



The lipoxygenase gene *AfLOX4* of *Amorpha fruticosa* L. is a potential regulator of drought stress tolerance pathways under saline and alkaline conditions

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Abstract

Lipoxygenase (LOX, EC1.13.11.12) catalyzes the oxygenation of polyunsaturated fatty acids and further generates oxylipins via enzymatic or non-enzymatic pathways, thus participating in the regulation of growth and development, stress response, and other processes. The objective of this research was to characterize the function and evaluate the mechanism of lipoxygenase gene *AfLOX4*'s role in enhancing drought tolerance. Transcriptomic data were generated and analyzed for the LOX gene, under different concentrations of drought (polyethylene glycol-6000) (PEG6000) and abscisic acid (ABA) stress treatment, to determine their effects on the expression of the *AfLOX4* gene and its role in plant physiology. The localization of the *AfLOX4* protein was detected through protoplast transformation. The expression of *AfLOX4* in *Amorpha fruticosa* tissues was determined by quantitative real-time PCR (qRT-PCR). Six *LOX* genes of *A. fruticosa* that showed an upregulated pattern under different concentrations of drought (PEG6000) stress were found via transcriptomic analysis and were named *AfLOX1* ~ 6. The *AfLOX4* protein was localized in the cytoplasm. *AfLOX4* was expressed in the stems, roots, flowers, and leaves of *Amorpha fruticosa* L., and its expression level was the highest in leaves. The expression of *AfLOX4* increased with the prolongation of stress time under different stresses. After ABA treatment, the germination rate of tobacco lines overexpressing *AfLOX4* was significantly increased, while under NaHCO_3 stress, the tolerance of tobacco plants overexpressing *AfLOX4* was higher than that of wild-type tobacco. The tobacco with overexpression of the *AfLOX4* gene detected by the natural drought method had a higher survival rate in the drought stress recovery experiment, and improved the drought tolerance of tobacco. This study reveals the function of the *AfLOX4* gene in resisting drought stress and lays a solid theoretical foundation for the genetic improvement of *Amorpha fruticosa* L. plant varieties, as well as improvement of resource utilization.

Keywords Lipoxygenase · *Amorpha fruticosa* L. · Saline-alkali stress · Drought stress · Transgenic tobacco

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Introduction

The growth and development of plants under stress mainly depends on the mechanism of their continuous adaptation to environmental changes—especially their ability to activate the specific defense mechanism of drought tolerance, take the initiative to adjust their physiology, and obtain the structure required to cope with environmental changes under environmental stress. In the physiological response of plants, there is a group of metabolic substances that play a basic role, namely, lipid derivatives produced by further transformation of oxidized fatty acids (Vellosillo et al. 2007).

Lipoxygenase (lipoxygenase, LOX, EC1.13.11.12) is a kind of non-heme ferritin that plays a key role in plants' fatty acid oxidation pathways. LOX often uses linolenic acid and linoleic acid as substrates to catalyze the addition of molecular oxygen to polyunsaturated fatty acids containing a 1 (Z, Z)-1,4-pentadiene system, so as to catalyze the reaction to produce unsaturated fatty acids and hydrogen peroxide, which contain β -barrel domain and carboxyl-terminal α -helix domain monomer proteins. Lipoxygenase is the first reported lipoxygenase in soybeans with linoleic acid and linolenic acid as substrates (Andre and Hou 1932). According to its protein structure and amino acid sequence similarity, lipoxygenase can be divided into two subfamilies: type I and type II. Type I lipoxygenase lacks chloroplast transfer signal peptides, including 9-, 13-, and 9-mer 13 LOX, while the type II N-terminal contains a chloroplast transfer signal peptide (Vick and Zimmerman 1987). At present, the *LOX* gene has been isolated from many plants, such as *A. thaliana* (*AtLOX1* ~ *AtLOX6*) (Bannenberg et al. 2009). Studies have shown that *AtLOX1* is involved in hormone stress response, and its mRNA expression is upregulated by ABA and jasmonic acid (JA) (Melan et al. 1993), while *AtLOX2* is a specific enzyme involved in JA biosynthesis (Bell et al. 1995). Sequence alignment and phylogenetic analysis of *Arabidopsis* lipoxygenase and other plant lipoxygenases have shown that there are four main families of plant lipoxygenase. The results show that *AtLOX-1* and *AtLOX-5* are clustered with the 9 s-lipoxygenase of other plants, as well as with double 9/13 lipoxygenase (group C). It has also been found that 9 s-lipoxygenase in *Arabidopsis* is clustered with 9 s-lipoxygenase in some dicotyledons (almonds, tobacco), while 9 s-lipoxygenase in monocotyledons forms a separate subgroup, and all D-cluster species are legumes (Bannenberg et al. 2009).

Under biotic and abiotic stress, the physiological defense of plants can be induced by the lipoxygenase pathway, and the oxidation step leads to a cascade of reactions (called the LOX pathway), whereby hydroperoxide

(HPO) produced by LOX activity is the matrix of HPO lyase and olefin oxide synthase (Koberlein and Munden 2021). HPO is highly reactive and degrades rapidly into metabolites, which are precursors of jasmonic acid, methyl jasmonate, conjugated dienoic acid, and volatile aldehydes. Studies have shown that changes in LOX activity are closely related to fruit ripening and senescence (Droillard et al. 1993), and HPO secondary metabolites also play an important role in plant defense (Wasternack et al. 1998). For example, mechanical injury can promote the accumulation of JA, and in *LOX-2*-knockout *A. thaliana* mutants, the expression of JA is seriously inhibited, indicating that mechanical-injury-induced JA synthesis requires the presence of *LOX-2* (Bell et al. 1995). With the continuous occurrence of chilling injury, LOX was continuously activated, and the relative content of linolenic acid decreased gradually, which may have been the result of the oxygenation of linolenic acid catalyzed by LOX and its hydroperoxide (Gao and Rao 2007). Under the condition of low temperature, non-cold-sensitive figs had higher LOX activity and cell membrane fluidity and had no chilling injury symptoms, while the opposite was true for cold-sensitive cucumbers. At the same time, the *LOX* gene expression profiles of the two plants were consistent with their enzyme activity, so it is considered that the activation of gene expression is one of the characteristics of low-temperature-tolerant plants (Lee et al. 2005). Recent studies have shown that the addition of exogenous LOX can directly increase the content of JA. Water stress not only changes the activity of LOX in plants, but also changes the concentration of endogenous JA in tissues and cells, and the addition of LOX to a cell-free system can directly cause the accumulation of JA. This also indicates that LOX is likely to be a key enzyme in the biosynthesis and accumulation of JA induced by stress (Gong et al. 2003). JA, as a kind of plant hormone, can induce the expression of plant defense genes. Plants can effectively regulate LOX expression through JA information transmission. JA can promote respiration and stomatal closure, and it makes plants respond to environmental stresses. At present, little is known about *Amorpha fruticosa* L. LOX as a key enzyme of JA synthesis in response to water stress.

Amorpha fruticosa L. is a deciduous shrub native to North America and has been introduced to China as an ornamental plant (Wang et al. 2002). *Amorpha fruticosa* L. can tolerate dry soils and is most abundant on riverbanks, roads, and flooded forest edges—even in areas with occasional flooding (Kozuharova et al. 2017). The high tolerance to various habitat conditions and strong reproductive capacity of *Amorpha fruticosa* L. promote invasive behavior outside its native range (Kozuharova et al. 2017). Understanding the drought tolerance mechanisms of *Amorpha fruticosa* L. is important for the study of

plants' drought tolerance. Drought stress is one of the most prevalent environmental factors limiting plant growth.

In recent years, there have been several studies on the physiological characteristics and function of *Amorpha fruticosa* L. at home and abroad, and studies on the salt tolerance and drought tolerance ability of *Amorpha fruticosa* L. at the seedling stage show that there is a certain phenomenon of cross-adaptation (Sun et al. 2021). In one such study, RNA-sequencing and other techniques were used to conduct an in-depth study of the drought tolerance function of the lipoxygenase protein, connect the physiological index of *A. fruticosa* with molecular genetic regulation pathways, and further study the drought-tolerance-related genes (Sun et al. 2021). We investigated the growth characteristics of tobacco overexpressing AfLOX4 under saline stress and measured the physiological indicators of transgenic tobacco. We found that AfLOX4 could improve the tolerance of tobacco to saline stress, which was achieved through the ABA pathway. This study provides an experimental basis for the mechanism of AfLOX4's involvement in plant growth under adversity, lays the foundation for the genetic improvement of *Amorpha fruticosa* L., and provides genetic resources for the utilization of soda saline land in the Songnen Plain. To explore the effects of related metabolites of AfLOX4 on the drought tolerance of *A. fruticosa* through the catalytic pathway of lipoxygenase is of great significance for further revealing the effects of the induction mechanism of ABA signaling on the increase in lipoxygenase activity, providing an experimental basis for the drought tolerance regulation mechanism and molecular breeding of *A. fruticosa*, and it is of great significance for improving ecological environments and contributing stress-resistant germplasm resources for forest cultivation.

Materials and methods

Plant materials

Ben's tobacco (*Nicotiana tabacum*) and *A. fruticosa* L. seeds were donated by Bu Qingyun Research Group of the Northeast Institute of Geography and Agricultural Ecology, Chinese Academy of Sciences.

Strains and vectors

Escherichia coli. JM109 and *Agrobacterium tumefaciens* EHA105 were preserved in our laboratory, and the vectors pQB-V3 and pGWB18 were donated by Bu Qingyun Research Group of the Chinese Academy of Sciences.

Laboratory reagents

The cloned DNA polymerase KOD FX polymerase was purchased from TOBOY Biotechnology Co., Ltd.; DNA ligase T4 and Invitrogen LR enzyme were purchased from Zhongyi Biological Co., Ltd.; hygromycin (Hyg), rifampicin (Rif), and acetosyringone (As) were obtained from Sigma Company; and chloramphenicol (Chl) and carbenicillin (Carb) were obtained from Shanghai Bioengineering Co., Ltd. The DNA gel recovery kit was purchased from Kangwei Reagent Company, while the mixed powder of yeast extract, sucrose, tryptone, agar powder and Murashige and Skoog (MS) medium was purchased from Zhongyi Biological Company, and PEG6000, sodium bicarbonate, sodium hypochlorite, CTAB, Tris base and EDTA- Na_2 were all domestic pure reagents for chemical analysis.

Analysis of the expression characteristics of AfLOX4-specific genes under drought stress

In this study, 20% PEG6000 solution was used to irrigate the roots of 4-week-old *Amorpha fruticosa* L. seedlings grown (temperature 25 ± 2 °C; relative humidity $60 \pm 5\%$; light intensity $150 \mu\text{mol m}^{-2} \text{s}^{-1}$; light:dark cycle 16:8) in sand culture. The roots, stems, and leaves of the *Amorpha fruticosa* L. seedlings were treated with liquid nitrogen for 48 h. Total RNA was extracted and diluted 50 times after reverse transcription into cDNA, and six genes from the plants were selected for qRT-PCR validation. qRT-PCR analysis was performed on an Agilent Mx3000P QPCR system (Agilent, USA) using $2 \times$ Brilliant III SYBR Green qPCR Master Mix (Agilent, USA), and the specific primers of 6 genes of the AfLOX family and Afqublin primers of internal reference genes were designed using Primer Premier 5.0 (<http://www.PremierBiosoft.com>) and were synthesized by Invitrogen (Carlsbad, USA) (Supplementary Table 1). The PCR reaction procedure was 95 °C for 30 s (denaturation), 58 °C for 30 s (annealing), 72 °C for 30 s (extension), and 40 cycles to detect the relative expression of genes after PEG6000-simulated drought stress. From each of the three biologically independent cDNA samples, two independent technical replicates were performed and averaged for further calculations. Relative transcript abundance calculations were performed using CFX Maestro software (Bio-Rad, Hercules, CA, USA). Quantification of gene expression was performed by the comparative $2^{-\Delta\Delta CT}$ method (Guan et al. 2016).

As above, in this study, 4-week-old *Amorpha fruticosa* L. seedlings were treated with 150 mM NaCl and 60 mM NaHCO_3 . At the same time, the aboveground stems and leaves were harvested, and the underground roots were frozen in liquid nitrogen. The relative expression of the AfLOX4 gene was detected by qRT-PCR.

Statistical analysis

Statistical analysis was completed using SPSS 17.0 and Microsoft Excel 2010 software. All experimental data were expressed as means $\pm$ standard deviations (SD), and differences between groups and controls were analyzed using one-way ANOVA with Tukey's test. $p < 0.05$ was set as the threshold for statistically significant differences. Graphs were produced using Microsoft Excel 2010 and Origin 8.0 software.

Cloning of *AfLOX4* gene by RT-PCR

In this study, the reverse-transcribed cDNA of RNA from the whole *A. fruticosa* plants treated with PEG6000 was used as a template, the primers *AfLOX4*-1F and *AfLOX4*-2R (Supplementary Table 1) were used for guided amplification, and the ORF region of the *AfLOX4* gene was amplified by PCR under the action of KOD FX polymerase. The reaction system and procedure were carried out according to the manufacturer's instructions. The PCR amplification products were subjected to 0.1% agarose gel electrophoresis, and the electrophoresis results were detected with the gel imager. The target gene was recovered with a gel recovery kit connected to the pBQ-V3 vector under the action of T4 DNA ligase and was sent to Kumei Biotechnology Company for sequencing.

Bioinformatics analysis of the *AfLOX4* gene

Conserved domains of proteins were obtained using the website InterPro analysis. Physicochemical properties of proteins were analyzed using the website <http://web.expasy.org/protparam/>, and the transmembrane regions of protein sequences (<https://embnet.vital-it.ch>) and protein signal peptide analysis and phosphorylation sites were predicted using the website <http://www.cbs.dtu.dk>. Amino acid sequence homology was analyzed using BioEdit software, and a phylogenetic tree was constructed using MEGA 5.0 software (An et al. 2020).

Detection of the subcellular location of *AfLOX4*'s leukocyte-implanting protein

The amino acid sequence of the *AfLOX4* gene was input into the PSORT website to predict the subcellular localization of its protein. In order to verify the location where the *AfLOX4* protein functions in cells, a pGWB18-GFP-*AfLOX4* fusion expression vector was constructed. The protoplast cells of *A. thaliana* rosette leaves were extracted by three treatments. Arabidopsis protoplasts were genetically transformed by the PEG6000 transformation method. After being cultured at room temperature for 14–16 h (Wu et al. 2009), protoplasts

were prepared. The location of green fluorescence expression in the 35S-GFP-*AfLOX4* fusion protein was observed under a laser confocal microscope, and the subcellular localization of the *AfLOX4* protein was determined.

Genetic transformation of transgenic tobacco with overexpression of the *AfLOX4* gene

Construction of plant expression vectors

In this study, pGWB18 and pQB-V3-*AfLOX4* plasmids were mixed at a molar ratio of 1:3, and then LR ligase and Gateway reaction buffer were added and reacted at a constant temperature of 25 °C for 2 h, stimulated with heat, and transferred into *E. coli* JM109 coated on LB screening medium with 50 mg/L antibiotics, after which the monoclonal antibody was isolated and cultured, and the plasmids were extracted after expansion to *AfLOX3F, 4R* primers for PCR amplification (Supplementary Table 1). The pGWB18-*AfLOX4* vector was successfully connected via electrophoresis.

Acquisition of overexpressed tobacco

A. tumefaciens EHA105 was transformed by electric shock, and *A. tumefaciens* containing plasmid DNA of the vector PGWB18-*AfLOX4* was expanded and cultured to $OD_{600} = 0.05$. Then, 0.5 cm² leaves of wild-type tobacco (*N. tabacum*) were infected for 10–20 min. The bacterial solution was sucked dry on the filter paper, co-cultured in the dark on 0.5 mg/L Murashige and Skoog (MS) medium supplemented with 0.1 mg/L 6-benzyl adenine (BA) and 20 mg/L 1-naphthaleneacetic acid (NAA) for 48 h, before being sterilized with carboxybenzyl penicillin solution, and then differentiated and cultured under the screening of 50 mg/L hygromycin to obtain resistant buds, which were rooted and cultured into seedlings. The seeds of T1 transformants were harvested, and the genomic integration of the 35 s-*AfLOX4* gene was detected by PCR. Then, the seeds were further germinated in 50 mg/L of hygromycin medium, and the T2 generation was screened until the T3 generation of transgenic tobacco seeds were harvested, and the expression of the *AfLOX4* gene in the transformed tobacco was detected by qPCR with *AfLOX4*-1F, 2R primers (Supplementary Table 1).

Analysis of germination characteristics of tobacco with overexpression of *AfLOX4* under ABA stress

In this study, the germination characteristics of tobacco lines overexpressing the *AfLOX4* gene were detected on the medium supplemented with ABA. The seeds of wild-type (WT) and overexpressed lines were disinfected and sterilized, then seeded in groups of five on 1/2 MS solid medium

supplemented with 10, 15, and 20 μM ABA, in triplicate. After 14 days of treatment, the differences between the WT and overexpressed lines were compared and analyzed, and the germination, growth phenotype, fresh weight, and root length growth were investigated (Tian et al. 2015).

Analysis of tolerance to alkaline stress in tobacco overexpressing the *AfLOX4* tolerance gene due to alkaline salt stress

In this study, WT tobacco lines and those overexpressing the *AfLOX4* gene were germinated on 1/2 MS medium. After 7 days, five of the two-leaf seedlings were inoculated on a medium supplemented with the alkaline salt NaHCO_3 . The treatment concentration was 0, 2, 4, and 6 mM, repeated three times. After 7 days of stress culture, the differences between the WT and overexpressed lines were compared and analyzed, and their growth phenotype, fresh weight, and chlorophyll content were detected (Tian et al. 2015).

Analysis of the drought stress tolerance of tobacco at the flowering stage

Overexpressed and wild-type *AfLOX4* tobacco seeds were sown in flower pots mixed with vermiculite and grass carbon soil, then transferred to 7×7 cm flower pots at the four-leaf stage and cultured in a plant culture room for 3 weeks until individual plants began to lose water and natural drought when they developed buds. The phenotypes were investigated at 7 days, 10 days, and 14 days of drought, and the fresh weight of the individual plants was detected after 14 days of natural drought.

Results

Expression characteristics of the *AfLOX4* gene under drought and saline–alkaline stress

In this study, under drought (PEG6000), salt (NaCl), and alkaline (NaHCO_3) stresses, qRT-PCR analysis of the *AfLOX4* gene showed that the transcriptional expression of the *AfLOX4* gene increased with the extension of treatment time (Fig. 1). Under 20% PEG6000 root irrigation treatment, the expression of the *AfLOX4* gene in leaves increased significantly compared to that at 0 h; the expression of the *AfLOX4* gene increased at first and then decreased under salt (NaCl) induction at 6 h and 12 h after treatment, respectively; and the expression of the *AfLOX4* gene increased significantly under alkaline salt (NaHCO_3) stress. The results showed that the *AfLOX4* gene was induced and its expression increased. It is speculated that the *AfLOX4* gene is related to PEG6000, NaCl, and NaHCO_3 stresses. Interestingly,

although the overall expression of the *AfLOX4* gene showed an upward trend, it increased steadily under PEG6000 and salt stresses, but it increased at first and then decreased under alkaline stress, so we are more interested in the *AfLOX4* gene's response to drought and salinity. Therefore, the *AfLOX4* gene may play an important role in the drought tolerance and saline–alkaline tolerance of plants.

Cloning of the *AfLOX4* gene

In this study, the DNA bands were cloned by RT-PCR to about 2600 bp (Fig. 2), and the genes were recovered and purified, ligated at the flat end, inserted into the pQB-V3 vector, and transformed into *E. coli*, completing the construction of the pQB-V3-*AfLOX4* vector. The plasmid DNA was sequenced by Kumei Biotechnology Co., Ltd., and the sequence was analyzed using BioEdit software and compared with the sequence obtained for the *Amorpha fruticosa* L. transcriptome. It was proven that the *LOX4* gene of *A. fruticosa* was successfully cloned, which provided a genetic material basis for the study of the *AfLOX4* gene.

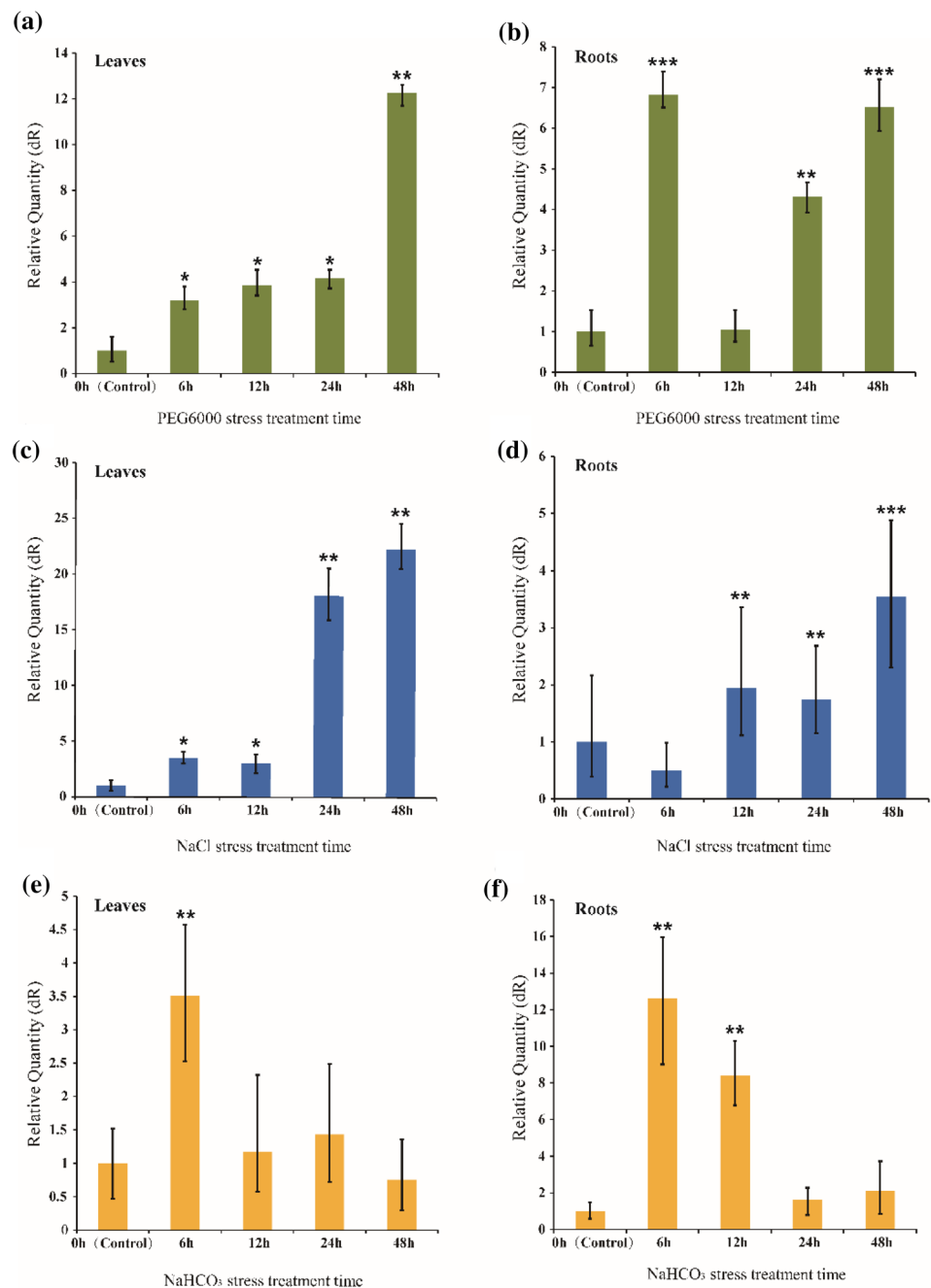
Biological and physicochemical characteristics of the *AfLOX4* gene

We analyzed the nucleotide sequence of the *AfLOX4* gene. The open reading frame (ORF) encodes a protein of 867 amino acids (Supplementary Fig. 1). Furthermore, the physical and chemical properties of the *AfLOX4* protein were analyzed online using the ProtParam tool, yielding the following results—molecular formula: $\text{C}_{400}\text{H}_{6844}\text{N}_{1178}\text{O}_{1286}\text{S}_{10}$, total number of atoms: 13718, molecular weight: 97142.46 Da, isoelectric point: PI 6.36, aliphatic index: 90.44, average hydrophilicity (GRAVY): -0.308, total number of negatively charged residues (Asp + Glu): 98, total number of positively charged residues (Arg + Lys): 92. The calculated value of the instability index (II) was 42.59, so it was classified as an unstable protein. The probability of the existence of the *AfLOX4* protein's signaling peptide was 0.0025.

Bioinformatics analysis of *AfLOX4*

Online prediction of the *AfLOX4* protein's secondary structure indicates that the protein is mostly composed of random curls (50%), followed by 45% α -helices and β -rotations, and at least 5% extended chains. In total, 44 aligned proteins have 6 PDB-matching amino acids, as shown in Supplementary Fig. 2. The tertiary structure of the protein (Fig. 3a) showed that the qmean was -3.13, C β was -1.19, all atoms was -0.81, solvation was -0.53, and the torsion was -2.68. The comparison of the number of amino acids and the size of residues showed that the consistency with lipoxigenase 4 was 76.53%. The conformational detection of the protein's

Fig. 1 Expression patterns of *AfLOX4* in *Amorpha fruticosa* L. under different abiotic stresses: **a** leaves under 20% PEG6000 solution treatment; **b** roots under 20% PEG6000 solution treatment; **c** leaves under 150 mM NaCl treatment; **d** roots under 150 mM NaCl treatment; **e** leaves under 60 mM NaHCO₃ treatment; **f** roots under 60 mM NaHCO₃ treatment. Total RNA was extracted and qRT-PCR was performed to detect the expression level of the *AfLOX4* gene, using *Afqbublin* as an internal reference gene. The values at 0 h (Control) were the same and were set as 1. Values are means $\pm$ SD; $n=3$. Statistical analyses were performed using Student's *t* test: * $p < 0.05$, ** $p < 0.01$



structural region reflected by the amino acid residues' dihedral angles ψ and ϕ of the protein's Lagrangian diagram proved to be reasonable (Fig. 3b). Figure 3c shows the tertiary structural model of the *AfLOX4* protein. The *AfLOX4* protein has two conserved domains (Fig. 4a): one is a highly conserved domain of lipoxygenase 2 (lipoxygenase homology 2 (beta-barrel) domain (LH2)) with 175 amino acids, and the other is a highly conserved domain of lipoxygenase (lipoxygenase (LOX)) with 186-850 amino acids. The iron atoms in lipoxygenase are bound by four ligands, three of which are histidine residues. Six histidines are conserved in

all lipoxygenase sequences, and five of them are clustered in 40 amino acids; this region contains two of the three iron ligands. The other histidine has been shown to be important for lipoxygenase activity. When the transmembrane structure of the *AfLOX4* protein was analyzed using online soft detection, no transmembrane domain was found (Fig. 4a). Through homologous nucleotide alignment, the first 20 genes with high homology were found, and the maximum proximity method was used to construct the phylogenetic tree (Fig. 4b). *AfLOX4* (lipoxygenase 4 C193915), *Lupinus angustifolius* Linn., and linoleate 13 s-lipoxygenase are in

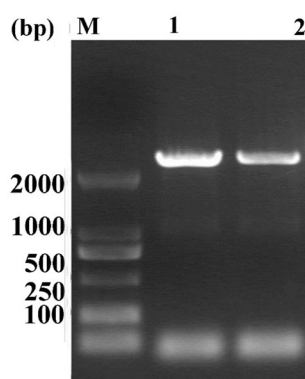


Fig. 2 Electrophoresis diagram of the cloned *LOX4* gene of *Amorpha fruticosa* L. M for DNA Maker 2000; 1 and 2 are the genes cloned with cDNA as template

the same branch, and are highly homologous, followed by *Tribulus* (*Tribulus terrestris*), alfalfa (*Medicago truncatula*), and chickpea (*Cicer arietinum*). Lipxygenase similarity is very high.

Functional position of the AfLOX4 protein

Using the subcellular localization network of the AfLOX4 protein, the probability of the AfLOX4 protein being in the peroxisome is 0.505, that in the cytoplasm is 0.450, and that in the mitochondrial matrix and chloroplast thylakoid membrane is 0.1. In terms of probability, the protein is most likely to be located in the peroxisome and cytoplasm. Only through confirmatory experiments can it be accurately located. In this study, the pGWB18-GFP-AfLOX4 expression vector was constructed using the Gateway system, and the correctly sequenced plasmid DNA was transformed into *Amorpha fruticosa* L. protoplasts. The protoplast cells cultured for

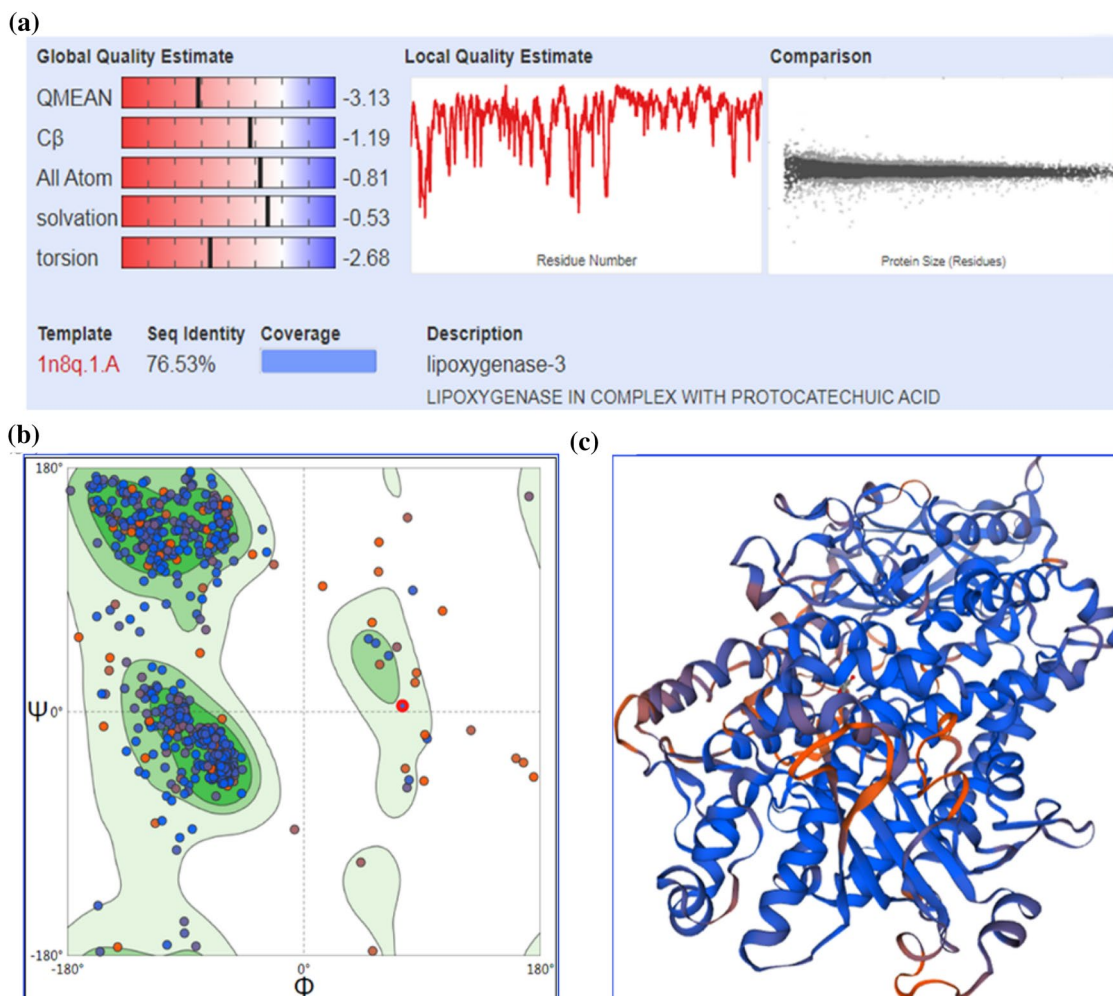


Fig. 3 The AfLOX4 protein's tertiary structure analysis diagram: **a** mass assessment of the total protein; **b** Rasch diagram of the protein; **c** 3D structural model

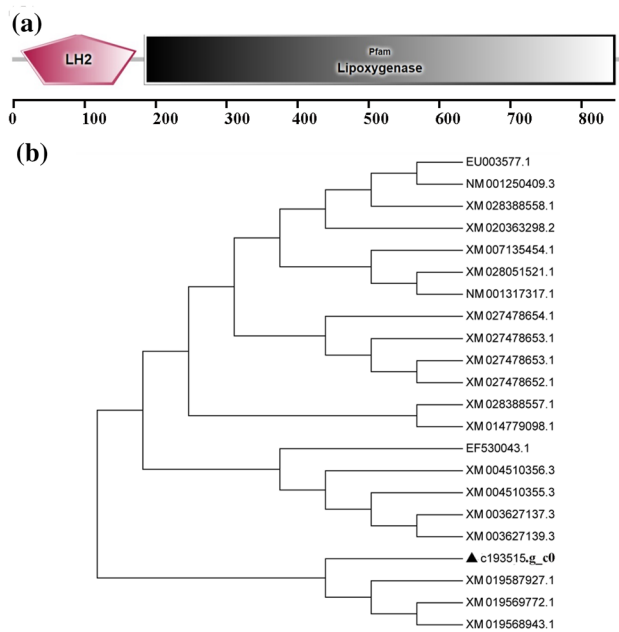


Fig. 4 Conserved structural domains and phylogenetic tree of the AfLOX4 protein: **a** Lipoxigenase homology 2 (beta-barrel) domain (12-175) and lipoxigenase (186-850) were found in the amino acid sequence. **b** Phylogenetic tree based on the AfLOX4 gene sequence, and GenBank database homologous sequences with *Lupinus angustifolius* Linn. and linoleate 13 s-lipoxygenase in the same branch

14 h and 16 h were smeared on grooved slides and covered with cover slides to detect the cell integrity under confocal microscopy, along with green fluorescence channels to detect the green fluorescence of GFP. The coincidence of the 35S-GFP-AfLOX4 fusion protein was detected by merging channels (Fig. 5). The experimental results showed that the AfLOX4 fusion protein glowed in the cytoplasm, and the

subcellular function of the AfLOX4 protein was verified in the cytoplasm.

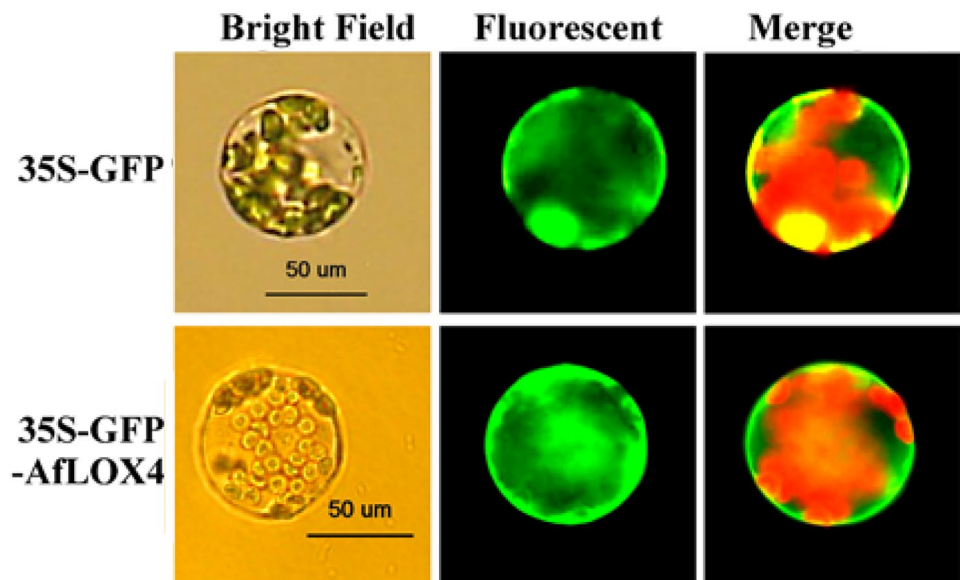
Overexpression of the AfLOX4 gene due to drought and salt tolerance in tobacco

Identification of tobacco overexpressing the AfLOX4 gene

The resistant buds differentiated from the infected and transformed tobacco leaves were selected by hygromycin to take root and grow into resistant plants. Genomic DNA was extracted from the leaves of 1-month-old resistant potted plants. PCR detection showed that T0#2~7 lines of resistant transformed seedlings had the same gene band as the positive control (CK). In T0-#1 relative to CK, there was no target gene band, indicating that the AfLOX4 gene was integrated in the genome of the T0-#2~7 lines. Potted plants were cultured, and T1 generation transgenic seeds were harvested (Fig. 6a).

The T3 generation of transgenic seeds screened by hygromycin were sown on 35 mg/L hygromycin plates and cultured for 21 days. The growth status of T3#1,2,3 roots of the transformed line was significantly better than that of wild-type (WT) plants, which did not take root, but the rooting development of the transgenic line was normal. The total RNA of each strain and WT was extracted to 1 µl RNA that was reverse-transcribed into total cDNA. qRT-PCR results showed that the expression of the three strains was significantly higher than that of WT; that of T3-#1, -#2, and -#3 was 85.8524, 74.5958, and 76.7283 times higher than that of the WT strain, respectively (Fig. 6b). The results showed that the T3-#1, -#2, and -#3 lines overexpressed the AfLOX4 gene, driven by 35S promoter, which provided transgenic plant materials for follow-up research.

Fig. 5 Subcellular localization of the 35S-GFP-AfLOX4 fusion protein, as observed by laser scanning confocal microscopy in *Arabidopsis* protoplasts



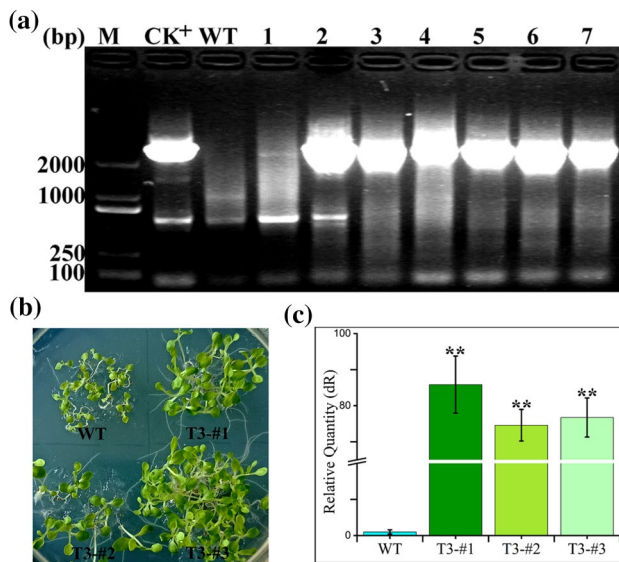


Fig. 6 Identification of transgenic *AfLOX4* tobacco and expression levels of the *AfLOX4* gene in transgenic T3 generation strains: **a** PCR identification of transgenic tobacco, with CK⁺ as the positive control and WT as the negative control; 1 ~ 7 are the transgenic *AfLOX4* gene strains tested. **b** Growth status of WT, T3#1, T3#2, and T3#3 on 1/2 MS medium containing 35 mg/L hygromycin. **c** Relative expression levels of the *AfLOX4* gene in WT, T3#1, T3#2, and T3#3. Statistical analyses were performed using Student's *t* test; ***p* < 0.01

Germination and growth characteristics of *AfLOX4*-overexpressing tobacco under ABA stress

In this study, the germination tolerance of *AfLOX4*-overexpressing tobacco lines to ABA stress showed that with the increase in the ABA treatment concentration, the germination and growth of overexpressing and WT lines were inhibited, and the overexpressing lines had greater tolerance compared with the WT controls. It could be seen from their appearance that the plant size and root elongation were different. The whole plant weight of overexpression lines #1, #2, and #3 under ABA treatment was more than five times that of the wild-type lines. The fresh weight of seedlings grown under stress for 14 days showed that the growth of the overexpressing lines was faster than that of the WT lines, indicating that the inhibition of ABA was weak. Therefore, overexpression of the *AfLOX4* gene improves tolerance to ABA (Fig. 7).

Growth characteristics of tobacco seedlings overexpressing *AfLOX4* under NaHCO₃ stress

The T3 strains (#1,2,3) with overexpression of *AfLOX4*, along with the two-leaf seedlings of WT lines, were inoculated on the 1/2 MS stress medium supplemented with 0, 2, 4, or 6 mM NaHCO₃. The phenotype and fresh weight after 14 days of growth were detected. The results showed that NaHCO₃ stress inhibited the growth of the tobacco, but

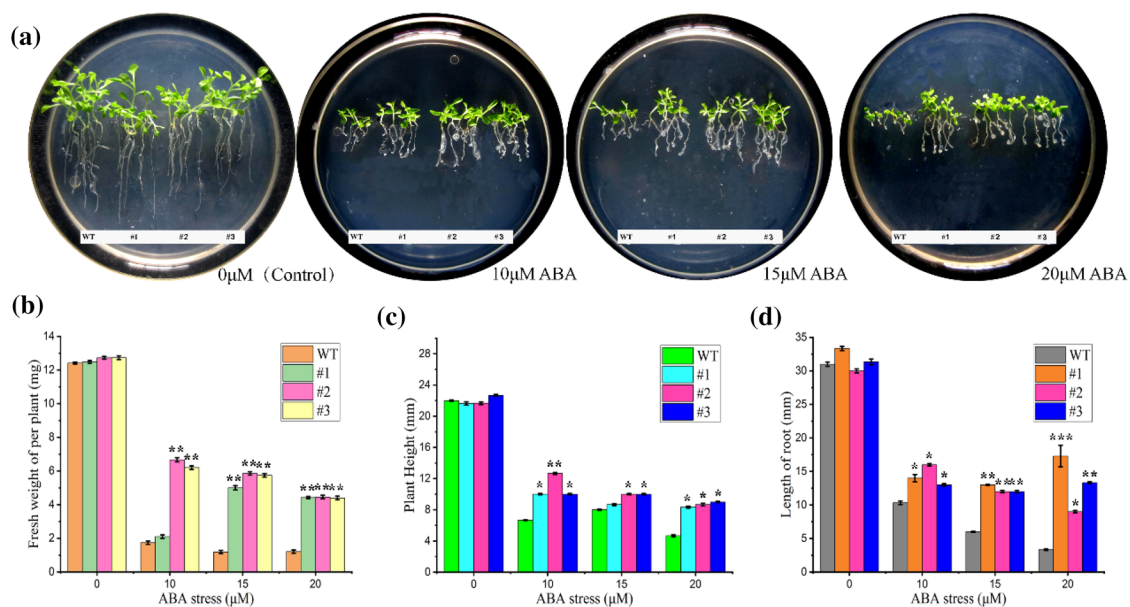


Fig. 7 Analysis of fresh weight, plant height, and root length of transgenic *AfLOX4* tobacco strains under ABA stress: **a** Phenotypic comparison of wild-type (WT) and T3 generation transgenic lines under different concentrations of ABA (0 μM, 10 μM, 15 μM, or 20 μM) stress. **b** Fresh weight of WT, #1, #2, and #3 strains under different concentrations of ABA stress. Values are means ± SD; *n* = 3 plants.

c Plant height of WT, #1, #2, and #3 strains under different concentrations of ABA stress. Values are means ± SD; *n* = 3 plants. **d** Root length of WT, #1, #2, and #3 plants under different concentrations of ABA stress. Values are means ± SD; *n* = 3 plants. The assays were repeated three times. Statistical analyses were performed using Student's *t* test: **p* < 0.05, ***p* < 0.01

the *AfLOX4*-overexpressing tobacco strain showed greater tolerance than the WT strain, and the damage caused by NaHCO_3 stress to the tobacco increased with the increase in concentration. The overexpressed #2 and #3 tobacco lines were more tolerant, and had certain growth advantages compared to the WT, and their fresh weight was 4–6 times that of the WT. When treated with 6 mM, the plants were inhibited and no longer grew; the injured leaves lost their green color, and the chlorophyll content of the overexpressed #1 and #2 tobacco lines was significantly higher than that of the WT (Fig. 8).

Tolerance of tobacco plants overexpressing *AfLOX4* to drought stress

AfLOX4-overexpressing and wild type (WT) tobacco were transplanted into 7×7 cm flowerpots mixed with vermiculite and grass carbon soil at the four-leaf stage and cultured in a plant culture room. After 3 weeks, individual plants were treated with natural drought when they developed buds. Phenotypes were observed after 7 days, 10 days, and 14 days of drought, and the leaves wilted and curled after 7 days of drought treatment. The degree of leaf shrinkage of the overexpressed lines was slightly slighter than that of the WT. The wilting condition of the WT leaves after 10 days of natural drought was more serious than that of the overexpressed

lines, and their water loss was higher. After continuing the natural drought stress to the 14th day, the plant damage was the same; the leaves wilted, the bottom leaves became yellowed, and the degree of dehydration and drying was similar. After watering and rewatering on the 14th day, there was a significant difference between the overexpressed and WT lines after one week of rewatering. The growth recovery ability of the overexpressed lines was better than that of the WT, and the fresh weight of the overexpressed lines was significantly higher than that of the WT. The results showed that the tolerance of *AfLOX4*-overexpressing lines to natural drought stress was stronger than that of the WT (Fig. 9) and revealed the role of the *AfLOX4* gene in improving tobacco's defense against drought stress.

Discussion

There are many branching pathways downstream of the lipoxygenase pathway, which play key roles in many processes, such as plant growth and development, stress response, etc. At present, the pathways of propylene oxide synthase and hydroperoxide lyase are clear (León-morcillo et al. 2012). Lipoxygenase in the above pathway is the key enzyme involved in the regulation of oxidation balance in response to upstream enzymes. In recent years, the research on the regulation of plants' reactive

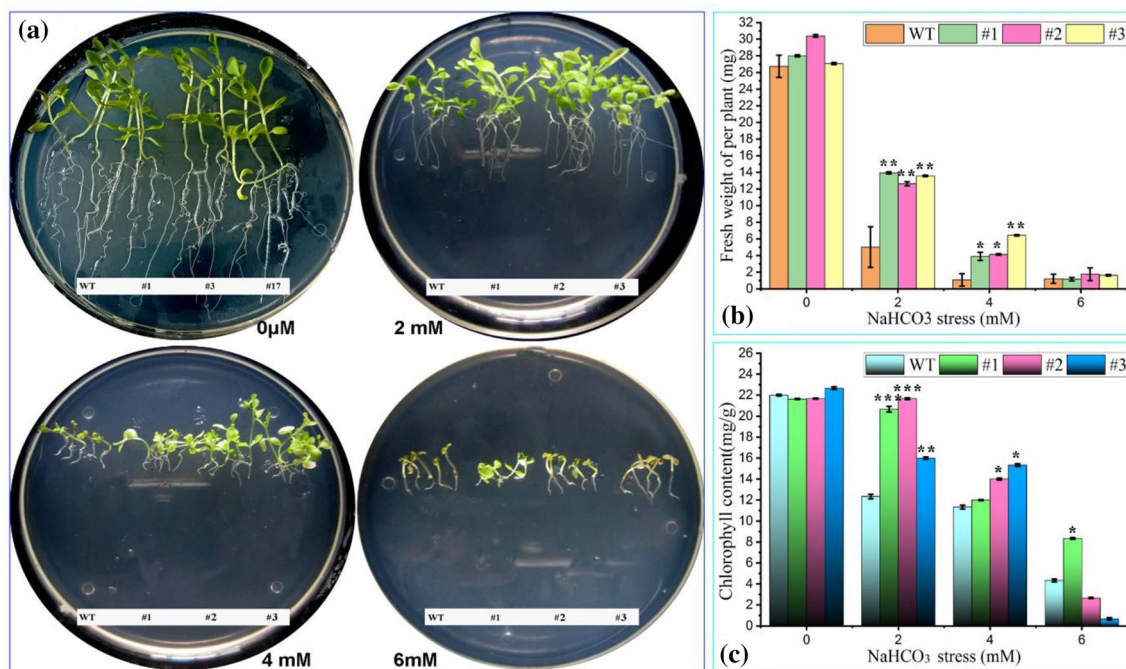
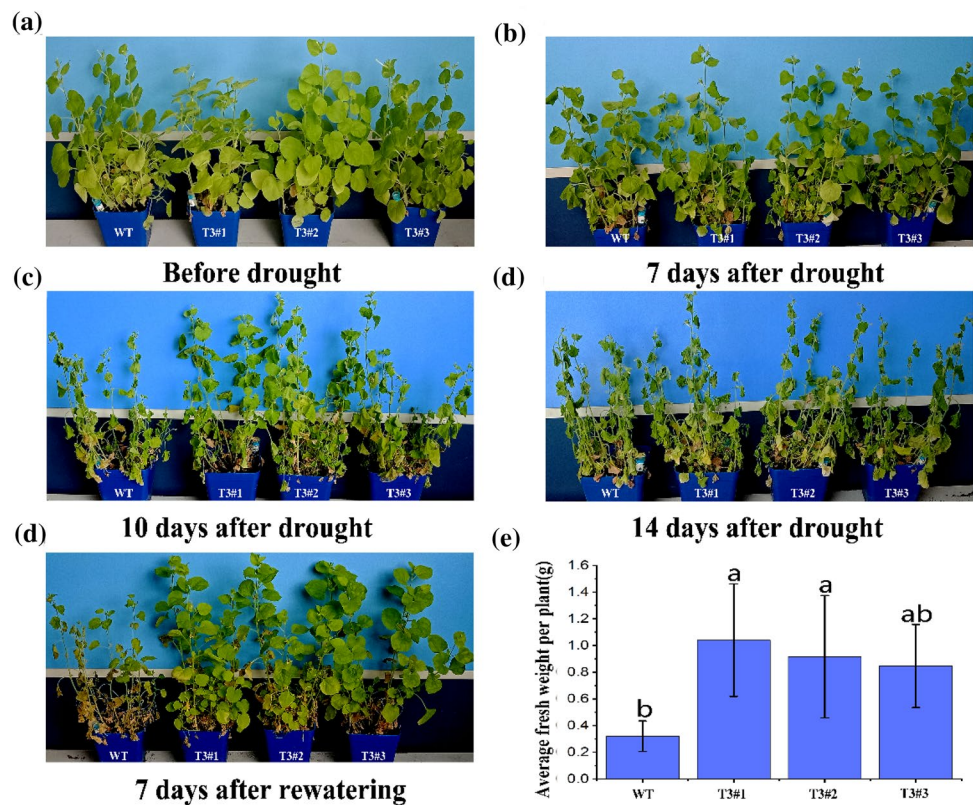


Fig. 8 Analysis of fresh weight and chlorophyll content of transgenic *AfLOX4* tobacco strains under NaHCO_3 stress: **a** Phenotypic comparison of wild-type (WT) and T3 generation transgenic strains under different concentrations of NaHCO_3 (0 mM, 2 mM, 4 mM, or 6 mM) stress. **b** Fresh weights of WT, #1, #2, and #3 lines under dif-

ferent concentrations of NaHCO_3 stress. Values are means $\pm$ SD; $n=3$ plants. **c** Chlorophyll contents of WT, #1, #2, and #3 lines under different concentrations of NaHCO_3 stress. Values are means $\pm$ SD; $n=3$ plants. The assays were repeated three times. Statistical analyses were performed using Student's *t* test: * $p < 0.05$, ** $p < 0.01$

Fig. 9 Growth characteristics of tobacco overexpressing the *AfLOX4* gene under natural drought conditions: **a** before natural drought treatment (WT, T3#1, T3#2, T3#3); **b** natural drought treatment for 7 days (WT, T3#1, T3#2, T3#3); **c** natural drought treatment for 10 days (WT, T3#1, T3#2, T3#3); **d** natural drought treatment for 14 days (WT, T3#1, T3#2, T3#3); **e** natural drought treatment followed by rehydration for 7 days (WT, T3#1, T3#2, T3#3); **f** fresh weight per plant after rehydration (WT, T3#1, T3#2, T3#3); $p < 0.05$ by one-way analysis of variance



oxygen species and stress has gradually become a hotspot. It has been found that the lipoxygenase gene has a significant conserved domain in plants (Suzuki et al. 2003), which has the functions of stress defense, regulating metabolism, mediating lipids, and interaction with membrane-binding proteins (Sharanya et al. 2020). In this study, the lipoxygenase 4 gene of *A. fruticosa* (*AfLOX4*) with a 2604 bp nucleotide was isolated and cloned by RT-PCR. The protein with an aliphatic index of 90.44 and the ability to catalyze peroxidation at the C13 site was isolated and cloned. The gene has two conserved domains (Fig. 4a): one is the lipoxygenase 2 conserved domain (LH2), and the other is the LOX conserved domain (LOX) at 186–850 bp. The phylogenetic tree (Fig. 4b) shows that *AfLOX4* is in the same branch as the linoleic acid ester 13 s lipoxygenase of lupin. Lipoxygenase is a polygenic family. Transcriptome sequencing under drought stress shows that there are six members of the *A. fruticosa* lipoxygenase family (Sun et al. 2021). Studies have shown that the expression of the *AtLOX6* gene in *Arabidopsis* 13-lox mutants and 12-opda function deletion mutants is sensitive to infiltration and drought (Grebner et al. 2013). The *AtLOX2* gene increases mRNA expression in leaves induced by MeJA. ABA is an important signaling molecule that is an integrator in response to environmental changes; it plays a role in regulating seed germination and root growth, as well as adaptive responses to various abiotic stresses (Whitley et al. 1999). ABA, as a regulator of plant stress response, slightly reduced *AtLOX2* expression (Bell

and Mullet 1993). Lipoxygenases catalyze the production of oxylipins, which play a key role throughout the whole growth period of plants, such as during seed germination and plant growth—especially against abiotic stresses (RoyChowdhury et al. 2016; Upadhyay and Mattoo 2018; Mosblech et al. 2009). Similar results were also obtained in this study. Exogenous ABA induces *AfLOX4* gene expression and is also involved in ABA signaling during seed germination, seedling growth, and signal transduction. Overexpression of *AfLOX4* in tobacco strains improved the plants' tolerance to ABA stress, and the germination and growth development of both *AfLOX4*-overexpressing and WT strains were inhibited with increasing concentrations of ABA treatment, but the overexpressing strains were more tolerant than the WT controls. Several studies have shown that the synergistic effects of multiple gene interactions in plants ultimately lead to a rapid increase in the accumulation of endogenous ABA in plants (Xiong et al. 2002). Thus, ABA in transgenic plants may initiate more biosynthesis by regulating many stress-responsive genes induced by endogenous ABA, resulting in a further increase in the plants' stress tolerance. This could also explain the longer root length and greater stress tolerance of the transgenic tobacco under salt stress in this experiment. ABA is involved in physiological processes such as stomatal closure, osmolyte accumulation, and the synthesis of stress-related proteins (Hoekstra et al. 2001). Therefore, *AfLOX4* may be involved in stomatal regulation, which reduces water loss and, thus, confers drought

tolerance to the plant body. The expression of the *AfLOX4* gene was significantly increased after being induced by drought (PEG6000), salt (NaCl), and alkaline (NaHCO_3) stress. In particular, the expression in the stems and leaves of *A. fruticosa* under PEG6000 and alkaline salt (NaHCO_3) stress was up to 12.56 and 15.89 times higher than that without treatment, respectively (Fig. 1). It is speculated that the *AfLOX4* gene is related to *A. fruticosa*'s tolerance to drought, salt, and alkaline stresses. The LOX protein mainly exists in chloroplasts (Caifeng et al. 2010; Vick 1987), liposomes (Bell et al. 1995), vacuoles (Tranberger et al. 1991), plastids (Todd et al. 1990), etc. In this study, transgenic tobacco had higher chlorophyll content under salt (NaHCO_3) stress (Fig. 8), so *AfLOX4* may also play a role in plant photosynthesis, thus increasing plants' vigor under stress. Under natural drought stress, the transgenic tobacco showed better growth compared with the wild type, and the phenotype was more pronounced after rehydration (Fig. 9), so it is speculated that *AfLOX4* is associated with water uptake and utilization efficiency under drought stress, which needs to be further investigated. The Mdlox1a protein is located on the cell membrane and nucleus. In this study, the green fluorescence of the 35 s-GFP-AfLOX4 fusion protein showed that the AfLOX4 protein is subcellularly located in the cytoplasm, which also makes it convenient for it to participate in the regulation of a variety of life activities. *Amorpha fruticosa* L. plants overexpressing the woody tomato plant *DkLOX3* gene showed high tolerance to osmotic stress (i.e., high germination rate, high root growth rate) and enhanced tolerance to high salt and drought (Hou et al. 2015). In the present study, heterologous overexpression of the AfLOX4 gene in tobacco improved plants' responses to drought and alkaline salt stresses.

AfLOX4 may play an important role in the regulation of tolerance to alkaline stress and salt stress. Furthermore, AfLOX4-overexpressing tobacco had more chlorophyll under stress compared with the controls, and it is speculated that AfLOX4 may be involved in the regulation of the photosynthetic electron transport pathway, which helps to improve plants' tolerance to alkaline salts. The present study provides a theoretical and practical basis for the potential pathway of AfLOX4's regulation of plants' tolerance to alkaline salt stress. However, further studies are needed to elucidate the pathways involved in this AfLOX4-mediated mechanism.

Conclusion

Saline stress is a complex form of salt stress dominated by carbonates and characterized by high sodium ion concentrations and high pH. The AfLOX4 gene was seen to be induced and its expression increased under drought and saline stresses in

Amorpha fruticosa L. (Fig. 1), and it was found to be associated with drought (PEG6000), salt (NaCl), and alkaline (NaHCO_3) stresses. Moreover, in this study, we found that the growth of both AfLOX4-overexpressing and wild-type strains of tobacco after NaHCO_3 stress treatment was inhibited. In addition, the degree of damage increased with increasing stress intensity (Fig. 8). Under 2, 4, or 6 mM NaHCO_3 stress treatments, the AfLOX4-overexpressing lines had greater fresh weight and chlorophyll index than the wild-type strain. In addition, transgenic tobacco plants overexpressing AfLOX4 had improved physiological functions and enhanced tolerance to ABA stress. Under natural drought stress, the transgenic tobacco overexpressing AfLOX4 also grew better than the wild-type strain after water supply was restored (Fig. 9). Overall, the AfLOX4 gene is involved in enhancing the protective function of plants against drought and alkaline salt stresses. Therefore, this lays the foundation for further studies on how the LOX4 protein plays a regulatory role in oxidative stress caused by lipoxygenase pathway stress. The results of this study lay the foundation for growing plants in saline and drought-prone soils.

Author contribution statement YZ, and KW performed most of the experiments. ZW, XL, ML, FZ, and ZM assisted with the experiments. XL, and QG conceived and supervised the project. YZ and QG analyzed the data and wrote the article with contributions from XL, QG, and KW. All authors read and approved of its content.

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Data availability All relevant data are within the paper.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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