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SEQUENCE ANALYSIS OF SUNDEW CHITINASE GENE

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ABSTRACT

Progress in biotechnology has generated an interest towards the isolation and characterization of genes that should be used in strengthening of plant defence against biotic and abiotic stresses. Here we focused on sequence analysis of the chitinase gene isolated from untraditional genetic source, carnivorous plant sundew (*Droserarotundifolia*L.). The genomic sequence of *DrChit* indicated the coding region contained three exons and two introns.Splicing of introns predicted *in silico*was confirmed by experimental approach. Moreover, RACE (rapid amplification cDNA ends) analysis revealed the transcription start sitelocated 41 bp upstream of the ATG start codon. This is one of the first reports onthe isolation and molecular characterization of chitinase gene from the carnivorous sundew plant.

Keywords: Carnivorous plant, chitinase, sequence analysis, sundew

INTRODUCTION

Global food security is the major challenge for biological sciences. As the plants cannot avoid environmental stress they have developed defense mechanisms to alleviate their impact. The inducible defense responses include cell wall reinforcement, lignifications, induction of oxidative burst, hypersensitive cell death, the accumulation of antimicrobial secondary metabolites such as phytoalexins and expression of pathogenesis-related (PR) proteins **(Veluthakkal and Dasgupta, 2012)**. Chitinases (EC3.1.2.14) are particularly interesting plant enzymes because their substrate is not present in plant tissue *per se*. Their induction has been reported upon fungal infection. Moreover, the experiments performed under*in vitro* condition, confirmed the capability of some plant chitinases to inhibit growth of several fungal pathogens **(Schlumbaum*et al*., 1986; Theis and Stahl, 2004;Moravckova*et al*. 2004, 2007; Van Loon *et al.* 2006)**. Based on the primary structures and specific domains plant chitinase have been grouped into seven classes **(Collinge*et al*. 1993; Neuhaus 1999).** ClassI, II, IV, VI and VII have glycosyl hydrolase 19 catalytic domain. In addition to class I also class IV and VI harbor chitin-binding domain**.** Class III and V of enzymes are included in the family 18 of glycosyl hydrolases. They share the consensus sequence DXDXE, but otherwise show very low homology to each other **(Melchers *et al.*, 1994; Veluthakkal and Dasgupta, 2012).** The presence or absence of a C-terminal extension (CTE) divides chitinases into two subclasses: subclass a (CTE present) and subclass b (CTE absent). Its presence has been shown to be responsible for transport of the protein to the vacuole **(Neuhaus*et al.*, 1991; Renner and Specht,2012)**.
In addition to the role of chitinases in defense responses to pathogens class I chitinases have been shown to be important players in plant carnivory in the Caryophyllales. The presence of class I chitinases and their activity was demonstrated within the specialized carnivorous traps of sundew (*Drosera*) and tropical pitcher plants (*Nepenthes*) (**Matusikova*et al.*, 2005; Eilenberg*et al.*, 2006**). Recently we have isolated genomic sequence of *D. rotundifolia*chitinasewhile the TBLASTX confirmed its greatest consistency with the chitinases of *Droseraspatulata*(AY643483.1), *Allium sativum* (M94105.1) and *Nepenthes khasiana*(AY618883.1) with E values e^{-158}, e^{-133} and e^{-131}, respectively(**Ďurechová*et al.* 2013**). Here we bring the *in silico* analysis of this gene and experimental proof of splicing introns and transcriptional start site.

MATERIAL AND METHODS

Plant Material

Plants of *Droserarotundifolia* 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.

In silico analysis

The structure of *gDrChit*gene was analyzed using NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/(**Hebsgaard*et al.*, 1996**). Motif analysis was conducted using the InterProScansoftware(www.ebi.ac.uk/InterProScan). Predictions of transcription initiation sites were performed using the Neural Network Promoter Prediction (http://www.fruitfly.org/seqtools/promoter) (**Reese *et al.*,1997**).

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from *in vitro* sundew plants using the protocol described by **Békésiová*et al.*(1999)** and treated with RNase-free DNaseI (Thermo Fischer Scientific, USA). First strand cDNA was synthesized using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, USA) and oligo $(dT)_{20}$ primer. Primers ChitStartFOR 5´-AACCATGGGCATTACTATCATGCT-3´ and ChitSTOREV 5´-TGTTCTAGAAAAAGGACGCTGATTAT-3´were used to amplify cDNA sequence. PCR program involved one cycle at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 40s and one cycle at 72°C for 10 min. Next the PCR fragment was cloned into pGemT-easy (Promega, USA) was commercially sequenced.

Mapping of the transcription start site

The 5´end of chitinase transcript was mapped according to the protocol described by **Pinto and Lindblad(2010)**. Total RNA 8.5 µl (1 µg) was supplemented with 1 µl of 10 mMdNTP and 1 mM ChitREV255 5´-GCTGACATCACCACCTCCACTAGGAGAA-3´ primer denaturated at 65 ^{0}C for 5 min, and quickly cooled on ice. Then the mastermix containing 1 µl of 25 mM $MgCl_2$, 20 U RNase inhibitor, 3 µl of 5x buffer and 100 U Maxima H Minus reverse transcriptase (Thermo Scientific, USA) was added to the denaturated RNA and cDNA extension proceeded for 60 min at 50 ^{0}C. Next to the mixture was added prewarmed template-switching mastermix containing 1 µl of 25 mM $MgCl_2$,100 mM $MnCl_2$, 100 U reverse transcriptase, 1 µl of 5x buffer and 2 µl of 10 mM of TSOprimer (5´-GTCGCACGGTCCATCGCAGCAGTCACAGGGGG-3´), temperature was lowered to 42 ^{0}C and reaction was incubated for additional 120 min. The reaction was inactivated at 85^{0}C for 5 min. The 5´end products were synthesized in two step PCR, using U-sense primer (5´-GTCGCACGGTCCATCGCAGCAGTC-3´) and the gene specific primer ChitREV200 5´GCTGGTGGTGGAGAGCTGCCACCACACT3´. In subsequent PCR reaction U-sense primer and ChitREV146 5´-

GCTGGTGGTGGAGAGCTGCCACCACACT-3´were used. The conditions for the first and second PCR were one cycle at 98°C for 3 min, followed by 45 cycles at 98°C for 15 s and 72°C for 60 s and one cycle at 72°C for 10 min.The 5´end products were cloned into pGemT-easy (Promega, USA) and sequenced to identify the transcription start site.

RESULTS AND DISCUSSION

Analysis of genomic *Drchit* sequence

The1666 bp DNA fragment of chitinasewas amplified from the ATG start codon to the termination codon. The NetGene2 online program detected the presence of three exons and two intronswith the size 114 bp and 572 bp, respectively (figure1).

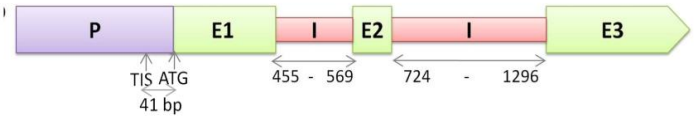

Figure 1 Structural characterization of the *Drchit* gene. The introns were *in silico* and experimentally proven at position 455 – 569 bp and 724 - 1296 bp from the ATG start codon.

To confirm splicing of the introns, the PCR on cDNA template was performed with ChitStartFor and ChitStopRev primers surrounding the *Drchit*gene. Following the amplification, the fragment of the length ~950 bp was detected on agarose gel (figure 2).

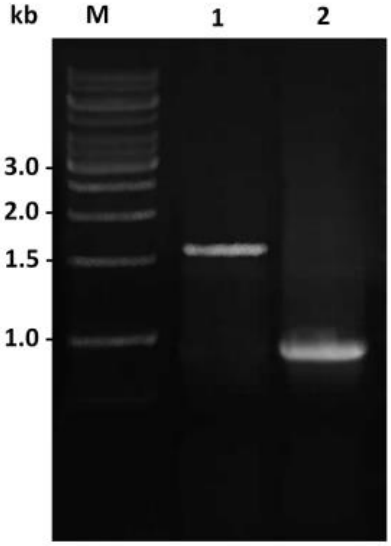

Figure 2 PCR splicing of introns in *DrChit* gene. PCR with ChitStartFOR and ChitStopREV primers resulted in amplification of~1600 bp fragment on the genomic DNA template (lane 1) and ~950 bp fragment on the cDNA template (lane 2) (Marker - 1 kb DNA ladder SM0312 ThermoScientific).

Analysis of PCR ampliconsequence data confirmed the accordance of intron splicing with results of *in silico*, performed by NetGene 2 program.Both introns were AT rich (60.7% and 65.2%, respectively) and posses consensus splice junction of 5´-G|GT and 3´-AG|G for the first intron and 5´-A|GT and 3´-AG|C for the second intron. On the other hand, *in silico* splicing of introns performed by GeneScan program predicted the first and third exon in accordance with an experimental approach, but the second exon differed in itslength.

When experimentally proven intron pattern of the *DrChit* was compared with other homologous genes, the similarity with other dicots chitinases was reported.Both introns were surrounded by the DNA sequences thatcorresponded at their 5´and 3´ends to amino acids FATT and GWPTA; QIST and NYNY, respectively. Generally, most of the genes encoding plant chitinases are interrupted by an intervening sequence. For example the *Arabidopsis* class I chitinases have one intron (**Samac*et al.*, 1990**) tobacco chitinase genes have two introns (**Van Buuren*et al.*, 1992**), while rice chitinase genes were intronless (**Zhu and Lamb, 1991**).

The nucleotide sequence around the predicted ATG start codon region, AACCATGG is in agreement with the Kozak consensus initiator ANNATGG **(Lutzke*etal*,.1987)** proposed for the translation start of plant genes.

When the 5´upstream sequence from ATG was investigated by Neural Network Promoter Prediction program for the presence oftranscription start site (TSS) two possible starts of transcription with the position 41 bp and 66 bp from the ATGwere predicted (**Ďurechová*et al.*, 2014**).To determine functional TSS and 5´untranslated region of mRNA in *in vivo*, the template-switch-based 5´RACE was performed.Following the isolation of total RNA from sundew leaves, cDNA synthesis and two-round PCR,a specific fragment of the length ~ 200 bp was isolated from the agarose gel, cloned into the pGemT-easy vector and sequenced (figure 3).The sequencing data obtained were in accordance with the transcription start site predicted *in silico* at the position 41 bp upstream of the ATG.

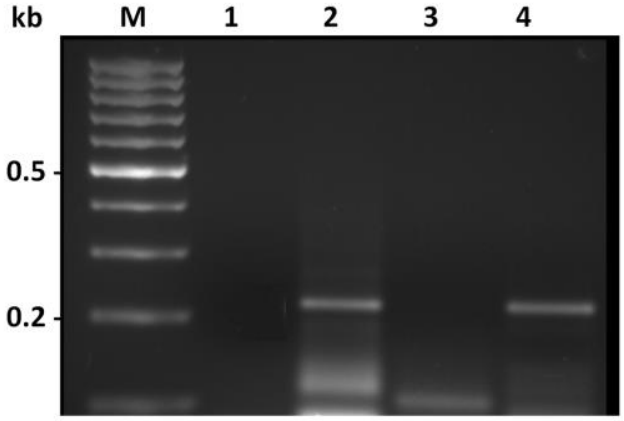

Figure 3 Agarose gel analysis of template-switch-based 5´RACE -PCR products. 5'-RACE-PCR were performed on first-strand cDNA prepared from total RNA of leaves *Drosrerarotundifolia*plants using the U-sense primer and gene specific primers of ChitREV200 and ChitREV146 and the templates prepared with (lanes 2 and 4) or without reverse transcriptase (lanes 1 and 3) (M- 100 bp DNA ladder SM1143 Thermo Scientific).

The 5´UTR of mRNAs are known to be involved in the translation of genes in response to the environmental stress (**Floris *et al.*, 2009**).Analysis *in silico*showed that within this short sequence the motifs ACGTATERD1 (**Simpson*et al.*, 2003**), MYB2CONSENSUSAT (**Abe*et al.*, 2003**) and MYBCORE (**Urao*et al.*, 1993**) playinga role in dehydrationstresswererevealed. Thepresenceof*cis*-acting elements in the untranslated region of the PR genes has not been reported so far (**Veluthakkal and Dasgupta, 2012).**

CONCLUSION

In conclusion we were able to isolate the chitinase gene from carnivorous plant *Droserarotundifolia* and make some deducation on the gene architecture through the use of bioinformatic tools. The isolation of total RNA from sundew and amplification of 5´end ofchitinase confirmed the expression of this gene in sundew plants and revealed the transcription start site 41 bp upstream from the ATG. The template-switch-based 5´RACE system used in our experiment for identification of 5´end of chitinase have been shown as time-in-efficient and inexpensive comparing to the other commercially available kits.

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