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Original Article

Molecular cloning and characterization of heat-responsive LcOPR1, a gene encoding oxophytodienoic acid reductase in lentil

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Abstract

Improving crop plants using biotechnological implications is a promising and modern approach compared to traditional methods. High-temperature exposure to the reproductive stage induces flower abortion and declines grain filling performance, leading to smaller grain production and low yield in lentil and other legumes. Thus, cloning effective candidate genes and their implication in temperature stress tolerance in lentil (Lens culinaris Medik.) using biotechnological tools is highly demandable. The 12-oxophytodienoic acid reductases (OPRs) are flavin mononucleotide-dependent oxidoreductases with vital roles in plants. They are members of the old yellow enzyme (OYE) family. These enzymes are involved in the octadecanoid pathway, which contributes to jasmonic acid biosynthesis and is essential in plant stress responses. Lentil is one of the vital legume crops affected by the temperature fluctuations caused by global warming. Therefore, in this study, the LcOPR1 gene was successfully cloned and isolated from lentils using RT-PCR to evaluate its functional responses in lentil under heat stress. The bioinformatics analysis revealed that the full-length cDNA of LcOPR1 was 1303 bp, containing an 1134 bp open reading frames (ORFs), encoding 377 amino acids with a predicted molecular weight of 41.63 and a theoretical isoelectric point of 5.61. Bioinformatics analyses revealed that the deduced LcOPR1 possesses considerable homology with other plant 12-oxophytodienoic acid reductases (OPRs). Phylogenetic tree analysis showed that LcOPR1 has an evolutionary relationship with other OPRs in different plant species of subgroup I, containing enzymes that are not required for jasmonic acid biosynthesis. The expression analysis of LcOPR1 indicated that this gene is upregulated in response to the heat-stress condition and during recovery in lentil. This study finding might be helpful to plant breeders and biotechnologists in LcOPR1 engineering and/or plant breeding programs in revealing the biological functions of LcOPR1 in lentils and the possibility of enhancing heat stress tolerance by overexpressing LcOPR1 in lentil and other legume plants under high temperature.

Keywords: Lens culinaris, Climate change. Global warming. Oxophytodienoic acid reductase. Gene expression. Heat tolerance.

1. Introduction

Temperatures fluctuate continuously worldwide and severely affect plants [1]. Extreme crop production reductions are expected, threatening future food supply and security [2,3]. Any plant, however, responds to heat stress via multiple pathways and regulatory networks, requiring

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coordination between several subcellular compartments [4–6]. Therefore, improving temperature stress (TS) tolerance in plants through boltechnological approach is highly demandable in this context.

As vital crops worldwide, legumes are subjected to various biotic and abiotic stress conditions. However, the abiotic stresses severely affect legumes' adaptability and productivity [7,8]. Lentil is a vital cool-season legume crop that encounters numerous stresses and is known to be highly sensitive to rising temperatures [9–11]. Heat stress in this legume is associated with cellular membrane damage, a significant reduction in relative leaf water content, and a substantial decrease in the chlorophyll concentration and fluorescence, resulting in a reduced photosynthetic rate [10,12]. Thus, understanding molecular mechanisms with the development of high temperature-tolerant genotype and temperature-resilient crops using a biotechnological approach is highly desirable.

Jasmonates (jasmonic acid (JA), methyl jasmonate, 12-Oxo-phytodienoic acid (OPDA), and related cyclopentenones) are lipid-derived compounds that play a role in plant development signaling. They are also implicated in the responses to biotic and abiotic stresses [13–15]. Jasmonate biosynthesis originates from releasing polyunsaturated fatty acids like linolenic or hexadecatrienoic acids from chloroplast-membrane lipids [16]. They are first oxygenated by 13 lipoxygenases (13-LOX) to produce their hydroperoxy derivatives. By the consecutive action of allene-oxide synthase and allene-oxide cyclase, the hydroperoxy-fatty acids are converted to the first cyclic intermediate of the pathway, i.e., 12-oxophytodienoic acid (OPDA). The subsequent reduction of the cyclopentenone ring of OPDA to the corresponding cyclopentanone is afforded by peroxisomal 12-OPDA reductase (OPR3) to yield OPC-8:0 (3-oxo-2-(2'-pentenyl)-cyclopentane1-octanoic acid. Finally, the alkanoic acid side chain of OPC-8:0 is shortened in three cycles of β-oxidation, resulting in the formation of JA [17].

The oxylipin 12-OPDA was first described and synthesized in 1978 [18]. For a long, 12-OPDA has been considered to act only as a JA precursor. However, accumulating evidence points toward the involvement of 12-OPDA in signaling functions in different developmental processes of plants, such as germination, seed dormancy, and embryogenesis [19]. Several publications elucidate the physiological role of 12-OPDA in wounding plant response [20–23]. In addition, it is becoming established that 12-OPDA functions independently as an effector in plant defense. In one such study, 12-OPDA enhanced the resistance of maize against corn leaf aphid *Rhopalosiphum maidis* [24]. In contrast, in another study, rice mutants lacking 12-OPDA seemed to have a varying defense against *Magnaporthe oryzae*. [25]. Moreover, 12-OPDA increased Arabidopsis resistance to various pathogens without JA/JA-isoleucine (JA-Ile) [21,26]. Other research groups have also revealed the role of OPDA in ameliorating the accumulation of callose in host plants and limiting infection caused by pathogens [27,28]. In addition, subjecting Arabidopsis (wild-type) to heat stress causes enhanced 12-oxo-phytodienoic acid (OPDA) accumulation accompanying JA and a JA-Ile conjugate [29].

However, research has also been conducted on 12-OPDA reductases (OPRs) and their role in plant development and response to biotic and abiotic stressors [16,17]. Multiple genes encode this gene family, and its members can be grouped according to substrate specificity into OPRI and OPRII [30]. Members preferentially reduce cis-(-) OPDA over cis-(+) OPDA belonging to class OPRI. At the same time, OPRII members are directly related to jasmonic acid biosynthesis (such as OPR3) since they catalyze the reduction of cis-(+) OPDA [30]. Most recently, Chini et al. [31] identified an alternative pathway for JA synthesis that is peroxisomal OPR3-independent and entails Arabidopsis OPR2 (OPRI member) in atopr3 mutant plants reducing 4,5-dihydro-JA in the cytoplasm.

OPR gene family has been thoroughly examined in the case of Arabidopsis, and their physiological role in alleviating photooxidative stress was suggested. In barley, the expression profile of two OPRI genes suggests their role in response and defense to abiotic stresses [32,33]. Additionally, it was discovered that AsOPR1 controls the development and production of nodules in *Astragalus sinicus* and influences endogenous JA metabolism[34]. Transcriptome analysis performed by [35] in inbred lines of maize revealed differing responses to drought stress, where three members of the OPRI subgroup (ZmOPR1, 2, and 3) were found to be upregulated in maize roots when drought-sensitive seedlings were subjected to water deficiency (drought) stress for 24, 48 and 72h. Furthermore, *ZmOPR1* and *ZmOPR2* were upregulated when drought-tolerant seedlings were subjected to similar conditions. Moreover, in *Arabidopsis thaliana* and tomato, six and three genes were identified, respectively [36,37], 13 OPR genes were reported in the rice genome [38], six OPR genes were characterized in pea, the model legume [39], and 48 OPR genes were recently identified and described in wheat [40].

Plant defense against biotic and abiotic stresses is complex. Hence, revealing the role of OPRI in this process is crucial to understanding the role of OPDA-related pathways other than the JA biosynthesis pathway. However, information about this gene family in legumes is restricted to peas and alfalfa [39,41]. Thus, we believe that cloning and subsequent functional characterization of LcOPR1 will explore a new avenue to legume breeders for developing temperature-tolerant lentil genotypes with high-temperature resilient smart crop production.

2. Materials and Methods

2.1. Plant material and heat stress treatment

Lentil *(Lens culinaris* Medik.) seeds were grown in pots containing soil/perlite mixture (3:1) under a controlled greenhouse environment. Three-week-old seedlings were subjected to 40 °C for 0, 0.5, 1, 2, 3, and 4h. Following 4 h of heat stress (HS) treatment at 40 °C, lentil plants were allowed to recover HS at 22 °C for 2, 4, 24, and 48 h. The control and HS and HS-recovered plant samples were collected, then quickly frozen with liquid N2 and kept at -80 °C until further molecular analysis.

2.2. Cloning of LcOPR gene cloning and bioinformatics analysis

RNA isolation from lentil plants and cDNA synthesis were done with Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) and PrimeScript™ RT Master Mix (Takara, Japan). A pair of gene-specific primers Table 1 represents the primer details which were synthesized based on the contig sequence of *Lens culinaris* OPR gene (l.culinaris_csfl_reftransV1_0021166) available from Cool Season

Table 1. List of primers used in the present study.

Purpose	Primer name	Sequence (5′–3′)
cDNA cloning	LcOPR-F	TCCCACAGTAATTGAAGGTCTCC
	LcOPR-R	CACATATTGCTGAGCTGACACA
qRT-PCR	qLcOPR-F	GGAGACTCTAACCCTCAAGCTT
	qLcOPR-R	AAGCTACCAAATCTGCACCATC
	qLcOPR2-F	ACAGCATCATTGTGGTAAAGGG
	qLcOPR2-R	TGTGTGCATTTCGATTAGGGTC

Food Legume Genome Database (https://www.coolseasonfoodlegume.org). Further, we performed PCR to amplify the coding sequence of *LcOPR* using cDNA from heat-stressed lentil plants. The amplified PCR product was cloned using pGEM-T Easy vector (Promega, USA) and then sequenced.

ORF finder of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.htm) was employed to study LcOPR nucleic acid sequence, and ExPASy was used to translate LcOPR ORF (http://web.expasy.org/translate/). The expected isoelectric point and the molecular weight of the deduced LcOPR protein were obtained using ProtParam (http://web.expasy.org/protparam/). Prediction of protein subcellular localization was performed using TargetP 2.0 (http://www.cbs.dtu.dk/services/TargetP/), CELLO (http://cello.life.nctu.edu.tw/), and ProtComp 9.0 onlintool(http://linux1.softberry.com/berry.phtml?topic=protcompplandgroup=programsandsubgroup=proloc) were used to predict subcellular localization of protein. Conserved domains of *LcOPR* protein were predicted using http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi [42].

Homologs of the *LcOPR* protein from different plant species were retrieved by NCBI BLAST search, and the Clustal-Omega program [43] was employed to execute multiple sequence alignments. The dendrogram was constructed using MEGA 7.0 [44] software, where the neighbor-joining method with a bootstrap value of 1000 replicates was applied.

2.3. LcOPR1 Gene Expression Analysis

The changes in LcOPR1 expression under diverse stress treatments were evaluated using quantitative qRT-PCR using KAPA SYBR® FAST qPCR Kit (KAPA BIO, USA). Primers used in qRT-PCR were designed using Primer3 software [45] and are listed in Table 1. The amplification of the *LcOPR1* gene was performed using a q-RT PCR system (CFX-96™). The LcOPR1 gene was amplified based on the program used previously[46]. The qRT-PCR data were normalized using the *LcRPL2* (ribosomal protein L2) gene (GeneBank accession number: YP_009141575), which is the most stable reference gene in lentils under abiotic stresses and among different developmental stages [47].

2.4. Statistical analysis

The observation was repeated three times, and a two-tailed t-test ($p \leq 0.05$) was used to calculate the data.

3. Results

3.1. Confirmation of *LcOPR1* cloned gene

The cDNA of putative *LcOPR* was amplified by RT-PCR using a pair of specific primers synthesized based on contig sequence information of *lentil* OPR gene (*l.culinaris*_csfl_reftransV1_0021166) accessible from Cool Season Food Legume Genome Database given above. Sequencing results indicated that *LcOPR* cDNA of full length was 1303 bp, comprising an 1134 bp ORF with a 96-bp 5′ UTR and 73-bp 3′ UTR (Figure 1). This gene was designated *LcOPR1* and deposited in the GenBank (GenBank accession no. MH491550). The LcOPR1 ORF encodes a protein of 377 amino acids. Analysis of deduced protein using the ProtParam tool showed that LcOPR1 has a predicted molecular weight of 41.63 and a theoretical isoelectric point of 5.61.

Three online tools, namely CELLO, TargetP, and ProtComp, were used to predict LcOPR1 subcellular localization. The results revealed that LcOPR1 was located in the cytoplasm, indicating the lack of any known organelle-localization signals in LcOPR1. However, this protein targeting prediction of LcOPR1 should be further explored in vivo. OPR isozymes can be found in either the peroxisome or the cytoplasm.

The results of the NCBI BLAST search indicated that LcOPR1 had high sequence similarity percentages with homologous proteins of the OPRI group, such as MtOPR1 in *Medicago truncatula* (93%), GmOPR1 in *Glycine max*

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tcccacagtaattgaaggtctccaaattcacaatatatctggaaaagagagcaggtgtga

gaatggaaaccagttgctggaagagtataactatggatggctaaagttgtgttggaagaa
                                    M  A  K  V  V  L  E  E
gagaacaagaaaatggtgaaaggaaatgaagtgataccctcctcaccccatacaacatg
 E  N  K  K  M  V  K  G  N  E  V  I  P  L  L  T  P  Y  N  M
ggaaaatttcatctatcccacaggatagttttggcaccattaaccagaacaagatcatat
 G  K  F  H  L  S  H  R  I  V  L  A  P  L  T  R  T  R  S  Y
aaatttgtggctcaaccacatgctattctctattactctcaaagaacaacaaagggtggc
 K  F  V  A  Q  P  H  A  I  L  Y  Y  S  Q  R  T  T  K  G  G
ctattgattggggaagcatcagggatatcagacactgcacaaggtatccaaatacacct
 L  L  I  G  E  A  S  G  I  S  D  T  A  Q  G  Y  P  N  T  P
ggtatttggacaaaagagcaggtagaagcatggaaaccaattgttgaagctgttcataaa
 G  I  W  T  K  E  Q  V  E  A  W  K  P  I  V  E  A  V  H  K
aaaggaggcatttttcttttgtcaactttggcatgctggaagagtatccaactatggttac
 K  G  G  I  F  F  C  Q  L  W  H  A  G  R  V  S  N  Y  G  Y
caaccaggtggacagcctccaatctcgagcacagacaaagcactacaaaaggaaggtagc
 Q  P  G  G  Q  P  P  I  S  S  T  D  K  A  L  Q  K  E  G  S
agctctaagtacccgcctcctcgacgtctcgcaatcgatgaaatctcggatgttgtaaat
 S  S  K  Y  P  P  P  R  R  L  A  I  D  E  I  S  D  V  V  N
gacttcaaaatggcagcaaaaaatgccatagaagccggttttgatggagtggagatacac
 D  F  K  M  A  A  K  N  A  I  E  A  G  F  D  G  V  E  I  H
ggagctaatggatacttgcttgatcaattcttaaaagacagagtaaacgatcgagaggac
 G  A  N  G  Y  L  L  D  Q  F  L  K  D  R  V  N  D  R  E  D
gcgtatggaggaagcttagaaaatcgatgtcgctttccactagaggttgttaaagcggtt
 A  Y  G  G  S  L  E  N  R  C  R  F  P  L  E  V  V  K  A  V
gctgatgagatcggagctgaaaaagtcggtgttaggctctctccttttgctgattactgt
 A  D  E  I  G  A  E  K  V  G  V  R  L  S  P  F  A  D  Y  C
ggctgtggagactctaaccctcaagctttgggagtttacatggctaattcgttgagccaa
 G  C  G  D  S  N  P  Q  A  L  G  V  Y  M  A  N  S  L  S  Q
ttaggaattctctactgtcatgtcattgaacctaggatgcaaaccatgtttgataaagat
 L  G  I  L  Y  C  H  V  I  E  P  R  M  Q  T  M  F  D  K  D
gacagtaatgtgtctttgatgccgatgcgaaaggctttcgatggaacatttattgttgct
 D  S  N  V  S  L  M  P  M  R  K  A  F  D  G  T  F  I  V  A
ggtggatatgataggagtgatggaaacaatgtgttggagaatgatggtgcagatttggta
 G  G  Y  D  R  S  D  G  N  N  V  L  E  N  D  G  A  D  L  V
gcttatggtcgattgtttttggcaaatccagatttgcctagaagatttgagttggatgct
 A  Y  G  R  L  F  L  A  N  P  D  L  P  R  R  F  E  L  D  A
gaattgaataaggctgataagtccacttttttacaccagtgatcctgttgttggatataca
 E  L  N  K  A  D  K  S  T  F  Y  T  S  D  P  V  V  G  Y  T
gattatccatttcttgaaaatgctagctagaacataaatgctacctgaaattgacactat
 D  Y  P  F  L  E  N  A  S  *
gcctagttgaaatttattgtatgtgtcagctcagcaatatgtg
```

Fig. 1. cDNA sequence of *LcOPR1* and its deduced amino acid sequence. A single-letter code designates amino acids under the middle nucleotide of each codon. The start codon is underlined, and an asterisk marks the stop codon.

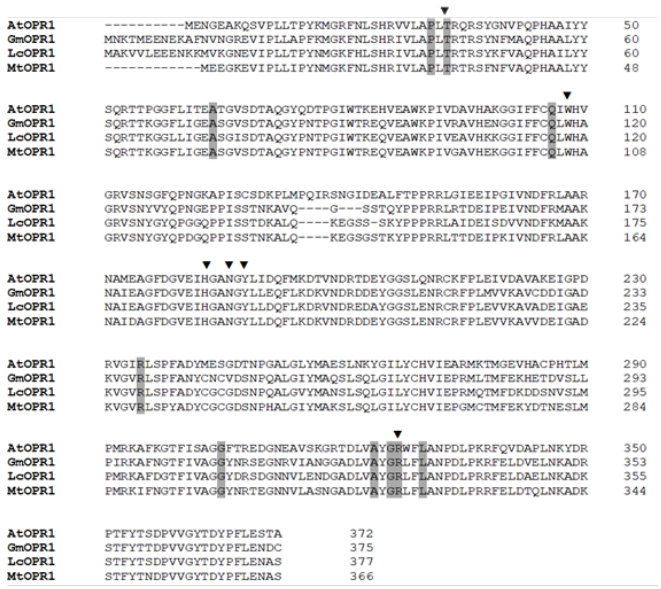

Fig. 2. Multiple sequence alignment of lentil LcOPR1 (MH491550) with related OPR proteins from Arabidopsis (AtOPR1, AAM65337), Soybean (GmOPR1, XP_006581134.1), and barrel clover (MtOPR1, XP_013462297.1). A gray background indicates the conserved FMN binding sites. The putative conserved residues implicated in the binding of substrates are depicted with triangles.

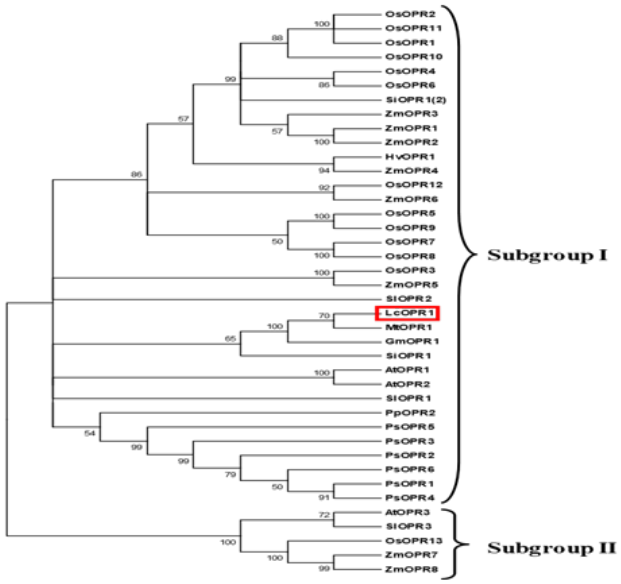

Fig. 3. Neighbour-Joining phylogenetic tree of OPR proteins from different plant species. The phylogenetic tree was created using MEGA 7.0 with 1000 bootstrap replicates.

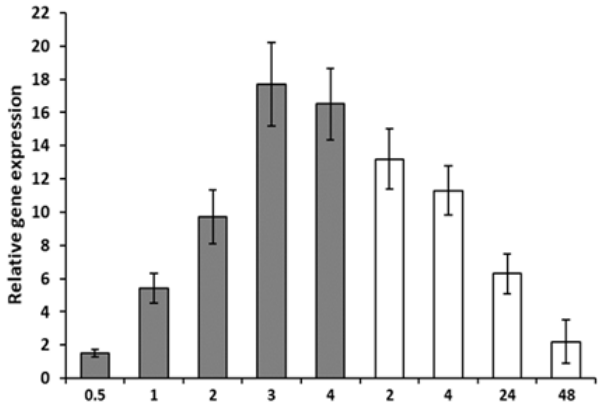

Fig. 4. Relative expression levels of *LcOPR1* in response to heat stress (HS) and during recovery. qRT-PCR assay was performed to examine the expression of *LcOPR1* in three-week-old seedlings subjected to HS at 40 °C for 0, 0.5, 1, 2, 3, and 4h (black bars). After 4 h of treatment at 40 °C, lentil plants were allowed to recover at room temperature for 2, 4, 24, and 48 h (white bars). The qRT-PCR data were normalized using *the LcRPL2 (ribosomal protein L2)* gene, and a ratio proportional to the untreated seedlings was indicated at each time point, set at 1.

(90%), PsOPR1 in *Pisum sativum* (83), SlOPR1 in *Solanum lycopersicum* (83%), AtOPR1 in *Arabidopsis thaliana* (80%), ZmOPR1 in *Zea mays* (80%), and OsOPR1 in *Oryza sativa* (78%). The present homology analysis indicated that LcOPR1 belongs to the OPR gene family. Multiple sequence alignment was performed with LcOPR1 and another OPRI protein member, including AtOPR1, GmOPR1, and MtOPR1. The results showed the presence of conserved residues responsible for binding substrate and flavin mononucleotide (FMN) (Figure 2).

To explore the evolutionary divergence of LcOPR1 compared to other OPR proteins, a phylogenetic tree (dendrogram) was designed using the LcOPR1 protein sequence and 39 other OPR proteins from different plant species (Figure 3). The dendrogram showed that these proteins are divided into subgroups, subgroup I and subgroup II. In the present investigation, LcOPR1 was clustered in subgroup I. Thus, LcOPR1 may not be required for JA biosynthesis. However, since an alternative pathway of JA biosynthesis through a peroxisomal OPR3-independent pathway has been described recently in *Arabidopsis* [31], this assumption remains to be tested in the cytosolic LcOPR1. LcOPR1 was most closely related to OPRs of *Medicago truncatula* and *Glycine max*, both of which belong to the *Fabaceae* family.

3.2. Expression patterns of LcOPR1 gene

The expression pattern of the *LcOPR1* gene indicated it responded after 0.5 h exposure to heat (40 °C), and the highest expression was observed at 3h (Fig. 4). However, the gene expression was gradually declined following a consecutive reduction of temperature with time intervals (Fig. 4). Compared to the untreated control, the expression level of *LcOPR1* increased to 5.4-fold after 1 h under the HS condition. Then, the expression steadily increased with an increase in the treatment time and reached the expression peak of 17.7-fold at 3 h, then slightly dropped afterward at 4 h (Figure 4). Therefore, the observed enhanced *LcOPR1* expression is expected to cope with the improved level of reactive carbonyls produced due to heat-stress-associated oxidative damage. After moving heat-stressed seedlings to room temperature for a 48 h recovery period, the expression levels of *LcOPR1* were relatively high at 2 and 4 h of recovery treatment. They scored 13.2 and 11.3-fold over untreated control, respectively. On the other hand, LcOPR1 expression dropped at 24 and 48 h of recovery and reached 2.2-fold at the end of the recovery treatment (Figure 4). This result suggested that activation of *LcOPR1* genes gradually declined due to the reduction of temperature in lentil.

4. Discussion

This study implies that *LcOPR1* was successfully clo-

ned, and the gene was fully active in response to temperature stress in lentil. Thus, the clone's efficiency is apparent and encourages us to apply deep biotechnological application in lentil and other legumes. Plant OPRs were first isolated in *Arabidopsis* and tomato [36,48], and many OPR genes have since been identified and characterized in different crop species [32,39,49–51]. Strassner et al. [36] demonstrated that OPRI proteins from *Arabidopsis* and tomato are cytoplasmic, whereas OPRII members, in contrast, possess C-terminal peroxisomal targeting signals and are therefore targeted to peroxisomes. OPR proteins are known to noncovalently bind FMN as cofactors [52], which NADPH reduces, indicating that these proteins are flavin-dependent oxidoreductases. Since NADPH reduces FMN, indicating that OPR proteins are flavin-dependent oxidoreductases. The phylogenetic tree illustrated that these proteins could be divided into two subgroups, in which cluster analysis classified subgroups I and II. Members of the OPRI subgroup are more likely to be involved in eliminating cis-(-) OPDA than cis-(+) OPDA. Previous received have revealed that OPR family members are categorized into 2 subgroups (OPRI and OPRII) according to their different preferences for stereoisomers of OPDA, and each subset contains members of both monocot and dicot plants [30,36,49,50,53]. Subgroup members of OPRI are preferentially involved in reducing cis-(-) OPDA than cis-(+) OPDA. Compared, cis-(+) OPDA are catalyzed by OPRII subgroup members and are directly related to jasmonic acid biosynthesis [30].

Heat stress is a major abiotic factor limiting legume growth and productivity [54]. The severity and impacts of heat stress are expected to exaggerate with climate change [55]. Therefore, exploring gene expression patterns of defense genes would help understand the molecular responses of plants to heat stress and aims to develop heat-resilient crops. Heat stress stimulates the accumulation of reactive oxygen species (ROS). Uncontrolled production of ROS mediates non-enzymatic lipid peroxidation, accumulating an array of lipid peroxide-derived α and β-unsaturated reactive carbonyls [56]. Plants have developed a detoxifying system against toxic reactive carbonyl, consisting of aldehyde dehydrogenase, aldo-keto reductase, and aldehyde reductase [57]. The expression pattern and function of 12-oxophytodienoic acid reductase (OPR) family genes are promising. In the same study, it was explored that *OPR3* triggers the reduction of the long spectrum of electrophilic species (ES) that leads to the reactivation of glutathione and ascorbate, and *OPR3* was also capable of generating ascorbate. Several lines of evidence have indicated that proteins of the OPRI subgroup reduce the double bonds in α and β-unsaturated carbonyl compounds [58,59]. In our current study in lentil, the considerable upregulation pattern of the *LcOPR1* gene under heat stress indicates that *LcOPR1* is a temperature-responsive gene. Further, it suggested that the LcOPR1gene was fully active lentil under temperature stimuli that effectively balanced plants' redox homeostasis and heat stress tolerance. This insight will help us understand molecular mechanisms that lead to heat-resilient crop development.

5. Conclusions

This study implies a biotechnological insight into *LcOPR1* mediating lentil plants' temperature tolerance. Successful cloning and functional characterization of the *LcOPR1* gene in lentil seedlings under heat stress suggest that the LcOPR1 is a temperature-responsive candidate gene fully active in response to temperature in lentil. This insight might be helpful to plant biotechnologists and lentil breeders for enhancing temperature tolerance in lentil and producing sustainable and smart lentil production under high-temperature conditions.

Conflict of interest

The authors declared they have no competing conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Saeid Abu-Romman , Sonia Mbarki, El Sabagh Ayman , designed, conducted the research, analyzed the data, and prepared the manuscript. Saurabh Pandey, Abdulrahman Alasmari , Fahad M. Alzuaibr, Mohamed Sakran , Sezai Ercisli, Mohamed El-Sharnouby, also contributed during writing the manuscript and advised scientific suggestions as well as revised/edited the manuscript.

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