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A PARTIAL SEQUENCE OF LIPOXYGENASE GENE FROM ROUGH LEMON (*CITRUS JAMBHIRI L.*)

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Abstract

A full sequence of lipoxygenase (LOX) genes were available from various plants, however, nucleonic or enzymatic examinations had not been reported for any citrus genera nor species. In this study, we have amplified the partial region of transcripts encoding LOX of rough lemon (*Citrus jambhiri* L.) by reverse transcription-polymerase chain reaction (RT-PCR) with several primers designed from the alignment analysis of published plant LOX genes. The sequence of partial LOX coding region from rough lemon transcripts was identified by a direct sequencing from the RT-PCR product (601bp), and compared for homology with other plant LOX genes. Blast analysis indicated that the RT-PCR products encoding rough lemon LOX gene were homologous to other plant LOX genes known, and higher homology was found toward the defense reaction related LOX genes of *Arabidopsis* or rice.

Key words : Lipoxygenase, RT-PCR, Citrus, Rough lemon (*Citrus jambhiri L.*).

Introduction

The lipoxygenases (EC 1.13.11.12) catalyzing the hydroperoxidation of specific pentadiene moieties in long-chain fatty acids, are enzyme families which widespread in higher plants and animals. It has been reported that an increase of lipoxygenase activity correlated with processes in development, maturation, and senescence[1], and also implicated in responses to wounding or attack of microbes[1,2,3,4]. Biosynthesis of signaling and/or regulatory compounds such as the jasmonates and hexenals[2,3,5,6], can be considered as the enzymatic role of the lipoxygenases in such diverse processes in plants.

Sequence information for the LOX genes is available from various plants, such as *Arabidopsis*[7,8], barely[9], cucumber[10], lentil[11], pea[12], potato[13], rice[12], soybean[15,16,17], tomato[18,19], however, no nucleonic nor enzymatic examination had been reported for any citrus genera nor species. As the first step toward understanding the role and function of LOX in citrus plants, we generated multiple PCR primers by the alignment analysis of plant LOX sequences described above, and amplified a partial region of rough lemon (*Citrus jambhiri* L.) LOX gene with these primers from mRNA of rough lemon leaves by using a reverse transcription-polymerase chain reaction (RT-PCR). Sequence data of the partial region of the rough lemon LOX gene by a direct sequencing of the RT-PCR products, and the homology analysis to other plant LOX genes will be described.

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Materials and Methods

Plant and Fungal Materials

Young trees or seeds of rough lemon (*C. jambhiri* L.) was kindly provided from the Ministry of Agriculture, Forest & Fish, Kuchinotsu Experimental Station, Ehime Prefectural Fruit Research Station, and Tokushima Prefectural Fruit Research Station. The seeds were germinated and grown in vermiculite until the highs of the seedlings became about 10 to 15cm under a greenhouse condition with a minimum temperature of above 15℃. The seedlings were then transferred to pots and maintained under greenhouse conditions.

Non-pathogenic strain (0-94) of *Alternaria alternata* against rough lemon, was grown on a PDA plate for 10 to 14 days at 24℃, and the spores of the fungi were removed by washing the mycelia grown on the plate with water. The spores were then collected by centrifugation at 800g for 5 min after passing through the four layers of cheesecloth, and the concentration of spore suspension was adjusted at 5×10^5 spores/ml with water by a haemocytometer (Thoma).

RT-PCR

For the template preparation of RT-PCR, total RNA was isolated by a hot borate method[20] from young leaves (1g) of rough lemon harvested at 6 hr after spraying with about one ml of the spore suspension (5×10^5 spores/ml) of non-pathogenic strain (0-94) of *A. alternata* prepared by the method described above. The spore-inoculated leaves were incubated in a moist chamber at 24℃ during the 6 hr incubation period. RT-PCR was performed by using the Superscript One-Step RT-PCR System (GIBCO/BRL) as manufacture's instruction, with various combinations of the following primers : LOX#1/5'-AGCCATTGGTTAAATACTCA-3' ; LOX#2/5'-CACCCAATTTA(T/C)AAGCTTCT-3' ; LOX#3/5'-ATAGTTCTCAAATAAGCCTT-3' ; LOX#4/5'-CC(A/G)AA(A/G)TTNACNGCNGC(A/G)TG-3' and 500 ng of the total RNA as the template. LOX#1 and #2 are forward primers, and LOX#3 and #4 are reverse primers, respectively. One tenth of the RT-PCR products was loaded on 1% agarose gel and the size of the RT-PCR products was determined by comparing with DNA molecular marker (lambda/Hind Ⅲ) (Takara).

Direct Sequencing

One tenth of the RT-PCR products was used as a template for sequencing. Cycle sequencing technic with Thermo Sequenase (Amersham) was used for the sequencing of the DNA fragment with the method of the GATC-Biocycle Sequencing Kit (GATC) described by the manufacturer. Either the LOX#1 primer or the LOX#4 primer was used to sequence the ends of the amplified products. Internal primers were then constructed to complete the sequencing of the entire gene. These sequencing-primers were : LOX SEQ#1/5'-ATTCCATCAGCATTAACA-3' ; LOX SEQ#2/5'-GAAGAGCTCCAAGCTTGG-3' ; LOX SEQ#3/5'-AAAACAATGGGTTACCGA-3', respectively. The dideoxy-biotin labeled fragments were separated on electrophoresis, transferred onto the membrane (Hybound N+, Amersham), and visuclized ladders of the fragments with streptavidin-alkaline phosphotase conjugate (GATC) by the GATC-1500 Direct Blotting Electrophoresis System (GATC) with the method of manufacturer's instruction.

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Sequence Analysis and LOX Motif Examination

LOX motifs were examined by comparing the amino acid sequences of plant LOX. Rough lemon LOX sequence was compared with other plant LOX sequences available in a databank. Sequence analysis was performed by using the Genetyx-Mac program (Software Development), and other several different programs communicated through an internet. The programs used were : SIM-alignment tool (http://expasy.hcuge.ch/sprot/sim-prot.html) ; Translation analysis/The Protein Machine (http://www.ebi.ac.uk/~tomm aso/translate.html) ; Homology (BLAST) analysis (http://www.ddbj.nig.ac.jp), respectively.

Southern Blot Analysis

RT-PCR products with various combinations of the primers (LOX#1 to #4) were loaded on a 1% agarose gel, and transferred after electrophoretic separation to membrane (Hybound N+, Amersham) by a capillary transfer[21]. Hybridization was performed at 68℃ overnight with non-isotope labeled probe (10 ng/ml) with the Digoxigenin System (Boehringer Mannheim). The probe was made from the RT-PCR product with a primer set of LOX#1 and LOX#4. The blot was washed at 68℃ in an excess of 2X SSC, 0.1% (w/v) SDS for 15 min, then in 0.1X SSC, 0.1% SDS for a further 15 min. The hybridized bands were visualized with anti-digoxigenin antibodies-alkaline phosphotase conjugate (Boehringer Mannheim) on X-ray film by the method described by manufacturer's instruction of Digoxigenin Detection Kit (Boehringer Mannheim).

Results and Discussion

In order to generate primers which amplify a partial LOX gene of rough lemon, we analyzed alignments of amino acid sequences of plant LOX genes available in a databank. Accession numbers of these LOX genes from a databank were indicated as the followings : U01843 and L23968 for *Arabidopsis*[7,8], L35931 for barely[9], U25058 for cucumber[10], X71344 for lentil[11], X17061 for pea[12], X79107 for potato[13], D14000 for rice[14], J02795, J03211 and X13302 for soybean[15,16,17], U09026 and U09025 for tomato[18,19]. The alignment analysis identified four LOX motif sequences of SHWLNTH, HPIYKL, KAYLRTI, and HAAVNF (Fig. 1), and four PCR primers (20 mers) : LOX#1/5'-AGCCATTGGTTAAATACTCA-3' for the motif SHWLNTH ; LOX#2/5'-CACCCAATTTA(T/C)AAGCTTCT-3' for the motif HPIYKL ; LOX#3/5'-ATAGTTCTCAAATAAGCCTT-3' for the motif KAYLRTI ; LOX#4/5'-CC(A/G)AA(A/G)TTNACNGCNGC(A/G)TG-3' for the motif HAAVNF, were synthesized. The motif KAYLRTI was mostly found in soybean LOXs but not in LOXs of other plants (Fig. 1). Although attempts of LOX gene amplification from genomic DNA of rough lemon leaves by PCR using these primers did not succeed (data not shown), we could amplify a partial region (about 600 bp) of rough lemon LOX gene by RT-PCR using primer set of LOX#1 and LOX#4, from total RNA of rough lemon leaves inoculated with non-pathogenic strain (0-94) of *A. alternata* (Fig. 2).

Non-pathogenic fungus was inoculated because LOX is considered to take a part in the defense mechanism of plants and a high level of LOX transcripts are often associated with the response to wounding or attacks of microbes[1,2,3,4]. Direct sequencing of the RT-PCR product identified 601 bp of DNA sequences (Fig. 3), and the deduced amino acid sequence started from the LOX motif of SHWLNTH (Fig. 1) was determined (Fig. 3). Blast analysis of this deduced amino acid sequence (Fig. 3) indicated high homology to other plant LOX sequences (Table 1), suggesting that our RT-PCR product using LOX

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A.

Concensus		S	H	W	L	N	T	H
Arabidopsis	LOX1	*	*	*	M	Q	*	*
Arabidopsis	LOX2	*	*	*	*	R	*	*
barley	LOXA	*	*	*	*	*	*	*
cucumber	LOX1	*	*	*	*	*	*	*
lentil	LOX	*	*	*	*	*	*	*
pea	LOX	*	*	*	*	*	*	*
potato	LOX	*	*	*	*	*	*	*
rice	LOX	T	*	*	*	R	*	*
soybean	LOX1	*	*	*	*	*	*	*
soybean	LOX2	*	*	*	*	*	*	*
soybean	LOX3	*	*	*	*	*	*	*
tomato	LOXA	*	*	*	*	*	*	*
tomato	LOXB	*	*	*	*	*	*	*

B.

Concensus		H	P	I	Y	K	L	L
Arabidopsis	LOX1	*	*	V	F	*	*	*
Arabidopsis	LOX2	*	*	*	*	*	*	*
barley	LOXA	*	*	V	H	*	*	*
cucumber	LOX1	*	*	*	H	*	*	*
lentil	LOX	*	*	*	H	*	*	*
pea	LOX	*	*	*	*	*	*	*
potato	LOX	*	*	*	F	*	*	*
rice	LOX	*	*	V	H	*	*	*
soybean	LOX1	*	*	*	*	*	*	*
soybean	LOX2	*	*	*	*	*	*	*
soybean	LOX3	*	*	*	*	*	*	*
tomato	LOXA	*	*	*	H	*	*	*
tomato	LOXB	*	*	*	H	*	*	*

C.

Concensus		K	A	Y	L	R	T	I
pea	LOX	*	*	*	*	*	*	*
soybean	LOX1	*	*	*	*	*	*	*
soybean	LOX2	*	*	*	*	K	*	*
soybean	LOX3	*	*	*	*	K	*	*

D.

Concensus		H	A	A	V	N	F	G
Arabidopsis	LOX1	*	*	*	*	*	*	*
Arabidopsis	LOX2	*	*	*	*	*	*	*
barley	LOXA	*	*	*	*	*	*	*
cucumber	LOX1	*	*	*	*	*	*	*
lentil	LOX	*	*	*	*	*	*	*
pea	LOX	*	*	*	*	*	*	*
potato	LOX	*	*	*	*	*	*	*
rice	LOX	*	*	*	*	*	*	*
soybean	LOX1	*	*	*	*	*	*	*
soybean	LOX2	*	*	*	*	*	*	*
soybean	LOX3	*	*	*	*	*	*	*
tomato	LOXA	*	*	*	*	*	*	*
tomato	LOXB	*	*	*	*	*	*	*

Fig. 1 The motif examination of plant LOX genes by alignment analysis

Alignment analysis of amino acid sequences of LOX genes was performed by using Genetyx-Mac and SIM programs. The sequence data was obtained from a databank, and the accession number of each sequence was described in the text. The symbols of A, B, C, D in this figure indicate the motif 1, motif 2, motif 3, and motif 4 of LOX, respectively.

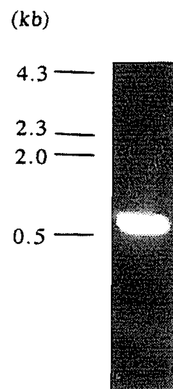

Fig. 2 Amplification of rough lemon transcripts by using RT-PCR with a primer set of LOX#1 and LOX#4

One tenth of RT-PCR product from total RNA of rough lemon harvested at 6 hr after spraying with non-pathogenic strain (0-94) of *A. alternata*, was loaded and separated on 1% agarose gel. The numbers on the left indicate the position of DNA mol. wt. markers in kb.

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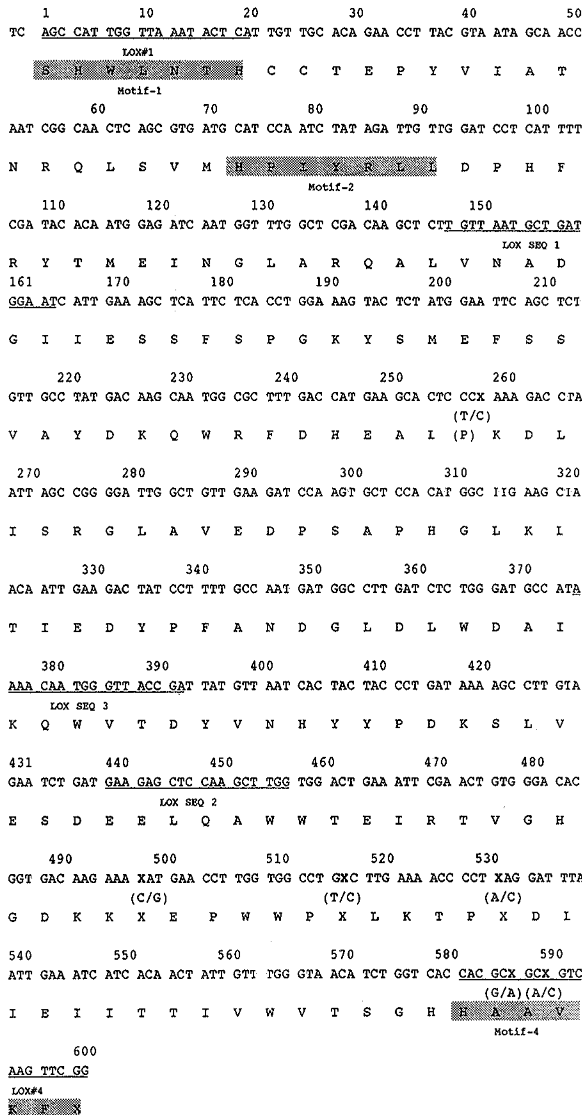

Fig. 3 Nucleotide and deduced amino acid sequences of the RT-PCR product from rough lemon transcripts

Annealing sites of primers LOX#1 and LOX#4, and sequencing-primer sites are underlined and the names of the primers are tagged. Plant LOX motifs examined in Fig. 1 are shaded and the names of the motifs are also tagged. The sites which could not identified as a single nucleotide are indicated as X.

motif primers is confirmed to be a part of LOX gene. In the deduced amino acid sequence of the rough lemon LOX gene, we could find three motifs like sequences of SHWLNTH, HPIYRL, HAAVKF but not KAYLRTI (Fig. 3). However, combinations of primers which could amplified the partial region of this rough lemon LOX gene, were the LOX#1 corresponding motif 1 (SHWLNTH) and LOX#4

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Table 1 High-scoring genes by BLAST analysis to RT-PCR product generated from transcripts of rough lemon leaves

Accession number in a databank	High-scoring gene	Source	Score
L23968	lipoxygenase Lox2	*Arabidopsis*	830
L04637	lipoxygenase Lox1	*Arabidopsis*	650
D14000	lipoxygenase	rice	547
S44940	lipoxygenase	potato	432
S18906	lipoxygenase	kidney bean	422
S18612	lipoxygenase	soybean	418
S13381	lipoxygenase	soybean	418
S22153	lipoxygenase	kidney bean	416
S57964	lipoxygenase	common bean	404

Deduced amino acid sequence (described in Fig. 3) of RT-PCR product amplified from rough lemon transcripts with a primer set of LOX#1 and LOX#4 was analyzed by BLAST at DDBJ site (http://www.ddbj.nig.ac.jp).

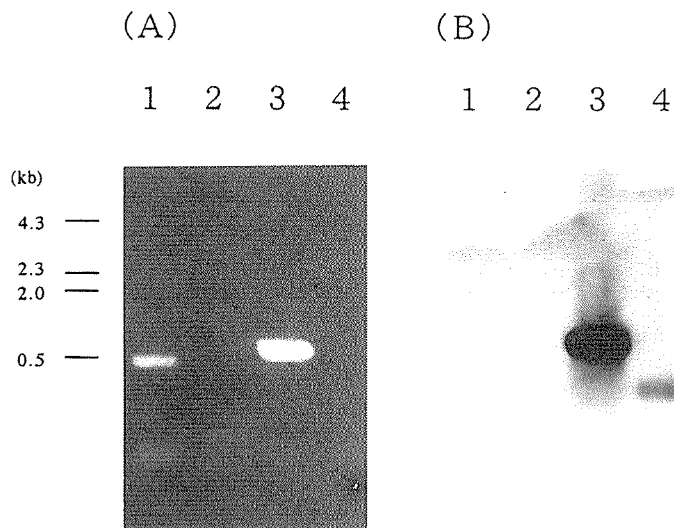

Fig. 4 Amplification of rough lemon LOX gene by RT-PCR with multiple combinations of LOX primers
(A) indicates agarose gel profile of the RT-PCR product ; and (B) indicates a southern blot analysis of the RT-PCR products by using the product of LOX#1 and LOX#4 as the probe. RT-PCR products with different combinations of the primers were loaded on each lane as the following orders : Lane 1 ; primer set of LOX#2 and LOX#4, Lane 2 ; primer set of LOX#2 and LOX#3, Lane #3 ; primer set of LOX#1 and LOX#4, Lane 4 ; primer set of LOX#1 and LOX#3, respectively. The numbers on the left indicate the position of DNA mol. wt. markers in kb.

corresponding motif 4 (HAAVNF) (Fig. 2 and 4), and surprisingly LOX#1 and LOX#3 corresponding motif 3 (KAYLRTI) (Fig. 4). Although there is no identical amino acids to the KAYLRTI motif exist, a putative annealing site of the LOX#3 primer is the position 529 to 549, based on the size of the

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amplified product by RT-PCR and a partial identity on their sequences (Fig. 4). The direct sequence data had several sites (positions at 259, 498, 517, 531, 587, 590) which could not be sequenced as the single nucleotide by this method (Fig. 3), indicated that the RT-PCR product was amplified from multiple transcripts of different isoform genes of LOX, which share the same size at this amplified region but several sites in the sequences are different. LOX is an essential enzyme catalyzing the dioxygenation of polyunsaturated fatty acids, and the presence of multiple isoforms of LOXs in different tissues and different stages of development was also reported in a large number of plants[1,2,3]. The *Arabidopsis* Lox gene[7,8] showed the highest homology of 73% to the rough lemon LOX, and the rice LOX gene[14], showed the second highest homology of 67% in SIM aligment and BLAST analysis (Table 1). Both genes were reported to be activated in defense reaction against attacks of pathogens[5,12]. A homology of rough lemon LOXs toward these defense-related LOX genes of other plants may reveal that the function of our LOX amplified by RT-PCR is also involved in some resistant mechanisms.

This study shows that a partial region of rough lemon LOX gene was successfully amplified by RT-PCR from the transcripts of rough lemon leaves, which stimulating the defense mechanisms by inoculation with non-pathogenic fungus. The RT-PCR product is now suitable to be a probe for screening of the full cDNA or genomic clone from libraries. Our data also indicated the presence of multiple transcripts from different isoforms of LOX genes, and identification of each sequence of these multiple transcripts will be necessary to elucidate the role and functions of each isoform of LOXs in rough lemon cells.

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ラフレモン(*CITRUS JAMBHIRI* L.)リポキシゲナーゼ遺伝子の部分配列

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要　　約

リポキシゲナーゼ（ＬＯＸ）遺伝子は多くの植物から単離されその全域の配列が明らかにされているが，カンキツＬＯＸ遺伝子に関してはまったく報告がない．そこで本研究においては，現在までに報告された植物ＬＯＸ遺伝子のアライメント解析をもとに合成したプライマーを用いて，ラフレモンＬＯＸ遺伝子の転写物の部分領域をRT-PCRにより増幅し，ダイレクトシーケンス法でその配列（601bp）を決定した．このRT-PCR産物の配列を相同性解析した結果，他の多くの植物ＬＯＸ遺伝子，特に*Arabidopsis*やイネの病害抵抗性に関与したＬＯＸ遺伝子と高い相同性を示した．