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Pepper 9- and 13-lipoxygenase genes are differentially activated by two tobamoviruses and by hormone treatments

Csilla Juhász, István Tóbiás, Attila L. Ádám, György Kátay, Gábor Gullner*

Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó út 15, 1022 Budapest, Hungary

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ABSTRACT

Two novel pepper 13-lipoxygenase (*LOX*) genes were cloned and their expressions were compared with those of three 9-*LOX* genes in pepper leaves inoculated with two different tobamoviruses. *Obuda pepper virus* (ObPV) inoculation led to a massive induction of pathogenesis-related genes and to the development of hypersensitive reaction (incompatible interaction), while *Pepper mild mottle virus* (PMMoV) inoculation resulted in a compatible interaction. Both virus infections markedly activated the expression of the two novel *13-LOX*s. The magnitudes of induction of 13-*LOXs* did not differ substantially between the ObPV- and PMMoV-inoculated leaves. The induction of three *9-LOX*s was markedly more robust and rapid in ObPV-inoculated leaves than in PMMoV-inoculated ones. *LOX*s were very differentially activated in pepper leaves treated with defense hormones. A large number of hormone-related *cis*-regulatory elements were identified in the promoter regions of *LOXs*. ObPV inoculation resulted also in the substantial up-regulation of an omega-6-fatty acid desaturase gene. Our results suggest that 9-LOX-dependent pathways are more probably involved in the suppression of virus replication than 13-LOX-dependent plant responses.

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1. Introduction

Plant lipoxygenase (LOX) enzymes are ubiquitous, non-heme iron-containing dioxygenases, which catalyze the peroxidation of polyunsaturated fatty acids (PUFAs) by using molecular oxygen. The most common LOX substrates in plants are linoleic acid and α-linolenic acid. LOX enzymes have diverse functions in plant development and stress responses [1,2]. LOXs are encoded by small gene families in higher plants. In the genomes of *Arabidopsis thaliana*, rice, grape, cucumber and poplar 6, 14, 18, 23 and 20 *LOX* genes were identified, respectively [3–7]. Plant LOX isoenzymes can be generally divided into two groups, they are either 9-lipoxygenases (9-LOXs, E.C. 1.13.11.58) or 13-lipoxygenases (13-LOXs, E.C. 1.13.11.12), which produce 9- and 13-fatty acid hydroperoxides, respectively [1,2]. Both 9- and 13-fatty acid hydroperoxides can serve as substrates for at least seven different enzyme families in plants, which transform them into a wide variety of compounds. These fatty acid-derived metabolites are collectively called oxylipins, the most well known of which are jasmonic acid, hydroxy-fatty acids, divinyl ethers, and volatile C_6 fragments [8,9].

LOX enzymes have often been shown to play substantial roles in plant defense reactions against various microbial pathogens [10–15]. Two defense functions of LOXs are already well characterized: a) their contribution to hypersensitive host cell death and b) the production of bioactive oxylipins initiated by LOXs. The hypersensitive reaction (HR) was characterized by a massive production of PUFA hydroperoxides together with typical tissue dehydration [16–18]. On the other hand, numerous LOX-derived oxylipins possess antimicrobial or signaling functions in bacterium- and fungus-infected plants [8,9]. Limited information is available about the role of LOXs and the oxylipin pathways in virus-infected plants. *Tobacco mosaic virus* (TMV) inoculation led to a rapid saturation of microsomal phospholipid-bound fatty acids and to increased LOX activity [19,20] as well as to the accumulation of divinyl ethers [21] in tobacco leaves. Montillet et al. [16] demonstrated the accumulation of 9-LOX-produced PUFA hydroperoxides

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CMV, *Cucumber mosaic virus*; CP, coat protein; DES, divinyl ether synthase; FAD, fatty acid desaturase; ERE, ethylene-related promoter elements; EST, expressed sequence tag; hpi, hours post-inoculation; HR, hypersensitive response; LOX, lipoxygenase; MeJA, methyl-jasmonate; NaSA, sodium-salicylate; ObPV, *Obuda pepper virus*; PMMoV, *Pepper mild mottle virus*; PR, pathogenesis-related; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; TC, tentative consensus sequence; TMV, *Tobacco mosaic virus*.

* Corresponding author.
E-mail address: gullner.gabor@agrar.mta.hu (G. Gullner).

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in TMV-infected tobacco leaves by a temperature-shift method. The TMV-elicited increase of total LOX activity was more rapid and robust in resistant Xanthi-nc tobacco leaves than in the susceptible Samsun-nn cultivar [22]. *Cucumber mosaic virus* (CMV) inoculation led to up-regulated expression of five *LOX* genes in *A. thaliana* leaves. In addition, CMV inoculation increased the expression of two genes participating in jasmonic acid biosynthesis [23]. LOX activity and oxylipin metabolism were found to positively regulate the process of systemic necrosis during compatible plant–virus interactions [24].

To obtain a deeper knowledge about the role of LOX-dependent pathways in antiviral resistance mechanisms, we have investigated the activation of *LOX* genes in tobamovirus-infected pepper (*Capsicum annuum* L.) plants. Several viruses belonging to the genus *Tobamovirus* are major pathogens of pepper plants. In *Capsicum* species the resistance against tobamoviruses is conferred by four allelic forms of a resistance gene ($L^1 - L^4$, numbered in order of increasing effectiveness) at the locus L of chromosome 11 [25,26]. Resistance conferred by the recently sequenced L^3 gene [27] is very efficient against most tobamoviruses except for some closely related isolates of *Pepper mild mottle virus* (PMMoV), which are able to overcome this type of resistance and to cause systemic infection [26,28,29]. PMMoV is a positive-sense, single-stranded RNA virus with a relatively small (approx. 6400 bp) monopartite genome that encodes four proteins including two replication proteins, a movement protein, and a coat protein [30]. PMMoV can cause serious economic losses in both field and greenhouse-grown peppers [25,31]. Visible disease symptoms on PMMoV-infected leaves can be mild chlorotic spots but often no symptom appears. However, mottling, mosaic or curling symptoms can appear on those leaves that develop after inoculation. Infected plants can be stunted and the fruits are usually severely deformed, mottled or blotched [26,29,31]. *Obuda pepper virus* (ObPV) is also a positive-strand RNA virus, which belongs to the genus *Tobamovirus*. The genome organization of ObPV is similar to that of PMMoV [32]. However, in contrast to PMMoV, ObPV can not break the L^3 gene-mediated resistance, because the defense mechanisms of pepper are effectively activated. Thus ObPV inoculation leads to the development of local necrotic lesions (hypersensitive response, HR) [28,33,34]. The accumulation of pathogenesis-related proteins and the marked production of ethylene were also detected in ObPV-infected pepper leaves [28].

During our earlier investigations we observed that ObPV inoculation leads to a 51-fold induction of total LOX enzyme activity and to the up-regulation of several *9-LOX* genes in leaves of a pepper cultivar harboring the L^3 resistance gene. In addition, the expression of a 9-divinyl ether synthase (*9-DES*) gene was massively up-regulated in ObPV-inoculated leaves [35]. PMMoV exerted only a negligible effect on *DES* and *LOX* expression levels [35]. In addition, ObPV inoculation resulted in early membrane leakage, partial desiccation, increased heat emission as well as to declining chlorophyll *a* levels and diminished photosynthetic activity [36]. In the present study we have further investigated the same pepper-virus pathosystems. We have cloned two novel *13-LOX* genes and compared their up-regulation with that of three already known *9-LOX* genes in pepper leaves during an early phase following ObPV and PMMoV inoculations, between 4 and 48 h post-inoculation (hpi). We have characterized also the inducibility of these genes by defense-related plant hormones.

2. Materials and methods

2.1. Pepper variety, virus inoculations and hormone treatments

The pepper (*C. annuum* L.) cultivar TL 1791 harboring the L^3 resistance gene was used for all experiments. Seeds were kindly provided by Dr. Lajos Zatykó (Research Institute of Vegetable Crops, Budatétény, Hungary). Pepper plants were grown as described earlier [36] and 55–60-day-old plants were used for all experiments. ObPV, PMMoV and mock inoculations were carried out as described earlier [36]. The ObPV strain was isolated in Hungary (formerly used synonym: Ob strain of *Tomato mosaic virus*) [28,33,34] whereas the L^3-resistance-breaking strain of PMMoV was isolated in Louisiana, USA (formerly used synonym: Samsun latent strain of *Tobacco mosaic virus*) [28,34,37]. Virus-inoculated, and mock-inoculated control plants were kept at 25 °C in a growth chamber with 16/8 h light/dark cycles. Leaf samples for total RNA extraction were taken from virus-inoculated and corresponding mock-inoculated leaves after various early time periods between 4 and 48 hpi. In separate experiments three middle leaves of two-month-old pepper plants were treated with 1 mM sodium salicylate, 1 mM methyl jasmonate and 5 mM 1-aminocyclopropane-1-carboxylic acid (ACC) solutions by gentle brushing. Treatment with distilled water used as control. Treated and control plants were incubated at 22 °C in a growth chamber, and leaf samples were taken for total RNA extractions and LOX activity measurements at different intervals following hormonal treatment.

2.2. Cloning and sequencing of novel LOX genes

To identify novel pepper *13-LOX* genes nucleotide BLAST searches were conducted in the pepper expressed sequence tag (EST) databases of the GenBank and the Gene Index Project [38] with a soybean *13-LOX* sequences as a query (GenBank J02795). Identified EST sequences were *in silico* assembled into larger tentative consensus (TC) sequences. TC sequences were experimentally verified by RT-PCR with specific primer pairs (Table 1) by using total RNA extracts obtained from ObPV-inoculated leaves. Consecutively internal fragments were also amplified by specific primer pairs (Table 1). An alternative method was also used for the identification of novel genes. In this case a degenerate primer pair (Table 1) designed on well-conserved *LOX* nucleotide segments was used for RT-PCR. The 3′ terminal sequence of a novel *LOX* gene fragments was identified by a 3′-RACE kit (Invitrogen, Carlsbad, CA, USA) with specific forward primers (Table 1). All PCR products of the expected length were purified and ligated into pJET 1.2/blunt cloning vector with a CloneJET PCR Cloning Kit (MBI Fermentas, Vilnius, Lithuania) and cloned in competent *Escherichia coli* cells by standard methods. Recombinant plasmids containing the PCR fragments were purified by High-Speed Plasmid Mini Kit (Geneaid, Taipei, Taiwan) and made sequenced by Macrogen (Amsterdam, The Netherlands).

2.3. RNA extraction and gene expression analysis by RT-PCR

To analyze the expression of viral coat protein genes and pepper defense genes a reverse transcription - polymerase chain reaction (RT-PCR) procedure was applied. Total RNA was extracted from 0.1 g virus-inoculated and mock-inoculated pepper leaves ground under liquid nitrogen with a Total RNA Miniprep kit (Viogene, Sunnyvale, CA, USA). Reverse transcription (RT) of 2.5 μg total RNA was carried out with a RevertAid H Minus First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) using an oligo(dT) primer. The reverse transcription of viral RNAs was carried out by using the reverse primer of the virus coat protein (CP)-specific primer pairs instead of an oligoT primer (Table 1). Semiquantitative PCRs for assaying gene expression levels were conducted with a PTC 200 DNA Engine extended with an ALS-1296 sample holder (Bio-Rad, Hercules, CA, USA). The PCR reaction mixtures contained 4 pmol of

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