BMC Plant Biology

RESEARCH Open Access

Genome-wide exploration and characterization of the RALFs and analysis of its role in peanut (*Arachis hypogaea L.*)

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Abstract

Background Rapid alkalinization factors (RALFs) are small peptides hormones that regulate plant growth and stress responses. Although RALFs have been identified in a broad range of land plant species, their roles in peanuts (*Arachis hypogaea* L.) remain largely unexplored.

Result A total of 24 *AhRALF* genes we identified in the peanut genome and classified them into three clades through phylogenetic analysis. Whole genome duplication (WGD) or segmental duplication primarily drives the expansion of *AhRALFs*. Gene transcription analysis revealed that two genes from clade II (*AhRALF1* and *AhRALF12*) and three from clade III (*AhRALF8*, *AhRALF10*, and *AhRALF21*) are highly expressed across 18 different tissues. Notably, *AhRALF11* and *AhRALF24*, paralogous genes from clade II, are specifically expressed in immature buds and flowers. Additionally, *AhRALF1*, *AhRALF12*, *AhRALF8*, and *AhRALF21* exhibited elevated expression under aluminum (Al) stress. Functional analysis of *AhRALF1* confirmed its secretory function and inhibitory effect on root growth in *Arabidopsis*. Moreover, *AhRALF1*-silenced plants displayed reduced tolerance to Al stress, with altered antioxidant enzyme activities and increased oxidative damage.

Conclusion This study provides a comprehensive analysis of the *AhRALF* gene family in peanut, highlighting their roles in growth regulation and stress responses. The function of *AhRALF1* in enhancing peanut tolerance to Al stress was preliminary revealed. Our findings provide valuable insights into the roles of *AhRALFs* in peanuts and lay the groundwork for future functional studies and breeding programs.

Keywords Peanut, RALF, Expression level, Growth and development, Aluminum stress

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Background

Rapid alkalinization factors (RALFs) are small secreted peptides, typically 80–120 amino acids in length. First identified over twenty years ago due to their rapid alkalinization effect in tobacco cell suspension culture [1]. RALFs have since been found in a wide range of plant species [2–5]. According to Abarca et al. [6], the model plant *Arabidopsis* contains 37 RALF genes. Subsequently, RALFs have been identified in numerous economically significant crop plants, with their numbers varying from a few to hundreds [2, 7]. The widespread presence and diversity of RALFs underscore their importance in plant growth and development.

The function of proteins is inherently linked to their primary structure. RALF propeptides contain a signal peptide and three conserved motifs or residues: a site-1 protease (S1P) cleavage site, a conserved dibasic RRXL site necessary for the maturation and release of active RALF peptides [8, 9], and a YISY motif crucial for alkalinization activity and receptor binding [10]. Additionally, four conserved cysteines at the C-terminal are involved in forming disulfide bridges, maintaining the three-dimensional structure and biological activity of RALFs [11, 12].

Studies have highlighted the significance of RALFs in various physiological processes, particularly in root and root hair growth, pollen tube development, nitrogen-fixing nodule formation, and inulin accumulation [11, 13, 14]. For instance, the binding of the RALF1 peptide to FER inhibits root growth [15], while RALF32 regulates seedling growth through its interaction with FER [8]. Pollen tube reception and rupture are mediated by RALF6/7/16/36/37 peptides in receptor complexes [16]. Recent research by Lan et al. [17] demonstrated that in Brassicaceae, pollen tube penetration is controlled by stigmatic or pollen RALF peptides interacting with receptor-like kinases and cell-wall proteins, regulating hybridization barriers on the stigma. In *Medicago truncatula*, overexpression of *MtRALFL1* resulted in abnormal nodule growth and a significant reduction in nodule numbers [18]. In rice, *OsRALF17* and *OsRALF19* mediate reactive oxygen species signals through *OsMTD2*, crucial for pollen tube germination and integrity [19]. In *Phaseolus vulgaris*, *PvRALF1* and *PvRALF6* control nodule numbers relative to nitrate availability [20]. Jia and Li [21] identified *RALF* genes in both legume and non-legume species and found that rhizobial treatment significantly influenced the expression of most *RALFs* in soybean. Additionally, seven *GmRALFs* were implicated in rhizobial release within cortical cells. Beyond their roles in growth regulation, RALF peptides are also involved in abiotic and biotic stress responses, with RALF23 and RALF33 participating in pathogen-induced immune responses [8] and RALFL8 in cell wall remodeling under various stress conditions [22]. Furthermore, 18 out of 27 *GmRALFs* were found to be responsive to *Fusarium oxysporum* infection in soybean [48].

Genome-wide investigations of RALF gene families across different species provide evolutionary insights and functional information. To date, RALF gene families have been identified in several plant species, including *Arabidopsis*, rice, corn, soybean, cotton, and strawberry [3, 23–25]. The peanut (*Arachis hypogaea* L.) is a vital oil and cash crop essential for global economic and agricultural development. However, its yield is significantly impacted by Al toxicity and water deficit [25, 27]. Our study identified homologous genes of FERONIA, a receptor of RALFs, in peanuts and their response to Al stress [28]. Nonetheless, the identification of RALF members in peanuts and their functional roles remain unexplored. This study identifies *AhRALFs* in peanuts, analyzes their chromosomal location, structure, and evolution, and confirms the secretory function and bioactivity of *AhRALF1*, an Al-responsive gene. These results establish a foundation for further exploration of *AhRALF* functions in growth regulation and stress responses in peanuts.

Materials and methods

Identification of RALF genes in *Arachis hypogaea*

The *RALF* gene sequences of *Arabidopsis thaliana* were systematically retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and The Arabidopsis Information Resource (http://www.Arabidopsis.org/). Detailed sequence information is provided in Additional File 1: Table S8. The genome and transcriptome data for *Arachis hypogaea* were obtained from NCBI (https://www.ncbi.nlm.nih.gov/assembly/?term=PEANUT). The genome sequence file was processed using the Genome Length Filter tool in TBtools, applying a minimum length threshold of 51,897,010 bp to exclude scaffold sequences. Only sequences assembled onto chromosomes were retained for further analysis [29]. A local BLAST search was performed using the Blast GUI Wrapper tool (TBtools v1.108) to identify potential *AhRALF* genes in the *Arachis hypogaea* genome [29]. The 36 *Arabidopsis thaliana RALF* sequences were used as query sequences, while the filtered genome sequence file served as the subject. The BLAST parameters were set as follows: output format (Outfmt): Table, number of threads (NumofThread): 2, E-value threshold: 1e-5, maximum number of hits (Num of Hits): 500, and maximum number of alignments (Num of Aligns): 250. In the first round of BLAST analysis, 74 candidate sequences were identified. These sequences were then used as query sequences for a second round of BLAST, with the SwissProt protein database as the subject. The BLAST output format was set to *BlastXML*, while all other parameters remained at their default settings. Among the results, 24 sequences were annotated

as *RALF*-like genes and classified as *AhRALF* genes (Table 1).

Phylogenetic analysis of RALFs in peanut and Arabidopsis

The RALF peptide sequences from *Arabidopsis* and peanut were aligned using MEGA 11.0.13 with default parameters. Phylogenetic trees were constructed using the neighbor-joining method, and iTOL v6 (https://itol.embl.de/) was utilized to visualize the generated tree.

Chromosomal location and evolutionary analysis of *AhRALF* genes

TBtools was used to filter and visualize the chromosomal locations of *AhRALF* genes in peanuts [29]. Collinearity analysis of the *AhRALF* genes was performed using the one-step MCScanX feature of TBtools with default parameters [29].

The Simple Ka/Ks calculator was used to determine the ratio of the synonymous substitution rate (Ks) to the nonsynonymous substitution rate (Ka) (TBtools v1.108) [29]. Divergence time was estimated using the formula $T = Ks/2\lambda$, where λ is 1.5×10^{-8} [28].

Gene structure, conserved motifs, and promoter cis-regulatory elements analysis

The annotation files for *AhRALFs* obtained from NCBI were filtered using TBtools. The conserved motifs of *AhRALF* proteins were examined using MEME (https://meme-suite.org/meme/doc/meme.html), with parameters set to identify a maximum of five motifs and allow zero or one occurrence per sequence. Gene Structure View in TBtools was used to display the conserved motifs [29]. Cis-regulatory elements predicted by PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html) were visualized using the Simple Biosequence Viewer in TBtools v1.108 [29].

Gene expression analysis and validation

To elucidate the tissue-specific and Al-responsive expression patterns of *AhRALF* genes, RNA-seq datasets obtained from the NCBI Sequence Read Archive (SRA) database (accession numbers: PRJNA484860 and PRJNA525247) were analyzed. Expression profiles were systematically assessed using TBtools software (v1.108). Specifically, raw sequencing reads were processed and quantified using the Kallisto Super GUI Wrapper module in TBtools, which employs a pseudo-alignment-based algorithm for efficient and accurate transcript

Table 1 Characteristics of the identified *AhRALF* genes

Gene	mRNA ID	Number of Exon	Num of Intron	Number of Amino Acid	MW (Da)	pI	Signal peptide length (aa)	Mature peptide length (aa)	RRXL domain	XIXY domain	Conserved cysteine residues
AhRALF1	XM_025755590.2	1	0	129	14,365.36	9.22	28	49	RRIL	YISY	CCCC
AhRALF2	XM_025816602.2	1	0	129	14,876.10	8.48	24	59	RRIM	YISY	CCCC
AhRALF3	XM_025835954.2	1	0	112	12,184.73	8.41	19	55	RRIL	IISN	C-CC
AhRALF4	XM_025837415.1	1	0	126	13,341.46	9.5	22	62	\	VIGN	CCCC
AhRALF5	XM_025837417.1	1	0	126	13,620.80	9.23	22	62	\	VIGN	CCCC
AhRALF6	XM_025837418.1	1	0	126	13,539.72	9.34	22	62	\	VIGN	CCCC
AhRALF7	XM_025841590.1	1	0	119	13,414.43	8.47	14	58	RRIL	YISY	CCCC
AhRALF8	XM_025842906.2	1	0	139	15,548.91	8.25	28	56	RRSL	YISY	C-CC
AhRALF9	XM_025748302.2	1	0	118	13,161.17	8.86	22	50	RRIL	YISY	CCCC
AhRALF10	XM_025747904.2	1	0	130	13,901.84	8.68	17	49	RRIL	YISY	CCCC
AhRALF11	XM_025770196.1	1	0	110	12,621.45	9.42	19	52	RRTL	YISY	CCCC
AhRALF12	XM_025774317.2	1	0	129	14,319.31	9.2	23	49	RRIL	YISY	CCCC
AhRALF13	XM_025785630.2	1	0	112	12,240.84	8.41	19	55	RRIL	IISN	C-CC
AhRALF14	XM_025785211.2	1	0	130	14,989.22	8.17	20	60	RRLM	YISY	CCCC
AhRALF15	XM_025789710.2	1	0	118	13,589.16	5.69	\	55	RRFL	YISY	C-CC
AhRALF16	XM_025792670.1	1	0	126	13,341.46	9.5	26	62	\	VIGN	CCCC
AhRALF17	XM_025792671.1	1	0	126	13,620.80	9.23	26	61	\	VIGN	CCCC
AhRALF18	XM_025792672.1	1	0	126	13,539.72	9.34	26	62	\	VIGN	CCCC
AhRALF19	XM_025789225.1	1	0	119	13,417.32	8.6	18	58	RRIL	YISY	CCCC
AhRALF20	XM_025806044.1	1	0	121	13,051.08	9.06	27	57	\	VIEN	CCCC
AhRALF21	XM_025806685.2	1	0	130	13,890.77	8.86	21	49	RRIL	YISY	CCCC
AhRALF22	XM_025805261.1	1	0	116	12,866.85	9.07	26	50	RRIL	YISY	CCCC
AhRALF23	XM_025814073.1	1	0	114	12,757.32	9.36	\	53	RRNI	YISY	CCCC
AhRALF24	XM_025828768.1	1	0	110	12,651.47	9.42	23	51	RRTL	YISY	CCCC

quantification. This analysis pipeline included quality filtration of raw reads followed by pseudo-alignment to the reference transcriptome of cultivated peanut. The resulting expression values were normalized as Transcripts Per Million (TPM). The normalized expression data of *AhRALF* genes were then extracted using the Table Row Extractor and Filter tools (TBtools v1.108). The spatiotemporal and stress-responsive expression patterns were visualized using the Heat Map tool in TBtools (v1.108) [29]. The expression data were log_2-transformed before visualization to improve the dynamic range representation.

To validate the reliability and reproducibility of the RNA-seq analysis, quantitative real-time PCR (qRT-PCR) analysis was performed on a subset of representative *AhRALF* genes. Peanut seeds were surface-sterilized and germinated in moistened perlite for three days at 28 °C. The germinated seeds were then transferred to Hoagland nutrient solution for hydroponic culture, as described in a previous study [28]. Once seedlings developed 1–3 leaves, they were sequentially treated with $CaCl_2$ solution (Hoagland nutrient solution supplemented with 0.1mmol/L $CaCl_2$, pH 4.2) for 24 h, followed by Al treatment (Hoagland nutrient solution containing 0.1mmol/L $CaCl_2$ and 0.1mmol/L $AlCl_3$, pH 4.2). After treatment, root tips were collected at 0 (CK), 12, and 24 h, snap-frozen in liquid nitrogen, and stored at -80 °C. Each sample consisted of three different root tips, and three biological replicates were performed.

Total RNA was extracted using the RNAprep Pure Plant Plus Kit (TIANGEN, Beijing, China). First-strand cDNA was synthesized using the Hifair® Advance-Fast 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen, Shanghai, China) following the manufacturer's instructions. Gene-specific primers for *AhRALF1*, *AhRALF8*, *AhRALF12*, and *AhRALF15* were designed using Primer Premier 5 and confirmed for specificity using NCBI BLAST analysis. The *Ahβ-actin* gene served as the reference for normalization. All primer sequences are provided in Additional file 1: Table S1. qRT-PCR was conducted using ArtiCanATM SYBR qPCR Mix (Tsingke, Beijing, China), and relative gene expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method. Each sample was analyzed in triplicate, and statistical significance was evaluated using Student's t-test.

Verification of the AhRALF1 secretory function

To determine the subcellular location of *AhRALF1*, tobacco (*Nicotiana tabacum*) leaf epidermal cells were injected with the recombinant plasmid 35 S:*AhRALF1*-GFP and an empty GFP vector as a control. After injection, the plants were cultivated under normal conditions for approximately 48 h. A fluorescence microscope was then used to observe the fluorescent signals.

To confirm the secretory properties of *AhRALF1*, yeast strain YTK12 was employed. The coding sequence of *AhRALF1* was cloned into the pSUC2 vector, and the primer information is listed in Additional file 1: Table S2. The pSUC2-*AhRALF1* construct was transformed into yeast strain YTK12 using the lithium acetate method [30]. The growth status and color reaction with 2, 3, 5-triphenyltetrazolium chloride (TTC) were observed in the transformed strain cultured on CMD-W and YPRAA media, respectively [31].

In vitro bioactivity assay of AhRALF1

The AhRALF1 peptide (FWRRMKYYISYGALSANRIP-CPPRSGRSYYTHNCFKARGPAHPYTR GCSIITRCRR) was synthesized by Shanghai Hong Tide Biotechnology Co., Ltd., Shanghai, China.

After surface-sterilization, seeds of *A. thaliana* Col-0 were planted on 1/2 MS medium containing 30 g/L of sucrose and 7 g/L of agar (pH 5.7). The seeds were stratified at 4 °C for two to three days and then transferred to growth cabinets set at 23 °C with a 14 h/10 h light/dark photoperiod for three to four more days. Subsequently, seedlings were transplanted to 1/2 MS solid medium containing 1 μM *AhRALF1* peptide and cultured for an additional three days. A control group was maintained on 1/2 MS solid medium without the *AhRALF1* peptide. Root lengths were measured three days later. Three experimental replicates were performed for each treatment, with 15 seedling root lengths measured per replicate. The variance was assessed using Excel 2019, and significance was evaluated using Student's t-test.

Overexpression of *AhRALF1* in Arabidopsis

The cDNA fragment of *AhRALF1* was amplified by PCR using gene-specific primers, and the expression vector pCAMBIA1300 was constructed. The pCAMBIA1300-*AhRALF1* construct was then transformed into *Agrobacterium tumefaciens* strain GV3101. Colony PCR was used to identify positive *Agrobacterium* colonies carrying the desired gene. The floral dipping method was employed to transform *A. thaliana* Col-0 plants. Positive transgenic plants were selected on 1/2 MS medium containing 40 μg/mL hygromycin. At least nine independent transgenic lines were produced up to the T3 generation for further study. The overexpression efficiency of different transgenic lines was verified using qRT-PCR. ArtiCanATM SYBR qPCR Mix (Tsingke, Beijing, China) was used for the qRT-PCR. The primer pairs utilized are listed in Additional file 1: Table S1.

Virus-induced gene silencing (VIGS) analysis

The pTRV2:AhRALF1 construct was generated for VIGS analysis. A 378 bp fragment of AhRALF1 coding sequence was amplified using gene-specific primers

(Forward: 5'- TGCTCTAGATCTCACCAATGGCTGC AGTT – 3', Reverse: 5'- CGGGGTACCCGGGTCCTC TTGCCTTGAAA – 3') and cloned into the pTRV2 vector between XbaI and KpnI restriction sites. The empty pTRV2 vector served as a negative control. The resulting constructs (pTRV1, pTRV2, and pTRV2:AhRALF1) were transformed into *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method. *Agrobacterium* strains were cultured in LB medium containing appropriate antibiotics at 28 °C until OD_{600} reached 1.0. The bacterial cells were collected by centrifugation (4,500 rpm, 5 min) and resuspended in infiltration buffer (10 mM MgCl2, 10 mM MES, pH 5.6, and 100 μM acetosyringone). Equal volumes of *Agrobacterium* cultures harboring pTRV1 and pTRV2 or pTRV2:AhRALF1 were mixed and incubated at room temperature for 3 h. For transformation, peanut seeds were wounded using a sterile blade and immersed in the *Agrobacterium* suspension. The infected seeds were incubated in the dark at 28 °C with gentle shaking (120 rpm) for 16 h, followed by transfer to moistened vermiculite for germination. After five days, the germinated seedlings were transferred to Hoagland nutrient solution for hydroponic culture. The infiltrated plants were maintained in a growth chamber at 28 °C with a 16/8 h light/dark photoperiod. At 2–3 weeks post-infiltration, root samples were collected from both control (pTRV2) and silenced (pTRV2:AhRALF1) plants for RNA extraction and qRT-PCR analysis to verify the silencing efficiency. The silenced plants were then subjected to Al treatment as described above to investigate the role of AhRALF1 in Al stress response. Each treatment included at least 10 individual plants, and the experiment was repeated three times independently. Plants showing successful silencing of *AhRALF1* were selected for phenotypic analysis, including plant height, fresh weight, and root length measurements. The silenced plants were subsequently subjected to Al treatment as described above to investigate the role of *AhRALF1* in Al stress response. Each treatment included at least 10 individual plants, and the experiment was repeated three times independently.

Histochemical staining and physiological measurements

For hematoxylin staining, fresh root tips were immersed in 0.1% hematoxylin solution containing 0.02% KIO_3 for 30 min, followed by rinsing with deionized water until the solution was clear. Stained roots were observed and photographed using an Olympus optical microscope (CX33, Olympus Corporation, Tokyo, Japan).

For enzyme activity and MDA content measurements, fresh leaf samples (approximately 0.1 g) were collected and immediately processed. Superoxide dismutase (SOD) activity was determined using the Superoxide Dismutase (SOD) assay kit (WST-1 method) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Peroxidase (POD) activity was measured using a Peroxidase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Catalase (CAT) activity was assessed using the Catalase (CAT) assay kit (Visible light) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The malondialdehyde (MDA) content was determined using the Malondialdehyde (MDA) assay kit (TBA method) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Three biological replicates were conducted, each including three technical replicates.

Results

Identification and phylogenetic analysis of RALF genes in peanut genome

We identified RALF genes in peanuts through a BLAST similarity search. Two rounds of BLAST led to the identification of 24 putative RALF-like peptides (*AhRALFs*) in the *Arachis hypogaea* genome, named *AhRALF1-24* (Table 1). The amino acid lengths of *AhRALF* preproproteins ranged from 110 to 139, with predicted molecular weights between 12.18 and 15.55 kDa. Additionally, 95.83% (23/24) of *AhRALF* proteins exhibited high isoelectric points (pIs) ranging from 8.17 to 9.50, except for *AhRALF15* (5.69). The mature peptides of *AhRALFs* ranged from 49 to 62 amino acids, indicating conservation.

To understand the evolutionary relationships of *AhRALFs*, we created a phylogenetic tree using the protein sequences. The analysis revealed that the 61 RALF protein sequences from *Arabidopsis* and peanut clustered into four distinct clades (I-IV), with 8, 11, 20, and 22 members, respectively (Fig. 1). *AhRALFs* were distributed across clades I–III, suggesting that the evolution of *AtRALF* genes in clade IV occurred after the divergence of these two lineages. Furthermore, the distribution of *AhRALFs* across clades was uneven: 87.5% (7/8) in clade I, 60% (12/20) in clade III, and only 45.45% (5/11) in clade II. Paralogous gene pairs and clusters were common among *AhRALFs*, such as *AhRALF1/12*, *AhRALF2/14*, *AhRALF3/13/15*, *AhRALF4/16*, *AhRALF5/17*, *AhRALF6/18*, *AhRALF7/19*, *AhRALF9/22*, *AhRALF10/21*, *AhRALF11/24*. Similar phenomena are observed in other plant species like rice, poplar, and *Arabidopsis* [3, 23]. Expression analysis revealed that these gene pairings exhibited either similar or divergent expression patterns. According to Campbell and Turner [2], these paralogous RALFs likely originated from tandem or segmental duplication events in multiple species.

Chromosomal locations and duplication events of the *AhRALFs*

The chromosomal locations of the *AhRALFs* were analyzed (Supplementary Fig. 1). The 24 *AhRALFs* were unevenly distributed across 12 out of 20 peanut

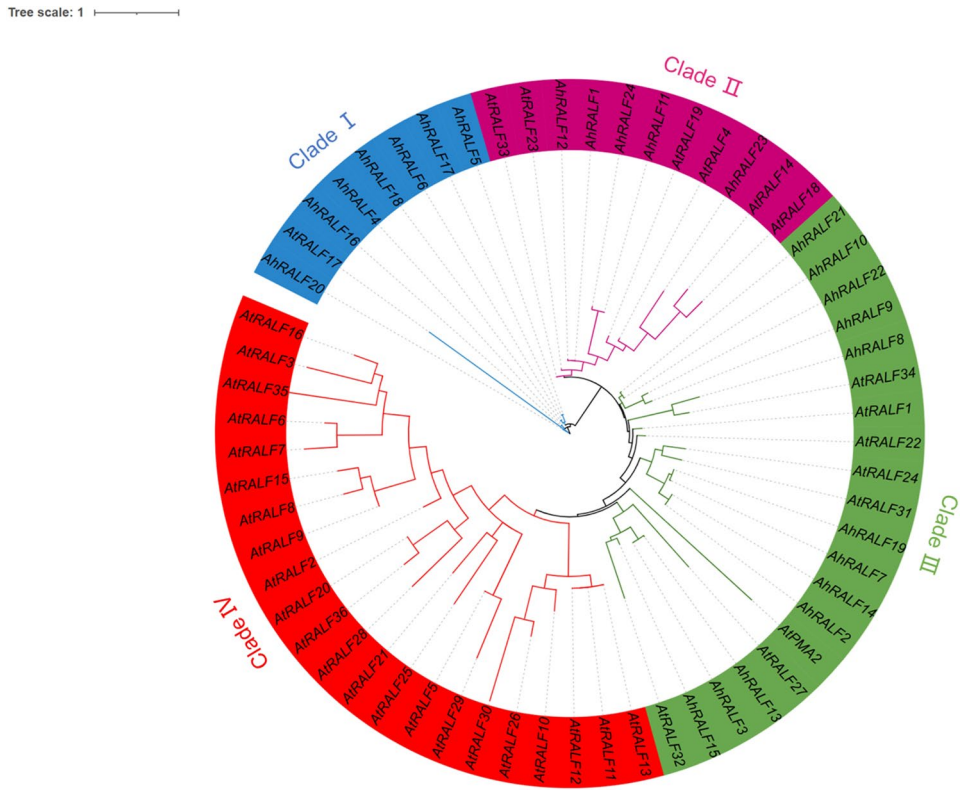


Fig. 1 Phylogenetic analysis of the RALFs in peanut and *Arabidopsis*. The circular phylogenetic tree illustrating the evolutionary relationships of RALF proteins in peanut and *Arabidopsis*. The tree is divided into four distinct clades (I - IV), each color-coded for clarity

chromosomes. Five *AhRALF* genes were located on chromosome NC_037631.1, followed by four genes on chromosome NC_037632.1. Three genes were found on chromosome NC_037633.1, and two genes each on chromosome NC_037620.1, NC_037623.1, and NC_037630.1. The remaining five chromosomes contained one gene each, all belonging to clade II.

To assess the evolutionary relationships of *AhRALF* genes, we examined duplication events. Eighteen *AhRALFs* resulted from whole genome duplication (WGD) or segmental duplication, while four arose from tandem duplication (Fig. 2, Additional file 1: Table S3). Tandem duplication events were confined to the *AhRALF* I subfamily, while all members of clades II and III resulted from segmental duplications. Except for two segmental duplication gene pairs for which values could not be calculated, all duplication gene pairs had Ka/Ks values less than 1. Tandem duplication events primarily occurred between four to seven million years ago, while WGD or segmental duplication events, accounting for 68% (17/25) of the total, occurred predominantly around twenty-five million years ago (Additional file 1: Table S4).

Gene structures and conserved motifs of *AhRALF* proteins

Gene structure analysis using TBtools revealed that all 24 *AhRALFs* in peanuts contain one exon each, with no introns present. To further characterize *AhRALFs*, we used MEGA 11 for peptide sequence alignment and Simple MEME Wrapper in TBtools for motif prediction. Among the 24 *AhRALFs*, fifteen contained the conserved RRXL and YISY domains, while all *AhRALFs* contained the C-terminal RGC(5 N)C domain. The RRXL (X represents any amino acid) domain, a conserved S1P cleavage site crucial for the maturation of the peptide, was present in all members of clades II and III but absent in clade I proteins (Table 1; Fig. 3). MEME motif scanning identified five distinct motifs (motif 1 to motif 5) (Fig. 4; Additional file 1: Table S5). Motifs 1 and 2 were conserved across all *AhRALFs*, while motif 4 was unique to clade I. Motifs 3, 4, and 5 were only present in preproproteins, whereas motifs 1 and 2 were present in both preproproteins and mature peptides. SignalP 5.0 predicted signal peptides in 22 out of 24 *AhRALFs* (Fig. 3), with *AhRALF15* and *AhRALF23* lacking signal peptides, suggesting a potential lack of secretory function.

Cis-regulatory elements analyses of peanut *AhRALFs*

Cis-regulatory elements in the 2 kb upstream sequences of *AhRALFs* were analyzed using the PlantCARE database. Eighty-seven cis-regulatory elements were identified in the promoter regions of *AhRALFs* (Table 2; Additional file 1: Table S6). As shown in Fig. 5, elements

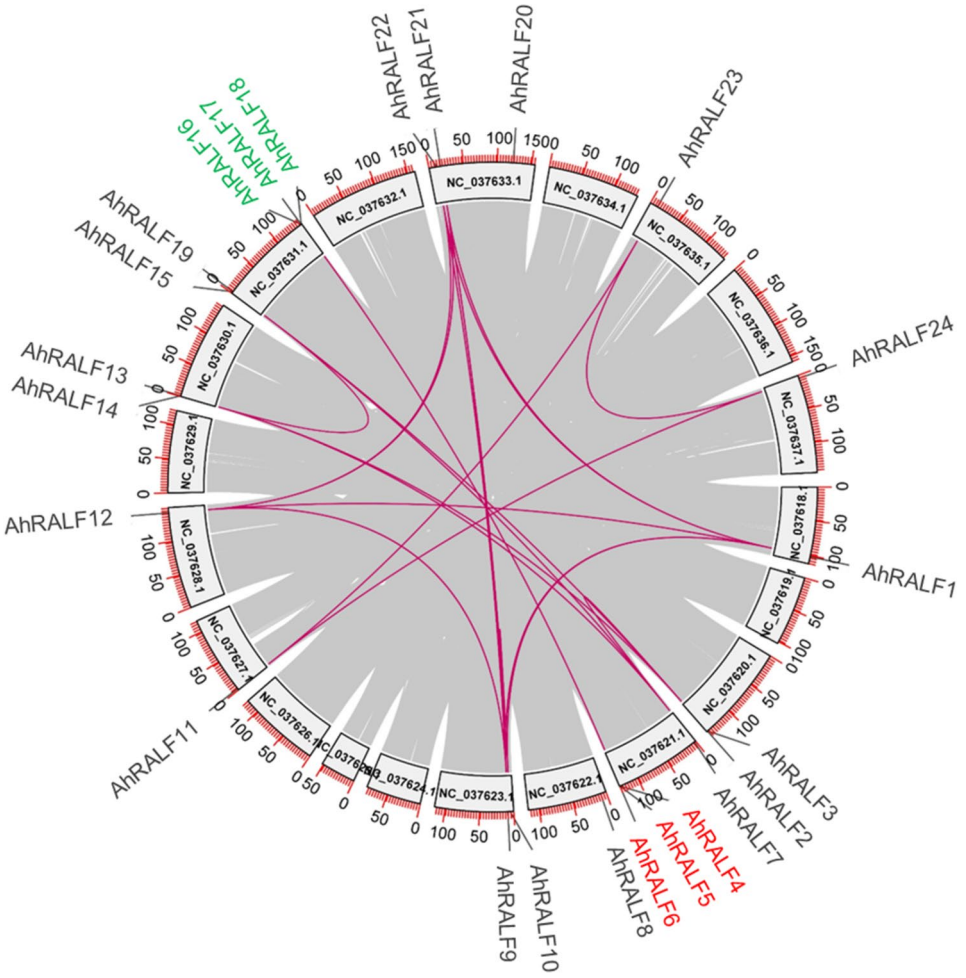


Fig. 2 *AhRALFs* duplication events analysis. Peanut chromosomes 01–20 are shown on the outer ring, with segmental duplication gene pairs indicated by purple lines connecting corresponding chromosomal regions. This highlights the extensive duplication events that have contributed to the expansion and diversification of the *AhRALF* gene family

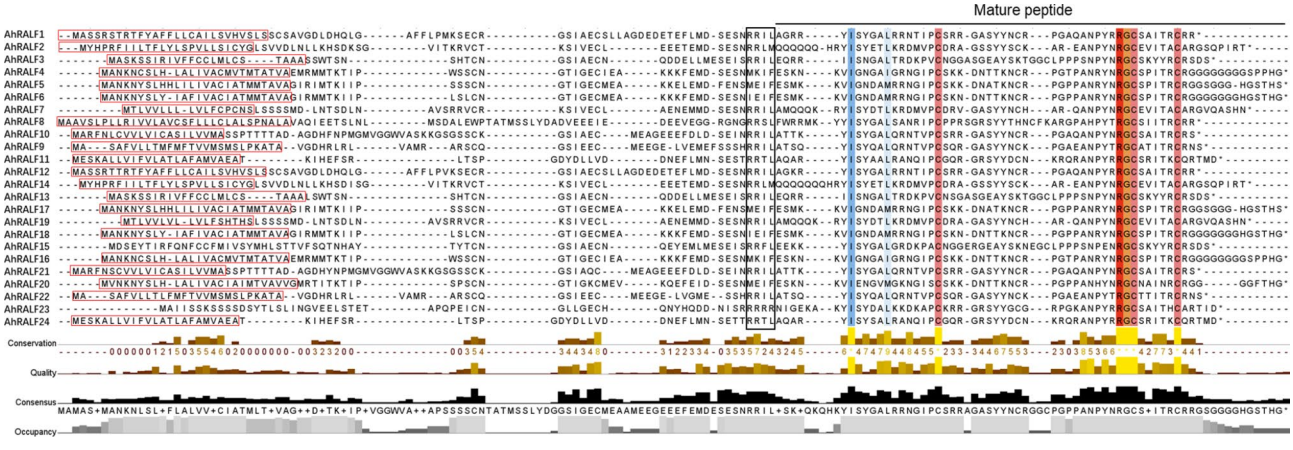


Fig. 3 Amino acid alignment of AhRALFs. The black frame indicates the S1P processing site. The red frame indicates the signal peptide. The alignment highlights conserved regions among the *AhRALF* proteins, providing insight into their functional domains and potential processing sites

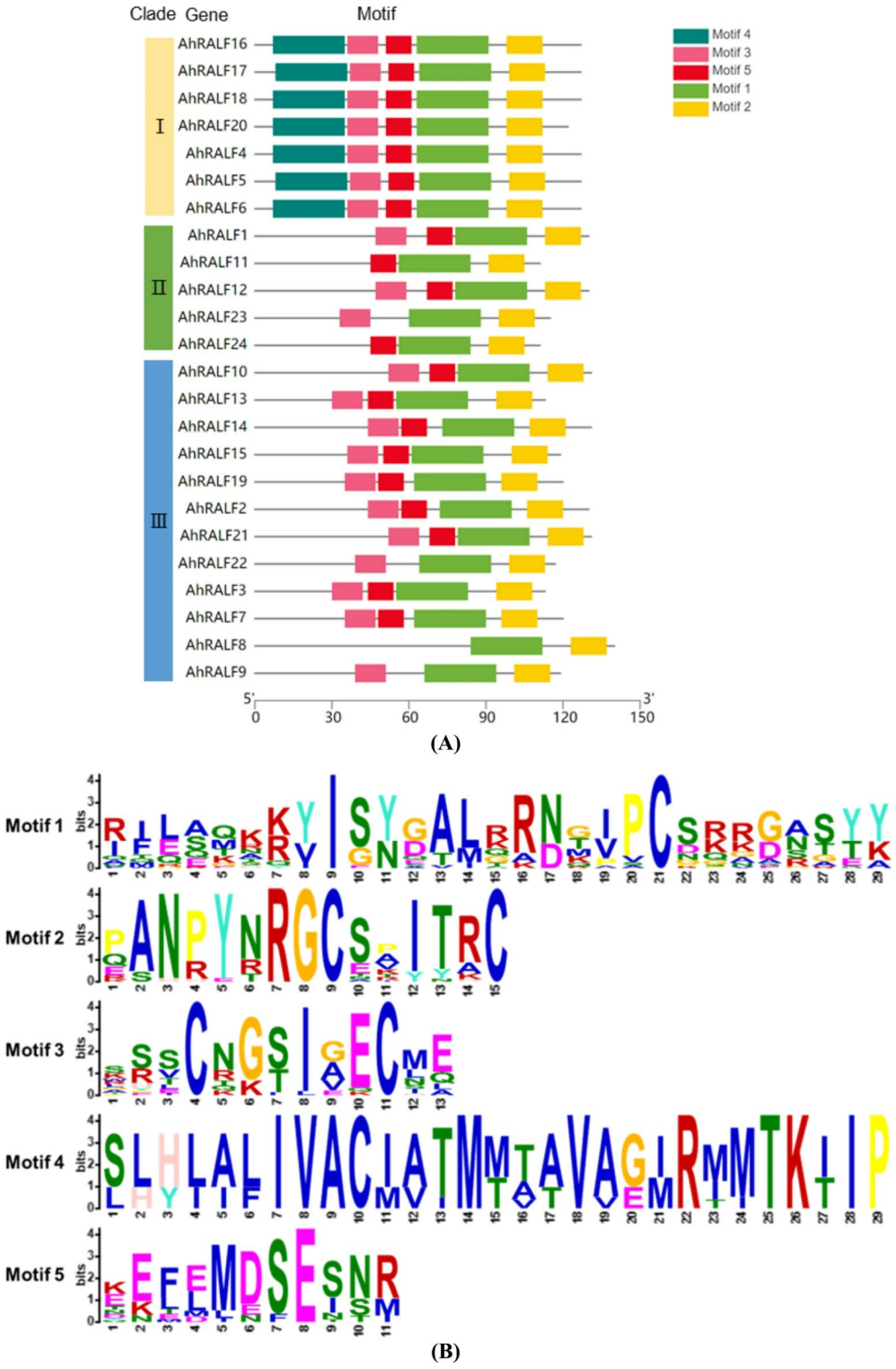


Fig. 4 Motif patterns of all AhRALFs (**A**). The five boxes represent distinct MEME motifs identified in the AhRALF proteins; Logos of the five motifs (**B**)

related to plant hormone responses were widespread in the promoter regions of *AhRALFs*. The largest number of *AhRALFs* contained elements related to abscisic acid response (16/24), followed by elements responsive to auxin and methyl jasmonate (MeJA). All seven *AhRALF* I genes contained auxin-responsive elements, with six also containing elements responsive to abscisic acid and salicylic acid. Most *AhRALF* II genes contained elements

Table 2 The number of cis-regulatory elements identified in the promoter regions of each *AhRALF* gene

Clade	Gene name	Abscisic acid responsive	Auxin responsive	Low temperature responsive	Defense and stress responsive	MeJA responsive	Salicylic acid responsive	Gibberellin responsive	Drought inducibility
I	*AhRALF4*	4	1	2	0	0	1	0	0
I	*AhRALF5*	2	1	0	0	2	1	0	0
I	*AhRALF6*	2	1	0	1	2	1	0	0
I	*AhRALF16*	4	1	2	0	0	1	0	0
I	*AhRALF17*	2	1	0	0	2	1	0	0
I	*AhRALF18*	2	1	0	1	2	1	0	0
I	*AhRALF20*	0	2	1	0	0	0	1	0
II	*AhRALF1*	5	0	1	0	2	1	0	2
II	*AhRALF11*	4	1	1	0	0	0	0	0
II	*AhRALF12*	2	0	1	0	4	0	2	2
II	*AhRALF23*	0	2	0	1	4	2	1	1
II	*AhRALF24*	3	0	0	0	2	0	0	1
III	*AhRALF2*	1	0	1	0	0	0	2	0
III	*AhRALF3*	0	0	0	0	0	0	4	0
III	*AhRALF7*	1	1	0	0	0	1	2	0
III	*AhRALF8*	0	1	1	0	2	0	0	0
III	*AhRALF9*	2	0	0	2	0	0	1	3
III	*AhRALF10*	0	1	0	0	0	0	3	0
III	*AhRALF13*	0	0	0	0	0	1	3	0
III	*AhRALF14*	0	0	1	0	2	0	0	0
III	*AhRALF15*	0	1	0	0	2	0	3	1
III	*AhRALF19*	1	0	0	1	2	0	2	0
III	*AhRALF21*	4	2	0	0	4	0	0	0
III	*AhRALF22*	2	0	0	1	0	0	1	3

responsive to abscisic acid (4/5), MeJA (4/5), and drought inducibility (4/5). Most *AhRALF* III genes contained elements responsive to gibberellin (9/12).

Expression patterns of *AhRALF* genes in different organisms and under al stress

Based on RNA-seq data from various peanut tissues and under Al stress, the expression levels of *AhRALF* genes were investigated (Fig. 6; Additional file 1: Table S7). *AhRALF* genes from clade I were not expressed in any of the 18 tissues examined nor under Al stress. In contrast, two genes from clade II (*AhRALF1* and *AhRALF12*) and four from clade III (*AhRALF8*, *AhRALF10*, *AhRALF15*, and *AhRALF21*) showed high expression levels in most tissues. A few *AhRALFs* exhibited strict tissue specificity; for instance, *AhRALF11* and *AhRALF24* were highly expressed in immature buds and flowers but barely expressed or not expressed in the other 16 tissues. Under Al stress, two clade IIgenes (*AhRALF1* and *AhRALF12*) and five clade IIIgenes (*AhRALF3*, *AhRALF8*, *AhRALF13*, *AhRALF15*, and *AhRALF19*) showed significant inhibition. Further research focused on these Al-responsive genes. qRT-PCR analysis of *AhRALF1*, *AhRALF8*, *AhRALF12*, and *AhRALF15* confirmed the transcriptome analysis results (Fig. 7).

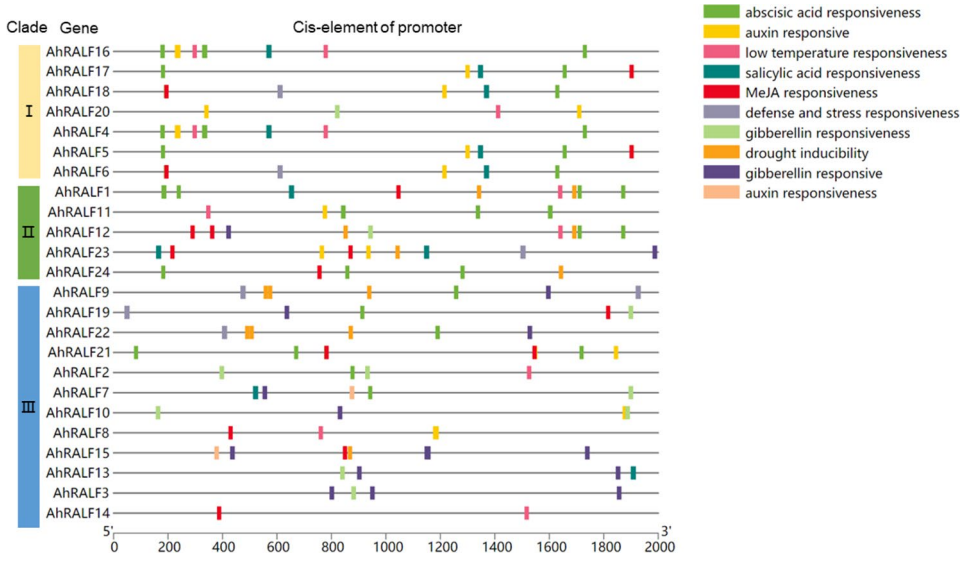


Fig. 5 Cis-regulatory elements in the RALF promoter regions in peanut

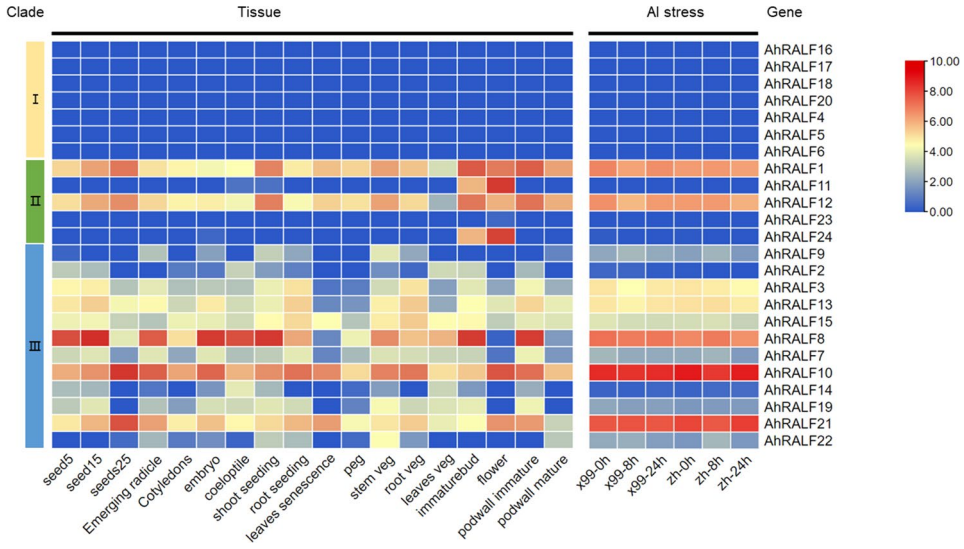


Fig. 6 RNAseq expression level of the *AhRALF* genes across 18 tissues and under Al stress. The heatmap shows expression levels, with blue indicating low expression and red indicating high expression

Analysis of the secretory function of *AhRALF1*

Based on RNA-seq and RT-qPCR analyses, *AhRALF1* was selected for further investigation. To confirm the secretion of *AhRALF1*, vectors encoding the 35 S: *AhRALF1*-GFP fusion protein were constructed. The epidermal cells of tobacco leaves expressing the *AhRALF1* fusion proteins exhibited a fluorescent signal at the cell periphery under a fluorescence microscope (Fig. 8A and B). The secretory function of *AhRALF1* was further verified using the yeast mutant strain YTK12. The *AhRALF1* peptide fused in the pSUC2 vector allowed yeast strain YTK12 to grow on both CMD-W and YPRAA media (Fig. 9A), indicating its secretory function. The color reaction results showed that pSUC2-*AhRALF1* induced a red color reaction with 0.1% TTC (Fig. 9B), further validating the secretory function of *AhRALF1*.

In vitro and in planta bioactivity assays of *AhRALF1*

To verify the bioactivity of *AhRALF1*, in vitro assays were conducted to determine its effect on inhibiting *Arabidopsis* root growth. The relative root elongation of *Arabidopsis* treated with exogenous *AhRALF1* peptides was 27.69% compared to the control treatment (Fig. 10A). To further verify the bioactivity of *AhRALF1*, transgenic *Arabidopsis* plants overexpressing *AhRALF1* were used for in planta assays. Two transgenic lines (OE1-4 and OE1-5) with the highest expression levels of *AhRALF1* were selected for phenotypic analysis. Both transgenic

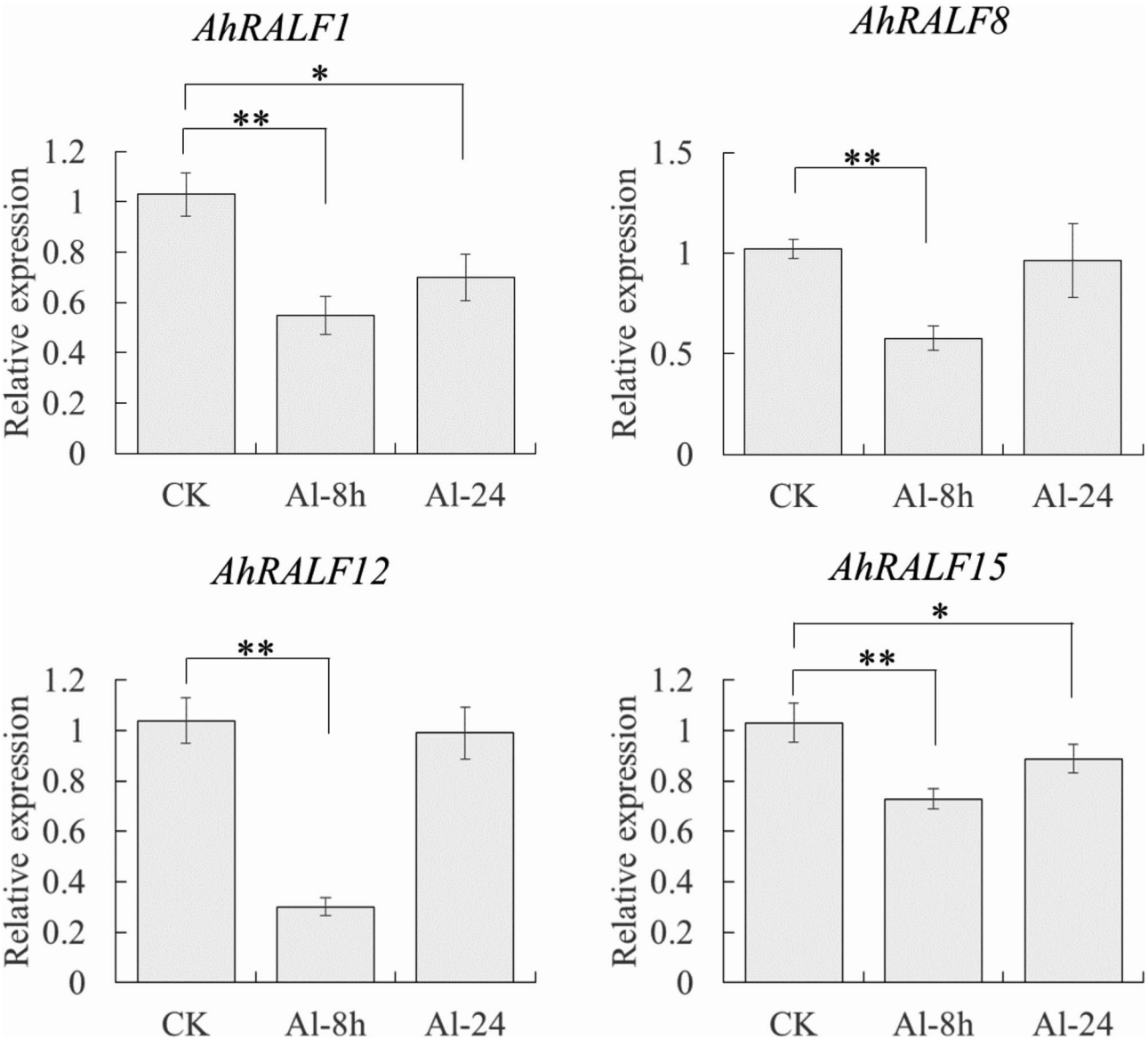


Fig. 7 qRT-PCR analysis of selected *AhRALFs* under Al stress. Relative expression levels of *AhRALF1*, *AhRALF8*, *AhRALF12*, and *AhRALF15* under different conditions: CK (control, without Al stress), Al-8 h (peanut seedlings treated with Al stress for 8 h), and Al-24 h (peanut seedlings treated with Al stress for 24 h). Asterisks indicate statistically significant differences (*: $P < 0.05$, **: $P < 0.01$). The results demonstrate the changes in expression levels of these *AhRALF* genes in response to Al stress over time

lines exhibited significantly shorter roots than the wild-type plants (Fig. 10B).

AhRALF1 affects al tolerance in transgenic Arabidopsis and peanut

To investigate the role of *AhRALF1* in Al stress responses, transgenic *Arabidopsis* lines (OE1-5) were subjected to Al stress treatment. After growing on MS medium containing 0.2 μM Al for 4 days, the root length of transgenic *Arabidopsis* remained shorter than that of the wild-type. However, the relative root elongation of OE1-5 was 159.68%, higher than that of the wild-type (86.09%) (Fig. 11A and B). Trypan blue staining showed that root damage in the wild-type was more severe than in OE1-5 when grown on MS medium containing 0.2 μM Al (Fig. 11C).

Virus-induced gene silencing was performed to confirm the function of *AhRALF1* in Al tolerance in peanuts. Compared to the wild-type, *AhRALF1*-silenced plants exhibited shorter root lengths and reduced plant height (Fig. 12). Additionally, the activities of SOD, POD, and CAT, along with the MDA content in the leaves of *AhRALF1*-silenced plants, remained elevated. After Al treatment for 2 days, the activity of SOD in wild-type seedling leaves increased 1.36-fold compared to controls without Al treatment, but no significant changes

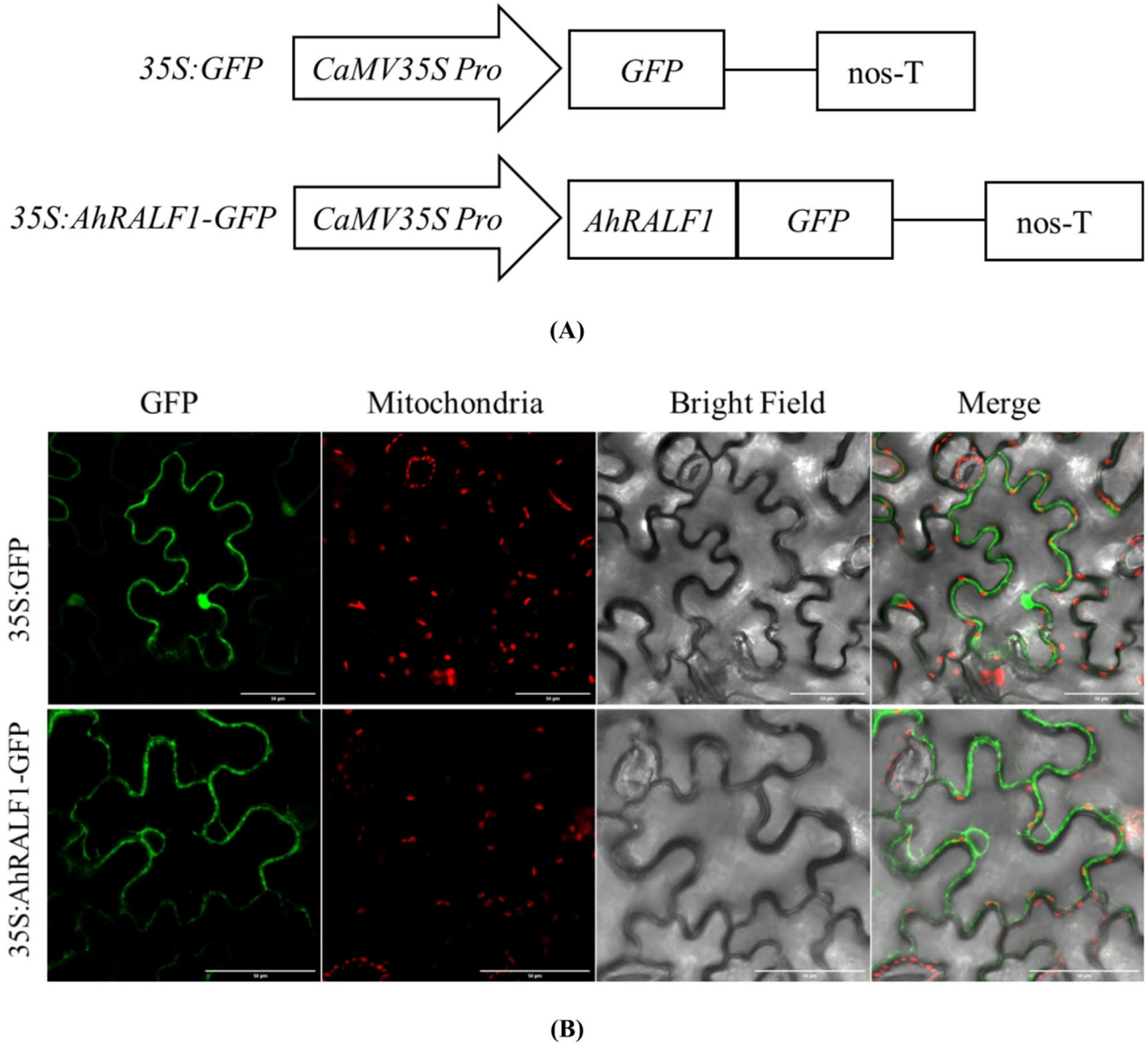


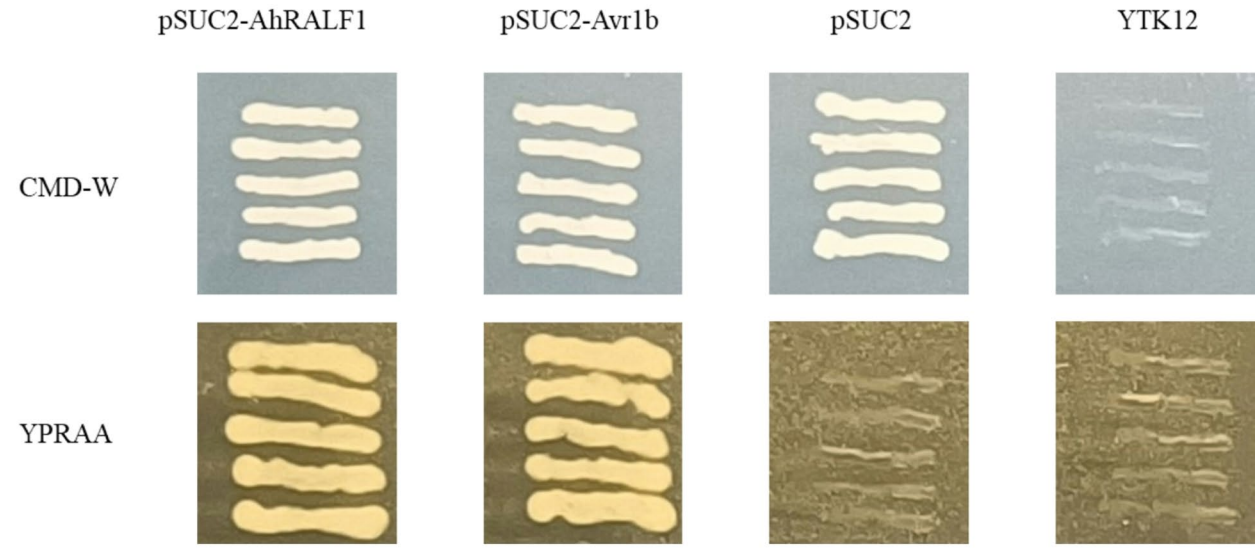
Fig. 8 Subcellular localization analysis of AhRALF1 proteins. **A**: Diagrammatic illustration of the control (*35 S: GFP*) and *35 S: AhRALF1-GFP*. **B**: Transient expression of *35 S: GFP* and *35 S: AhRALF1-GFP* after 48 h of transformation. The images show green fluorescence from GFP or *AhRALF1*-GFP, red fluorescence indicating mitochondria, bright field images of the cells, and merged images combining all channels to illustrate the subcellular localization of the proteins

were observed in *AhRALF1*-silenced plants. The activities of POD and CAT, and the content of MDA were 2.08, 3.26, and 2.40-fold higher than controls in wild-type, respectively, but only 1.29, 1.75, and 1.38-fold higher in *AhRALF1*-silenced plants. Hematoxylin staining showed deeper root staining in wild-type plants compared to *AhRALF1*-silenced plants after Al treatment (Fig. 13).

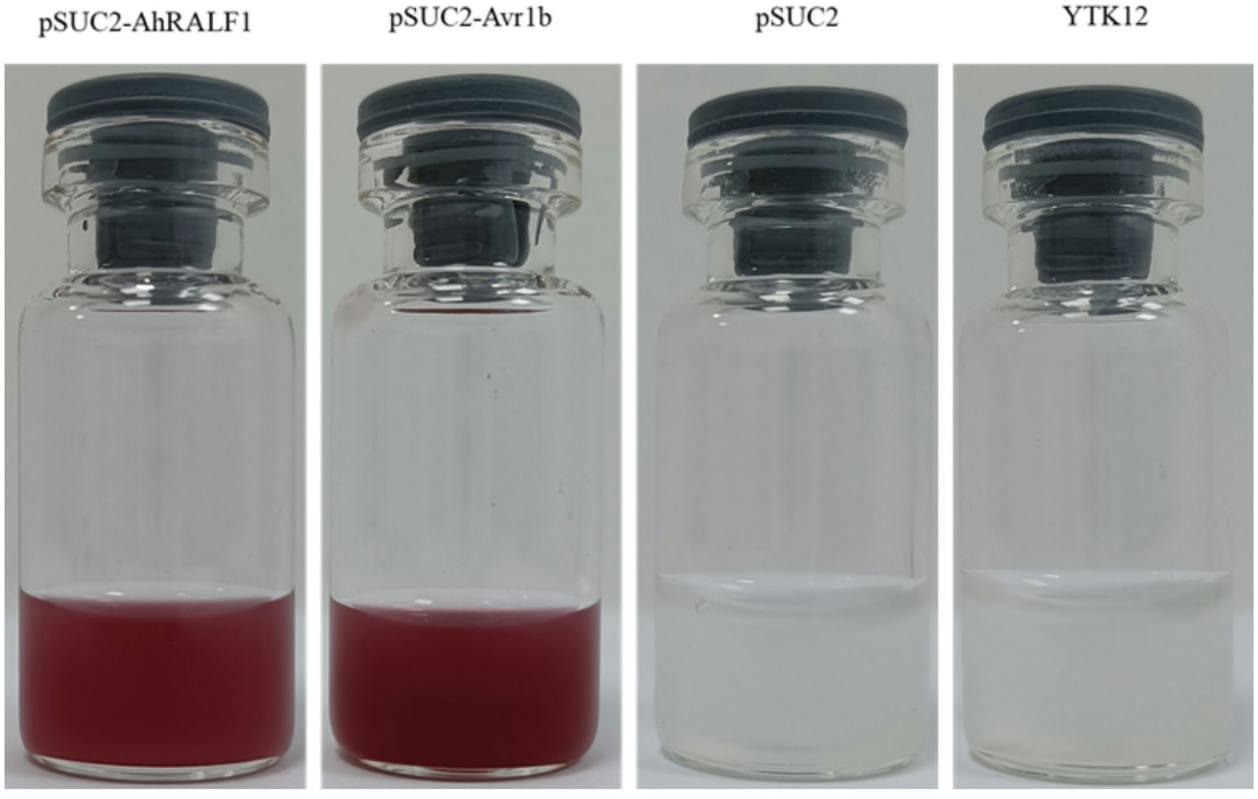
Discussion

Identification and characteristics of the RALF family in peanut

The RALFs are small peptide hormones in plants involved in various physiological and developmental processes, including cell expansion and immune response regulation [11]. Numerous studies have expanded our understanding of the character, structure, evolution, and function of RALFs across different plant species. The number of RALF genes varies among species: 37 in *A. thaliana*, 32 in *Brassica rapa*, 61 in *Brassica napus*, 23 in *Hevea brasiliensis*, and 32 to 120 in different species of

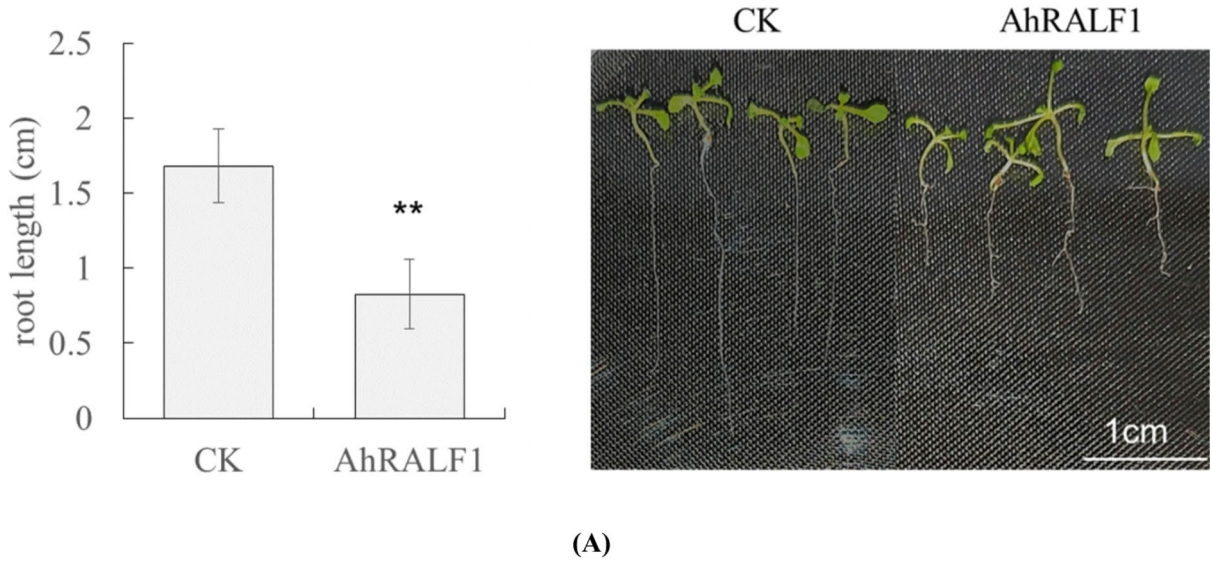


(A)

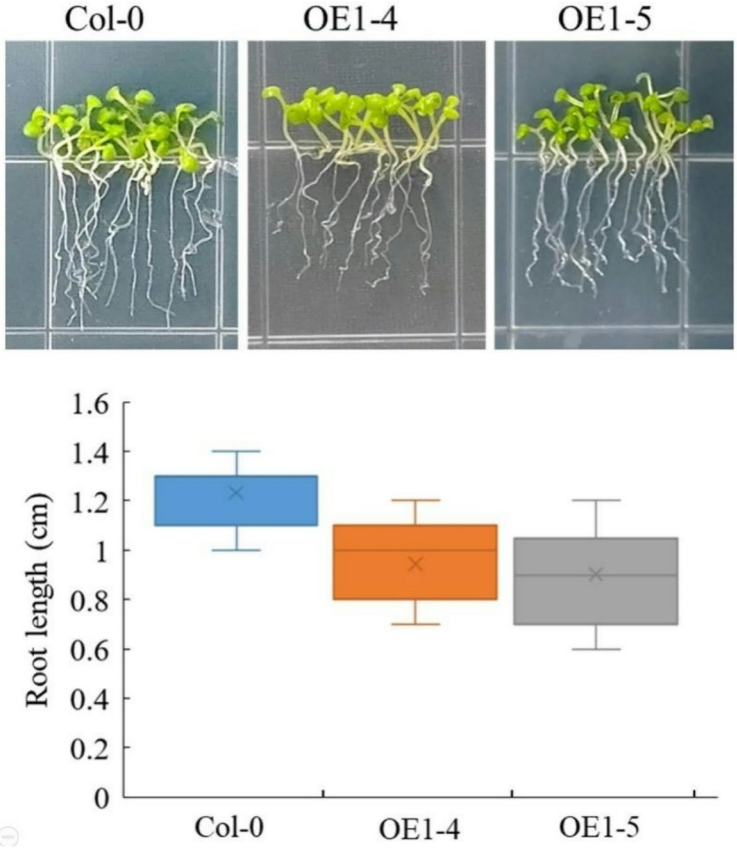


(B)

Fig. 9 Analysis of the secretion of AhRALF1. **A**: growth condition of YTK12; **B**: color reaction of 2,3,5-Triphenyltetrazolium Chloride (TTC). **YTK12**: A mutant strain of yeast devoid of genes responsible for sucrose invertase and tryptophan production. **pSUC2**: YTK12 strain containing the pSUC2 empty plasmid. **pSUC2-Avr1b**: YTK12 strain serving as a positive control in this study, containing an Avr1b signal peptide fragment fused in the pSUC2 vector. **pSUC2-*AhRALF1***: YTK12 strain carrying *AhRALF1* peptides fused in the pSUC2 vector



(A)



(B)

Fig. 10 Bioactivity of AhRALF1 peptides and transgenic *Arabidopsis* overexpressing AhRALF1. (**A**) Phenotype and root length analysis of *Arabidopsis* seedlings treated with AhRALF1 peptides (1 μM). (**B**) Phenotype and root length analysis of Col-0 and two *AhRALF1* overexpressing transgenic lines (OE1-4 and OE1-5)

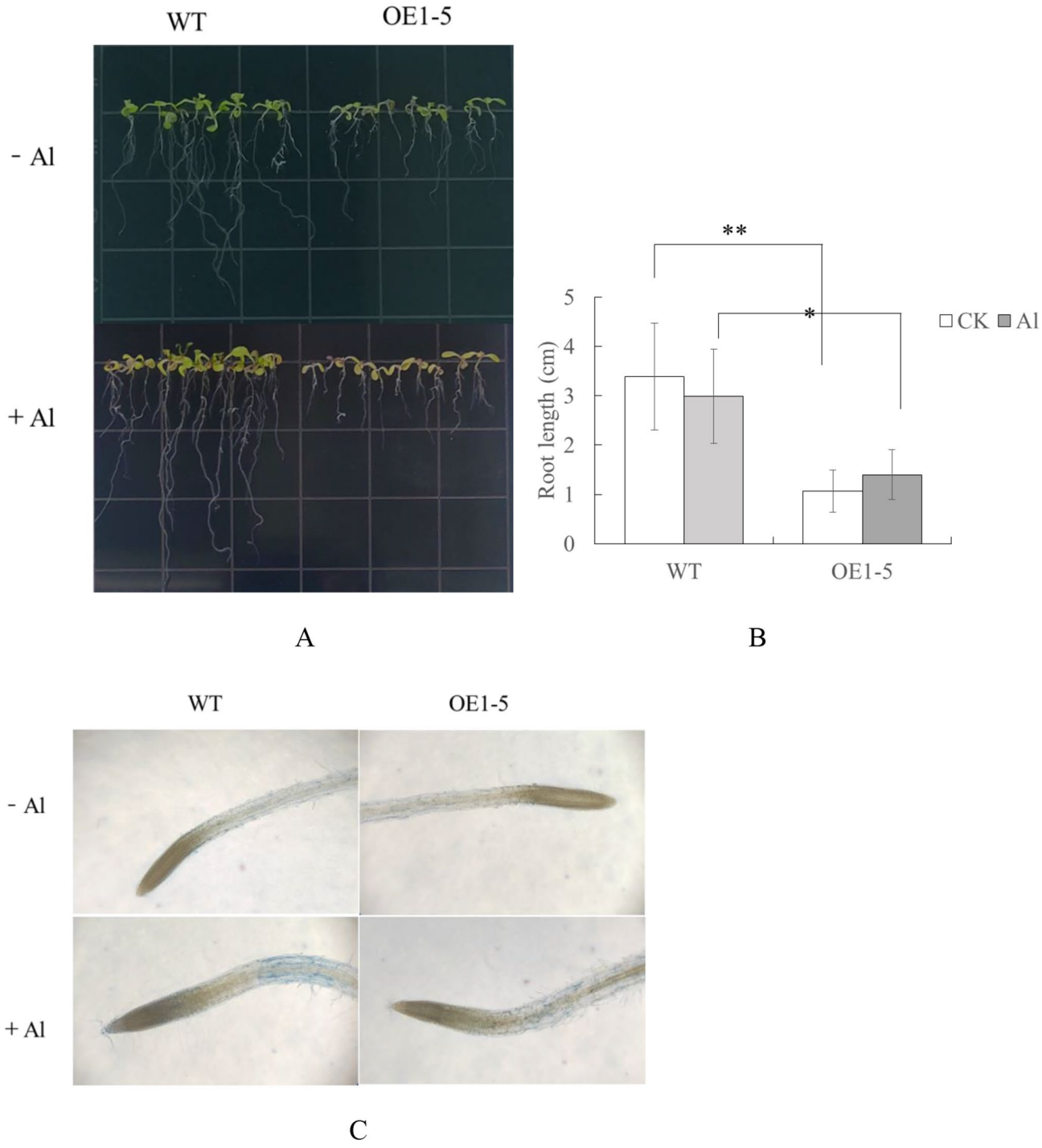


Fig. 11 Effects of Al tolerance in transgenic *Arabidopsis*. **A** and **B** is growth condition and root length of *Arabidopsis* (WT: wild-type, OE1-5: transgenic lines); **C** is trypan blue staining of *Arabidopsis* root

the *Gossypium* genus [2, 6, 23, 32, 33]. In this study, 24 *AhRALFs* were identified in *Arachis hypogaea*. Campbell and Turner [2] found a positive correlation between the number of RALF genes and genome size in most plants, with genome duplication driving the expansion of RALFs. Throughout the evolution of land plants, RALF family members have diversified significantly. Tandem duplication plays a dominant role in RALF evolution [2, 3], while Jia and Li [21] demonstrated that segmental duplication primarily drives the expansion of the RALF gene family in legume species. In this study, WGD or segmental duplication was the main expansion type of *AhRALFs*,

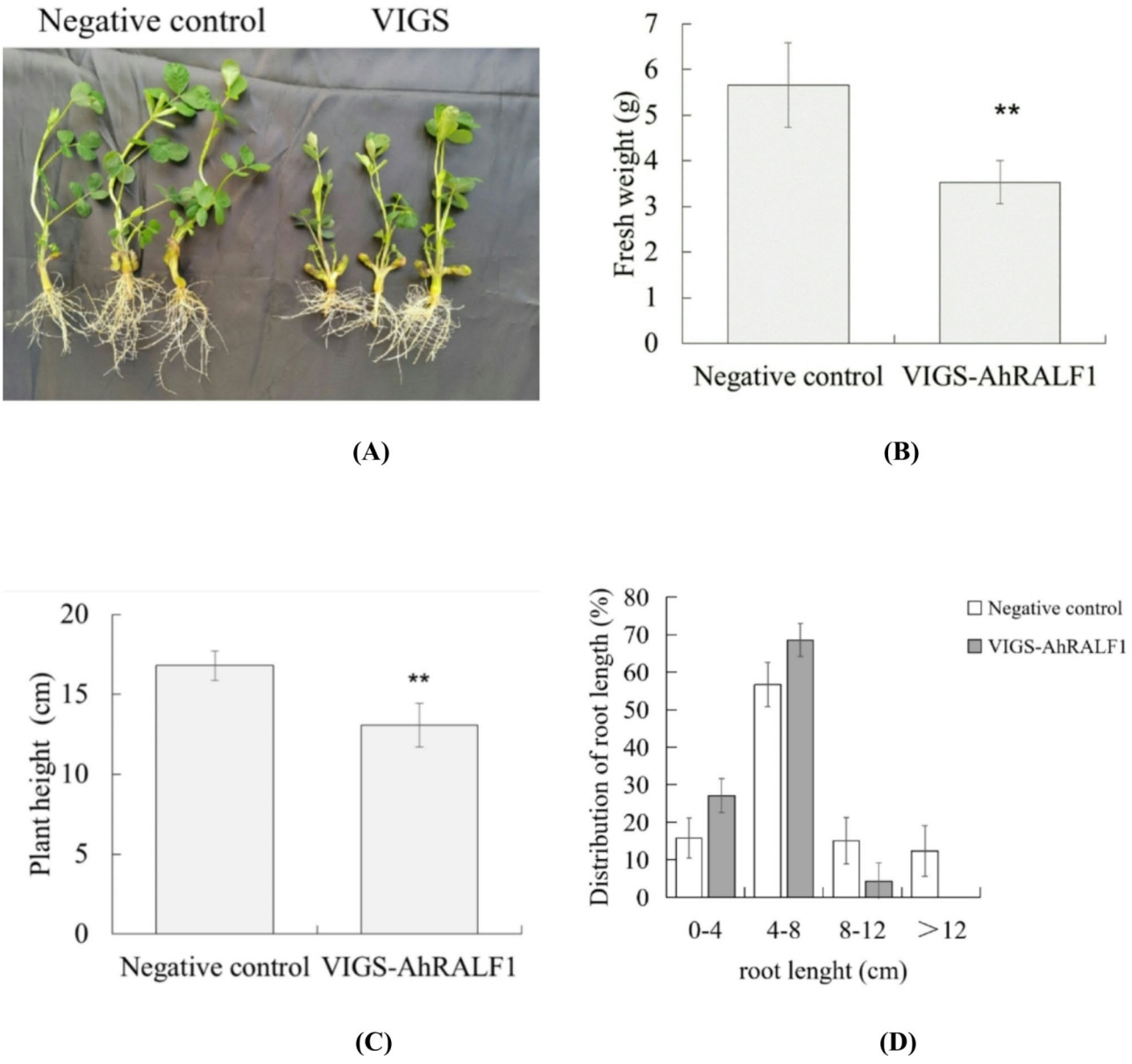
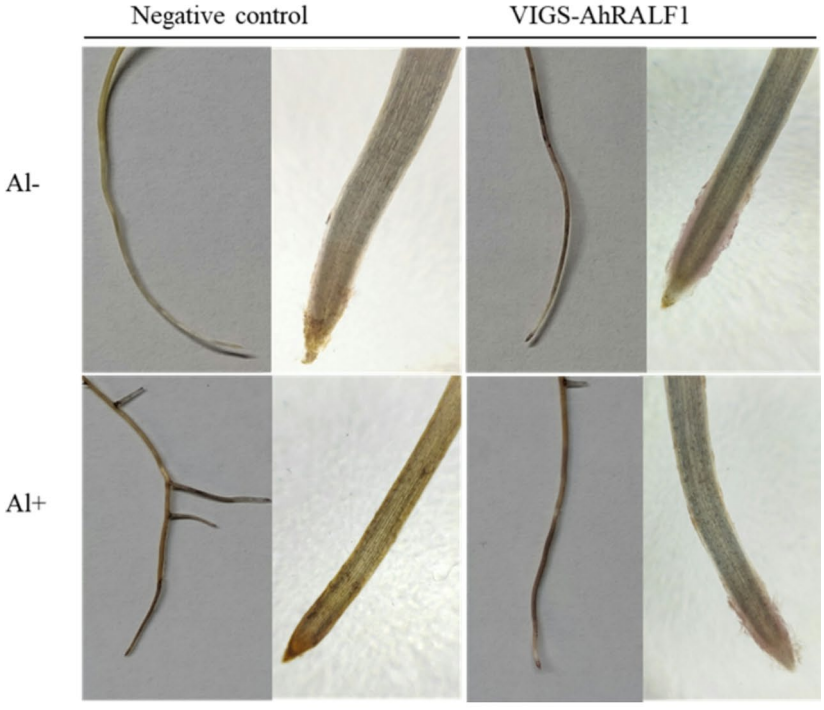


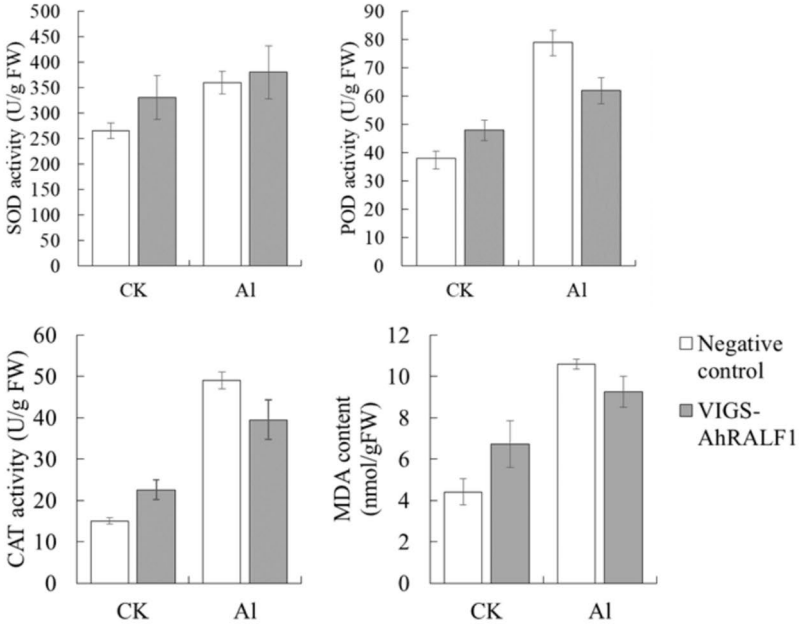
Fig. 12 Phenotype (**A**), fresh weight (**B**), plant height (**C**), and distribution of root length (**D**) analysis of Negative control and VIGS-AhRALF1 peanut seedlings

occurring approximately 25 million years ago. Paralogous gene pairs and clusters were prevalent among *AhRALFs*, suggesting that genome duplication events contributed to the majority of *AhRALFs* in peanuts. This may relate to the origin of cultivated peanuts through polyploidization and continuous selection in breeding, leading to a highly conserved genome. Phylogenetic analysis split the 61 RALF genes from peanut and *Arabidopsis* into four clades, with *AhRALFs* present in clades I–III but absent in clade IV, indicating species differentiation before the emergence of clade IV in *Arabidopsis*.

Alkaline proteins typically have negative (alkaline) charges and an average isoelectric point (pI) of 8.37 [34, 35]. *AhRALFs* have a higher average pI value of 8.82, suggesting stronger alkaline activity. The conserved recognition site (RRIL) for plant subtilisin-like serine protease S1P is essential for proper processing of RALF propeptides to acquire alkalinization activity and inhibit plant immunity [8, 36, 37]. The RR motif is present in all RALF precursor proteins of clades II and III in peanuts but absent in clade I members, suggesting that clade I *AhRALFs* may require additional maturation processing through a mechanism independent of RRXL. It remains to be determined whether these genes are involved in immune modulation and alkalinization during plant growth and symbiosis. The YISY motif near



(A)



(B)

Fig. 13 Al tolerance analysis of peanut *AhRALF1*-silenced lines. (**A**) Hematoxylin staining of peanut roots treated with 0.1mmol/L Al for two days. The staining highlights the extent of root damage caused by Al stress, comparing *AhRALF1*-silenced lines with wild-type plants (**B**) Analysis of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as the content of malondialdehyde (MDA) in peanut leaves treated with 0.1mmol/L Al for two days

the N-terminus and the C-terminal RGC(5 N)C motif at the C-terminus are conserved in mature RALF peptides and are necessary for their biological functions and receptor binding [10, 12, 38]. The C-terminal RGC(5 N) C motif is highly conserved in all *AhRALFs*, and the YISY motif is present in all clade II *AhRALFs* and 83.33% of clade III *AhRALFs*, indicating their functionality as RALF peptides.

AhRALFIs might pseudogene that has no bioactivity

The typical features of RALFs include the RRXL cleavage site, YISY motif, and four conserved cysteines at the C-terminus. In this study, these features were observed in all members of clades II and III. However, in clade I, although all members contained the signal peptide and four conserved cysteines, the S1P cleavage site and YISY motif were absent, replaced by XXIF and VIXN motifs. These clade I genes were not expressed in any of the 18 tissues examined nor under Al stress. RALF23 and RALF33 are activated by proteolytic cleavage following pathogen-induced immune responses [8], whereas RALF17 and RALF32, which lack a canonical S1P processing site, cannot inhibit ROS generation in RALF-mediated immune responses [8]. Some RALFs without the RRXL motif can still prevent multiple pollen tubes from targeting the ovule [16]. Whether clade I *AhRALFs* respond to other signals or are expressed in specific, unexamined organisms remains unclear. Atypical RALFs lacking the four conserved cysteines, YISY motif, and/or canonical RRXL are widely distributed in plants [2]. There is still much to learn about the mechanisms and roles of these unusual RALFs.

AhRALFs are important for the growth and development of peanuts

Members of the RALF family play crucial roles in various physiological and developmental processes, including pollination [39], root elongation [6], root hair polar growth [13], regulation of flowering time [40], responses to salt stress [9, 41], and modulation of immune responses [31]. Our findings indicate that *AhRALF11* and *AhRALF24*, paralogous gene pairs from clade II, show high expression levels in immature buds and flowers. Phylogenetic studies place these genes in the same clade as *RALF4* and *RALF19*. It has been demonstrated that *RALF4* and *RALF19* are ligands for ANXUR1 (ANX1), ANX2, and BUDDHA'S PAPER SEAL (BUPS) 1 and 2, essential for sperm release, pollen tube growth, and integrity maintenance [42, 43]. Despite their different origins, genes within the same cluster can perform similar biological activities [44], suggesting that *AhRALF11* and *AhRALF24* may also modulate pollen tube growth. Another paralogous gene pair from clade II, *AhRALF1* and *AhRALF12*, are evolutionarily closer to *RALF23* and *RALF33* of *Arabidopsis*. Besides regulating root growth, *RALF23* and *RALF33* are involved in plant immune signaling and pollen hydration and germination in a FER-dependent manner [8, 39, 45]. In this study, *AhRALF1* and *AhRALF12* showed high expression levels across 18 examined tissues. The most studied RALF member in *Arabidopsis*, *RALF1*, is involved in various physiological functions, including hormone signaling, RNA and energy metabolism, cell development, and monitoring cell wall integrity [46]. Three members of clade III (*AhRALF8*, *AhRALF10*, and *AhRALF21*), which are in the same clade as *RALF1*, were abundantly expressed in most analyzed tissues, indicating their significance in plant growth and development.

The potential role of *AhRALFs* in al stress response

Numerous studies have shown the critical role of RALFs in both biotic and abiotic stress responses. According to He et al. [32], RALFs modulate hormone signaling, which is vital for plant immunity. Zhou et al. [47] demonstrated that *RALF4* and *RALF19* are essential for maintaining cell integrity in response to mechanical stress. Zhao et al. [9] found that *RALF22/23*, FER, and LRX3/4/5 proteins form a signaling pathway crucial for salt resistance. However, there is limited research on the role of RALFs in Al tolerance. In this study, two clade II members (*AhRALF1* and *AhRALF12*) and five clade III members (*AhRALF3*, *AhRALF8*, *AhRALF13*, *AhRALF15*, and *AhRALF19*) were significantly inhibited by Al stress. Considering the findings from cis-regulatory element analysis, which showed elements generally related to plant stress responses, we deduced that these genes react to Al stress. qRT-PCR analysis of *AhRALF1*, *AhRALF8*, *AhRALF12*, and *AhRALF15* further confirmed these results.

The secretory function and bioactive of *AhRALF1* were conserved

RALFs are small secreted peptides that act as extracellular signals in plants [1]. The secretory function of RALFs is crucial for their activity. Nearly all RALF propeptides in *Arabidopsis* contain signal peptides. For instance, mature *RALF23*, released into the extracellular space to control growth-retarding activity, is created when PRORALF23 is cleaved by S1P [37]. The secretory function of two GmRALFs, *GmRALF4* and *GmRALF24*, was validated in soybeans [48]. Based on gene structure and qRT-PCR analysis, *AhRALF1* was selected for further investigation. We validated the secretory function of *AhRALF1* using the yeast mutant strain YTK12 system and confirmed its inhibition of root growth through in vitro and in planta assays. These results suggest that *AhRALF1* is involved in various biological processes and exhibits bioactivity similar to well-studied RALFs in other plants.

AhRALF1 enhances al tolerance

RALF peptides are crucial regulators in plant adaptation to various environmental stimuli, such as drought [49], salinity [50], and heavy metal stress [51]. Richter et al. [51] suggested that CrRLK1L receptors, which correspond to RALFs, enhance *Arabidopsis* tolerance to heavy metal stress by forming complexes with demethylated pectin. In this study, we observed that *AhRALF1*-silenced plants exhibited greater susceptibility to Al stress compared to wild-type plants, suggesting a crucial role for AhRALF1 in modulating Al tolerance.

Notably, wild-type plants displayed increased superoxide dismutase (SOD) activity under Al stress, consistent with its known role in mitigating oxidative damage [52]. However, this increase in SOD activity was not observed in VIGS-*AhRALF1* plants, implying that *AhRALF1* silencing alters the antioxidant response. This finding aligns with previous studies demonstrating that RALF peptides regulate antioxidant enzyme activity in response to environmental stresses [51]. Furthermore, the activities of CAT and POD were significantly reduced in VIGS-*AhRALF1* plants under Al stress compared to wild-type plants. Given that both enzymes play essential roles in reactive oxygen species (ROS) scavenging, their decreased activity suggests that *AhRALF1* silencing compromises the plant's ability to manage ROS accumulation, leading to greater oxidative damage. Additionally, MDA levels, an indicator of lipid peroxidation and cellular damage, were significantly higher in wild-type plants under Al stress. In contrast, MDA levels in VIGS-*AhRALF1* plants were comparatively lower, possibly reflecting a weakened oxidative stress response due to disrupted antioxidant regulation.

The results of this study indicate that *AhRALF1* plays a critical role in modulating antioxidant enzyme activities, particularly SOD, CAT, and POD, in response to Al stress. These findings align with previous reports showing that RALF family members interact with receptor-like kinases to regulate ROS levels and antioxidant enzyme activities, thereby influencing plant responses to environmental stressors. Further research should explore the molecular mechanisms through which AhRALF1 interacts with ROS signaling pathways and its potential role in enhancing Al stress tolerance in plants.

Conclusion

In this research, we performed a comprehensive investigation of the peanut *AhRALF* genes, examining their expression levels across various tissues and under Al stress. The secretory function and bioactivity of a candidate Al stress response gene, *AhRALF1*, were analyzed using in vivo and in planta assays. Functional studies revealed that *AhRALF1* plays a significant role in the response of peanuts to Al stress. This work lays the groundwork for further investigations into the role of *AhRALF* genes in Al stress resistance and the underlying mechanisms.

Abbreviations

RALFs	Rapid Alkalinization Factors
TTC	2, 3, 5-Triphenyltetrazolium Chloride
WGD	Whole Genome Duplication
MeJA	Methyl Jasmonate
POD	Peroxidase
CAT	Catalase
MDA	The Content of Malondialdehyde
SOD	Superoxide Dismutase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06356-6.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

We thank Guiyang Watch Biotechnology for providing the advice on data collection and analysis.

Author contributions

QQ analyzed the data and wrote the original manuscript. ZR, FX and QW: carried out the analysis. SF and LS performed the experiments. XD and HL designed the experiments and revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the founding of Natural Science Foundation of Guangxi Province, (Grant No. 2020GXNSFBA297036).

Data availability

In this study, datasets that were publicly accessible were evaluated. Those datasets are available in the NCBI SRA with accession numbers PRJNA484860 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA484860), and PRJNA525247 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA525247). All data evolved in this investigation are included in this published article (Additional file 1).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 9 August 2024 / Accepted: 5 March 2025
Published online: 15 March 2025

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