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IN SILICO ANALYSIS OF CHITINASE PROMOTER ISOLATED FROM DROSERA ROTUNDIFOLIA L.

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ABSTRACT

Chitinases occur in dozens of genes in the individual plant species and play diverse roles in plant growth and development, and during the plant defense to biotic and abiotic stress. Here we focused on isolation and *in silico* characterization of regulatory sequences of chitinase gene that belongs to the first isolated gene sequences from *D. rotundifolia* overall. For the isolation of the 739 bp sequence of chitinase promoter the genome walking approach was applied. The authenticity of the obtained fragment(s) was verified by sequencing and sequence alignment ClustalW program. The core of the chitinase promoter was predicted by Neural Network Promoter Prediction program. In total the PLACE online available database identified 66 various *cis*-regulatory elements in the analyzed sequence. Some of them might be potentially bound by specific transcription factors, and regulate gene expression in specific plant tissues during the plant development or upon the pathogen attack, dehydration, cold or high salinity stress. However, further analyses are needed to reveal which out of predicted *cis*-elements participate in the true expression profile of isolated promoter in origin and transgenic plant organism.

Keywords: Carnivorous plant, chitinase, hydrolytic enzymes, sundew, promoter

INTRODUCTION

Chitinases (EC 3.2.1.14) are hydrolytic enzymes that break down β-1,4-glycoside bonds in chitin. As chitin is a component of the cell of fungi and exoskeleton of some animals (including worms and arthropods), chitinases are generally found in organisms that either need to reshape their own chitin or digest the chitin of fungi or animals. Chitinases are also present in plants. In the individual plant species they occur in dozens of genes that play diverse roles in plant growth and development, including microsporogenesis, embryogenesis, germination, flowering and abscission **(López *et al.*, 2009)**. Despite the fact that chitin as a substrate is missing in plants, there is strong evidence that plant chitinases catalyze the hydrolytic decomposition of plant arabinogalactan proteins. It is supposed that other *N*-acetylglucosamine-containing glycoproteins occurring in cell walls can be also endogenous substrates for plant chitinases **(Kasprzewska, 2003)**. In addition, many chitinases belong to the pathogenesis-related (PR) proteins and participate in processes of plant defense against biotic and abiotic stress. Chitinase activity was found also in the mucilage from several carnivorous plants including sundew (*Drosera rotundifolia* L.) **(Matusikova *et al.*, 2005).** Here, destroying of a strong insect chitin containing cytoskeleton supposes the expression of chitinase gene(s) with a strong hydrolytic potential. The transcription activity of genes (involving chitinase genes) is regulated by corresponding promoter sequences that lie upstream of the adjacent coding region where the transcription factors and RNA polymerases can bind. Transcription factors recognize the specific DNA sequence/motifs which are known as the *cis*-regulatory elements and regulate the expression of genes in different conditions **(Wray *et al.*, 2003).** Here we focused on isolation and *in silico* characterization of regulatory sequences of chitinase gene that belongs to the first isolated gene sequences from *D. rotundifolia,* overall **(Matušikova *et al.*, 2004)**. Based on identification of specific *cis*-elements in the promoter sequence we are able to speculate a possible role of corresponding chitinase in plant organism.

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)** at 20 ± 2^{0}C with a day length of 16 h under 50 μEm^{-2} s^{-1} light intensity.

Isolation of promoter sequence

Isolation of 5´end of chitinase gene was performed using a Genome Walking kit (Clontech). For this two libraries were made by digesting the sundew genomic DNA with restriction enzymes EcoRV (Library EcoRV) and PvuII (Library PvuII) in separate tubes. After digestion blunt ends of DNA were ligated to Genome Walker Adaptor, that have complementary sequences of AP1 and AP2 primers.
The first extension of the 5´ end of chitinase gene was made by nested PCR on the EcoRV library with AP1 (5´-GTAATACGACTCACTATAGGGC-3´), GSP1REV (5´-CCCTGTTCTTGCTTGAAGCAATA-3´) and AP2 (5´-ACTATAGGGCACGCG
TGGT-3´), GSP2REV (5´-TAACTATGGCACTGTATGGCGCTA-3´) primers. The second extension of the 5´end of chitinase gene was performed on the PvuII library by nested PCR with AP1 (5´-GTAATACGACTCACTATAGGGC-3´), GSP3REV (5´- AGAAGGGCTGACACAGTTAATGT-3´) and AP2 (5´-ACTATAGGGCACGCGTGGT-3´), GSP4REV (5´-ACGGACGCAAGATGAATT
GAGT -3´) primers. The specific bands of both nested PCR were eluted from the gel and sequenced.
The full chitinase promoter sequence was amplified on sundew genomic DNA with the primers DrPRChitFOR 5´AGGAAGCTTTGTCCAATCGTCCCA 3´ and DrPRChitREV 5´ AGCATGATAGTAATGCCCATGGTT 3´. The PCR reactions were carried out in 50 μl reactions containing 100 - 200 ng of DNA template, 15 pmol of each primer, 200 μM dNTPs, 1 × PCR buffer and 1 unit of Taq DNA polymerase (Finnzymes, Finland). The first PCR step of 94^{o}C for 4 min was followed by 30 cycles of 94^{o}C for 45 s, 64^{o}C for 45 s and 72^{o}C for 2 min. The last step was performed at 72^{o}C for 7 min. The PCR product was eluted from the gel and sequenced.

In silico analysis

Obtained sequences were analyzed according to the following programs: http://www.ch.embnet.org/software/ClustalW.html; Neural Network Promotor Prediction http://www.fruitfly.org/seq_tools/promoter.html; and PLACE http://www.dna.affrc.go.jp/PLACE/signalscan.html.

RESULTS AND DISCUSSION

Isolation of chitinase promoter sequence

Here we focused on the isolation of the promoter sequence of chitinase gene from carnivorous plant *Drosera rotundifolia* L. and its *in silico* characterization. Previously the PCR with degenerative primers were used for isolation of the chitinase fragment gene of length of 987 bp **(Libantova *et al.*, 2007)**. In our experiments the Genome Walking approach was used to extend the 5´upstream chitinase/promoter fragment. Figure 1 shows schematic representation of DNA walking technique. The PCR amplicons were verified by sequencing. The overlap of the obtained sequences was revealed by the ClustalW program. Finally the chitinase promoter was amplified as a one fragment of length 739 bp using the specific primers, cloned into the pGemT easy vector and re-sequenced.

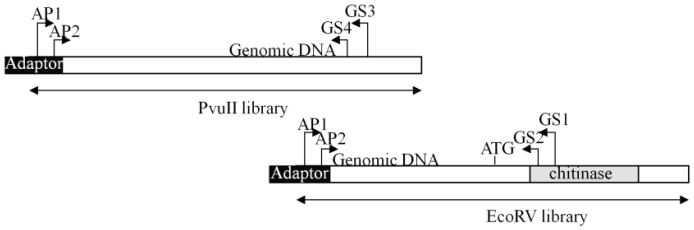

Figure 1 Schematic representation of DNA walking technique used for the sundew chitinase promoter isolation.

Promoter *in silico* analysis

The Neural Network Promoter Prediction program predicted the occurrence of two functional promoter cores in the analyzed 739 bp long sequence; with position 659 to 709 nt and 634 to 684 nt with the score 0.99 and 0.83, respectively (Figure 2). Putative promoters were evaluated based on the presence of the TATA-, GC-, CAAT-boxes and the transcription start site as these are essential for transcription factors and other proteins involved in the polymerase binding processes. Promoter estimated accuracy of the score 0.99 represents 0% detection of false positives **(Reese, 2001)**. Despite the fact that transcriptional initiation start was predicted at the position 709 with high probability only the experimental verification by RACE (rapid amplification of the cDNA ends) approach can definitely define the transcription start site of the adjacent chitinase gene.

Promoter predictions for sundew

Start	End	Score	Promoter Sequence
634	*684*	*0.83*	TTGTTCAATTTAAAAATCTGAGCAGGAAATCCGTATATATAGACCCCATG
659	709	0.99	GAAATCCGTATATATAGACCCCATGTATCCTTCAAACTTCACCAAGTTAC

Figure 2 Evaluation of putative transcription start sites of sundew chitinase gene using the Neural Network Promoter Prediction program.

Next the fragment of length 739 bp, carrying the isolated chitinase promoter, we explored by PLACE online available database **(Hugo *et al.* 1999)**, which contains the list of *cis*-acting regulatory DNA elements and their description, all retrieved from previously published reports. These motifs could potentially bind different transcription factors, and their occurrence in the promoter may coincide with specific functions of transcribed genes during the development and pathogen defense. Table 1 lists only the *cis*-elements that are related to the tissue specificity (1), or necessary for hormone (2) and stress (3) induced response.

The first category included the *cis*-regulatory elements that may be responsible for expression in mesophyll cells (YACT), root (ATATT, TACTATT), developing pollen (GTGA) or seed (CANNTG). According to **Gowik (2004)** the YACT motif is a key component of Mem1 (mesophyll expression module 1) found in the promoter of the phosphoenolpyruvate carboxylase (ppcA1) of the C4 plant *Flaveria trinervia*. We identified 8 duplications of this motif in the analyzed chitinase promoter of sundew that belongs to the C3 plants. However, this motif occurs also in another C3 plant – rice; in the regulatory sequences of phosphate transporter genes, that are up-regulated in rice root upon inoculation with mycorrhizal fungus *Glomus intraradices* **(Hatograngan *et al.* 2009)**. This implies that the YACT motif in C3 plant can be involved in different metabolic processes than in C4 plants. Chitinase promoter contains also two root specific motifs ATATT and TACTATT. The former was identified for the first time in *rolD* promoter of *Agrobacterium rhizogenes* and it was proven to be responsible for expression of the *gus* gene in roots of transgenic tobacco plants **(Elmayan *et al.* 1995)**. The latter, identified in β-amylase promoter of sweet potato, showed to be responsible for the adjacent gene activity in tuberous roots **(Ishiguro *et al.*, 1996)**. Finally in the category of tissue-specific motifs we have identified one pollen-specific (GTGA) and one seed-specific (CANNTG) *cis*-regulatory element.

The category of the ABA-inducible elements contained four *cis*-regulatory sequence motifs (TAACTG, YAACKG, ACCGAC, CANNTG). Generally, ABA-induced genes are involved in a myriad of biological processes including plant growth or plant response to environmental stress such as cold, drought and pathogens **(Wang *et al.* 2011)**. For example the ACCGAC *cis*-acting element was found in many cold-, high salt- and cold-responsive promoters in *Arabidopsis* and rice and provide the binding site for DRE transcription factors **(Suzuki *et al.* 2005)**; Ont the other side the identified WAACCA, YAACKG, TAACTG, CANNTG are often present in the promoters of drought-inducible genes. Their transcription is triggered by MYB and MYC transcription factors that function in mediating drought and ABA-regulated gene expression **(Abe *et al.* 1997)**.

Table 1 Putative *cis*–elements found in the analyzed chitinase promoter fragment. The 739 bp region upstream of the translation initiation was examined using the PLACE and Plant Care databases.

Motif name	Sequence	Function	Motif No
Tissue			
CACTFTPPCA1	YACT	Element related to mesophyll expression	8
ROOTMOTIFTAPOX1	ATATT	Root specific element from rolD gene	1
SP8BFIBSP8BIB	TACTATT	Tuberous root specific element	1
GTGANTG10	GTGA	An element found in late pollen gene gt10	2
MYCCONSENSUSAT/ EBOXBNNAPA	CANNTG	MYC binding site involved in the response to dehydration ans and ABA, seed specific element	1
Hormone ABA			
MYB1AT	WAACCA	Element involved in dehydration stress	1
MYB2AT	TAACTG	Element involved in the water stress and ABA	1
MYB2CONSENSUSA	YAACKG	MYB recognition site involved in dehydration and ABA response	1
DRE2COREZMRAB17	ACCGAC	Element involved in dehydration and ABA stress	1
MYCCONSENSUSAT/ EBOXBNNAPA	CANNTG	MYC binding site involved in the response to dehydration ans and ABA, seed specific element	1
Stress			
WBOXATNPR1	TTGACC	W box, response to wounding and pathogen attack	1
WBOXNTERF3	TGACY	W box; response to wounding	1
WRKY71OS	TTGACC	W box; response to wounding	2
MYBCORE	CNGTTR	Element involved in response to water stress	3
CCATBOX1	CCAAT	HSE (heat shock element)	2
CBFHV	RYCGAC	Involved in dehydration stress	1
GT1GMSCAM4	GAAAAA	Pathogenesis and salt-induced element	2

Legend: Y = C/T; K = G/T, N; W = A/T; R= A/G;

Last category included eight *cis*-acting elements that are related to biotic and abiotic stress. In general, stress responsive genes can be expressed either through an ABA-dependent or ABA-independent pathway. Regarding to the second option a group of the TTGACC, TGACY, TTGACC *cis*-regulatory elements with W box motif (TGAC) was found in the analyzed chitinase promoter. Rapid and systematic accumulation of WRKY transcription factors that bind to the W box element have been previously reported immediately upon wounding, salicylic acid signaling and responses to diseases, freezing, oxidative stress, drought, salinity, cold and heat **(Wang *et al.*, 2009)**. In addition to W-box elements, in the analyzed chitinase promoter, there were identified other stress –related *cis*-

regulatory elements. For example the ACGT sequence motif that was previously found in the promoter of erd1 (early responsive to dehydration) gene of Arabidopsis and shown to be required for etiolation-induced expression **(Simpson *et al*., 2003)**. The chitinase promoter also contained the CCAAT *cis*-regulatory element, the role of which in heat shock stress response was described by **Rieping *et al*. (1992)**. The category of stress induced elements included also the RYCGAC motif, the involvement of which in the cold acclimation in barley albina mutant was described by **Svensson et *al.* (2006)**; and the GAAAAA box playing the role pathogen- and salt –induced gene expression **(Park *et al*., 2004)**. In total the analyzed 739 bp sequence contained 66 various *cis*-regulatory elements revealed by PLACE database. Here we evaluated only the motifs that could be essential for isolated chitinase promoter expression profile in the respect of biotic and abiotic stress and tissue specificity. To find out which out of above-mentioned sequence motifs are involved in transcription of adjacent gene, the RT-PCR on the sundew mRNA isolated from various types of tissues and application of several stress inductors should be performed.

CONCLUSION

Here we bring the isolation of a 739 bp sequence carrying the chitinase promoter using the genome walking approach. The authenticity of the obtained fragment was verified by sequencing and sequence alignment Clustal W program. The core of the chitinase promoter was predicted by Neural Network Promoter Prediction program. In total the PLACE online available database identified 66 various *cis*-regulatory elements in the isolated chitinase sequence. Some of them might be potentially bound by specific transcription factors, and regulate gene expression in specific plant tissues or during the plant defense against biotic and abiotic stress. However, further analyses are needed to reveal which out of predicted cis-elements participate in the true expression profile of isolated promoter in origin and transgenic plant organism.

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