

BRIEF COMMUNICATION

Cloning and characterization of two osmotin isoforms from *Piper colubrinum*

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Abstract

In the present study, we report the cloning and sequence characterization of two isoforms of *osmotin*, an antifungal PR-5 gene homologue, from a salicylic acid-induced subtracted cDNA library earlier generated in *Piper colubrinum*. The larger form of the gene is 693 bp long, encoding a 21.5 kDa protein. The smaller form comprises a 543 bp long coding sequence which code for a protein of 16.4 kDa. A notable feature of the smaller form was a prominent internal deletion of 150 bp besides certain point mutations. Cloned isoforms of osmotin from resistant species could be candidates for molecular breeding for the improvement of black pepper as well as candidates for the study of structure based mechanism of antifungal activity attributed to PR-5 family.

Additional key words: black pepper, *Phytophthora capsici*, PR proteins.

Osmotin is a potent antifungal protein with varied roles in defense and development in plants. It has shown antifungal activity *in vitro* as well as *in planta* against many phytopathogens (Liu *et al.* 1994, Abad *et al.* 1996, Liu *et al.* 1996, Hu and Reddy 1997). Pathogenesis related (PR) proteins of PR-5 family are also cited as antifreeze proteins and inhibitors of insect proteases and α -amylases (Hon *et al.* 1995, Richardson *et al.* 1987). Fungi of different taxa and even molecules of fungal origin are shown to induce a range of PR proteins in sugar cane (Ramesh Sundar *et al.* 2008). Structural similarity of osmotin to mammalian adiponectin and its capability to bind the adiponectin receptors has highlighted its potential as a therapeutic protein that could be employed in treatment against diseases like diabetes (Kadowaki and Yamauchi 2005).

We report the cloning and sequence characterization of two osmotin isoforms from *Piper colubrinum*, a wild relative of commercially important black pepper, *Piper nigrum*. *P. colubrinum* is resistant to many of the fungal diseases affecting cultivated black pepper. The gene osmotin is found to be differentially over expressed in

this plant in response to the quick wilt disease causing pathogen, *Phytophthora capsici* and defense signaling molecules like ethylene and salicylic acid (Dicto and Manjula 2005).

Healthy plants of *Piper colubrinum* Link, maintained in the greenhouse of Rajiv Gandhi Centre for Biotechnology, Trivandrum, India (16-h photoperiod, irradiance of 200 - 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, day/night temperature of 20 - 25 °C and relative humidity 50 - 60 %) were used for the present study. Young leaves from 6-month-old cuttings were treated with ethylene (1 mg cm^{-3}) in the form of ethephon (*Sigma*, St. Louis, USA) and kept covered using plastic bags. Total RNA was isolated 24 h post treatment from 100 mg of leaf sample using *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA) as per the manufacturer's protocol. The isolated RNA sample was checked for integrity on 2 % EtBr-agarose gel and quantified by a spectrophotometer (*Biospec1601*, *Shimadzu*, Kyoto, Japan) at 260 nm. 5' and 3'RACE ready cDNA synthesis was carried out using *BD Power Script* reverse transcriptase following the standard reaction procedure given by the

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Abbreviations: PCR - polymerase chain reaction; PR - pathogenesis related; RACE - rapid amplification of cDNA ends.

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manufacturer (*BD SMART RACE* cDNA amplification kit, *Clontech*, CA, USA). The reaction was carried out at 42 °C for 1.5 h and terminated at 72 °C for 10 min. The standard RACE PCR protocol using the *P. colubrinum* osmotin (EB104044) gene specific primers (5'-CCGTGT TTAAGACTGACCAGTATTG-3' for 3'RACE and 5'-CAACTCCTTATTTTACCAACTGATC-3' for 5'RACE) and universal primer (5'TAATACGAC TCACTATAGGGCAAGCAGTGGTATCAACGCAG AGT-3') provided in the kit (*Clontech*) was carried out as per manufacturer's instructions. The PCR was carried out in an *Eppendorf* (Hamburg, Germany) *Mastercycler eps* gradient PCR machine under the following conditions: 95 °C (4 min), 25 cycles of 95 °C (15 s), 63 °C (1 min), and 63 °C (2 min). Gene specific primers were also used for positive control reactions besides the RACE PCR. Negative PCR reaction included either the gene specific or the universal primer alone. The products were checked on 1.2 % EtBr-agarose gel. The PCR products were purified from the gel using *GFX* columns (*Amersham Biosciences*, Little Chalfont, UK) and were cloned into T/A cloning vector *pGEMT-EASY* (*Promega*, Madison, WI, USA). The ligated products were introduced into *E.coli* strain JM109 and propagated on ampicillin (100 µg cm⁻³) supplemented LB agar plates. Plasmids isolated from selected clones were sequenced in an *ABI* prism automated sequencer (*Applied Biosystems*, Foster City, CA, USA) using the sequencing primers SP6 (5'-TATTTAGGTGACACTATAG-3') and T7 (5'-TAA TACGACTCACTATAGGG-3') from both the ends of the insert. The nucleotide sequence data obtained were entered into the *BLAST* query and searched for sequence identity and similarity. Primers designed based on the

sequences of the RACE products were used to amplify the gene in full length as a single product which could facilitate further validation process (forward primer 5'-CTTGCTATCACTCATCTCCACCTCTG-3', reverse primer 5'-CCATTAAGCTTTCATGGGCAGAAG-3'). The PCR cycling parameters were as follows: Initial denaturation at 95 °C for 2 min, followed by 25 cycles of 95 °C for 15 s and 66 °C for 1 min and a final extension of 66 °C for 5 min.

Good quality RNA was obtained using the *Trizol* method and a spectrophotometric reading of 1.88 as *A*₂₆₀/*A*₂₈₀ ratio was obtained on an average. The standard RACE PCR protocol gave amplification in the positive and the 5' as well as 3'RACE reactions. The 5'RACE PCR produced a 900 bp product and the positive control reaction gave an intense band of 300 bp (Fig 1). The 3'RACE product was approximately 500 bp. (Fig. 1). *BLAST* analysis revealed the identity of the sequence of PCR products as osmotin. The 5'RACE clone contained the entire coding sequence of the protein with start and stop codons and 5' and 3'UTRs. Primers designed based on the start and stop sequences of the RACE products were used to clone the gene in full length as a single PCR product which could facilitate further *in vitro* characterization procedures. Two distinct products were obtained as a result of the PCR using gene specific primers (Fig. 1) which were further cloned into T/A vector *pGEMT-EASY* for sequence characterization.

Both products were identified as proteins belonging to the PR-5 family. The sequence of both the genes revealed their identity as *osmotin* homologues. The larger form of *osmotin* comprised of 693 bp from start codon to the stop codon whereas the other form was shorter with only

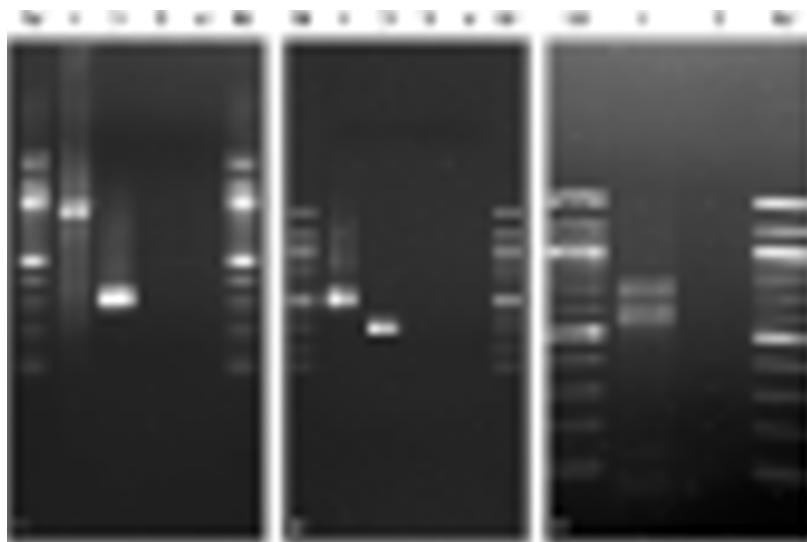


Fig. 1. The standard RACE PCR protocol. 1 - 5'RACE: lane 1 - the RACE product of 900 bp, lane 2 - positive control reaction with gene specific primer, lanes 3, 4 - negative control reaction with universal primer and gene specific primer, respectively. 2 - 3'RACE: lane 1 - the RACE product of 500 bp, lane 2 - positive control reaction with gene specific primer, lanes 3, 4 - negative control reaction with universal primer and gene specific primer, respectively. 3 - amplification of osmotin isoforms: lane 1 - the two forms are amplified from the ethylene induced cDNA sample, lane 2 - negative control reaction. M - 100 bp marker. The products were separated on 1.2 % EtBr-agarose gel.

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osm2 ATGTCACATATAACAATATAGTAAACATGGCCCTTCCTCTCCTCTACCTCCTCCCCTTGCTA 60
osm1 ATGTCACATATAACAATATAGTAAACATGGCCCTTCCTCTCCTCTACCTCCTCCCCTTGCTA 60
*****
osm2 TCACATCATCTCCACCTCTGCCCATGCAGCCAACCTTCCTAATAAGGAACAACCTGCCCTAC 120
osm1 TCACATCATCTCCACCTCTGCCCATGCAGCCAACCTTCCTAATAAGGAACAACCTGCCCTAC 120
*****
osm2 ACCGTC TGGGCGCCGAGTCCCCGGAGGAGGCCGCGCTCGACCGCGGTGCCACATGG 180
osm1 ACCGTC TGGGCGCCG----- 136
*****
osm2 AGCCTCAACGTCCCGGCAGGCACCACCGGAGCCGAATCTGGCCCCGAACCAACTGCAAC 240
osm1 -----
*****
osm2 TTCGACGGCAGCGGCCGTGGACGTTGCCAGACCGGCGACTGCGGCGGCGTCTCCAATGC 300
osm1 -----GCGTCTCCAATGC 150
*****
osm2 ACAGCCTACGGCGCCCCGCCAACACCCCTCGCCGAGTACGCCCAACCAAGTCTCCAAC 360
osm1 ACAGCCTACGGCGCCCCGCCAACACCCCTCGCCGAGTACGCCCAACCAAGTCTCCAAC 210
*****
osm2 CTCGACTTCTTCGACATCTCCCTCGTTCGACGGCTTCAACGTACCCATGGACTTTAGTCCC 420
osm1 CTCGACTTCTTCGACATCTCCCTCGTTCGACGGCTTCAACGTACCCATGGACTTTAGTCCC 270
*****
osm2 GTTTC TAGCGGTTGCAGGGGGCCAAGGGCATGCACCGCCGACATCAATGGCCAGTGCCCG 480
osm1 GTTTC TAGCGGTTGCAGGGGGCCAAGGGCATGCACCGCCGACATCAATGGCCAGTGCCCG 330
*****
osm2 AACGTGCTCCGAGCGCCAGGCGGGTGCAACAACCCATGCACCGTGTTTAAGACTGACCAG 540
osm1 AACGTGCTCCGAGCGCCAGGCGGGTGCAACAACCCATGCACCGTGTTTAAGACTGACCAG 390
*****
osm2 TATTGTTGCAACTCCGGCAGCTGTTCCCGACGGACTACTCGAGGTTCTTCAAACTAGG 600
osm1 TATTGTTGCAACTCCGGCAGCTGTTCCCGACGGACTACTCGAGGTTCTTCAAACTAGG 450
*****
osm2 TGCCCGGATGCCATATAGCTACCCCAAGGATGATCCTACCAGCACTTTCACCTTGCCCCGGC 660
osm1 TGCCCGGATGCCATATAGCTACCCCAAGGATGATCCTACCAGCACTTTCACCTTGCCCCGGC 510
*****
osm2 GGCACAAATTACAGAGTTGTCTTCTGCCCATGA 693
osm1 GGCACAAATTACAGAGTTGTCTTCTGCCCATGA 543
*****

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Fig. 2. Sequence alignment of osmotin isoforms. Nucleotide sequence alignment showing the internal deletion and the single base differences (194th and 417th position in osmotin isoform; osm 1) in the coding sequences.

543 bp. The deduced amino acid sequence of both proteins revealed an N-terminal signal peptide comprising of 29 amino acids, and a calculated molecular mass of 21.5 kDa, and a pI value of 8.32 for the larger form whereas the smaller form encodes a 16.4 kDa protein which is more acidic in nature (pI 5.6). Single base sequence changes were observed in smaller form at positions 194 and 417 (Fig. 2). Nucleotide sequences of both the forms are submitted to Genbank, NCBI (EU271754, FJ009675). *Piper colubrinum* is a wild species showing high degree of resistance to many fungal pathogens including *Phytophthora capsici* and hence documented as a rich source of defense genes for genetic improvement of cultivated black pepper (Ravindran *et al.* 2000). Characterization of resistant species may require the study of morphological traits along with molecular characterization as there is strong correlation of certain morphological features of plants and fungal resistance (Egea-Gilabert *et al.* 2008). Earlier reports on the development of micropropagation methods in *Piper nigrum* including the recent report on cyclic somatic embryogenesis (Philip *et al.* 1992, Nair and Gupta 2006) will facilitate the genetic improvement of this crop through gene transfer techniques. In the present study, the

osmotin gene homologues were cloned from *P. colubrinum*. Osmotin is an antifungal PR-5 family protein, which was isolated for the first time from salt adapted tobacco cells (Singh *et al.* 1987). Earlier reports of induction of osmotin in plant-pathogen interaction (Bryngelsson and Gr en 1989, Hong *et al.* 2004, Ramesh Sundar *et al.* 2008), the *in vitro* inhibitory activity of the protein towards fungal pathogens (Roberts and Selitrenikoff 1990, Woloshuk *et al.* 1991, Vigers *et al.* 1992, Liu *et al.* 1996, Hu and Reddy 1997) and in transgenic plants (Liu *et al.* 1994) highlight the protein as a component of the defense response in plants. In *P. colubrinum* the over expression of osmotin under biotic/abiotic stress and the earlier report of its induction in response to salicylic acid indicates a possible role in defense response of the plant (Dicto and Manjula 2005). Thaumatin like proteins (osmotin) from different plant species are expressed in response to a variety of infections and stress, and sequence similarity does not necessarily mean identical functions. Hence, it is important to identify plant specific forms of the respective genes. It is interesting to note that the two osmotin isoforms differ sharply in their pI value which could determine its mode as well as site of action. The

formation of such isoforms, probably by alternative splicing, could suggest a different function of these proteins in defense as well as in development. The different osmotin isoforms cloned from plants could be

candidates for in depth study on structure based mechanism of antifungal action and could yield relevant information for authentication of its therapeutic potential.

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