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Identification, cloning and expression of a new GDSL lipase from *Carica papaya*

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ABSTRACT

Plants are a very interesting and new source of lipases for a variety of applications. *Carica papaya* is a remarkable source of lipases, as its genome reveals several putative lipolytic genes whose expression, function and applications remain undefined. In this study, total RNA from papaya leaves was isolated and used as a template to amplify the open reading frame (ORF) of a putative GDSL lipase gene, *CpLIP2*, by RT-PCR. It was found that *CpLIP2* expression is induced by inoculation of papaya plants with the fungal pathogen *Corynespora cassiicola*. The *CpLIP2* ORF was further cloned into a viral provector module for transient expression in *Nicotiana benthamiana* plants. The presence of *CpLIP2* transcripts in *CpLIP2*-transformed plants was demonstrated by RT-PCR. Importantly, lipase activity was only detected in extracts from plants transformed with the *CpLIP2* gene and not in those from plants transformed with an irrelevant gene used as a negative control, confirming that the gene was successfully expressed and that its product retained activity. A characterization of substrate specificity showed that *CpLIP2* has a preference for short-chain rather than medium- and long-chain triglycerides.

1. Introduction

Lipases are defined as triacylglycerol acylhydrolases capable of hydrolyzing carboxyl esters of long-chain acylglycerols into fatty acids [1,2]. Based on their conserved sequences, lipases can be classified into two families. The first one, the GXSXG lipase family, is characterized by a catalytic triad, Ser–His–Asp/Glu, that forms the active site, whose serine residue is located in a highly conserved Gly/Ala–X–Ser–X–Gly (X stands for His/Tyr) region in a turn between a β-strand and an α-helix [2]. The second lipase family, the GDSL lipase family, is characterized by the presence of a conserved serine-containing motif, Gly-Asp-Ser-(Leu) [GDS(L)], closer to the N-terminus [3] and is the most common type of lipase found in plants. Concerning their function, it has been reported that lipases are mostly involved in two major processes in plants: the obtainment of energy for plant growth and development [4–6] and the response to abiotic or biotic stress [7–11].

Recently, plants have been studied as a source of new lipases with interesting properties for industrial applications (for review see [12–14]). However, the purification of plant lipases becomes especially complicated when they are produced as secondary metabolites in latex [13]. Nonetheless, *Carica papaya* (*C. papaya*) latex has been extensively reported to possess lipolytic activity and successfully used as a biocatalyst for different reactions with industrial applications [13], such as the hydrolysis of triglycerides [15] and the synthesis of human fat milk analogs [16], biopolymers [17] and medium- and long-chain diesters of 2-oxoglutaric acid [18] as well as the enantiomeric resolution of naproxen [19] and 2-bromo phenylacetic acid octyl esters [20].

The *C. papaya* lipolytic enzymes that catalyze these reactions remain unknown, mainly because it is extremely difficult to purify

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these enzymes from latex due to their strong attachment to the insoluble fraction. Nevertheless, three different enzymes with lipolytic activity have previously been reported to be present in this latex [21–23]. As the *C. papaya* genome is now available [24], it is possible to look for genes that encode lipolytic enzymes that have not yet been reported and evaluate their expression kinetics, function, biochemical properties and possible applications. Some lipases might be expressed at low levels, which may impede their purification for further characterization, structure-function relationship and applications studies. To overcome this problem, heterologous expression systems can be used, in which case the selection of the proper host is a key step of the process. For this particular purpose, transient protein expression in plants using viral provector systems presents several advantages, such as rapid and easy cloning and expression of genes, which allows for fast characterization of the gene product, and high expression levels, which can constitute up to 80% of the total protein produced [25,26]. Recently, a member of the GDSL family from *Brassica napus* was transiently expressed in *Nicotiana benthamiana* [27].

In this study, a putative GDSL lipase gene from *C. papaya* was selected and transiently expressed in *N. benthamiana* through a viral provector-based system, and its lipolytic activity in crude extracts was assessed. The substrate preference of crude CpLIP2 extracts in the hydrolysis of triglycerides of different chain lengths was also investigated.

2. Materials and methods

2.1. *Selection of putative lipase genes from the* C. papaya *genome,* CpLIP2 *sequence analysis and three-dimensional structure prediction*

An informatics approach to identify genes that putatively belong to the GDSL family in the *C. papaya* genome (GenBank accession number: ABIM00000000) was performed by PFAM [28]. The selected GDSL lipase was analyzed with the BLAST program from the National Center for Biotechnology Information (NCBI). The amino acid sequence of *CpLIP2* was aligned with *Capsicum annuum* CaGLIP1, *Arabidopsis thaliana* GLIP7, *Oryza sativa* GID1 and *Pseudomonas aeruginosa* PaEst A domain (GenBank accession numbers: AY775336, At5G15720, Os05g0407500 and 3KVN_X, respectively) using CLC Main Workbench software (CLC Bio, Qiagen). The predicted three-dimensional structure model of *CpLIP2* was constructed based on predictions made by I-tasser software.

2.2. CpLIP2 *expression after fungal infection*

Carica papaya cv. Maradol seeds were germinated in trays containing peat moss. Four weeks after germination, the plants were transferred individually into 4″ pots containing a rich soil mixture. The plants were watered every 2 days, fertilized weekly with a 20-20-20 (N-P-K) soil drench solution (Scotts-Sierra Horticulture Products) and maintained in a growth chamber under controlled light and temperature conditions (16 h light at 28 °C/8 h darkness at 16 °C). Six-week-old plants with 4–5 expanded leaves were used in both assays.

A pure culture of the fungal pathogen *Corynespora cassiicola* was isolated from naturally infected papaya leaves according to Herradura et al. [29] and was periodically subcultured on potato dextrose agar (PDA) medium for 25 days at 25 °C.

Papaya plants were foliar-sprayed with a *C. cassiicola* suspension (2×10^5 spores/ml) or H_2O as a control treatment. The inoculated plants were maintained inside polythene bags to maintain a high humidity level. Foliar tissue was collected at 24, 48 and 72 h after inoculation and immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

RNA isolation was performed using Trizol reagent (Invitrogen) followed by DNase (Promega) treatment. Two micrograms of treated RNA was reverse transcribed with Superscript III (Invitrogen) using oligo–$dT_{(12–18)}$ primers. qPCR amplifications were performed using SYBR Green detection in a StepOne Plus system (Life Technologies). Reactions were prepared in a total volume of 15 μl containing 4 μl of diluted cDNA (1:200), 0.15 μl of each primer (5 mM) and 7.5 μl of Power SYBR Green PCR Master Mix (Life Technologies). The primers used were as follows: *CpLip2* 5′-GTTTTAGCCTTCCTGATG-3′, 5′-TGAAGCTGCTGGCATGTC-3′; and *EIF* 5′-GCCTTCATAGATAGTAGCTC-3′, 5′-GCAAGACAGGAAAGGGAT-3′ [30]. The cycling program was as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min. After PCR amplification, a melting curve analysis was immediately performed.

Three biological replicates and two technical replicates were analyzed. Baseline and threshold cycles (Ct) were automatically determined using the qPCR system. Relative expression was calculated using the comparative cycle threshold method [31]. The transcript abundance data were normalized against the average transcript abundance of the reference gene eukaryotic initiation factor (*EIF*).

2.3. CpLIP2 *cloning and transient expression in* Nicotiana benthamiana

cDNA generated as described above was used as a template for the amplification of the complete *C. papaya* lipase 2 gene (*CpLIP2*) with the primers 5′-ATGGATATCTTCTCAAACAAGCA-3′ and 5′-TCATAGAGTGACGAGTCGT-3′.

The amplified fragment was purified using a MinElute PCR Purification Kit (Qiagen) and cloned into the pCR2.1-TOPO cloning vector. The product was introduced into chemically competent TOP10 *E. coli* cells, which were plated on Luria Bertani agar medium containing 100 mg/l ampicillin and 32 mg/l X-Gal.

Plasmid DNA was extracted from several white colonies with a GeneJet Plasmid Miniprep Kit (Fermentas). The gene identity was verified by sequencing. The plasmid containing the *CpLIP2* gene was digested with KpnI and HpaI enzymes, purified with a MinElute Purification Kit (Qiagen) and further used as a template for amplification with the primers 5′TTTGGTCTCAAGGTATGGATATCTTCTCAAACAAGCA3′ and 5′TTTGGTCTCTAAGCTCATAGAGTGACGAGTCGT-3′, designed for subsequent gene cloning into the plant provector 3′ module pICH31070 (ICON Genetics) according to the provider's instructions.

The *CpLIP2* gene was PCR amplified with GoTaq DNA Polymerase (Promega) and purified using a MinElute PCR Purification Kit (Qiagen). Then, 50 ng of the purified PCR product was combined with 50 ng of the pICH31070 plasmid, 10 U of Eco31I enzyme (Fermentas) and 3 U of T4 Ligase (Promega) in a final volume of 10 μl and incubated at 37 °C for 4 h and then at 55 °C for 30 min. The reaction was stopped by an incubation at 80 °C for 15 min.

TOP10 *E. coli* cells were electroporated with 1.5 μl of the ligation product and plated on Luria Bertani agar medium containing 50 mg/l kanamycin and 32 mg/l X-Gal. Plasmid DNA was extracted from several white colonies with a GeneJet Plasmid Miniprep Kit (Fermentas) and confirmed by PCR.

The new plasmid, designated pICH-CpLIP2, was introduced into the *Agrobacterium tumefaciens* strain GV3101 by electroporation, and the cells were plated on selective LB agar plates supplemented with 50 mg/l kanamycin and 50 mg/l rifampicin.

For the expression of the *CpLIP2* gene in plants using viral provectors from ICON Genetics, six-week-old *N. benthamiana* plantlets grown at 25 °C in 16 h light/8 h dark were used. Strains carrying pICH-CpLIP2, pICH20111 and pICH14011 were cultured

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