

Characterization and Stress-Responsive Expression of the Lentil Manganese Superoxide Dismutase (LcMn-SOD) Gene in Mitigating Oxidative Stress

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Abstract

Plants exposed to various stress conditions, such as drought, have developed enzymatic defense mechanisms crucial for mitigating oxidative stress caused by the accumulation of reactive oxygen species (ROS) like superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Among these defense mechanisms, superoxide dismutase (SOD) plays an essential role in neutralizing ROS to prevent cellular damage. This study aimed to characterize the LcMn-SOD gene in lentil (*Lens culinaris*) and assess its expression under different stress conditions. The LcMn-SOD gene encodes a protein of 240 amino acids, with a molecular mass of 26.59 kDa and a theoretical isoelectric point of 7.86. The gene's cDNA length is 858 bp, with a 723 bp open reading frame encoding a full-length protein likely localized in the mitochondria. Sequence analysis showed high similarity with mitochondrial Mn-SOD proteins in other legumes, supporting its role in oxidative stress responses across plant species. Quantitative RT-PCR analysis in two-week-old seedlings indicated that LcMn-SOD is a key player in stress response, with notable regulation patterns: hydrogen peroxide significantly upregulated LcMn-SOD expression, while salicylic acid initially downregulated it, followed by a subsequent increase. Abscisic acid treatment led to a 14-fold upregulation after four hours. These findings underscore LcMn-SOD's role in managing oxidative stress, suggesting its potential in enhancing crop resilience to environmental stressors, particularly under changing climate and habitat disturbances. Future research could examine how diverse stresses regulate LcMn-SOD to improve crop resilience.

Keywords: Mn-SOD (Manganese Superoxide Dismutase), Oxidative Stress, Lentil (*Lens culinaris*), Drought Stress, Gene Expression, Phytohormones.

1. Introduction

Plants under biotic and abiotic stresses, such as drought, salinity, extreme temperatures, and heavy metal accumulation, face oxidative stress due to the overproduction of toxic reactive oxygen species, such as (O_2^-) and (H_2O_2), which cause cellular damage that led to impaired plant growth thus productivity and biomass as a consequence of reduction of photosynthetic capacity eventually lead to cell death (Zhang et al., 2021; Hasanuzzaman et al., 2020). As part of their defense mechanisms, plants have developed an enzymatic and non-enzymatic antioxidant network responsible for mitigating the harmful effects of toxic Reactive oxygen species, such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) (Sharma et al., 2012 Odat, 2018). Manganese superoxide dismutase (Mn-SOD) is one of the important enzymes in plants that play a vital role in mitigating effects of oxidative stress by catalyzing the dismutation of the superoxide radical (O_2^-) into oxygen and hydrogen peroxide (H_2O_2), which is further detoxified by other antioxidant enzymes such as catalase and peroxidase. Under stress conditions, plants increase the expression of ROS especially in mitochondria in order to enhance

tolerance to such conditions (Wang et al., 2010; Al-Faris et al., 2022). Lentil (*Lens culinaris*), an important legume crop, is often exposed to abiotic stresses conditions which lead to reduction in crop biomass and productivity (Noor et al., 2024; Sachdev et al., 2021). Like other plants, lentil relies on its antioxidant defense mechanism that involved the expression of different genes of various enzymes Mn-SOD genes that mitigate the damaging effects of oxidative stress (Odat, 2020). Several studies on SOD genes in plants provided evidence on their dynamic expression level under different stress conditions. For example, Mn-SOD expression is found to upregulated in response to drought and salinity in species such as Arabidopsis, rice, and barley, suggesting a common mechanism across plant. Additionally, abscisic acid (ABA) and jasmonic acid (JA) are shown to modulate SOD expression suggesting the role of the gene in adaptive response to stress (Nguyen & Nambara, 2023). Given the increased effect of environmental stresses and habitat perturbation due to increased climate change, studying Mn-SOD gene and its characteristic and expression patterns can provide valuable insight on enhancing stress tolerance of crops. Mn-SOD gene has been studied in several legumes' species, such as soybean and pea, yet in lentil the characteristics and expression level of this gene are less studied, especially

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under stress conditions. Therefore, studying the role of this gene in lentil can provide valuable information into its potential applications in breeding programs aimed at enhancing stress tolerance (Al-Tawaha 2022). This study aims to characterize the LcMn-SOD gene in lentil and investigate its expression patterns under various stress conditions. The study utilizes molecular techniques, such as qRT-PCR and bioinformatics tools, to understand how the LcMn-SOD gene contributes to lentil's defense mechanisms and how it can be harnessed to improve crop resilience

2. Materials and Methods

2.1. Experimental setup

Seeds of *Lens culinaris* Medik cultivar (Jordan 2) were sown in plastic pots (14×14 cm) containing a substrate of peat moss and perlite (1:1) ratio and maintained under greenhouse conditions with regular irrigation using distilled water for a duration of two weeks. The expression of LcMn-SOD was analyzed in lentil seedlings (two-week-old) treated with various stress:

1. Drought: Irrigation was withheld for different durations 3 days and 6 days.
2. Hormonal: Seedlings were treated with abscisic acid (100 μ M), jasmonic acid (100 μ M), or salicylic acid (1 mM) by foliar spraying.
3. Hydrogen peroxide (H_2O_2) treatment: Seedlings were foliar sprayed with 10 mM H_2O_2 .

For drought stress experiments, leaf samples were collected at zero, 3 days, and 6 days after the treatment. For hormonal and H_2O_2 treatments, leaf samples were collected at 0, 1, 2, and 4 hours post-treatment. Collected leaf samples were immediately frozen in liquid nitrogen and stored at -20°C until RNA extraction.

2.2. RNA isolation and cDNA synthesis:

Frozen lentil leaves were utilized for the extraction of total RNA using the Spectrum™ Plant Total RNA Kit (Sigma), following the manufacturer's protocol. Subsequently, the concentration and purity of RNA were assessed spectrophotometrically at wavelengths of 260 nm and 280 nm (Biochrom, Cambridge). RNA quality was tested by electrophoresis. cDNA was prepared using two micrograms of lentil leaf RNA and primeScript™ MasterMix (Takara, Japan). All cDNA samples were stored at -20°C until further analysis for gene expression.

2.3. Mn-SOD gene cloning:

To identify ORF of the Mn-SOD gene in lentil, a candidate gene approach was employed. Primers (Mn-SOD F: 5'-CATGGCCGCTCGAACCCTA-3', sense; Mn-SOD R: 5'-CGGCCAGATTGCTCAAGTTC-3', antisense) were designed using the known sequence of the pea Mn-SOD gene (GenBank accession No. X60170.1). The PCR reaction was conducted using iNtRON i-MAX™ II (iNtRON, Korea), with the following conditions: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. PCR products were resolved on 1% agarose gels. The specific PCR amplicon (855 bp for the Mn-SOD gene)

was purified, and the products were cloned into the PGEM®-T Easy Vector (Promega) and subsequently subjected to sequencing analysis.

2.4. Bioinformatics analysis

The ExpASY Translate tool (<http://web.expasy.org/translate/>) was employed to derive the protein sequences of the Mn-SOD from lentil. Subsequently, the ProtParam tool (<http://web.expasy.org/protparam/>) was utilized to analyze the physical and chemical parameters of the LcMn-SOD protein. Prediction of the protein targeting of LcMn-SOD was done using ProtComp 9 (Emanuelsson et al., 2000). Similarity analyses were conducted using BLAST, and the conserved domains of protein were predicted by NCBI Conserved Domain Database (Marchler-Bauer et al., 2009). Alignment of multiple sequence analysis was performed using Clustal Omega software (Larkin et al., 2007). Protein analysis of predicted proteins was done by neighbor-joining algorithm using MEGA 7 program (Kumar et al., 2016), with 1000 replicates.

2.5. Quantitative RT-PCR:

Gene expression analysis of the LcMn-SOD gene was conducted utilizing quantitative real-time polymerase chain reaction (qRT-PCR). Primers specific to the target gene were designed using Primer 3 software. The qRT-PCR primer pairs for Mn-SOD (sense: 5'-TTGTGCAGAAGAACCCTATCCCC-3'; antisense: 5'-GATTCGCCGCTAATGACAGG -3') yielded an amplicon of 162 base pairs. The genes sequence of Actin was considered as an internal control, with primers (sense: 5'-ATACCCCTGCCATGTATGTAGC-3'; antisense: 5'-AGCCAGATCAAGACGAAGGATG-3') designed accordingly.

Each PCR reaction mixture contained a total volume of 25 μ L, comprising 10 μ L of KAPA SYBR® FAST universal qPCR Kit (KAPA, USA), 0.4 μ L of each specific primer (10 μ M), 120 ng/ μ L of diluted cDNA as a template, and RNase-free water to adjust the final volume. Amplification was conducted with an initial denaturation step at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 54°C for 25 seconds, and extension at 60°C for 25 seconds, during which fluorescent signals were acquired. A final extension step was performed at 60°C for 2 minutes.

All qRT-PCR were repeated three times for each sample. The level of gene expression was determined by calculating the fold difference using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001). Differences between treatment times were evaluated by the least significant difference (LSD) at a 5% CI.

3. Results

3.1. Cloning and bioinformatics analyses of Mn-SOD gene in lentil (LcMn-SOD)

To identify the Mn-SOD gene in lentil, a candidate gene approach was used, based on the pea Mn-SOD gene sequence (PsMn-SOD; GenBank accession No. X60170.1). Primers were designed based on the sequence of the gene which used to amplify the lentil Mn-SOD gene from cDNA synthesized. PCR amplification with LcMn-SOD F and LcMn-SOD R primers produced an 858 bp

product, which was purified, cloned into the PGEM®-T Easy Vector, and sequenced.

The full-length LcMn-SOD cDNA is 858 bp, containing both a start and stop codon, indicating the gene is complete. It includes a 723 bp open reading frame (ORF), a 1 bp 5'-untranslated region, and a 134 bp 3'-untranslated region. The ORF encodes a protein of 240 amino acids figure 6, with a predicted molecular mass of 26.59 kDa and a theoretical isoelectric point of 7.86. The nucleotide sequence has been submitted to GeneBank.

Subcellular localization predictions using Target 1.1 and ProtComp 9.0 indicated that LcMn-SOD is likely localized in the mitochondria figure 7, with TargetP 1.1 providing a high confidence prediction. Sequence similarity analysis using BLASTP showed homology between LcMn-SOD and Mn-SOD proteins from other plant species. Multiple sequence alignment using Clustal Omega revealed conserved manganese-binding residues at positions H-64, H-109, H-105, and Asp-101 figure 8, shared with Mn-SOD proteins from pea, common vetch, soybean, chickpea, and barrelclover.

3.2. Gene expression under the stress condition studied

In this study, the involvement of Mn-SOD genes in lentil responses to abiotic stress was explored by analyzing gene expression in seedlings under drought and hydrogen peroxide (H_2O_2). Drought stress was induced by withholding irrigation, and LcMn-SOD gene expression was measured at 3 and 6 days after emergency. The results showed an upregulation of LcMn-SOD, with fold changes of 1.4 and 2.3 at 3 and 6 days, respectively (Fig. 1). Additionally, the plant was treated with 10 mM H_2O_2 , and gene expression was monitored at 1, 2, and 4 hours. qRT-PCR analysis revealed that LcMn-SOD was downregulated at 1 hour but showed significant upregulation (3.7-fold) at 4 hours (Fig. 2).

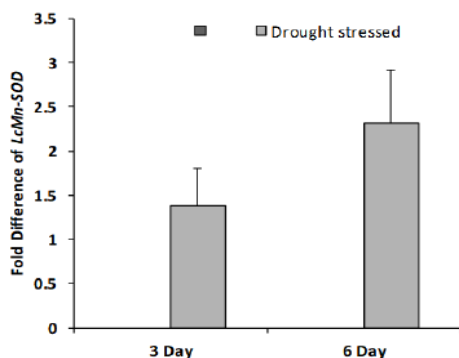


Figure 1. Expression of LcMn-SOD in lentil seedlings subjected to drought stress for 3- and 6-days post emergency (X axis is the days parameter), as measured by qRT-PCR. The LcACT1 was used as reference gene. Data represent the means of three replicates \pm SE. Significantly different values are indicated by different letters above the error bars.

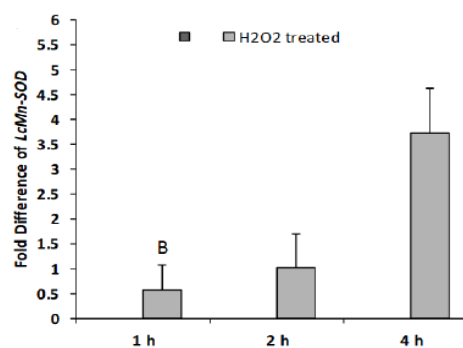


Figure 2. Expression of LcMn-SOD under H_2O_2 for 1, 2, and 4 hours, (X axis is the days parameter), as measured by qRT-PCR. The LcACT1 gene was used as a. Data represent the means of three replicates \pm SE. Significantly different values are indicated by different letters above the error bars

3.3. Expression under phytohormonal stressor

The effect of phytohormonal stimuli on LcMn-SOD gene expression was investigated using salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) treatments on two-week-old lentil seedlings. The expression profiles were analyzed via qRT-PCR at 1-, 2-, and 4-hours post-treatment.

Treatment with 1 mM SA led to downregulation of LcMn-SOD at 1 and 2 hours in contrast to the untreated control. However, by 4 hours, gene expression significantly increased, reaching a peak of 1.3-fold (Fig. 3). When the seedlings were treated with 100 μ M ABA, LcMn-SOD expression was initially downregulated at 1 hour but began to increase by 2 hours, although the changes were not statistically significant. A dramatic 14-fold upregulation of LcMn-SOD was observed at 4 hours (Fig. 4).

JA treatment (100 μ M) also significantly affected LcMn-SOD expression. The gene showed its highest expression at the 1-hour time point with a 1.82-fold increase, followed by a decline at 2 and 4 hours, reaching 30% and 10% of the initial peak, respectively (Fig. 5)

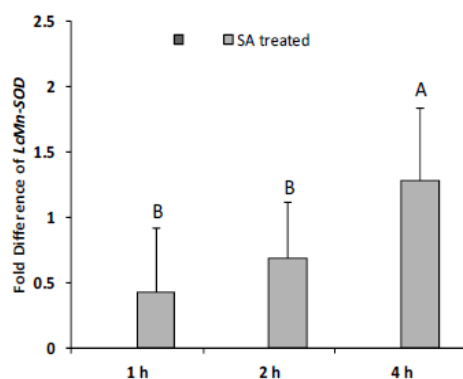


Figure 3. qRT-PCR analysis of LcMn-SOD expression at 1, 2, and 4 hours (X-axis shows time in days). Data represent the mean of three replicates \pm SE. Statistically significant differences are denoted by distinct letters above the error bars.

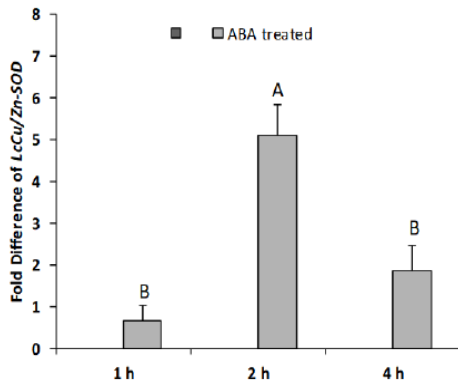


Figure 4. Relative expression of *LcCu/Zn-SOD* (a) and *LcMn-SOD* (b) in lentil seedlings treated with ABA for 1, 2, and 4 h (X axis is the days parameter), as measured by qRT-PCR. The expression of both genes was normalized to *LcACT1* reference gene, and then normalized with their expression in untreated control seedling (set to 1.0) at the corresponding time point. Data Significantly different values are indicated by different letters above the error bars.

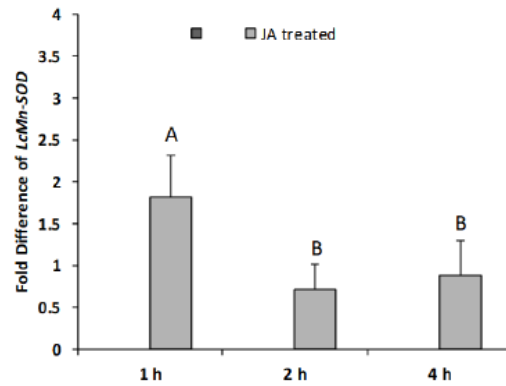


Figure 5. Relative expression of *LcMn-SOD* in lentil seedlings treated with JA for 1, 2, and 4 h (X axis is the days parameter), as measured by qRT-PCR. The expression of gene was normalized to *LcACT1* reference gene, and then was normalized with their expression in untreated control seedling (set to 1.0) at the corresponding time point. Data represent the means of three replicates ± SE. Significantly different values are indicated by different letters above the error bars.

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atggcgcgctcgaaccctattgtgcagaagaaccctatcccctgtcctccgcaacgatgca
M A A R T L L C R R T L S P V L R N D A
aaaccaatcggggcagccatagccgccgcacactcaatcccgtgggttacatgtcttc
K P I G A A I A A A S T Q S R G L H V F
acgctaccggatctcgtcttacgactacggagctttggagcctgtcattagcggcgaatc
T L P D L A Y D Y G A L E P V I S G E I
atgcaaatccatcatcagaaacaccaccagacttatattaccaactataacaaagctctc
M Q I H H Q K H H Q T Y I T N Y N K A L
gagcagcttcacgatgccgttggttaaagctgatacatctaccactgttaagctccagaat
E Q L H D A V G K A D T S T T V K L Q N
gccatcaagttcaacggcggagggtcatattaaccattccattttctggaaaaatctggct
A I K F N G G G H I N H S I F W K N L A
cctgttccgggaaggaggtggtgaaccaccaaaaggaatccctaggctgggccattgacaca
P V R E G G G E P P K E S L G W A I D T
aactttggatctttggaagcattgattcaaaagattaatgccgaagggtgcagctcttcag
N F G S L E A L I Q K I N A E G A A L Q
gggtctggatgggtgtggcttggctcttgacaaagatttgaagaggcttgtggttgaacc
G S G W V W L G L D K D L K R L V V E T
actgcaaaccaggacccactggctactaaaggagcaagtttggttccattgcttgggtata
T A N Q D P L V T K G A S L V P L L G I
gatgtttgggaacatgcctactacttacagtacaagaatgtagaccagactatttgaag
D V W E H A Y Y L Q Y K N V R P D Y L K
aacatttggaaagttattaactggaaacatgccagtgaaagtatatgagaaagagagctct
N I W K V I N W K H A S E V Y E K E S S
taatctgaagtgctgcttggatggaacttggataacaggttgcagcatgttggcgat
gaataaaatgatgtgaagtgatagataataccttcctatgatgtacttgtgctttagaa
cttgagcaatctggccg
    
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Figure 6. Nucleotide sequence and deduced amino acid sequence of *LcMn-SOD*. The amino acids are designated with single-letter code below the middle nucleotide of each codon. Start codon is shaded in gray and stop codon is shaded in black.

4. Discussion

4.1. Cloning and Bioinformatics Analysis of the SOD Gene in Lentil

Plants, in their natural environments, are subjected to various stressors that adversely affect their growth, productivity, and development, primarily due to the accumulation of reactive oxygen species (ROS) (Miller et al., 2008). To combat this, plants have evolved sophisticated antioxidative defense systems operating in nearly all cellular compartments (Halliwell et al., 2006, Odat, 2020b). Superoxide dismutases (SODs) are a crucial component of these systems, scavenging superoxide radicals in various organelles to mitigate oxidative stress (Paardekooper et al., 2019; Eraslan et al., 2007). Several SOD genes have been identified across a wide range of species, including Arabidopsis (Kliebenstein et al., 1998), and barley (Abu-Romman & Shatnawi, 2011). This study successfully isolated and characterized the Mn-SOD gene in lentil, designated as LcMn-SOD. Using bioinformatics tools, the LcMn-SOD protein was predicted to localize in the mitochondria, aligning with other studies where Mn-SOD isoenzymes are localized in both mitochondria and peroxisomes (Rio et al., 2003). Sequence similarity analysis revealed significant homology between LcMn-SOD and other legume species, with conserved manganese-binding residues critical for enzymatic activity (Kowalczyk et al., 2021).

4.2. Expression Analysis of LcMn-SOD Genes

Understanding how defense genes like LcMn-SOD are regulated under different environmental stressors provides valuable insight into their functional roles. In this study, drought stress resulted in upregulation of LcMn-SOD, correlating with increased ROS levels in chloroplasts and mitochondria, which disrupt photosynthesis due to stomatal closure (Postiglione & Muday, 2023). The upregulation of LcMn-SOD highlights its role in neutralizing ROS, as seen in other plants under abiotic stresses like drought and salinity (Chakrabarty et al., 2016; Kukreja et al., 2005, Al-Tawaha, et al., 2021). H₂O₂, a non-radical ROS, also significantly increased LcMn-SOD expression after 4 hours, suggesting its role as both a damaging agent and a signaling molecule, as observed in other species like maize and *Syzygium cumini* (Hu et al., 2006; Choudhary et al., 2012).

4.3. Phytohormonal Effects on LcMn-SOD Expression

Phytohormones like salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) play critical roles in plant stress responses by modulating antioxidant enzyme activities. This study demonstrated that SA, ABA, and JA upregulated LcMn-SOD expression at various time points, reflecting the complex signaling interactions these hormones trigger. ABA-induced upregulation aligns with its known role in ROS generation and antioxidant enzyme activation, while JA's early upregulation effect is consistent with the increased ROS levels following its application (Postiglione & Muday, 2023).

5. Conclusion and Future Directions:

This study successfully cloned and characterized the full-length Mn-SOD gene from lentil. Bioinformatics

analysis confirmed its mitochondrial localization and sequence conservation with Mn-SOD genes from other legumes. Expression analyses showed that LcMn-SOD is responsive to drought, H₂O₂ and phytohormonal treatments, highlighting its key role in lentil's defense against oxidative stress. Future research should focus on generating transgenic lentils overexpressing LcMn-SOD to assess its role in stress tolerance under combined abiotic and biotic stress conditions. Comparative studies across lentil cultivars and wild relatives could further uncover the regulatory mechanisms governing SOD gene expression in diverse environments.

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