horticulturae

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The Apple Lipoxygenase *MdLOX3* Regulates Salt Tolerance and ABA Sensitivity

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Abstract: Various abiotic stresses, particularly salinization, restrict plant growth and yield around the world. Lipoxygenases play essential functions in coping with various stresses. In the present study, we found an apple (*Malus domestica*) homolog of *Arabidopsis* lipoxygenase3, named *MdLOX3*. MdLOX3 has a typical conserved lipoxygenase domain. *MdLOX3* was expressed in all tissues of apple and was highly expressed in the root and flesh tissues by a qRT-PCR analysis. In addition, the promoter of *MdLOX3* consists of multiple response elements. Various abiotic stresses and ABA treatment can induce the expression of *MdLOX3*. The overexpression of *MdLOX3* in apple calli enhanced the ability to tolerate salt stress, and the heterotopic expression of *MdLOX3* in *Arabidopsis* elevated salt stress tolerance via enhancing the ability of scavenging ROS. Furthermore, the overexpression of *MdLOX3* in transgenic plants significantly reduced the sensitivity to ABA. Through the above, this work demonstrated that *MdLOX3* played an active position in salt resistance and decreased the sensitivity to ABA, providing a theoretical reference for studying the role of *MdLOX3* in abiotic stresses in apple.

Keywords: apple; lipoxygenase; *MdLOX3*; salinity; ABA

Citation: Chen, X.; Wang, D.; Zhang, C.; Wang, X.; Yang, K.; Wang, Y.; Wang, X.; You, C. The Apple Lipoxygenase *MdLOX3* Regulates Salt Tolerance and ABA Sensitivity. *Horticulturae* **2022**, *8*, 651. https://doi.org/10.3390/horticulturae8070651

Academic Editors: Maria G. Maleva, Alexander A. Ermoshin and Galina Borisova

Received: 19 June 2022
Accepted: 15 July 2022
Published: 17 July 2022

1. Introduction

As one of the most critical abiotic stresses, salt stress influences plant growth and development and reduces yield and quality [1,2]. In order to adapt to the complex and changeable environment, plants gradually evolved complex regulatory patterns [3,4]. In previous research, plant lipoxygenases (LOXs) were shown to function in physiological processes, including growth and stress-related responses [5,6].

LOX is a key rate-limiting enzyme in the metabolic pathway of plant fatty acids, which widely exists in animals, plants, and microorganisms [7,8]. According to the different oxygenation sites that are catalyzed, LOX can be classified into 9-LOXs and 13-LOXs, but some LOXs can oxygenate at two sites to produce two products [9,10]. *AtLOX1/5* belong to 9-LOX, and *AtLOX2/3/4/6* belong to 13-LOX in *Arabidopsis* [11].

It has been well proven that LOXs have important roles in biotic and abiotic stress tolerance. In dicotyledonous grasses, the overexpression of *AtLOX3* enhances salinity tolerance, and the salt sensitivity of *lox3* mutant can be supplemented by methyl jasmonate (MeJA) [12]. *NaLOX3* is a member of JA biosynthesis, which is more sensitive to mechanical injury and pest and disease infestation [13]. In monocotyledonous herbs, *ZmLOX3*-inactivated maize is more susceptible to *Aspergillus* [14]. *ZmLOX6* acts as a nutrient storage protein in mesophyll cells to store nitrogen and buffer the adverse effects of

drought stress in maize [15]. In dicotyledonous woody plants, *GhLOX12* and *GhLOX13* are negative regulators in salt tolerance by affecting the activity of superoxide dismutase (SOD) [16]. ABA responds to abiotic stresses by inducing many physiological and molecular alterations [17]. *AtLOX1* is induced by the expression of the stress-related hormone ABA [18]. *CaLOX1* can respond to drought and high salt stresses by regulating the expression of ABA-signaling-related genes (*RAB18* and *RD29B*) and stress-response genes (*COR15A*, *DREB2A*, *RD20*, *RD29A*, and *RD29B*) [19]. *DkLOX3* acts as a positive role in the stress response via regulating the accumulation of ROS and the expression of stress-response genes (especially *RD29A* and *RD29B*), and promotes ripening and senescence through lipid peroxidation [20].

Apple is among the most widely grown fruit trees worldwide. The quality and yield of apple are constrained by poor environmental conditions, including salt, drought, and low temperature stresses [21–23]. In our study, we isolated and identified a novel gene, *MdLOX3*, in *Malus domestica*. The overexpression of *MdLOX3* in apple calli enhanced salt tolerance, and the heterotopic expression of *MdLOX3* reduced the sensitivity to salt by regulating ROS scavenging in *Arabidopsis*. Additionally, we investigated its role in the ABA response, and the overexpression of *MdLOX3* decreased the sensitivity to ABA. This provides a reference for developing future genetic engineering assays to study the mechanism during abiotic stresses in apple.

2. Materials and Methods

2.1. Plant Materials and Treatment

To analyze the expression pattern of *MdLOX3* in different organs of apple, the samples (roots, stems, leaves, flowers, peels, and flesh) were taken from a seven-year-old 'Gala' apple tree (Taian, China). The apple culture seedlings were maintained on Murashige and Skoog (MS) medium containing 0.5 mg/L of naphthyl acetic acid (NAA) and 0.5 mg/L of 6-benzylaminopurine (6-BA) under long-day conditions (light intensity: 300 μmol·m^{-2}·s^{-1}, photoperiod: light 16 h/dark 8 h) [24]. Four-week-old apple seedlings were treated with NaCl (150 mM); PEG 6000 (10%); or ABA (100 μM), and sampled at 0, 1, 3, 6 or 12 h, respectively.

In the dark conditions, the apple calli were cultured in MS medium with 1.5 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.4 mg/L of 6-BA. The *Arabidopsis thaliana* (including wild type and *MdLOX3* ectopic-expression transgenic homozygotes T_3 generation) were cultured on MS medium and vernalized at a temperature of 4 °C for 3 days, then cultured under a 16-h light/8-h dark photoperiod at a temperature of 22 °C. Three-week-old seedlings of *Arabidopsis* were treated with 150 mM of NaCl at 7-day intervals.

2.2. Bioinformatics Analysis of the MdLOX3

The online software SMART (https://smart.embl.de/, accessed on 20 March 2022) was used to define the conservation domains in the *MdLOX3* [25]. A prediction of the secondary and tertiary structures of MdLOX3 was made according to the method that was described by Wang [26].

2.3. Phylogenetic Analysis and Sequence Alignment

The adjacency algorithm of MEGA version 11 was used to construct the LOX3 phylogenetic neighbor-joining trees of 12 plant species [27]. The sequence alignment of MdLOX3 and AtLOX3 was performed by the Cluster Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on 15 March 2022), and then visualized by Jalview (Jalview 2.11.2.2) [28].

2.4. RNA Extraction and Quantitative RT-PCR

The plant materials (including apple organs, apple calli, and *Arabidopsis*) were ground into powder in liquid nitrogen. Then, the total RNA of the samples was extracted via an established method [29]. A quantitative RT-PCR was carried out according to established protocols [30]. An analysis of the relative expression levels of *MdLOX3* was performed by

the (Ct)$2^{-\Delta\Delta CT}$ calculation method. For the apple samples, *18S* was used as an internal control, and *AtACTIN* was used for the *Arabidopsis* samples. All the primers that were used for the gene expression analysis are listed in Supplemental Table S1.

2.5. Apple Calli and Arabidopsis Transformation

The open reading frame of *MdLOX3* was linked with the pRI101 plasmids, in order to create the overexpression vector of *MdLOX3*. The overexpression of *MdLOX3* in apple calli (*MdLOX3-OE1*, *MdLOX3-OE2,* and *MdLOX3-OE3*) was obtained via the *Agrobacterium* strain EHA105 [31]. We transformed the *35S::MdLOX3* vector through the floral dip method through *Agrobacterium* strain GV4404 to obtain the transgenic lines (L1, L2, and L3) [32].

2.6. Determination of MDA and ROS

The content of malondialdehyde (MDA) was determined via the thiobarbituric acid (TBA)-based method [33]. The accumulated H_2O_2 and O_2^- were monitored by 3,3′-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) histochemical staining, respectively [34]. The H_2O_2 content and O_2^- production rates were quantified based on the corresponding test kits (Keming, Suzhou, China) using a spectrophotometry.

2.7. Statistical Analysis

Each set of experiments was performed at least three times, and three parallel technical replicates were performed. Different small letters above the bars represent significant differences using an ANOVA with a Tukey–Kramer test at $p < 0.05$ [35].

3. Results

3.1. Bioinformatics Analysis of the MdLOX3

The MdLOX3 sequence (MD10G1294000) was confirmed to be the closest apple homolog of the AtLOX3 sequence (AT1G17420), and the total length of its cDNA was 1341 bp, encoding 446 amino acids. As shown in Figure 1A, MdLOX3 contained a lipoxygenase domain spanning almost all of its length. We predicted that the secondary structure was consisted of 40.36% alpha helices, 11.43% extended strands, 4.26% beta turns, and 43.95% random coils (Figure 1B). According to the folding of the secondary structure, the tertiary structure was predicted (Figure 1C).

3.2. Phylogenetic Tree Analysis and Sequence Analysis of the MdLOX3 Protein

To investigate the evolutionary relationships between MdLOX3 and LOX3 proteins from other plant species, the phylogenetic tree was constructed by MEGA 11 (Figure 2A). The results showed that MdLOX3 and *Pyrus ussuriensis* × *Pyrus communis* (KAB2601216.1) were highly homologous. MdLOX3 was in close association with *Prunus mume* (XP_0082-25101.1); *Prunus persica* (XP_020417634.1); and *Prunus dulcis* (XP_034210916.1). We showed that AtLOX3 in *Arabidopsis* had the highest similarity and consistency with MdLOX3 in apple by a homology comparison. In addition, we compared the MdLOX3 and AtLOX3 protein sequences, and found that the two sequences were highly similar (Figure 2B).

3.3. Expression Pattern of MdLOX3 in Apple Tissues

To elucidate the biological functions of *MdLOX3* in plants, we investigated the expression of *MdLOX3* by a qRT-PCR assay (Figure 3A). The results showed that *MdLOX3* was expressed in all six organs of apple (roots, stems, leaves, flowers, peel, and flesh). *MdLOX3* was highly expressed in the roots and flesh, indicating its important function in highly expressed tissues. *MdLOX3* was expressed at relatively poor levels in the leaves and flowers.

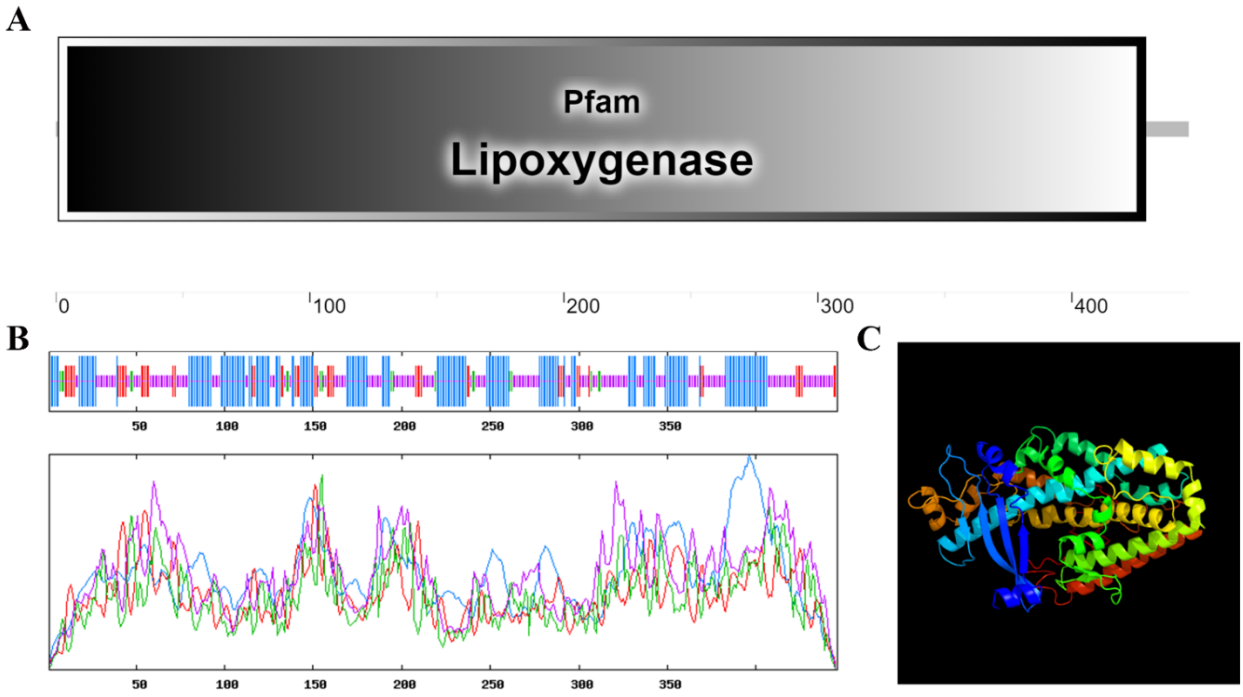

Figure 1. Bioinformatics analysis of the *MdLOX3* gene. (**A**) Conserved domain of MdLOX3 protein. The rectangle indicates the lipoxygenase domain. The numbers represent the length of amino acids. The predicted secondary (**B**) and tertiary (**C**) protein structures of MdLOX3. The numbers denote the length of amino acids. Blue, red, orange and green represent alpha helices, extended strands, random coils and beta turns.

3.4. MdLOX3 Promoter Cis-Regulation Analysis and Expression Patterns of MdLOX3 under NaCl, PEG, and ABA Treatments

The upstream promoter region of the *MdLOX3* was analyzed via the online software PlantCARE. The results revealed that the promoter region of the *MdLOX3* contained multiple plant stress-responsiveness and hormone-responsiveness elements; for instance, the abscisic acid responsive element (ABRE); the anaerobic induction (ARE); the MeJA-responsive elements (CGTCA-motif and TGACG-motif), and so on. In addition, the *MdLOX3* promoter region had various light-responsive elements, such as the ACE and G-Box (Table 1).

To examine whether *MdLOX3* responded to abiotic stresses in apple, the apple seedlings with 150 mM of NaCl, 10% PEG6000, or 100 μM of ABA were used, and the expression levels of *MdLOX3* were measured by a qRT-PCR assay (Figure 3B–D). Under NaCl treatment, the expression of *MdLOX3* dropped initially (but the decrease was not significant) and then increased at 12 h (Figure 3B). Under PEG treatment, *MdLOX3* expression peaked at 3 h, and then rapidly declined (Figure 3C). When treated with ABA, the expression levels of *MdLOX3* decreased only at 12 h, and the rest did not change significantly (Figure 3D).

3.5. MdLOX3 Improves the Resistance of Apple Calli to Abiotic Stresses

To investigate the roles of *MdLOX3* in coping with abiotic stresses, *MdLOX3-OE* was successfully transformed into apple calli (Figure 4A). The wild-type (WT) and *MdLOX3-OE* apple calli were cultured in different concentrations of NaCl (100 and 150 mM) or ABA (50 and 100 μM) for 15 days. As shown in Figure 4B, when treated with NaCl and ABA, *MdLOX3-OE* exhibited faster growth than the WT. However, WT and *MdLOX3-OE* exhibited a similar growth status under normal growth conditions. Consistent with the observed phenotype, *MdLOX3-OE* exhibited higher fresh weights and a lower MDA content than WT under the NaCl and ABA treatments (Figure 4C,D). Therefore, the overexpression of *MdLOX3* enhances tolerance to salt and reduces sensitivity to ABA in apple calli.

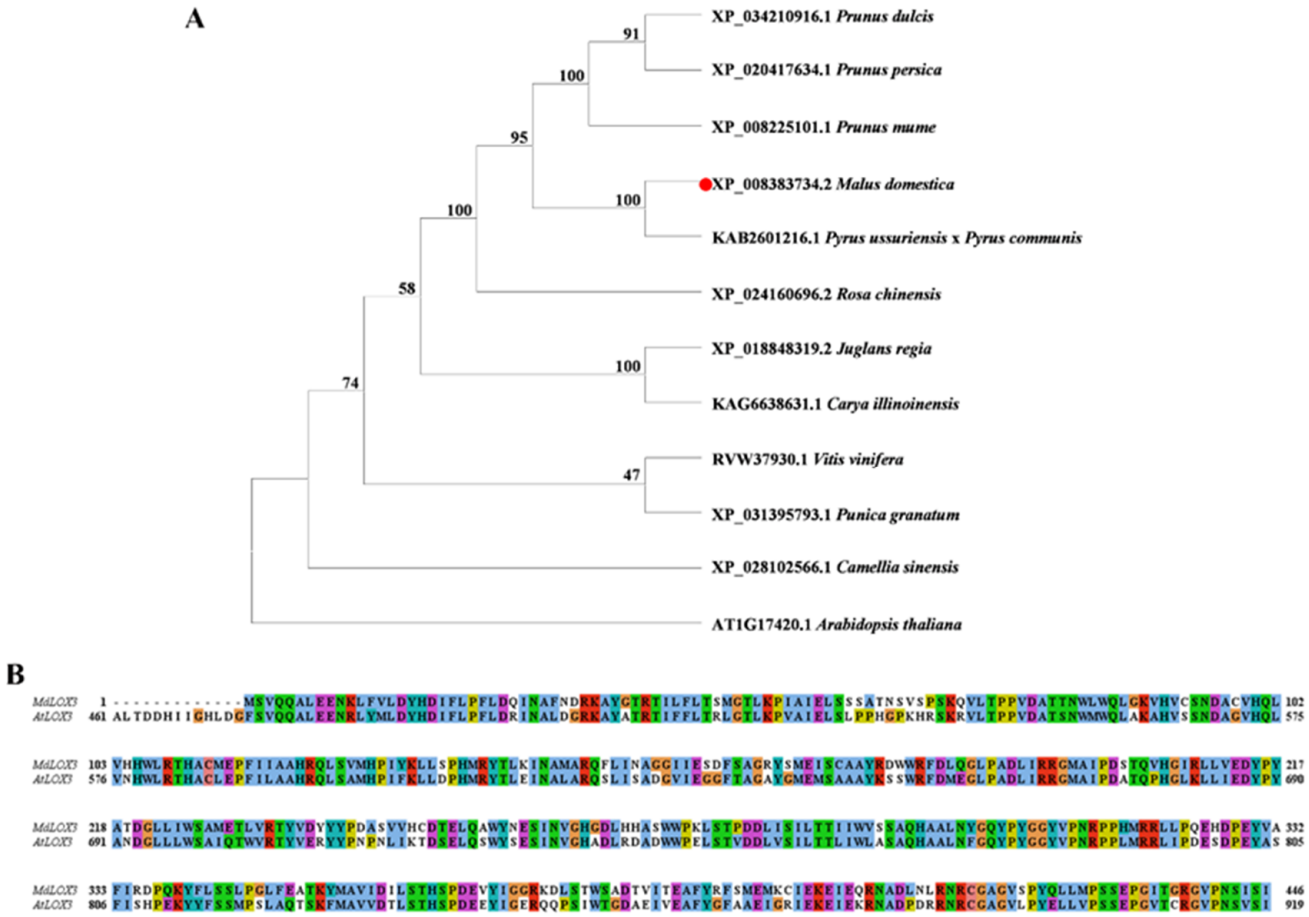

Figure 2. Phylogenetic tree analysis and amino acid sequence alignment of the MdLOX3 protein. (**A**) The phylogenetic relationship between MdLOX3 and LOX3 proteins from other plant species via MEGA 11. (**B**) Alignment of between MdLOX3 and AtLOX3 amino acid sequences. Red circles indicate MdLOX3 (*Malus* × *domestica*) protein.

3.6. MdLOX3 Enhances Tolerance of Arabidopsis to Abiotic Stresses

We obtained *MdLOX3* transgenic *Arabidopsis* in order to further test the role of *MdLOX3* in response to salt stress and ABA (Figure 5). *Arabidopsis* seedlings (WT and *MdLOX3* transgenic *Arabidopsis*) were shifted to medium with NaCl or ABA for 15 days. There was no remarkable difference in primary root length among the WT and transgenic seedlings under the control conditions (Figure 5A,B). After treatments with salt stress and ABA, the transgenic seedlings developed longer roots than the WT under the salt stress and ABA treatments.

In addition, we treated three-week-old WT and *MdLOX3* transgenic plants with 150 mM of NaCl solution in soil for 21 days. After salt treatment, *MdLOX3* transgenic *Arabidopsis* showed greater height and more branches and seeds than WT (Figure 5D,E). Taken together, these results show that *MdLOX3* plays a positive role in salt resistance.

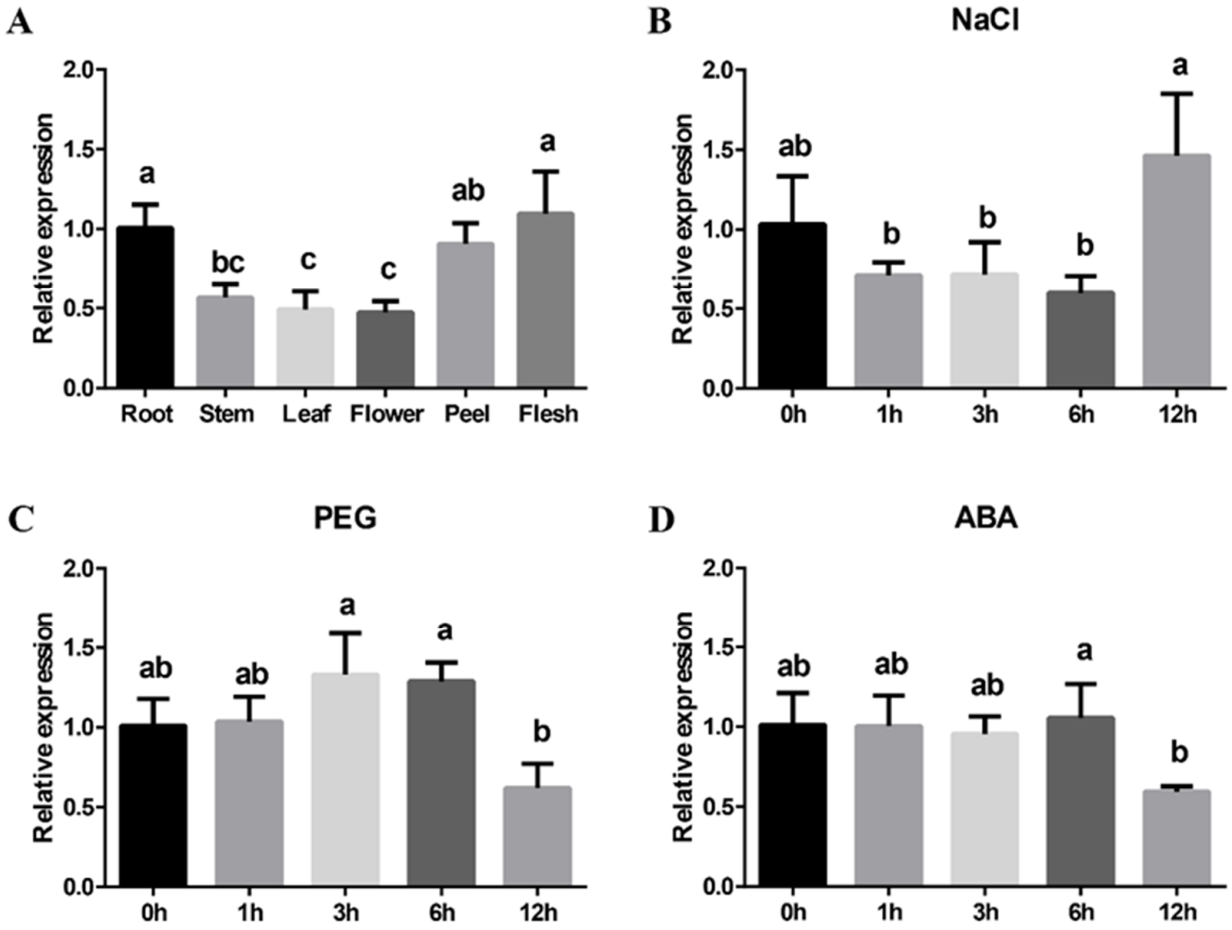

Figure 3. Expression patterns of *MdLOX3*. (**A**) Expression pattern of *MdLOX3* in different tissues of apple (roots, stems, leaves, flowers, peel, and flesh). Apple seedlings were treated with 150 mM of NaCl (**B**); 10% PEG6000 (**C**); and 100 μm of ABA (**D**) for 0, 1, 3, 6, and 12 h, respectively. Error bars, ±SD of three independent replicates. Different lowercase letters represent significant differences ($p < 0.05$).

Table 1. Promoter cis-elements analysis of *MdLOX3* region.

Cis-Element Name	Cis-Element Sequence (5′-3′)	Function	Location
ABRE	ACGTG	*cis*-acting element involved in the abscisic acid responsiveness	+1023
CGTCA-motif	CGTCA	*cis*-acting regulatory element involved in the MeJA responsiveness	+1365
TGACG-motif	TGACG	*cis*-acting regulatory element involved in the MeJA responsiveness	−127
GARE-motif	TCTGTTG	gibberellin-responsive element	−975
ARE	AAACCA	*cis*-acting regulatory element essential for the anaerobic induction	−1168
ACE	GACACGTATG	*cis*-acting element involved in light responsiveness	−961
G-Box	TACGTG	*cis*-acting regulatory element involved in light responsiveness	−786

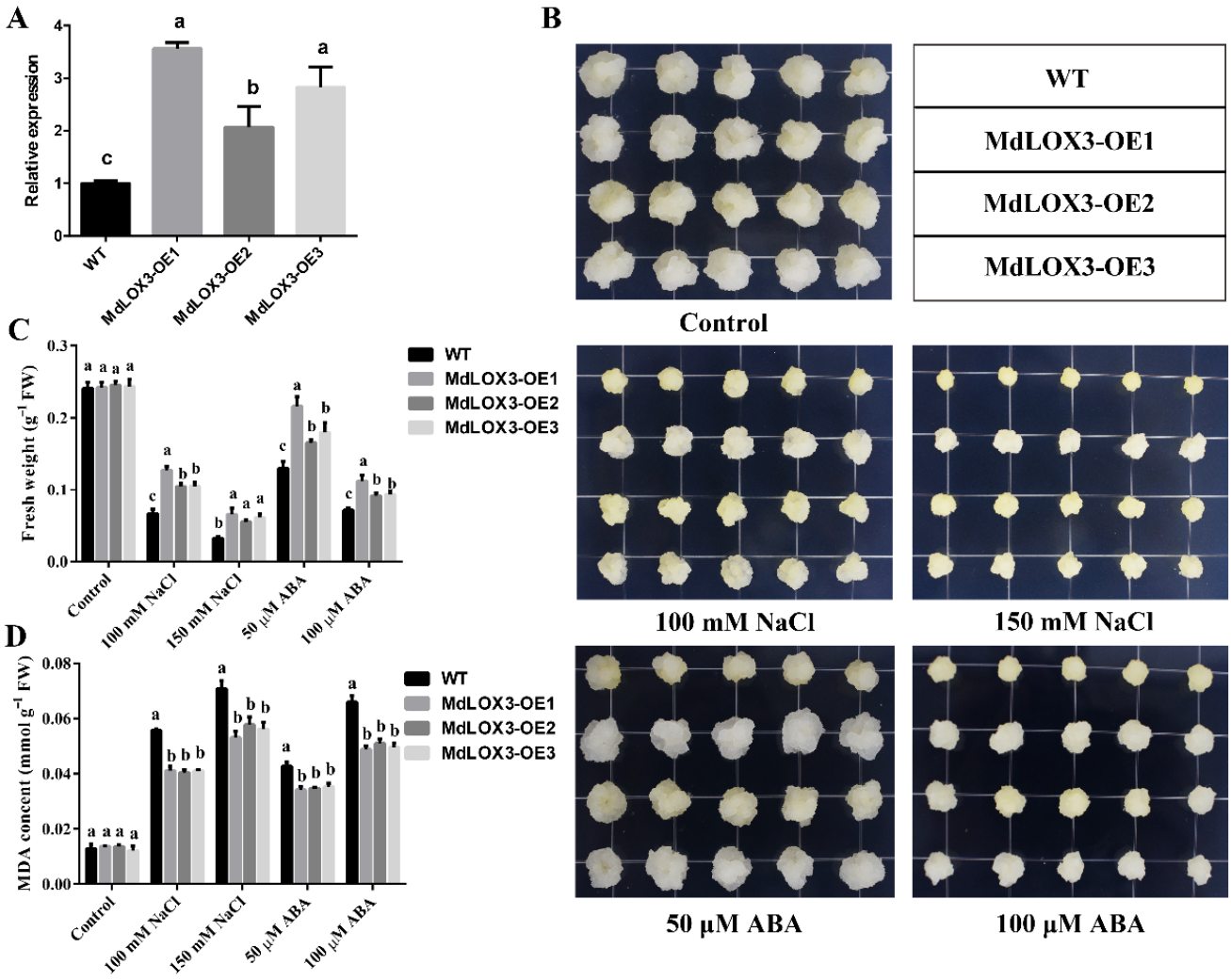

Figure 4. *MdLOX3* improves the resistance of apple calli to NaCl and ABA. (**A**) qRT-PCR analysis of *MdLOX3* transcript level in WT and transgenic calli. (**B**) The growth phenotypes of WT and *MdLOX3-OE* cultured on MS medium containing different concentrations of NaCl (100 and 150 mM) or ABA (50 and 100 μM) for 15 days. Fresh weight (**C**) and MDA content (**D**) of WT and *MdLOX3-OE*. Error bars, ±SD of three independent replicates. Different lowercase letters represent significant differences ($p < 0.05$).

3.7. Overexpression of MdLOX3 in Arabidopsis Increases ROS Scavenging Capacity under Salt Stress

Stresses usually lead to the excessive production of ROS (especially H_2O_2 and O_2^-), which causes ROS-induced cellular damage [36]. There was no significant difference observed between the three WT and *MdLOX3* transgenic lines before NaCl treatment. However, *MdLOX3* overexpression lines stained lighter compared with the wild type after NaCl treatment, suggesting that *MdLOX3* transgenic *Arabidopsis* accumulated less H_2O_2 and O_2^- than WT (Figure 6A,B). We also measured the accumulation of H_2O_2 and the production rate of O_2^- corresponding kits, and found that *MdLOX3* transgenic *Arabidopsis* had a relatively low accumulation of H_2O_2 and a lower rate of O_2^- production compared with WT (Figure 6C,D).

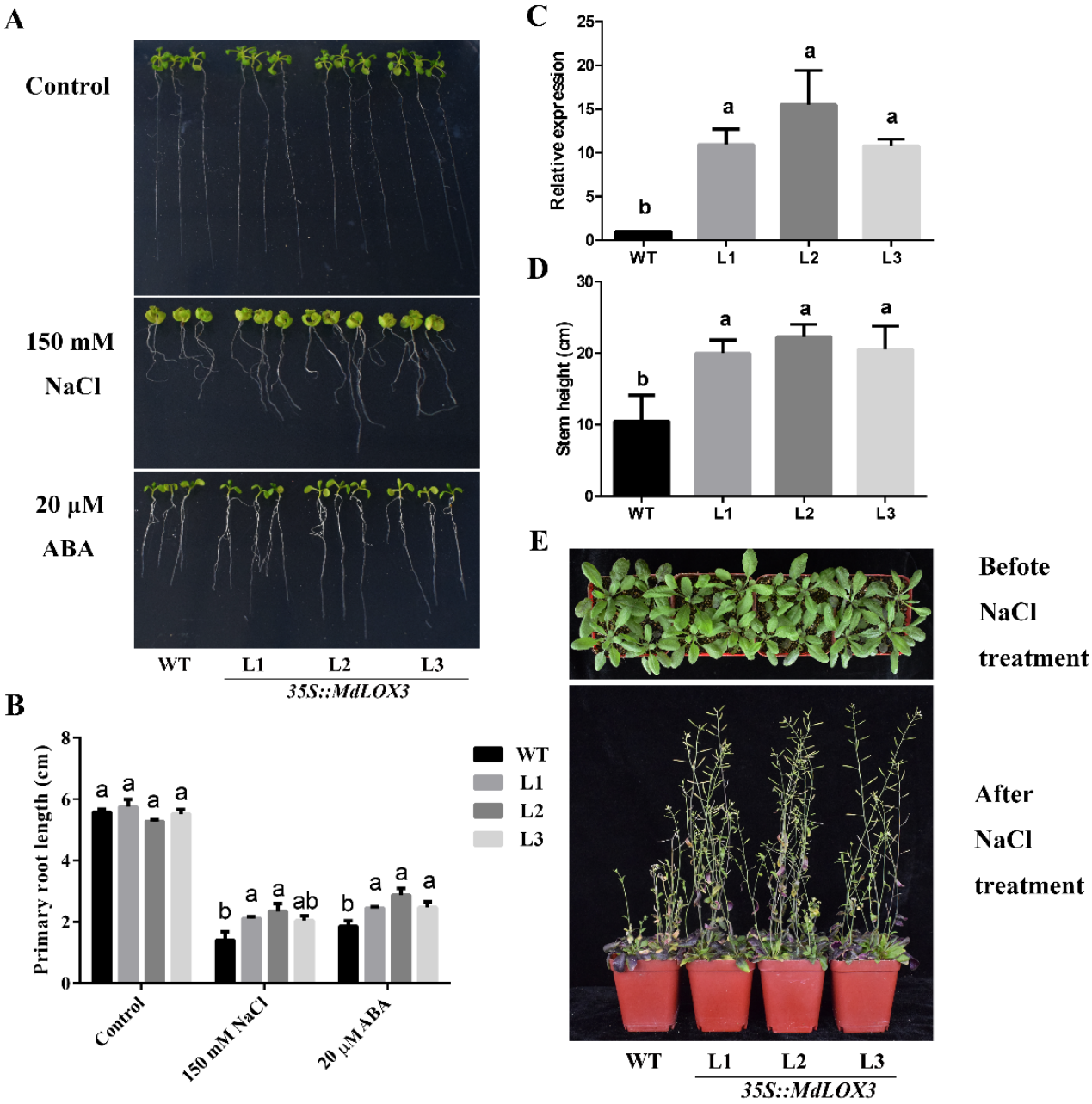

Figure 5. Ectopic expression of *MdLOX3* positively regulates resistance to salt and ABA. (**A**) Growth phenotypes of WT and transgenic *Arabidopsis* treated with nothing added or supplemented with NaCl (150 mM) or ABA (20 μM). (**B**) Analysis of primary root length in WT and transgenic *Arabidopsis* shown in (**A**). (**C**) Expression analysis of *MdLOX3* in WT and the transgenic *Arabidopsis*. (**D**) Analysis of stem height in WT and transgenic *Arabidopsis* shown in (**E**). (**E**) Phenotypes of *Arabidopsis* treated with 150 mM of NaCl after 21 days. Error bars, ±SD of three independent replicates. Different lowercase letters represent significant differences ($p < 0.05$).

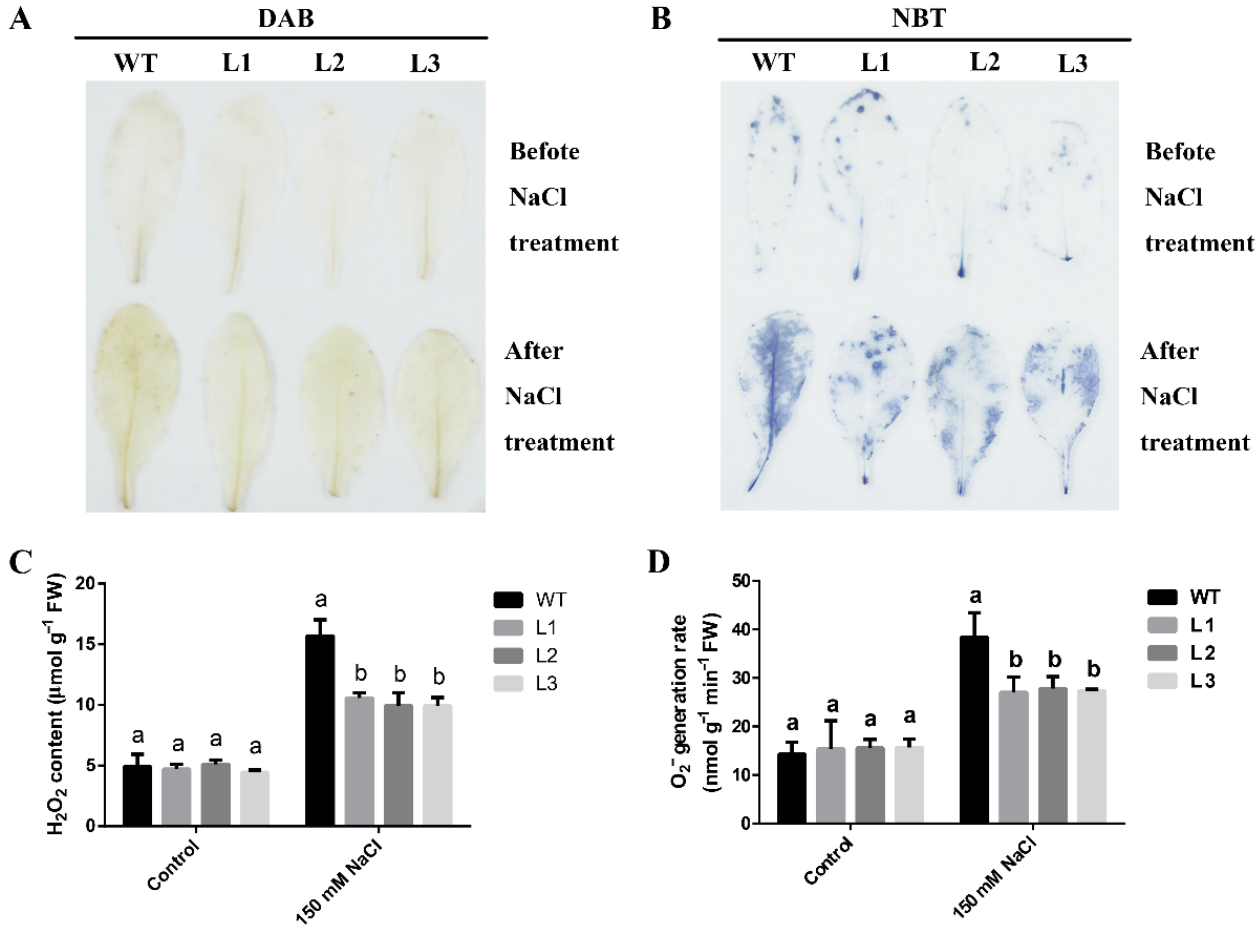

Figure 6. Overexpression of *MdLOX3* decreases ROS production under salt treatment for 21 days. DAB staining for H_2O_2 (**A**) and NBT staining for O_2^- (**B**) in WT and *MdLOX3* transgenic lines before and after NaCl treatments. The content of H_2O_2 (**C**) and production rate of O_2^- (**D**) in WT and *MdLOX3* transgenic lines before and after NaCl treatments. Error bars, ±SD of three independent replicates. Different lowercase letters represent significant differences ($p < 0.05$).

4. Discussion

In plants, LOXs have diverse functions in various processes, such as stress responses, vegetative growth, seed development, and germination [37]. To date, the functions of LOXs have been widely studied in *Arabidopsis* [38,39]. A total of six LOX genes were found in *Arabidopsis* [11] and 11 in apple [40], indicating that there are some differences between species. *MdLOX1a* can promote the synthesis of aroma substances, including ketones, alcohols, aldehydes, and esters [41]. Previous studies have demonstrated that LOXs in apple are not only associated with fruit ripening and senescence, but are also likely to play an important role in fruit cell division and expansion [42].Nevertheless, few studies have reported that apple LOXs are also involved in abiotic stresses. In our study, we cloned the *MdLOX3* and characterized its roles in the tolerance to salt stress in apple. MdLOX3 contains a lipoxygenase domain (Figure 1A), suggesting that it is a member of the LOX gene family. The MdLOX3 protein sequence is very similar to the AtLOX3 sequence (Figure 2B). Conserved amino acid sequences are important for the structure and function of proteins.

Salt stress affects crops' growth and development [43]. Therefore, an emphasis on stress-response genes and their functional properties is important to promote plant genetic improvement. In our study, *MdLOX3* had a relatively high expression level in apple roots (Figure 3A), suggesting that it may play a key role in abiotic stresses. The roots are the vital plant organ for water and nutrient uptake and sensing adverse environmental conditions

in the soil [44,45]. Previous studies have shown that *AtLOX3* is dramatically induced under salt stress [12]. The *MdLOX3* transcript levels fluctuated but did not change much under different stress conditions. There may be other regulatory modification pathways. In addition, there may be other time points of change that are not captured. In addition, Table 1 showed that the cis elements in the promoter region of *MdLOX3* had an ABA response element (ABRE). Under salt stress and ABA, the ABRE binding protein (DcAREB) binds to the ABRE element in the *Phytoene synthase 2* (*PSY2*) promoter to induce the expression of *DcPSY2* [46]. It has previously been studied that ABA plays important functions in a variety of abiotic stress responses and seed development [47].

In this study, the overexpression of *MdLOX3* improved tolerance to salt stress and exhibited an ABA-insensitive phenotype (Figures 4 and 5). Many studies have shown that ABA has an important relationship with plant resistance to abiotic stresses [48,49]. The MDA content of overexpressing *MdLOX3* calli was lower than WT, indicating that *MdLOX3* plays an active regulatory role in abiotic stresses (Figure 4D). The homeostasis of plant ROS (such as H_2O_2 and O_2^-) is disturbed by various abiotic stresses (including salt stress). Therefore, more ROS is produced under abiotic stress [50]. The accumulation of ROS leads to the peroxidation of the plasma membrane to produce MDA [51]. The degree of cell membrane damage can be determined by measuring some physiological indicators, such as MDA content [52]. Under salt stress, the overexpression of *MdLOX3* displayed significantly lighter levels of DAB and NBT staining, and lower H_2O_2 content levels and O_2^- generation rates (Figure 6). *CaLOX1* regulates ABA-responsive genes and reduces ROS accumulation to improve tolerance to salt and drought stresses [19]. Previous studies have shown that lipoxygenase may be involved in the synthesis of ABA to some extent [53].

In the present study, we discovered a novel gene, *MdLOX3*, and determined that its overexpression increased the ability of scavenging ROS to enhance salt stress tolerance. The results provide new perspectives for future research on tolerance to salinization and lay the foundation for an in-depth study on the function of MdLOX3 in abiotic stresses.

5. Conclusions

In summary, the overexpression of *MdLOX3* shows higher salt stress tolerance by reducing ROS accumulation. Moreover, the overexpression of *MdLOX3* exhibits less sensitivity to ABA, compared to WT. We speculate that *MdLOX3* may enhance tolerance to salt stress by regulating the ABA pathway or enhancing the scavenging ability of ROS. Our study provides a new insight into *MdLOX3*-mediated salt tolerance, which provides a theoretical reference for studying the role of MdLOX3 in abiotic stresses in apple.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8070651/s1, Table S1: Primers for quantitative real-time PCR.

Author Contributions: Software, Y.W. and X.W. (Xun Wang); writing—original draft preparation, X.C. and D.W.; conceptualization, C.Y. and X.C.; methodology, X.W. (Xiaofei Wang), C.Z. and K.Y.; funding acquisition, C.Y. and X.W. (Xiaofei Wang); resources, C.Y. and X.W. (Xiaofei Wang); writing—review and editing, X.C., X.W. (Xiaofei Wang) and D.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (32172538) and the major basic research project of Natural Science Foundation of Shandong Province (ZR2020ZD43).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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