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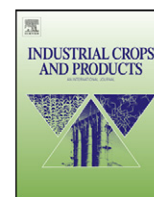

Castor (*Ricinus communis* L.) Rc-LOX5 plays important role in wilt resistance

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ABSTRACT

Phyto-oxylipins are a group of biologically active molecules that play an important role in plant defense. Their production begins with the oxygenation of polyunsaturated fatty acids by lipoxygenases (LOXs; EC 1.13.11.12) to form 9- or 13-hydroperoxides. These are substrates for several enzymes involved in the synthesis of final oxylipins, which can act as signal molecules and/or direct antimicrobials. Recent completion of the castor bean genome sequence now permits genome-wide analysis of the LOX gene family in castor as well as comparison with LOX in *Arabidopsis*. We identified 12 candidate *LOX* genes in the castor bean genome. Phylogenetic analysis indicated that these *LOX* members cluster into two groups, designated types 1 and 2, as expected from previous studies. Out of which six LOX gene specific primers were designed to amplify castor LOX genes i.e. LOX1, LOX2, LOX3, LOX4, LOX5 and Dox. Sequence analysis showed conserved five iron binding sites in the LOX domain of all the Rc-LOX, however, only LOX5 contained consensus (positions 547, 556, and 715) histidines residue. Expression analysis of LOX2, 3, 4, 5 and DOX genes in resistant and susceptible genotypes of castor at 0 days after infection (DAI), 5 DAI and 10 DAI (30 days after sowing) was carried out using quantitative real time (RT)-PCR during castor bean–*Fusarium oxysporium* f. sp. *ricini* interaction. Results suggest that 2 (LOX2 and LOX5) of 6 *Rc-LOX* genes were detectable. Resistant genotypes (48-1 and SKP-84) exhibited appreciably higher expression of LOX5 during castor bean–*F. oxysporium* interaction, which further suggest the participation of Rc-*LOX5*, a type-1 LOX predicted to be 9-LOX, in wilt resistance.

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1. Introduction

Castor (*Ricinus communis* L.) is highly remunerative industrially important non-edible oilseed crop belongs to family *Ephorbiaceae*. Castor seeds contain about 46–55% oil by weight. It is a raw material for paints, coatings, inks, lubricants and a wide variety of other products. Castor cake not suitable as an animal feed because of the presence of toxic protein called ricin which is commonly referred to as CBA (castor bean allergen), however, is widely used as organic manure in agriculture. None of the toxic components are carried into the oil, therefore, castor oil is used as laxative in human medicine. Another product formed from the modification of castor oil is sulfated castor oil which an active wetting agent and used extensively in dyeing and in finishing of cotton and linen (Ogunniyi, 2006). The major castor growing countries are India, China, Brazil and Russia. In India, Gujarat is leading castor growing state, which produces 82% of total production in India. Wilt disease of castor bean caused by *Fusarium oxysporum* f. sp. *ricini* is a major constrain of crop production in India. Monocropping has been followed due to its high economical return, which has established an endemic situation in the major castor growing area of Gujarat (Dange et al., 1997). More than 80% loss in crop yield have been reported due to the diseases compelling farmers to grow another crops which are less remunerative (Singh et al., 2011).

Resistance source is available in the local germplasm and is being introgressed in the elite germplasm. The disease is often observed in the compatible interaction of host and pathogen. However, resistance germplasm contains some gene responsible for the incompatible interaction. Lipoxygenase (LOX) gene is an important gene of incompatible interaction. The role of LOX in plant–microbe interactions was first time realized when arachidonic acid and eicosapentaenoic acid were isolated from the mycelium of a phytopathogenic fungi *Phytophthora infestans*, which was shown to elicit phytoalexin accumulation and hypersensitive cell death in potato tissues, probably via LOX action. Transgenic tobacco plants expressing antisense LOX clearly demonstrates the role of LOX in plant resistance to pathogens (Rance et al., 1998). The enzyme is able to generate molecules that can be divided into three groups with different functions: (a) hydroperoxides and free radicals that

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Table 1
Sequence of primers used for sequencing and expression analysis.

Primer	Sequence	NCBI No.
LOX1 F LOX1 R	5′-GGATTACTACATTCACTCCTGCAAC-3′ 5′-GGCAGATATGCCTTGTTAGTAAAGA-3′	XM_002512340.1
LOX2 F LOX2 R	5′-CAAGATGCAACACAGATACACTTTC-3′ 5′-TCTTTCCAACCTTGTTCTTTAAGTG-3′	XM_002513397.1
LOX3 F LOX3 R	5′-GGAATTAGAAAATTGTGCGATAGAA-3′ 5′-CAGAGACTTCTATTCTTCTGCCATC-3′	XM_002519024.1
LOX5 F LOX5 R	5′-AAGTATTACTCCAATAACCGCTGTG-3′ 5′-CATGGAAGGTAGGTCTTGTTAGAGA-3′	XM_002516725.1
Dox F Dox R	5′-GATCTAACGGATGACAAAGAAGCTA-3′ 5′-ACTTTCTGTGGTATTCACCCATTTA-3′	XM_002517356.1
LOX4 F LOX4 R	5′-ACAGATGACACTAAGGAGTCTCCAG-3′ 5′-TGGAACATTCTTTCTTTGACTTTTC-3′	XM_002519026.1
RcUBIQ F RcUBIQ R	5′-TCT TCT TAG GCC TTA ACT GAT TGC-3′ 5′-ATG GCT ATG GCT GGA TTG TACC-3′	XM_002530294.1

might be involved in the localized cell death observed during the hypersensitive response; (b) signal molecules such as jasmonic acid (JA) and its methyl ester that can trigger defense gene expression and amplify the initial response; and (c) antimicrobial compounds such as 2-trans-hexenal that constitute a direct defense against pathogen attack (Croft et al., 1993).

Diverse roles played by different LOXs during growth, development, and environmental stress can be explained by the versatile catalytic activities of LOXs and region specificity of various oxylipins in plant tissues (Gao et al., 2011). Recently, 12 putative OsLOX genes were studied in *Oryza sativa indica*. Gene expression, amino acid composition, protein secondary structure, and promoter region analysis revealed the role of OsLOX3 gene in providing resistance to blast fungus (*Magnaporthe grisea*) in rice plants (Marla and Singh, 2012). Nevertheless, there is no information on LOX gene analysis and expression during castor–*Fusarium oxysporium* f. sp. *ricini* interaction. Therefore, these observations reinforced to sequence LOX genes with further characterization at transcriptional level to confirm which LOX gene is important for castor wilt resistance.

2. Materials and methods

2.1. Castor genotypes and its inoculation with F. oxysporium f. sp. ricini

The investigation was carried out using the four castor genotypes. The seeds of two resistance genotypes (SKP-84, 48-1) and two susceptible genotypes (VP-1, VI-9) to castor wilt were procured from the Main Castor and Mustard Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardar Krushinagar, Dantiwada, Gujarat, India.

Seeds were sown on the surface of the fine sterilized sand and then covered with a 2 cm layer of sand. The crop was well irrigated regularly to avoid any physiological stress and to maintain high relative humidity condition. Highly susceptible genotypes VP-1, VI-9 and resistant genotypes SKP-84, 48-1 were sown in individual trays.

All the four genotypes were infected with *F. oxysporium* f. sp. *ricini* by mechanical method at 20 days after germination. Plants were pulled out gently from the sand and roots were washed with distilled water then roots from terminal sites about 1–2 cm were slotted out. Injured roots of castor seedlings were dipped in 1×10^{-6} fungal suspension for 10–15 min, while for control treatment the injured roots were dipped in distilled water. After that plants were transplanted in fresh sterilized pot containing sterilized soil and sand in 1:1 ratio. For the relative expression analysis of lipoxygenase genes, second fresh leaves were taken at 0, 5 and 10 days after infection (DAI) from both infected and non-infected plants, while for the sequencing of LOX genes leaves of resistant genotype 48-1 was used after 20 days of germination.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from infected and non-infected leaves with TRIzol reagent. RNA concentration was measured spectrophotometrically at 260 nm. The first strand cDNA synthesis reactions were performed using the Omniscript RT Kit (Qiagen) with oligodT 18 primers according to the manufacturer's instructions.

2.3. Amplification of lipoxygenase gene(s)

Total 12 LOX genes in castor were reported by Thakur et al. (2011) out of these, 6 gene sequences were used to designed gene specific primers. Primers were designed using primer 3 software (Table 1) and used to amplify each cDNA by RT-PCR.

The PCR amplification of lipoxygenase genes were performed using different reverse and forward primers i.e. LOX1, LOX2, LOX3, LOX5, DOX, LOX Putative (Table 1). The reaction mixtures composed of 5 μl 10 × PCR buffer, 0.2 mM dNTPs, 1 μM each primer, 200 ng cDNA template, 2.5 units *Taq* DNA polymerase and sterile deionized water to a final volume of 50 μl. PCR programme was as follows: initial denaturation at 94 °C for 5 min, followed by secondary denaturation (94 °C, 30 s), annealing (65 °C, 45 s), initial extension (72 °C, 1 min), and final extension at 72 °C for 5 min with 31 cycles. PCR product was stored at 4 °C for future use.

All the PCR amplified products were run on 0.7% agarose gel containing 5 μl ethidium bromide, in 0.5× TBE (5× TBE stock, 54 g Tris, 27.5 g boric acid, 20 ml of 0.5 M EDTA, pH8.0). Amplified product (5 μl) was mixed with 1 μl of 5× gel loading dye and loaded in the well. The electrophoresis was carried out at 60 mA (constant) to separate the amplified bands. The standard DNA marker (DNA ladder, 100 bp) was also run along with the samples. The single band were seen under UV light and photographed by gel documentation system.

2.4. DNA sequencing

Cycle sequencing of the PCR product was done using Big Dye sequencing kit 3.1v (Applied Biosystems). A 250 ng of the PCR product was used for cycle sequencing. Product of cycle sequencing (10 μl) was purified by mixing 12 μl of master mix I consisting 10 μl

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