Research, Society and Development, v. 11, n. 9, e57411932254, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i9.32254

Isolation and partial characterization of the lipoxygenase gene in black pepper (*Piper nigrum* L)

Isolamento e caracterização parcial do gene lipoxigenase em pimenta-do-reino (*Piper nigrum* L)

Aislamiento y caracterización parcial del gen de la lipoxigenasa en pimienta negra (*Piper nigrum L*)

Received: 06/00/2022 | Reviewed: 06/00/2022 | Accept: 07/12/2022 | Published: 07/19/2022

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Abstract
Black pepper is a spice that have an economic importance in the world by its wide application in industry and by medicinal properties. State of Pará is one of the largest producers of these spices, but the production of this species is committed by fusariosis, that causes root rot in plant. Study of molecular biology is important by support breeding improvement of black pepper. In this work we draw the primers Lox3R and Lox3F to amplify a larger fragment of the gene lipoxygenase (Lox) in black pepper. The amplified fragment using primers drawn was sequenced. The sequence isolated has 770 nucleotides that encode 258 amino acids. This sequence was characterized by comparison in biological databases and using computational programs. The analysis with BlastX showed that sequence isolated has high similarity with lipoxygenase proteins of *Persea americana*, *Parasponia andersonii* e *Vitis vinífera*. We verified that Lox of black pepper has the PLAT/ LH2 domain of plant lipoxygenase related proteins. Its description in black pepper is essential to clarify the molecular mechanisms of response of the plant to the Fungus and understand its paper in activation of the defense response, once the gene Lox is activated in plant to signal its defense in a possible attack against pathogen and may be precursors of metabolic regulators.
Keywords: Gene lipoxygenase; *Piper nigrum L.*; Defense; Response.

Resumo
A pimenta-do-reino é uma especiaria que tem importância econômica no mundo por sua ampla aplicação na indústria e pelas propriedades medicinais. O estado do Pará é um dos maiores produtores dessa especiaria, mas a produção dessa espécie é comprometida pela fusariose, que causa a podridão radicular na planta. O estudo da biologia molecular é importante para apoiar programas de melhoramento genético em pimenta-do-reino. Neste trabalho desenhamos os primers Lox3R e Lox3F para amplificar um fragmento maior do gene lipoxigenase (Lox) em pimenta-do-reino. O fragmento amplificado usando iniciadores desenhados foi sequenciado. A sequência isolada tem entorno de 770 nucleotídeos que codificam 258 aminoácidos. Esta sequência foi caracterizada por comparação em bancos de dados biológicos e utilizando programas computacionais. A análise com BlastX mostrou que a sequência isolada apresenta alta similaridade com proteínas lipoxigenases de *Persea americana*, *Parasponia andersonii* e *Vitis vinífera*. Verificamos que a Lox da pimenta-do-reino possui o domínio PLAT/LH2 de proteínas relacionadas à lipoxigenase vegetal. Sua descrição em pimenta-do-reino é essencial para esclarecer os mecanismos moleculares de resposta da

planta ao fungo e entender seu papel na ativação da resposta de defesa, uma vez que o gene Lox é ativado na planta para sinalizar sua defesa em um possível ataque contra patógeno e podem ser precursores de reguladores metabólicos.
Palavras-chave: Gene lipoxigenase; *Piper nigrum* L.; Defesa; Resposta.

Resumen
La pimienta negra es una especia que tiene importancia económica en el mundo por su extensa aplicación en la industria y sus propiedades medicinales. El estado de Pará es uno de los mayores productores de esta especia, sin embargo su producción se ve afectada por la fusariosis, que provoca la pudrición de la raíz de la planta. Los estudios de biología molecular son importantes para apoyar a los programas de mejoramiento genético en pimienta negra. En este trabajo fueron diseñados los primers Lox3R y Lox3f para amplificar un fragmento mayor del gen de la lipoxigenasa (Lox) en pimienta negra. El fragmento amplificado, empleando los primers diseñados, fue secuenciado. La secuencia aislada tiene alrededor de 770 nucleótidos que codifican 258 aminoácidos. Esta secuencia fue caracterizada por comparación, empleando bases de datos biológicos y programas informáticos. El análisis BlastX mostro, que la secuencia aislada presenta una gran similitud con las proteínas lipoxigenasas de *Persea americana*, *Parasponia andersonii* y *Vitis vinífera*. Se observó que Lox de pimienta negra tiene el dominio PLAT/LH2 de proteínas relacionadas con la lipoxigenasa vegetal. Su descripción en pimienta negra, es esencial para aclarar los mecanismos moleculares de respuesta de la planta al hongo, y entender su papel en la activación de la respuesta de defensa, ya que el gen de Lox se activa en la planta, para señalar su defensa, en un posible ataque contra un patógeno y pueden ser precursores de reguladores metabólicos.
Palabras clave: Gen de la lipoxigenasa; *Piper nigrum* L.; Defensa; Respuesta.

1. Introduction

The black pepper (*Piper nigrum L*) is a spice consumed world widely, that has a great economic importance. This species is widely used in the food industry, cosmetics industry (Liu et al., 2010) and in the traditional medicine due to its antioxidant, anti-inflammatory and anticancer properties (Nishimura et al., 2011).

In Brazil, the state of Pára is one of the main productor of black pepper (EMBRAPA, 2010), Nonetheless the production is affected, since that this species has great susceptibility to a disease called fusarium (EMBRAPA, 2010). This disease affects the root system and the aerial part of the plant, resulting in the yellowing and fall of the leaves and root rot and consequently reducing the lifespan of peppers (Chu et al., 1997). Therefore, up to the present time, there are only palliative measures against fusarium such as; the use of fungicide, use of disinfected tutors, eradication and burning of the diseased plants (Serrano et al., 2006).

In general, the works that contribute to understand the plants' molecular answers are scarce and considered of great relevance specially to support programs for the genetic improvement of the species. From the molecular perspective, the lipoxygenases are protein found in most plants associated with important physiological processes as a defense against pathogenic attack (Song et al., 2016). These enzymes catalyze the addition of molecular oxygen, forming hydroperoxides (Axelrod et al., 1981; Siedow, 1991).

The hydroperoxides derived from lipoxygenase may be metabolized giving rise to volatile aldehyde molecules and jasmonates in vegetables (Mosblech et al., 2009). These molecules have an important role in the process of signaling and healing plants' injuries (Andreou & Feussner, 2009) and are associated to important physiologic processes in living beings such as: biosynthesis of regulatory compounds, growth and development (Siedow, 1991), senescence (Rouet Mayer et al., 1992), seed germination (PARK et al., 1994), wound response (Vieira et al., 2001), protein of vegetative reserve (Stephenson et al., 1998) and in resistance to biotic and abiotic stress (Song et al., 2016).

The transcriptome studies of Black Pepper showed the altered expression pattern of several genes in response to the pathogen Fusarium solani, including a lipoxygenase gene (LOX) (Moreira et al., 2017). Therefore, the objective of this work was to isolate and characterize partially the Lipoxygenase gene in Piper nigrum, providing the base to understand the functioning of the plant's molecular response mechanisms.

2. Methodology

Biological material

Were used black pepper leaves (*Piper nigrum cv. Bragantina*), provided by the Brazilian Agricultural Research Corporation (EMBRAPA) Eastern Amazon (Belém - PA) around two months old. The leaves were disinfected using distilled water and then proceeded to extract the genomic DNA following the protocol of the Kit "PureLink Plant Total DNA Purification Kit", commercially available.

Design of the initiators

For the construction of the initiators were used sequences of nucleotides from different plant species available at NCBI (National Center for Biotechnology Information) (https://www.ncbi.nlm.nih.gov/). After obtaining the sequences, they were placed in fasta format and then aligned using the Multalin program (Corpet, 1988). From the alignment the Forward initiators were built (in the chemical sense 5'– 3') and reverse (in the chemical sense 3´- 5') using the program Primer3plus (Untergasser et al., 2007).

Amplification and sequencing

The gene was amplified by PCR with a total volume of 25 µl, being 2.5 µl of 10x Buffer, 0.5 µl of MgCl2 (Magnesium Chloride), 0.75 µl of dNTP, 1 µl for each forward primer (LoxPn3F 5'-ATCATTTGGGCGTCTCTGCT-3') and reverse (Lox3R 3'-GTGTGTTGCACTGCACTTCC-5') 0.25 µl of Taq polymerase enzyme, 2.5 µl of plant DNA and Milli-Q ultrapure water to complete the reaction. To the amplification of the gene, were used the following conditions: 1 cycle of denaturation at 94 °C for 4 minutes, then 35 cycles with 1 minute of denaturation at 94 °C, 1 minute of annealing at 55 °C and extension for 2 minutes at 72 °C. Finally, a final extension of 10 minutes at 72 °C. The partial sequence of the Lox gene was obtained from the sequencing performed by Moreira et al. (2017).

Lox gene characterization using biological databases

The nucleotides sequence of Lox of Piper nigrum L was analyzed by comparison in a biological database using the program Blast-X (Altschul et al., 1990). The program Expasy (Artimo et al., 2012) was used to identify possible reading frames and identify amino acid sequences. The evaluation of possible domains in the isolated sequence was performed using the Blast-X and pfam databases (Finn, 2016). The Phyre2 server (Kelly et al., 2015) was used to model and predict the secondary structure of the obtained protein. The PDB program (Berman et al., 2000) was consulted to visualize the three-dimensional structure of the lipoxygenase protein with greater similarity to that of Piper nigrum. Then the Lox sequence was predicted through comparative modeling using the Swiss Model server (Guex et al., 2009). The evaluation of the model's quality was observed from the GMQE (Global model Quality estimation) and Qmean scores.

3. Results and Discussion

Design of primers for partial isolation of the Lox in *P. nigrum*

After the isolation of the DNA, After DNA isolation, primers were designed by aligning lipoxygenase gene sequences from different plant species available in the database. NCBI. The species used, as well as their accession number in the database are shown in Table 1.

Table 1 - Nucleotide sequences used for the alignment of the lipoxygenase gene sequences obtained from the NCBI database.

ESPECIES	POPULAR NAME	ACCESS NUMBER
Salvia miltiorrhiza	Salvia vermelha	JX297420.1
Solanum tuberosum	Batata	Y18548.1
Momordica charantia	Melão	AM930395.1
Arabidopsis thaliana	*Arabidopsis thaliana*	L04637.1
Crocus sativus	Açafrão	X92890.1

Source: Moreira et al. (2022).

The alignment shown in table 1indicates a high degree of conversation between the sequences. The regions selected for the design of the primers Lox3R (in the 5'-3' chemical sense) and Lox3F (in the 3'- 5' chemical sense) are highlighted (Figure 1).

Figure 1 – Alignment of Lipoxygenase gene sequences in different plant species, the synthesized primers (F) Forward and (R) Reverse are indicated in green.

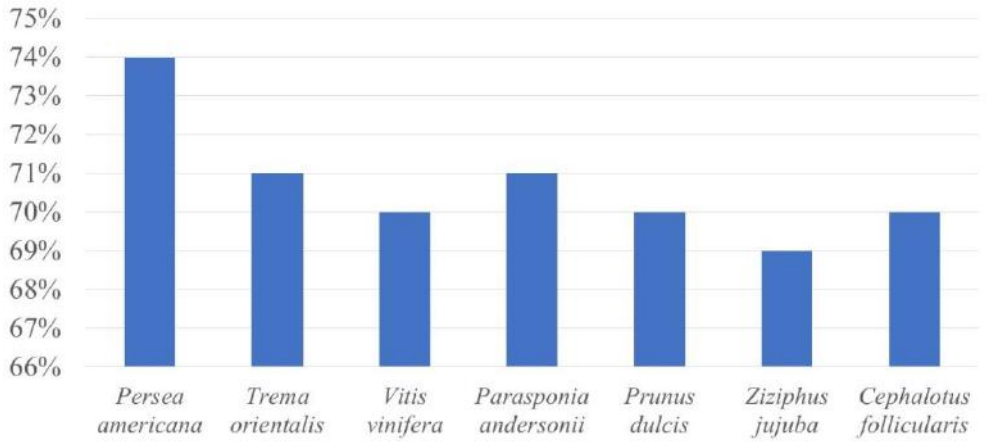
Source: Moreira et al. (2022).

Comparative analyzes of the isolated partial nucleotide sequence

The isolated sequence was obtained from the sequencing of Moreira et al. (2017) and was characterized through computer programs described below. The analysis of the nucleotide sequence was performed with the BlastX program, and

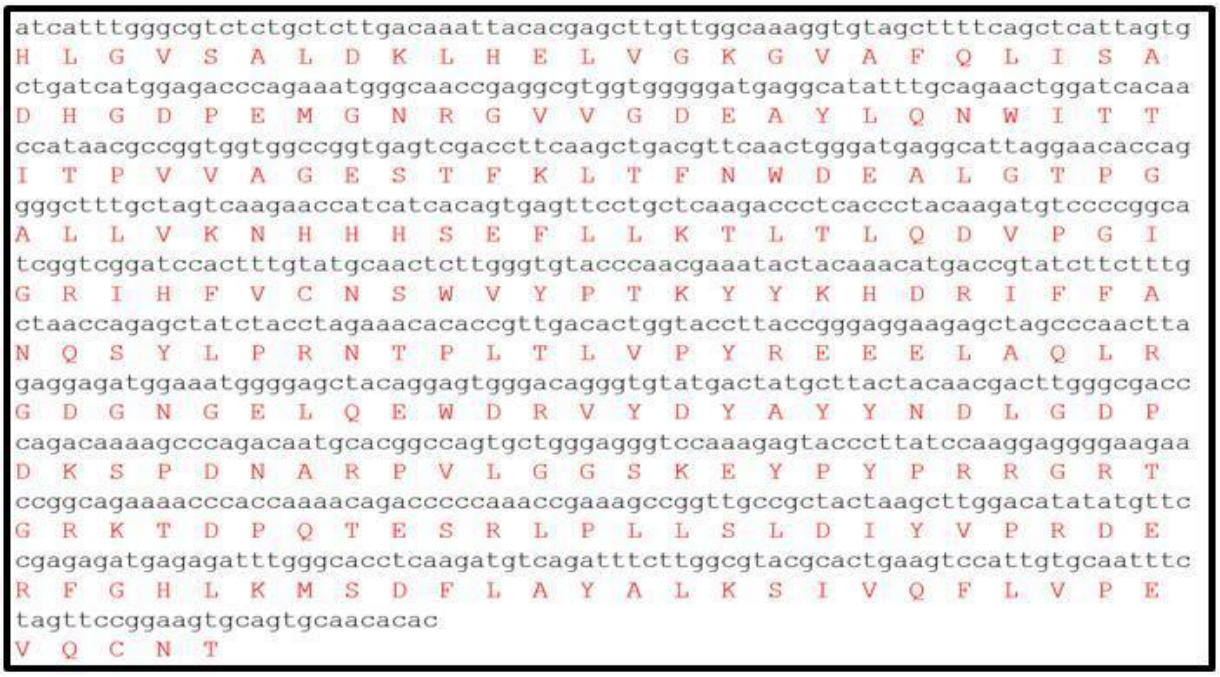
showed that it has identity with other lipoxygenase-type proteins from different plant species. Among the species that showed greater identity with the isolated partial protein, Persea americana, Parasponia andersonii, Vitis vinífera, Prunus dulcis, Trema orientalis, Cephalotus follicularis e Ziziphus jujuba (Figure 2). The analysis with the program Expasy showed that the isolated protein has 777 nucleotides, which encode 258 amino acids (Figure 3).

Figure 2 – Percentage of identity of the black pepper lipoxygenase gene compared in different species using the BlastX program.



Source: Adapted from BlastX.

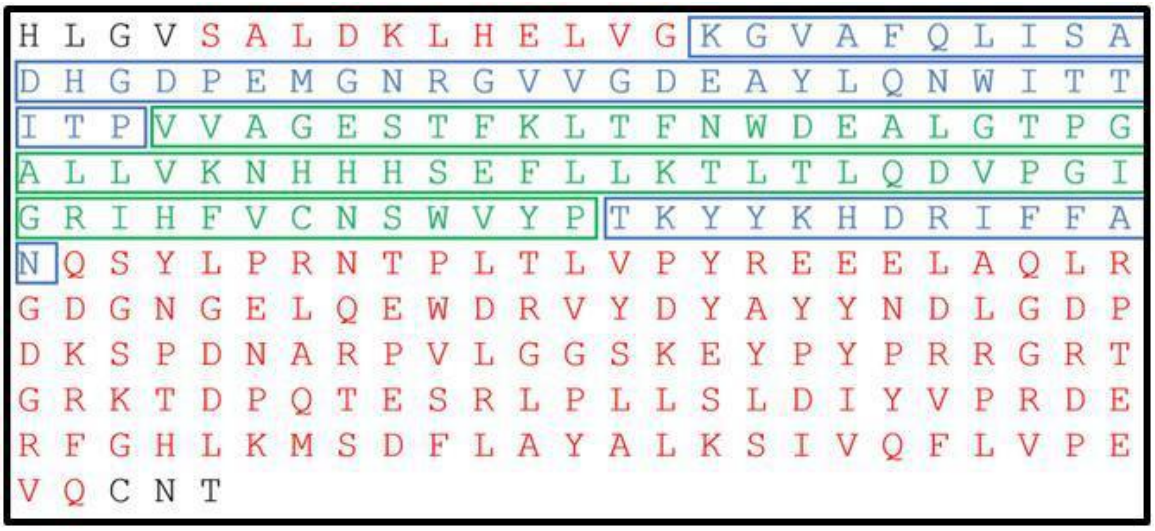
Figure 3 – Nucleotide sequence of the sequenced *P. nigrum* protein (in black) and the translated amino acid sequence (in red).



Source: Moreira et al. (2022)

The analyzes performed with Pfam and BlastX showed that the isolated protein has two domains called Plat-LH2 and lipoxygenase. The amino acid sequences that are part of the Lox and Plat domains of black pepper are shown in the figure below (Figure 4).

Figure 4 – Amino acid sequence of the lipoxygenase gene. The lipoxygenase domain is highlighted in red. In blue is the Plat domain. In green the LH2 domain.



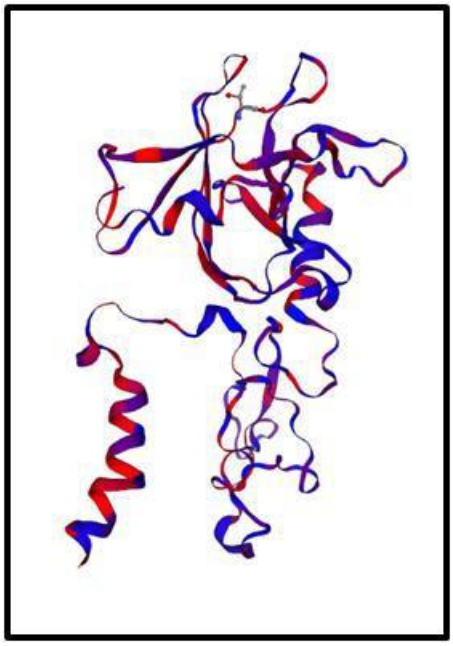
Source: Moreira et al. (2022)

Lipoxygenase-like proteins have a Plat-LH2 domain in the amino-terminal region and a lipoxygenase domain in the carboxy-terminal region (Song et al., 2016). The Plat domain is found in a large number of membrane-associated proteins or lipids and may present a single domain or be repeated (Bateman and Sandford, 1999; Hong et al., 2000; Minor et al., 2006; Shin et al., 2004). It is reported in the literature that this domain is always present in monocot and dicot plant species, in addition, a study with Arabidopsis suggests that the promotion of stress tolerance may be the general function of PLAT (Hyun et al., 2015), however, studies on the Plat domain for vegetables are still scarce or non-existent (Shin et al. 2004).

According to the analysis of the Lox domain of the isolated partial protein, we can say that it is a 9-lipoxygenase (9-LOXS) type protein. These 9-Loxs proteins catalyze the oxygenate at the C-9 position of fatty acids and have been reported to activate plant defense (Walper et al., 2016). Genetic studies show that the 9-Loxs pathway plays an important role in activating the local defense against pathogens (Hwang & Hwang, 2010; López et al., 2011; Montillet et al., 2013), in addition, this pathway helps in the triggering of systemic resistance (Vicente et al., 2012). Studies by Marcos et al., (2015) also show that derivatives of the 9-Loxs pathway activate cell wall-based defense responses, participating in the signaling of cell wall damage. Furthermore, the analysis of the secondary structure of the Lox domain of the partial protein performed in the Phyre2 program showed that it has great similarity to the soy protein Lipoxygenase (Data not shown).

Regarding the tertiary structure, the isolated protein is similar to the structure of the soy lipoxygenase protein (1rrl2.A). The tertiary structure model built for black pepper using the Swiss model server is shown in the figure below (Figure 5). The regions shown in blue are polar regions and in red are highlighted hydrophilic regions. The model built is a reliable model according to the QMEAN and GMQE measure around 0.77 observed. The GMQE is given by the number between 0 and 1 and reflects the expected accuracy of the model built. Higher numbers reflect greater reliability. The QMEAN Z-score around zero indicates good agreement between the model structure and the experimental structure of similar size.

Figure 5 – Tertiary structure of the lipoxygenase protein of *P. nigrum*.



Source: Moreira et al. (2022)

4. Conclusion

The work allowed the partial characterization of Lipoxygenase gene in the Black pepper (P. nigrum). The gene isolated has approximately 770 base pairs and identity with different plant species such as: *Persea americana*, *Parasponia andersonii* and *Vitis vinifera*. The gene encodes 258 amino acids and has the Plat-LH2 and Lipoxygenase domains, reported in the literature as important for plant defense against pathogens. The protein isolated is of the 9-Lipoxygenase type, important for activation of local defense, systemic resistance and activation of other plant defense responses. The results achieved represent a contribution regarding molecular data for *P. nigrum*, which can be considered important, as molecular information for this species is still scarce and these can help in the development of genetic improvement programs.

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