). Since there are different chitinases with variable enzyme and antifungal activities in the individual plant species, their isolation and characterization appears to be highly urgent for biotechnology programs. Round-leaf sundew (*Drosera rotundifolia* L.) from *Droseraceae* family, genus *Drosera* is a carnivorous plant that belongs among a few plant species with strong antifungal potential (**Grevenstuk *et al.*, 2012**). The study of **Matušíková *et al.*, (2005)** indicated that sundew chitinases play role during the insect prey digestion, when hard chitin skeleton is being decomposed. For this reason, we focused our attention on the isolation and *in silico* characterization of the sequence of sundew chitinase gene. Then this gene was fused to strong constitutive CaMV35S promoter and cloned into the plant binary vector pBinPlus and tested in *A. tumefaciens* LBA 4404 for its stability. Next, when transgenic tobacco plants are obtained, increasing of their antifungal potential will be tested.

In this work the isolation, *in silico* characterization and the vector construct preparation involving *Drosera rotundifolia* chitinase gene under the control strong constitutive *CaMV 35S* promoter was performed.

MATERIAL AND METHODS

Plant material

Plants of *Drosera rotundifolia* L. were cultivated *in vitro* on basal MS medium (DUCHEFA) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (**Bobák *et al.*, 1995**). The plantlets were cultivated at 20 ± 2^0C with a day length of 16 h under 50 μEm^{-2} s^{-1} light intensity.

Isolation of full genomic DNA sequence

In the case of 5´upstream sequence, the genomic DNA was at first digested with PvuII restriction enzyme. Then after ligation of adaptors to the restriction fragments, the library DNA was used for amplification of fragments, where forward primer was designed in the sequence of adaptor (delivered in kit) and reverse specific primer was composed as follows: Chit REV 5´ ATATGGACCATCTGGTGCAGTTGG 3´. The reaction mixture contained in 50 µl volume 0.075 µg of template library DNA, 0.2 µM FOR and REV primers, 1x PCR buffer, 0.8 mM dNTP mixture and 1U Taq polymerase (INVITROGEN). PCR program involved 1 cycle 94^0C 3 min; 35 cycles [94^0C 25 s, 62^0C 30s, 72^0C 90s]; 72^0C 10 min. In the case 3´downstream sequence the genomic DNA was digested with StuII restriction enzyme. After ligation of adaptors to the restriction fragments, the library DNA was applied in PCR, where reverse primer was designed in the sequence of adaptor (delivered in kit) and forward specific primer was composed as follows: Chit FOR 5´GCATCCGAATGATAATGACAGTGG 3´. The reaction mixture contained in 50 µl volume 0.075 µg of template library DNA, 0.2 µM FOR and REV primers, 1x PCR buffer, 0.8 mM dNTP mixture and 1U Taq polymerase (INVITROGEN). PCR program involved 1 cycle 94^0C 3 min; 35 cycles [94^0C 25 s, 62^0C 30s, 72^0C 90s]; 72^0C 10 min.
Subsequently PCR products were isolated from the gel using the Gel extraction kit (QIAGEN), cloned into pGem-T easy vector system (PROMEGA) and sequenced using the M13 primers. Based on obtained sequences the full genomic chitinase gene was amplified on sundew genomic DNA with the primers DrChitFOR 5´AACCATGGGCATTACTATCATGCT 3´ and DrChitREV 5´ AAGCTTAATTAACTGCAGGTCACTGGAT 3´.

***In silico* analysis**

Obtained sequences were analysed according to following online programs:

BLAST http://www.ncbi.nlm.nih.gov/BLAST/, CLUSTAL
http://www.ebi.ac.uk/Tools/msa/clustalw2/ and GENSCAN
http://genes.mit.edu/GENSCAN.html.

Preparation of plant binary vector for plant transformation

DNA manipulations were carried out as described by Sambrook *et al*. (1989). In first step NcoI – XbaI of chitinase fragment and XbaI – HindIII fragment of 35S terminator were cloned into pSL301 cloning vector. Then HindIII – NcoI fragment of CaMV35S promoter isolated from pCAMBIA1304 plasmid (**Robers et al., 2002**) and NcoI – PacI fragment of chitinase 35S terminator isolated from pRT100 cassette vector (**Topfer et al., 1987**) were ligated into pBinPlus binary vector (**van Engelen et al., 1995**) restricted by HindIII – PacI enzymes. The resulting plant binary construct pDD3 (Figure 1) were transformed into *Agrobacterium tumefaciens* LBA 4404 by triparental mating method (**Matzke and Matzke 1986**).

RESULTS AND DISCUSSION

Isolation of full length chitinase gene and *in silico* analyses

The presence of chitinases in the mucilage from carnivorous plant *Drosera rotundifolia* assumes their activity in digestive processes of captured insect prey and plant defence against phytopathogenic fungi or bacteria (**Matusikova *et al.*, 2005**). The experiments were therefore focused on isolation and identification of a chitinase gene from this untraditional plant species and subsequent *in silico* characterization.

Full length genomic chitinase sequence isolated from carnivorous plant *Drosera rotundifolia* using the Genome Walking approach was of the length 1665 bps. When the obtained sequence was analysed by BLASTX program the occurrence of the conserved ChtBD1_GH19_hevein domain [cd06921], and chitinase_glyco_hydro_domain19 [cd00325] were detected. The TBLASTX confirmed the greatest consistency of the translated analysed sequence with the chitinases of partial coding sequence of *Drosera spatulata* chitinase (AY643483.1), *Allium sativum* (M94105.1) and *Nepenthes khasiana* (AY618883.1) with E values e^{-158}, e^{-133} and e^{-131}, respectively. As shown in Table 1 *in silico* analyses using the GenScan program predicted three exons within the analysed chitinase sequence. Splicing of predicted introns by the ProtParam tool (**Gasteiger *et al.*, 2005**) showed that putative chitinase protein consists of 287 amino acids, has molecular weight of 299 kDa and pI 8.11**.**

Table 1 Output from the GenScan analyses

Genomic DNA chitinase						
Exon	**Type**	**Start**	**End**	**CodRg**	**P**	**Tscr**
1	Init	1	454	352	0,831	28,83
2	Intr	684	723	5	0,612	2,45
3	Term	1297	1666	307	0,640	22,22

Legend: Init - initial Intr - internal Term - terminal exon; CodRg (coding region), P (probability); Tscr (transcription score)

In the second part of our work we focused on preparation of plant expression unit consisting of chitinase gene under the control strong constitutive *CaMV 35S* promoter and its cloning into the plant binary vector pBinPLUS vector (**van Engelen et al., 1995**) resulting in construct pDD3 (**Figure 1**).

Figure 1 T-DNA structure of the plant binary vector construct pDD3

As a selectable marker the neomycin phosphotransferase (NPTII) regulated by the nopaline synthase (NOS) promoter and terminated by nopaline synthase terminator (NOS TER). Chitinase gene is driven by cauliflower mosaic virus promoter (35SP) and terminated by cauliflower mosaic virus promoter terminator (35S Term).

There are several binary vector systems such as pPZP (Hajdukiewicz et al. 1994), pCAMBIA derivatives (**Roberts et al., 2002**) as well as pCGN (**McBride and Summerfelt, 1990**), pGreen (**Hellens et al., 2000**) and pGPTV (**Becker et al., 1992**) families into which the plant expression units can be cloned, however we preferred the pBinPLUS that involves the *npt*II gene driven by *nos* instead of *CaMV 35S* promoter. When two identical promoters are present in T-DNA of the binary vector, after transformation in many regenerated plants the phenomena of gene silencing may occur (**Vaucheret et al., 1998**).
The correctness of cloning in *E. coli* was verified by restriction analyses using the enzymes *Hind*III–XbaI, HindIII–KpnI, PstI (**Figure 2**).

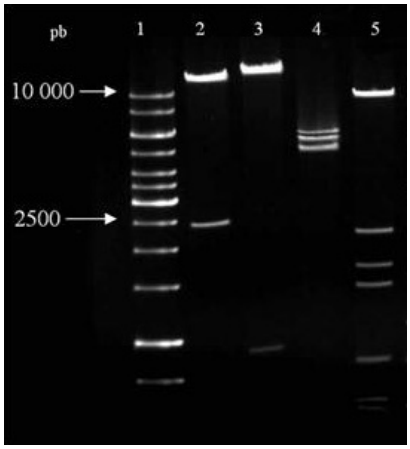

Figure 2 Restriction analysis of the plasmid pDD3

(Lane - Marker 1kbPlus (FERMENTAS). Lane 2 - HindIII– XbaI enzymes yielded the fragments of length 2400 bp and 11000 bp. Lane 3 - HindIII–KpnI enzymes yielded the fragments of length 11000 bp a 900 bp. Lane 4 - PstI enzyme yielded the fragments of length 5300 bp, 5000 bp and 4594 bp. Lane 5 - HindIII–NcoI–PacI enzymes yielded the fragments of length 492 bp, 528 bp, 762 bp, 1359 bp, 1565 bp, 2037 bp and 8201 bp.)

Stability of prepared binary vector construct in *A. tumefaciens* LBA 4404 was verified on one hundred plasmids isolated after re-transformation into *E. coli* and 98% of plasmids yielded with PstI restriction enzyme the correct restriction profile (**Figure 3**).

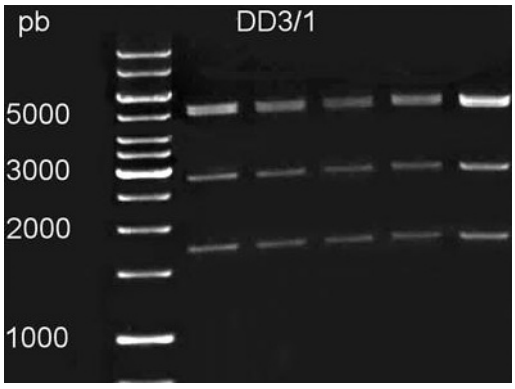

Figure 3 Restriction analysis pDD3 after re-transformation from *A. tumefaciens* into *E.coli.*

For verifying of stability the enzymes KpnI – PstI were used yielding the fragments of length 5091, 5406, 2844 a 1743 bp

CONCLUSION

In this work the isolation, *in silico* characterization and the vector construct preparation involving *Drosera rotundifolia* chitinase gene under the control strong constitutive *CaMV 35S* promoter was performed. Next this construct for transformation of

model plant – tobacco will be used. In transgenic tobacco plants enhancement of antifungal potential of crude protein extracts (containing also over-expressing sundew chitinase) will be studied.

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