SHORT COMMUNICATION

CLONING AND EXPRESSION OF A NOVEL LACCASE GENE FROM PHYTOPHTHORA CAPSICI

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SUMMARY

Phytophthora capsici is an aggressive plant pathogen that affects solanaceous and cucurbitaceous hosts. From this fungus, the novel laccase gene pclac1 was recovered and its corresponding full-length cDNA was cloned. The 1716 bp full-length cDNA of pclac1 encoded a mature laccase protein containing 571 amino acids. The deduced protein sequence showed high similarity with other known fungal laccases and contained four copper-binding conserved domains typical of laccase protein. Expression pattern of pclac1 in the host plant was evaluated, showing that expression levels increased until the 4th day post inoculation (dpi). Heterologous expression of PCLAC1 was achieved using the expression vector pPIC9K with the Pichia pastoris expression system. The purified recombinant PCLAC1 protein migrated as a single band in SDS-PAGE, with an apparent molecular weight of ca. 68 kDa. A high activity of purified PCLAC1 (88.46 U/ml), induced with methanol with 2,2'-azino-di-(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) as substrate, was observed at the 7th dpi. The reported data add new knowledge on P. capsici laccase multigene family and shed light on the potential function of individual laccase isoforms of oomycetes for biotechnological and industrial applications.

Key words: Phytophthora capsici, laccase, expression, purification.

Oomycetes are ubiquitous Protista belonging to the kingdom Stramenopile and comprising many pathogenic species (Baldauf et al., 2000). The genus Phytophthora contains a large number of phytopathogens (e.g. P. capsici, P. infestans, P. sojae) that cause blight, crown rot and stem, leaf, and fruit lesions in many plants. Phytophthora capsici, in particular, is known as a devastating pathogen of solanaceous and cucurbitaceous hosts including pepper, cucumber, eggplant, squash, pumpkin, tomato, melon, and zucchini (Lamour and Hausbeck, 2004).

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are a group of enzymes called blue copper oxidases, capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water. They are widely distributed in nature, have been found in plants, fungi, bacteria and insects and are believed to be involved in lignin degradation and biosynthesis (Thurston, 1994; Gianfreda et al., 1999). Moreover, laccases are very valuable enzymes in the global carbon cycle and for industrial applications, as they degrade a wide range of recalcitrant compounds and are used as biological bleachers in the paper industry (Hou et al., 2004). Recent studies on the biological function of fungal laccases suggest that these enzymes may play an important role also in fungal morphogenesis, virulence and pigmentation (Thurston, 1994; Baldrian, 2006).

Several laccase genes have been cloned from different fungal sources and heterologously expressed with the specific purpose of using laccases more efficiently in biotechnology (Galhaup et al., 2002; Xiao et al., 2006; Soden et al., 2002). Furthermore, a variety of heterologous proteins have been successfully produced for studying their biological functions (Cereghino and Cregg, 2000; Piscitelli et al., 2010). For instance, the expression in the host of a laccase from Cryptococcus neoformans was investigated for its pathogenic function (Williamson et al., 1998). However, to date, apparently there are no studies on the function of oomycete laccases.

In this investigation, a novel laccase gene from P. capsici cDNA was cloned, its expression pattern during infection of the host plant was analyzed, and the heterologous expression and purification of PCLAC1, the gene product, was achieved. PCLAC1 showed a high activity when ABTS [2,2'-azino-di-(3-ethylbenzothiazolin-6-sulfonic acid)] was used as substrate.

Phyc12, a virulent P. capsici strain was maintained at 25°C on 10% V8 juice agar medium as described by Tyler et al. (1995). After growing in 10% V8 liquid medium for three days Phyc12 mycelium was collected and ground in liquid nitrogen. For Pclac1 gene cloning, total RNA was extracted using a fungal RNA kit (OMEGA, USA) following the manufacturer’s recommendations. The primers lcc1-1 and lcc1-2 (Table 1) were designed using Primer Express 3.0 software, ac-
According to a laccase sequence derived from the genome sequence of *P. capsici* (http://genome.jgi-psf.org/PhycaF7/PhycaF7.download.html). PCR parameters were as follows: 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 57°C for 45 sec, 72°C for 60 sec, then 72°C for 10 min for a final extension. Purified PCR products were cloned into the plasmid vector pMD20-T (TaKaRa, Japan), and positive clones were verified by sequencing.

The pepper cv. Tianying was grown in a greenhouse at 25-30°C for inoculation experiments. Leaves detached from plants at the 5th to 6th leaf stage were inoculated with 2.5 µl of a zoospore suspension (1 × 10⁵ zoospores/ml) and placed in 1.5% (w/v) water agar plates in the dark for 5 days at 28°C (Feng et al., 2010). Control leaves were inoculated with distilled water. Samples were collected at 1, 2, 3, 4, and 5 days post inoculation (dpi), frozen in liquid nitrogen and stored at -80°C.

The expression pattern of *pclac* in the host-pathogen interaction process was analyzed by Real Time quantitative PCR with the ICycler IQ real-time PCR detection system (Bio-Rad, Denmark) using SYBR primer Script RT-PCR kit (TaKaRa, Japan). For PCR reactions, 2.5 µl of cDNA template were added to 12.5 µl of the 2X SYBR Green PCR master mix, 800 nM of each primer and double distilled water to a final volume of 25 µl. After a denaturation step at 95°C for 10 min, the cycling profile used was 10 sec at 95°C, 55 sec at 60°C, and 45 sec at 72°C for 45 cycles. The primers qPCR1 and qPCR2 (Table 1) were designed using Primer Express 3.0 software. The 18SrRNA was used as housekeeping gene.

*Pclac1* was expressed using an EasySelect Pichia Expression Kit (Invitrogen, USA) employing media and procedures according to the kit manual. To obtain the mature protein, the *pclac* cDNA was used as template with primers Lac1-UPtag (including XhoI in bold and his tag underlined) and Lac1-DNtag (including NotI in bold) (Table 1). PCR products were digested with XhoI and NotI, then cloned in XhoI-NotI-digested pPIC9K to generate expression vector pPIC9K/pclac1.

The recombinant vector pPIC9K/pclac1 was transformed into *Pi. pastoris* competent cells using a JY2000-1A Gene Pulser (Ningbo, China). Transformants (His+/Mut*) were selected as described previously (Feng et al., 2010). Resultant colonies were screened by PCR using primers 5’-AOX1/3’-AOX1 and Lac1-UPtag/Lac1-DNtag (Table 1). The obtained transformants were grown in 100 ml buffered methanol complex medium (BMMY) in 250 ml flasks and methanol was added to a final concentration of 1% (w/v) for 14 days induction at 30°C. The empty vector pPIC9K was used as a control.

For purification, the culture was centrifuged at 8000 g for 10 min. Then the supernatant was treated with solid ammonium sulfate at 70% saturation in a Ni-NTA column (Invitrogen, USA) according to Feng et al. (2010). The product was designated PCLAC1.

Laccase activity was determined with ABTS as substrate (Bourbonnais et al., 1998). The reaction mixture contained 0.5 mM substrate (ABTS), 2.8 ml of 0.1 M sodium acetate buffer of pH 4.5, 100 µl of PCLAC1 and was incubated for 5 min. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank. One unit was defined as the amount of the laccase that oxidized 1 µmol of ABTS substrate per minute. Purified PCLAC1 concentration was determined by the dye-

Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>lac1-1</td>
<td>CGGAGCGAGACGATGTCATTTGCAC</td>
</tr>
<tr>
<td>lac1-2</td>
<td>AACTCCACGACAAATCCGGCTCGG</td>
</tr>
<tr>
<td>qPCR1</td>
<td>CAGCTTGGTGGTAGTCCCTGA</td>
</tr>
<tr>
<td>qPCR2</td>
<td>CAGTGCATGATTACACAGCC</td>
</tr>
<tr>
<td>18SrRNAF</td>
<td>TTTTGCGTTCATATTAGTTGG</td>
</tr>
<tr>
<td>18SrRNAR</td>
<td>TTTGCGATTGTTCGTCTTTTC</td>
</tr>
<tr>
<td>Lac1-UPtag</td>
<td>CGTACTCGAGCACCCACCCACCCACACCGCTGTCGCCGCTACGACT</td>
</tr>
<tr>
<td>Lac1-DNtag</td>
<td>CGTACCGGCCGCTTACAGGGTGAAAGTTCACA</td>
</tr>
<tr>
<td>5’-AOX1</td>
<td>GACTGCGTTCCAATTGACAGC</td>
</tr>
<tr>
<td>3’-AOX1</td>
<td>GCAAATGGCATTTGACATCC</td>
</tr>
</tbody>
</table>
binding method, using BSA as standard (Bradford, 1976).

One complete sequence of the pclac1 gene was obtained and submitted to GenBank (accession No. JQ683128). The open reading frame of pclac1 contains 1,716 bp and encodes a polypeptide of 571 amino acid residues with a predicted molecular mass of 62.74 kDa (Fig. 1). Based on alignment with BLAST in the NCBI online database, pclac1 contains four copper-binding conserved domains of typical laccase: CuI (HGHEEVQ), CuII (HPFHLAHSP), and CuIV (MHCHIDWH), as reported by Piontek et al. (2002) and Fan et al. (2011).

The expression pattern of pclac1 gene was investigated every day from 1 to 5 dpi (Fig. 2). The pclac1 transcripts exhibited significant changes during the infection process, increasing from 1 to 4 dpi while decreasing at the 5th day. Transcription of LAC3 from Gaemnnomyces graminis was observed only when the fungus was grown in association with the host (Litvintseva and Henson, 2002) and laccases from Cryphonectria parasitica (Chung et al., 2008) and Fusarium oxysporum (Canero and Roncero, 2008) have been implicated in pathogenicity. Our data indicated that pclac1 may be involved in the host-plant interaction process.

A protein (PCLAC1) with an apparent molecular weight about 68 kDa was detected by SDS-PAGE in the culture within 1-14 days after methanol induction and there was no band in the negative control with empty pPIC9K (Fig. 3).

Fig. 1. Nucleotide and deduced amino acid sequences of pclac1. Signal peptides are underlined. Four conserved regions are boxed out, representing the CuI, CuII, CuIII and CuIV domain, respectively, as described (Hakulinen et al., 2002; Piontek et al., 2002). Seven potential N-glycosylation sites are doubly underlined.

Fig. 2. Real-time PCR analysis of pclac1 gene expression in pepper leaves inoculated with P. capsici from 1 to 5 dpi. 18S rRNA was chosen as an endogenous control. Data represent the average of three independent experiments with standard error.

Fig. 3. The recombinant protein expression of PCLAC1 in Pi. Pastoris GS115. Lane 1, low molecular weight marker; lane 2, pPIC9K (empty vector); lane 3, recombinant protein expression after induction; lane 4, purified PCLAC1 with Ni-NTA column.
Due to the 6^th His tag of PCLAC1, purification of the secreted PCLAC1 was easily obtained on a small scale using the His-bind Ni-NTA purification method (Pro-Bond, Promega, USA). The result of SDS-PAGE showed that PCLAC1 from P. capsici had a molecular weight of ca. 68 kDa (Fig. 3), while the predicted molecular mass was 62.74 kDa. This difference could be attributed, at least partially, to glycosylation of the protein, as the deduced sequence presents seven potential residues for N-glycosylation (Fig. 1) (Patrick et al., 2005; Shi et al., 2007).

The activity of purified PCLAC1 was measured daily in culture growth at 30°C from 1-14 days. Results showed that laccase activity increased at 1-7 days, and peaked at the seventh day, then decreased in the days that followed. The highest yield of PCLAC1 activity was 88.46 U/ml at day 7 (Fig. 4). High laccase activity has also been observed with ABTS as substrate by others (Eggert et al., 1996; Jung et al., 2002). No extracellular laccase activity was detected in culture supernatants of the negative control (pPIC9K).

In conclusion, PCLAC1 is a novel protein with possible biological functions in terms of interaction with plants and for biotechnological applications. Further studies are however necessary to uncover the function of laccases present in P. capsici and other Phytophthora species.

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REFERENCES


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