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Research article

Peanut genes encoding tetrapyrrole biosynthetic enzymes, AhHEMA1 and AhFC1, alleviating the salt stress in transgenic tobacco

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ABSTRACT

Glutamyl-tRNA reductase1 (HEMA1) and ferrochelatase1 (FC1) are both expressed in response to salt stress in the biosynthetic pathway of tetrapyrroles. Peanut (*Arachis hypogaea* L.) HEMA1 and FC1 were isolated by RT-PCR. The amino acid sequence encoded by the two genes showed high similarity with that in other plant species. The AhFC1 fusion protein was verified to function in chloroplast using *Arabidopsis* mesophyll protoplast. Sense and wild-type (WT) tobaccos were used to further study the physiological effects of *AhHEMA1* and AhFC1. Compared with WT, the Heme contents and germination rate were higher in AhFC1 overexpressing plants under salt stress. Meanwhile, overexpressing *AhHEMA1* also led to higher ALA and chlorophyll contents and multiple physiological changes under salt stress, such as higher activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX), lower contents of reactive oxygen species (ROS) and slighter membrane damage. In addition, the activities of CAT, POD and APX in the *AhFC1* overexpressing plants were significantly higher than that in WT lines under salt stress, but the activity of SOD between the WT plants and the transgenic plants did not exhibit significant differences. These results suggested that, peanut can enhance resistance to salt stress by improving the biosynthesis of tetrapyrrole biosynthetic.

1. Introduction

Soil salinization has become the main influencing factor for global agricultural production and ecological environment (Bray et al., 2000; Wang et al., 2003). High salinity seriously affects plant growth and development by initiating osmotic stress, ionic toxicity and oxidative stress (Zhu, 2001). Plants have formed a series of mechanisms including stomatal aperture, osmotic substances synthesis, excess ions compartmentation and reactive oxygen species scavenging to alleviate damage caused by salt stress (Tuteja, 2007; Zhang et al., 2012a,b). Unraveling the mechanism of plant salt resistance will be helpful for improving crop salt tolerance by genetic engineering.

Under salt stress, plant growth regulators (PGRs) is implicated in the physiological changes of plants, particularly with regard to activating the photosynthesis, stomatal movement and various enzymes activities (Hayat and Ahmad, 2007; Fu et al., 2015). 5-aminolevulinic acid (ALA), one of these PGRs, is formed from glutamate through three enzymatic reactions: first glutamate is converted to glutamyl adenylate and then transferred to specific tRNA to form glutamyl-tRNA, then the glutamyl-tRNA reductase (GluTR) catalyzed glutamyl-tRNA to Glu 1-semialdehyde (GSA) in a NADPH-dependent reaction, and then ALA is formed by GSA aminotransferase (Fang et al., 2016). Among these steps, the main regulating enzyme is Glutamyl-tRNA reductases, which is encoded by *HEMA* genes. So far three *HEMA* genes of Arabidopsis (*Arabidopsis thaliana*) and two cucumber (*Cucumis sativus*) *HEMA* genes have been cloned, respectively (Fang et al., 2016; Tanaka et al., 2011).

Abbreviations: ALA, 5-aminolevulinic acid; APX, Ascorbate peroxidase; CAT, Catalase; Chl, Chlorophyll; FC, Ferrochelatase; GSA, Glu 1-semialdehyde; MDA, Measurement of malondialdehyde; Mg, Magnesium; MS, Murashige and Skoog; PGRs, Plant growth regulators; POD, peroxidase; PSII, Photosystem II; REC, Relative electric conductivity; ROS, Reactive oxygen species; RT-PCR, Reverse transcription-polymerase chain reaction; SD, Standard deviation; SOD, Superoxide dismutase

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Both in these two plants, *HEMA1* gene was induced by illumination and preferentially expressed in photosynthetic tissues, but no transcripts were detectable in roots. On the other hand, *HEMA2* gene was found to express preferentially in nonphotosynthetic tissues in a light independent manner. The expression level of *HEMA3* in *Arabidopsis* was not significantly influenced by chilling, light and rhythm treatments (Czarnecki et al., 2011).

Aminolevulinic acid (ALA) is an important precursor necessary for heme and chlorophyll (Chl) synthesis in the tetrapyrrole biosynthesis pathway. One of the strict control points in the biosynthesis pathway is the branch point between heme and Chls. The protoporphyrin IX (Proto IX) serves as the common substrate at this branch (Nagahatenna et al., 2015). Insertion of Mg^{2+} into Proto prefers the branch of chlorophyll formation, on the contrary Fe^{2+} instead of Mg^{2+} was inserted into Proto leads to heme synthesis, catalyzed by Ferrochelatase (FC). To date, two groups of FC, 'FC1 type' and 'FC2 type', were identified. FC1 includes *Arabidopsis,* cucumber and barley ferrochelatase-1, whereas FC2 clusters mainly from cucumber, potato, rice and cyanobacteria (Suzuki et al., 2002). The *FC1* gene accumulate predominantly in roots relative to leaves and stems, and is light inducible, whereas *FC2* gene is only expressed in aerial parts of the plant (Scharfenberg et al., 2015). Although FC1 and FC2 share 83% similarity at the nucleotide level and 69% identity at the amino acid level, phylogenetic analysis revealed that FC1 and FC2 belong to distinct ferrochelatase family found in plants. Because FC1 and FC2 were both located only in the plastids, it is speculated that heme is mainly synthesized in plastids. Heme, as an essential plastid signal, is responsible for the signal transduction from chloroplast to cytoplasm and modulating the transcription factors necessary for stress tolerance (Woodson et al., 2011). However, how the functional heme is transported to the correct subcellular destination is still not known.

Heme plays a vital role in ROS detoxification as well as (1) the cofactor of cytochromes in respiratory chains (2) functions in oxidation process, playing roles in activating the ROS-related gene expression (3) a protein involved in oxygen homeostasis (4) activates the heme-activated transcription factors (HA-TFs), which was regulated by heme, including the nuclear factor family at the post transcriptional level. Taken together, the mechanism of *FC1* gene initiating salt acclimation is probably related to oxidative stress.

Peanut (*Arachis hypogaea* L.), as an important oil crop and nutrient, is mainly cultivated in Asia and Africa, with the largest production in China. Saline-alkali soil is an important factor limiting peanut production, thus identifying the mechanisms of peanut salt resistance is important. It has been reported that the growth of peanut such as the photosynthetic activity, osmotic adjustments, integrity of pigment content and plant membrane were disturbed under soil salinity and water-deficit complex abiotic stresses (Yadav et al., 2012; Datta et al., 2012; Sarkar et al., 2014). *HEMA1* and *FC1*, two key genes in the Chlorophyll synthesis pathway, were first isolated from peanut in this work. It was uncertain that whether these two genes could function in enhancing the tetrapyrrole biosynthesis and thus improve salt resistance. In addition, the physiological and biochemical mechanism to promote salt tolerance by these two genes is also unknown. Here, we constructed gene overexpression vector and transformed with tobacco plants to discuss the protective mechanism under salt stress condition.

2. Materials and methods

2.1. Plant materials, growth and treatments

Peanut (*Arachis hypogaea* L. cv. Huayu 22) and tobacco (*Nicotiana tabacum* cv. NC89) were used in this study. Tobacco seeds were sown on Murashige and Skoog (MS) medium after disinfection by ethanol and sodium hypochlorite. After germination, seeds were transplanted in aseptic soil and grown in the greenhouse for 4 weeks. For the salt-induced assay, peanut plants were treated with 200 mM NaCl solution for 3, 6, 12 and 24 h on peanut and qRT-PCR were performed. For the physiological indicators measurement experiments, preliminary experiments were carried out to find out the optimum salt concentration and treatment time. Moreover, FC functions downstream of HEMA in the signal pathway, thus salinity stress was performed by treating the seedlings with 200 mM NaCl solution for 24 (*AhHEMA1*) or 48 h (*AhFC1*) on tobacco plants. The treated plant leaves were frozen in liquid nitrogen and stored at −70 °C.

Table 1
Primer sequences used in cloning and expression analysis.

HEMA-F	5′-ATGGCTGTTTCGACGAGCTT-3′
HEMA-R	5′-GGAGAGTTGTTGATTCAGAAT-3′
FC-F	5′- AGGCTTGTGGTGCTACCGCTTT-3′
FC-R	5′- GCATCTGCCAGGTCTGTAATGAAGG-3′
TUA5-F	5′-CTGATGTCGCTGTGCTCTTGG-3′
TUA5-R	5′-CTGTTGAGGTTGGTGTAGGTAGG-3′
Actin-F	5′-TGGACTCTGGTGATGGTGT-3′
Actin-R	5′-TCAGCAATACCAGGGAACAT-3′

2.2. Isolation of AhFC1 and AhHEMA1

Total RNA was extracted from peanut leaves by Trizol reagent (Invitrogen, Shanghai, China) and used for reverse-transcription polymerase chain reaction (RT-PCR). gDNA Eraser was used to remove DNA from 1 μg sample of RNA at 42 °C for 2 min, and then the transcription reaction mix was added and incubated at 37 °C for 15 min and terminated at 85 °C for 5 s. To isolate the *FC1* gene from peanut, a 605 bp fragment was amplified from cDNA using primers FC-F and FC-R (Table 1) and cloned into the pEASY-T simple. The 5′- and 3′-fragments of *FC1* gene were obtained from cDNA using RACE Kit (TAKARA).

The full-length DNA sequence of the *AhHEMA1* gene was amplified by PCR using specific primers HEMA-F and HEMA-R according to the sequence from wild peanut (Table 1). The primers used in this study were synthesized by Sangon Biotech Company (Shanghai, China).

2.3. Plasmid construction and Agrobacterium mediated transformation of tomato plants

The full-length of *AhFC1* and *AhHEMA1* cDNA were cloned into the expression vector pCAMBIA2300-35S-OCS downstream of the 35S-CaMV promoter to form constructs. The 35S-CaMV-AhFC1 and 35S-CaMV-AhHEMA1 constructs were respectively introduced into *Agrobacterium tumefaciens* LBA4404 by the freezing transformation method and verified by PCR and sequencing. Leaf disk transformation using wild type plants was performed as described by Horsch et al. (1985). Disks infected with *A. tumefaciens* were incubated on a medium for inducing leaves. After a few weeks, the regenerated tissues were transferred to the medium for inducing roots. Both media contained cefotaxime sodium (250 mg mL^{-1}) and kanamycin (50 mg mL^{-1}). Transgenic plants were screened by semiquantitative RT-PCR and fluorescent quantitative real time RT-PCR.

2.4. Subcellular localization of AhFC1

The specific primers 5′-GGATCCTATTCTATGACCATGAACGCC-3′ and 5′-GTCGACGATTAGATTC CTGAATGCCGT-3′ were used to amplify the full-length cDNA of *AhFC1*. Two DNA constructions (p35S-GFP and p35S-AhFC1-GFP) were constructed to investigate the intracellular targeting of AhFC1 using transient expression in *Arabidopsis* mesophyll protoplasts. The complete coding region of AhFC1 was subcloned into the p35S-GFP vector between the BamHI and SalI sites, upstream and in frame with the green fluorescence protein (GFP) coding region. Arabidopsis mesophyll protoplasts were isolated, transfected with the above two constructions (Duan et al., 2012) and examined by dual channel confocal microscopy (LSM510 META, Zeiss, Germany).

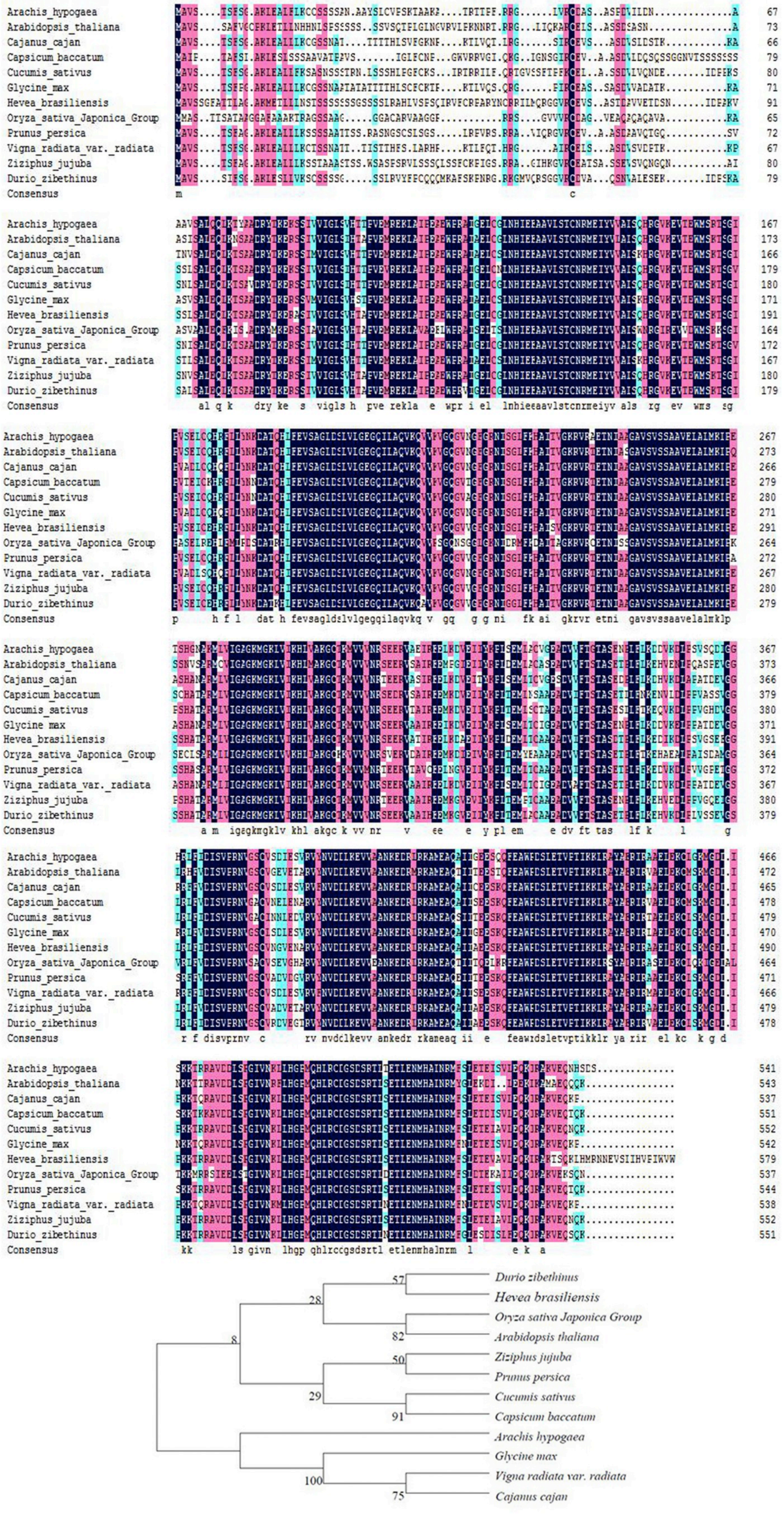


Fig. 1. Alignment of deduced amino acid sequences of HEMA1 from different plant species. The alignment was done using DNAman.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA extraction and reverse transcription was performed as previously described. qRT-PCR was performed on a ABI 7500 Real-time PCR System using SYBR Real Master Mix (Takara, Dalian) with the following PCR thermal cycle conditions: denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and 68 °C for 10 s *AhTUA5* was used as the actin in peanut while *NtActin* was used in tobacco. Template-free, negative, and single primer controls were established before the examination. The results were represented by three biological replicates (each with three technical replicates) for each sample, and a standard curve method was used for statistical analysis.

2.6. Determination of ALA and chlorophyll

0.5 g seedlings were incubated in 5 mL 50 mM Tris-HCl buffer (pH 7.2) which containing 40 mM levulinic acid for 4 h and centrifuged at 12,000 × *g* for 15 min. Repeated the above experiment once again and combined the supernatant of the two experiments to make a constant volume at 20 ml. The assay mixture consisted of 5 mL of supernatant, 2.35 mL of sodium acetate, and 0.15 mL of acetylacetone. The assay medium was mixed and heated in a boiling water bath for 10 min. The extract was then cooled at room temperature, and an equal volume of modified Ehrlich's reagent was added and vortexed for 2 min. Using distilled water as the control, the OD value of the reaction solution was measured at λ 553 nm (Morton, 1975).

Leaf chlorophyll were extracted with 95% ethanol and determined according to Arnon (1949).

2.7. Determination of heme content

HCl-Acetone method was used to extract heme from tobacco leaves, and quantification of the heme content in the extracts followed the protocols of Masuda and Takahashi (2006) and Takahashi and Masuda (2009).

2.8. Measurement of the activities of antioxidant enzymes and the content of reactive oxygen species

Leaf tissue (0.5 g) was grinded in phosphate buffer (PH7.8) containing 0.1 mM EDTA and 1% (g mL^{-1}) PVP. After centrifugation for 10 min at 4 °C, supernatants were used as enzyme extraction. When different reactants were added, SOD, CAT, APX and POD were measured, respectively (Giannopolitis and Ries, 1977; Aebi, 1984; Jimenez et al., 1997). For detection of H_2O_2, 0.5 g leaves were grinded with 3 mL precooled acetone, 2000 g centrifugation for 10min, the supernatant was deposited with 0.1 mL 5% titanium tetrachloride ($TiCl_4$) and concentrated ammonia. The deposition was washed 3–5 times with acetone then dissolved with 5 mL 2 mol $L^{-1}H_2SO_4$ completely. The H_2O_2 content was obtained by the standard curve at λ 415 nm colorimetric (Sairam and Srivastava, 2002). The assay for $O_2^{\bullet -}$ content was determined according to Wang and Luo (1990).

2.9. Measurement of malondialdehyde (MDA) and the relative electric conductivity (REC)

For measurement of MDA content, leaves (0.5 g) were grinded with liquid nitrogen in a cold mortar and fixed capacity to 5 mL with 0.05 mol L^{-1} pH7.8 phosphate buffer. After centrifugation at 4500 rpm for 10 min, 2 mL supernatant with 3 mL reagent (0.5% (m/v) TBA dissolved in 5% (m/v) TCA) was mixed, heated at 100 °C for 10 min, and then quickly cooled and centrifuged at 4500 rpm for 10 min. The absorbance of supernatant was determined at 450, 532, and 600 nm and MDA content was calculated using formula. MDA concentration (μmol L^{-1}) = 6.45 × (OD_{532}-OD_{600})-0.56 × OD_{450} and the MDA content (μmol g^{-1} FW) = MDA concentration*Extraction volume/Fresh weight of plant tissue.

0.3 g thin leaves (0.1 cm wide) from each line were immersed in 10 mL distilled water, vacuumed for 30 min, and then surged for 3 h to measure the initial electric conductivity (S1). After boiling for half an hour, the material is cooled to room temperature to measure the final electric conductivity (S2). The relative conductivity was calculated by the formula: REC = (S1-S0)/(S2-S0) × 100.

3. Results

3.1. Identification and characterization of AhFC1 and AhHEMA1

The full-length of *AhHEMA1* sequence was composed of 1626 bp nucleotides encoding 542 amino acids. Sequence alignment results indicated that the homology of Glutamyl-tRNA reductase in different species was high, and the similarity rate is 79.76% (Fig. 1). The FC1 middle fragment was cloned from peanut leaves by RT-PCR according to the sequence alignment results. Then 5′ and 3’ RACE were experimented respectively using TAKARA Kit. At last these obtained sequences were stitched to obtain the full-length sequence of the *FC1* gene, which was proved to belong to the *FC1* gene family by blasting in GenBank. Its open reading frame had 1449 bp and encoded a 483 residue polypeptide (Fig. 2).

3.2. AhFC1 targeting in chloroplast

In order to demonstrate the subcellular localization of AhFC1 protein, p35S-FC1-GFP fusion vector was constructed and transformed into *Arabidopsis thaliana* protoplast from leaf tissue (Fig. 3). As shown in Fig. 3A–D, the protoplasts transfected with p35S-GFP (only expressing the GFP coding sequence) as the control cells exhibited spontaneous red fluorescence, and the cytoplasm showed green fluorescence. The red and green fluorescence could not colocalize with each other. In contrast, the protoplasts transformed with p35S-AhFC1-GFP fusion protein showed red and green fluorescence, and these two fluorescences overlapped in chloroplasts. These results indicated that the fusion protein was localized in chloroplasts.

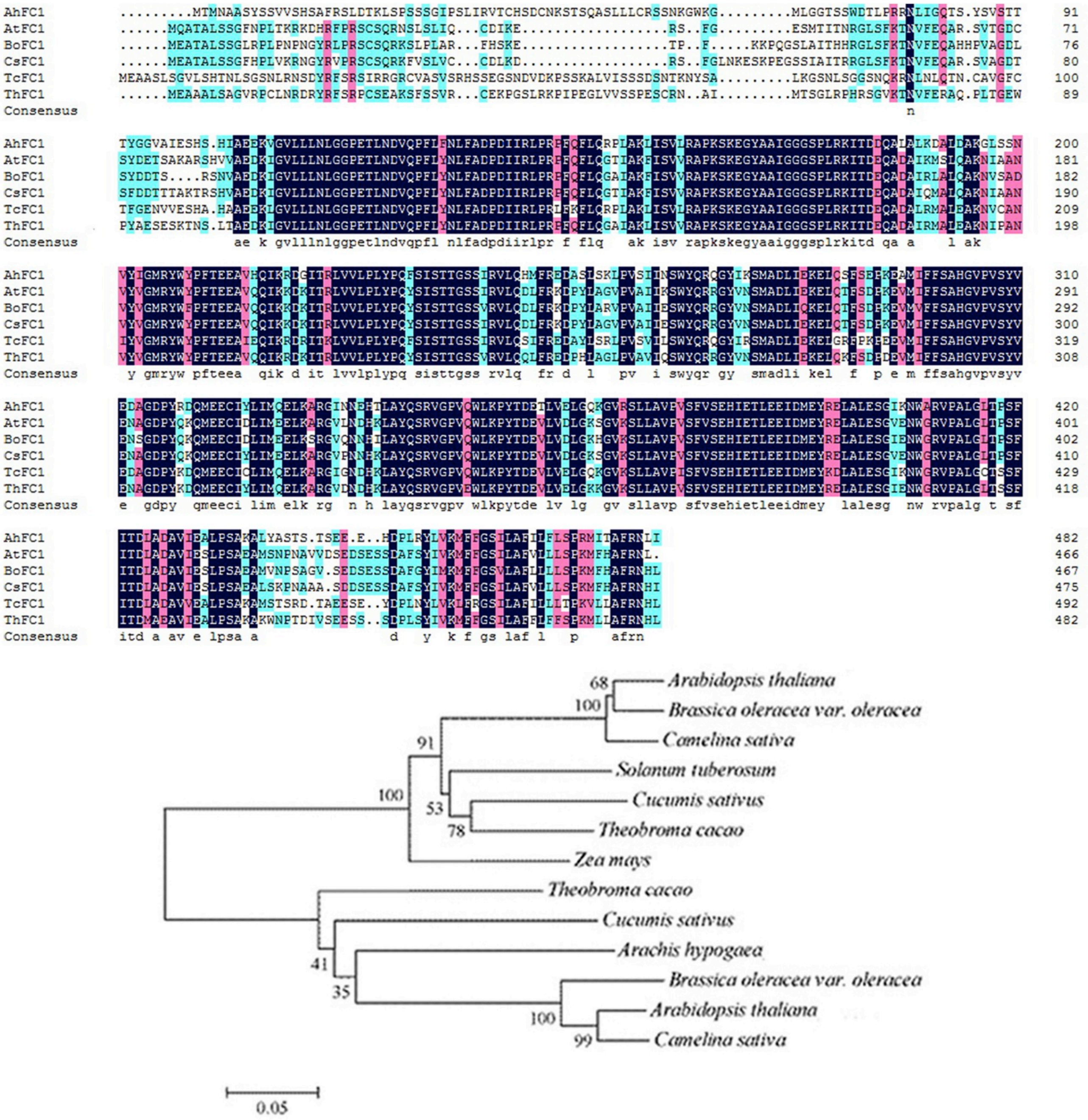
3.3. Expression parttern of AhHEMA1 and AhFC1 in peanut and tobacco plants

The transcript levels of *AhHEMA1* and *AhFC1* under salt stress were shown in Fig. 4. The level of *AhHEMA1* expression reached the maximum after treated with 200 mM NaCl for 3 h (Fig. 4a) while transcript of *AhFC1* at 12 h and then decreased (Fig. 4b).

29 (15 *AhHEMA1* lines, 14 *AhFC1* lines) transgenic tobacco lines can grow normally under tissue culture with kanamycin. These plants with kanamycin-resistant were named T_0 and the next generation obtained from T_0 were named T_1. Eight and five T1 lines were selected respectively for semiquantitative RT-PCR and fluorescent quantitative real time RT-PCR. The relative expression levels of *AhHEMA1* and *AhFC1* in the examined lines were obviously higher than that in WT (Fig. 5). These results indicated that *AhHEMA1* and *AhFC1* genes had been successfully integrated into the tobacco genome, respectively and were highly enhanced in transgenic lines.

3.4. Overexpression of AhFC1 promotes heme synthesis

Ferrochelatase takes part in catalyzing the heme synthesis. Overexpressing *AhFC1* in tobacco could increase the content of heme. After salt stress, the heme content in both transgenic plants and WT tobacco decreased. However, the heme contents of L2 and L6 were still higher than that of WT, which were 1.7 and 1.5 times of WT, respectively (Fig. 6).



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AhFC1     .........MTMNAASYSSVVSHSAFRSLDTKLSPSSSGIPSLIRVTCHSDCNKSTSQASLLLCRSSNKGWKG.........MLGGTSSWDTLPRRNLIGQTS.YSVSTT   91
AtFC1     ......MQATALSSGFNPLTKRKDHRFPRSCSQRNSLSLIQ...CDIKE..................RS..FG.........ESMTITNRGLSFKTNVFEQAR.SVTGDC   71
BoFC1     ......MEATALSSGLRPLPNPNGYRLPRSCSQRKSLPLAR...FHSKE..................TP..F.....KKPQGSLAITHHRGLSFKTNVFEQAHHPVAGDL   76
CsFC1     ......MEATALSSGFHPLVKRNGYRVPRSCSQRKFVSLVC...CDLKD..................RS..FGLNKESKPEGSSIAITRRGLSFKTNVFEQAR.SVAGDT   80
TcFC1     MEAASLSGVLSHTNLSGSNLRNSDYRFSRSIRRGRCVASVSRHSSEGSNDVDKPSSKALVISSSDSNTKNYSA.........LKGSNLSGGSNQKRNLNLQTN.CAVGFC  100
ThFC1     ......MEAAALSAGVRPCLNRDRYRFSRPCSEAKSFSSVR...CEKPGSLRKPIPEGLVVSSPESCRN..AI.........MTSGLRPHRSGVKTNVFERAQ.PLTGEW   89
Consensus                                                                                                 n

AhFC1     TYGGVAIESHS.HIAEEKVGVLLLNLGGPETLNDVQPFLFNLFADPDIIRLPRPFQFLQRPLAKLISVLRAPKSKEGYAAIGGGSPLRKITDDQALALKDALDAKGLSSN  200
AtFC1     SYDETSAKARSHVVAEDKIGVLLLNLGGPETLNDVQPFLYNLFADPDIIRLPRPFQFLQGTIAKFISVVRAPKSKEGYAAIGGGSPLRKITDEQADAIKMSLQAKNIAAN  181
BoFC1     SYDDTS....RSNVAEDKIGVLLLNLGGPETLNDVQPFLYNLFADPDIIRLPRPFQFLQGAIAKFISVVRAPKSKEGYAAIGGGSPLRKITDEQADAIRLALQAKNVSAD  182
CsFC1     SFDDTTTAKTRSHVAEDKIGVLLLNLGGPETLNDVQPFLYNLFADPDIIRLPRPFQFLQGTIAKFISVVRAPKSKEGYAAIGGGSPLRKITDEQADAIQMALQAKNIAAN  190
TcFC1     TFGENVVESHA.HAAEEKLGVLLLNLGGPETLNDVQPFLYNLFADPDIIRLPRLFKFLQRPLAKLISVLRAPKSKEGYAAIGGGSPLRKITDEQADALRMALEAKNVCAN  209
ThFC1     PYAESESKTNS.LTAEDKIGVLLLNLGGPETLNDVQPFLYNLFADPDIIRLPRPFQFLQGAIAKLISVVRAPKSKEGYAAIGGGSPLRKITDEQADAIRMALEAKNIPAN  198
Consensus               ae k gvlllnlggpetlndvqpfl nlfadpdiirlpr f flq   ak isv rapkskegyaaigggsplrkitd qa a    l ak

AhFC1     VYIGMRYWYPFTEEAVHQIKRDGITRLVVLPLYPQFSISTTGSSIRVLQHMFREDASLSKLPVSIINSWYQRQGYIKSMADLIEKELQSFSEPKEAMIFFSAHGVPVSYV  310
AtFC1     VYVGMRYWYPFTEEAVQQIKKDKITRLVVLPLYPQYSISTTGSSIRVLQDLFRKDPYLAGVPVAIIKSWYQRRGYVNSMADLIEKELQTFSDPKEVMIFFSAHGVPVSYV  291
BoFC1     VYVGMRYWFPFTEEAVQQIKKDKITRLVVLPLYPQYSISTTGSSVRVLQDLFRKDPYLARVPVAIIESWYQRRGYVNSMADLIQKELQTFSDPKEVMVFFSAHGVPVSYV  292
CsFC1     VYVGMRYWYPFTEEAVQQIKKDKITRLVVLPLYPQYSISTTGSSIRVLQDLFRKDPYLAGVPVAIIESWYQRRGYVNSMADLIEKELQTFSDPKEVMIFFSAHGVPVSYV  300
TcFC1     IYVGMRYWYPFTEEAIEQIKRDRITKLVVLPLYPQFSISTTGSSIRVLQSIFREDAYLSRLPVSVILSWYQRQGYIRSMADLIEKELGRFPKPEEVMIFFSAHGVPVSYV  319
ThFC1     VYVGMRYWYPFTEEAVQQIKRDKITRLVVLPLYPQYSISTTGSSVRVLQQLFREDPHLAGLPVAVIQSWYQRRGYVNSMADLIEKELQKFSDPDEVMIFFSAHGVPVSYV  308
Consensus  y gmryw pfteea  qik d it lvvlplypq sisttgss rvlq  fr d  l   pv  i swyqr gy  smadli kel  f  p e m ffsahgvpvsyv

AhFC1     EDAGDPYRDQMEECIYLIMQELKARGINNEHTLAYQSRVGPVQWLKPYTDETLVELGQKGVRSLLAVPVSFVSEHIETLEEIDMEYRELALESGIKNWARVPALGLTPSF  420
AtFC1     ENAGDPYQKQMEECIDLIMEELKARGVLNDHKLAYQSRVGPVQWLKPYTDEVLVDLGKSGVKSLLAVPVSFVSEHIETLEEIDMEYRELALESGVENWGRVPALGLTPSF  401
BoFC1     ENSGDPYQKQMEECIDLIMEELKSRGVQNNHILAYQSRVGPVQWLKPYTDEVLVDLGKHGVKSLLAVPVSFVSEHIETLEEIDMEYRELALESGIENWGRVPALGLTPSF  402
CsFC1     ENAGDPYQKQMEECIYLIMEELKARGVPNNHKLAYQSRVGPVQWLKPYTDEVLVDLGKSGVKSLLAVPVSFVSEHIETLEEIDMEYRELALESGVENWGRVPALGLTPSF  410
TcFC1     EDAGDPYKDQMEECICLIMQELKARGIGNDHKLAYQSRVGPVQWLKPYTDEVLVELGQKGVKSLLAVPISFVSEHIETLEEIDMEYKDLALESGIKNWGRVPALGCTSSF  429
ThFC1     ENAGDPYKDQMEECIYLIMQELKARGVDNDHKLAYQSRVGPVEWLKPYTDEVLVELGKKGVKSLLAVPVSFVSEHIETLEEIDMEYRELALESGIENWGRVPALGLTSSF  418
Consensus e  gdpy  qmeeci lim elk rg  n h layqsrvgpv wlkpytde lv lg  gv sllavp sfvsehietleeidmey  lalesg  nw rvpalg t sf

AhFC1     ITDLADAVIEALPSAKALYASTS.TSEE.E..HDPLRYLVKMFFGSILAFILFLSPRMITAFRNLI                                              482
AtFC1     ITDLADAVIESLPSAEAMSNPNAVVDSEDSESSDAFSYIVKMFFGSILAFVLLLSPKMFHAFRNL.                                              466
BoFC1     ITDLADAVIESLPSAEAMVNPSAGV.SEDSESSDAFGYIMKMFFGSVLAFLLLLSPKMFHAFRNHL                                              467
CsFC1     ITDLADAVIESLPSAEALSKPNAAA.SDDSESSDAFSYIVKMFFGSILAFVLLLSPKMFHAFRNHI                                              475
TcFC1     ITDLADAVVEALPSAKAMSTSRD.TAEESE..YDPLNYLVKLFRGSILAFILLLTPKVLLAFRNHL                                              492
ThFC1     ITDMAEAVIEALPSAKAKWNPTDIVSEESS..SDPLSYIVKMFFGSILAFFLFFSPKMLLAFRNHL                                              482
Consensus itd a av e lpsa a                d   y  k f gs laf l   p    afrn
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Fig. 2. Alignment of deduced amino acid sequences of FC1 from different plant species. The alignment was done using DNAman.

3.5. Germination of AhFC1 transgenic lines and WT under salt stress

The seeds of transgenic lines L2, L6 and WT were seeded on the 1/2 MS medium containing 0 mM, 150 mM and 200 mM NaCl respectively. After 20 days, there was no significant difference between transgenic tobacco and WT on germination rates under normal growth conditions. While the germination rate of transgenic tobacco and WT decreased under salt stress, however the germination rate of L2 and L6 was still significantly higher than that of WT. When salt concentration was 150 mM, the germination rate of L2 and L6 was 93% and 86%, the wild type was only 65%. When the salt concentration rose to 200 mM, the germination rate of L2 and L6 could reach 22% and 16%, and the wild type was only 7% (Fig. 7).

3.6. Changes of the ALA and chlorophyll contents in AhHEMA1 overexpressing tobacco seedlings

To investigate whether overexpression of *AhHEMA1* affected chlorophyll synthesis process, the contents of ALA and chlorophyll were detected from leaves of tobacco. Under normal growth conditions, ALA contents in H3, H14 and H16 transgenic lines were 1.6 times, 1.3 times and 2 times higher than that in WT, respectively (Fig. 8a). Similarly, the chlorophyll contents of H3, H14 and H16 transgenic lines increased by 26%, 15.1% and 31% compared with WT, respectively (Fig. 8b). After salt stress, ALA contents increased while chlorophyll contents decreased, but the contents of both ALA and chlorophyll in *AhHEMA1* overexpressing plants were still higher than that in WT.

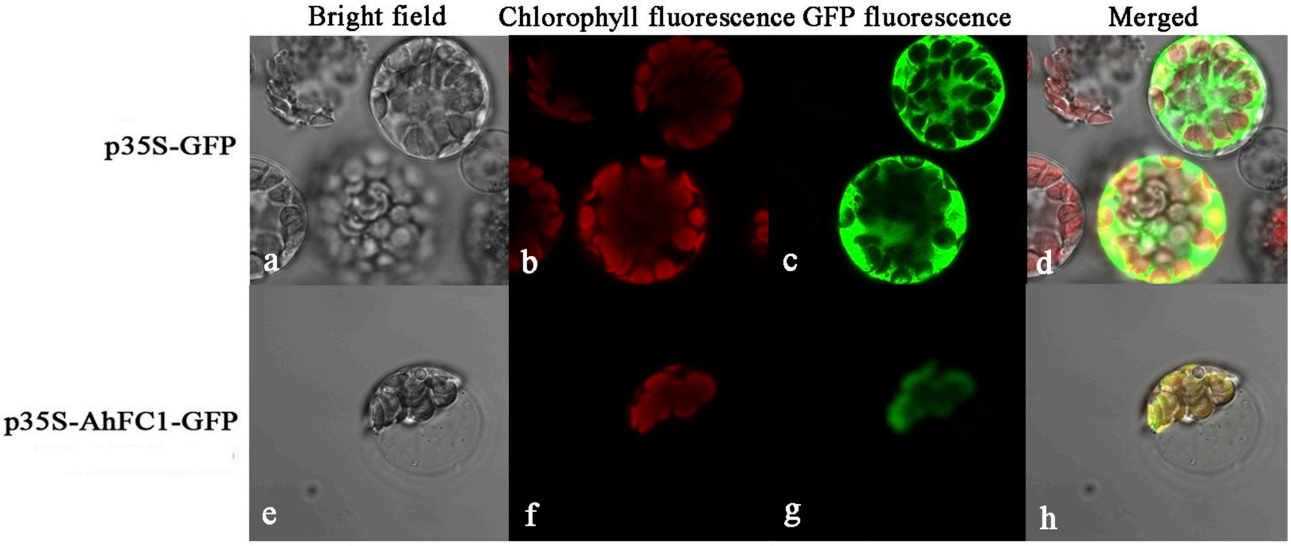

Fig. 3. Targeting of AhFC1 using *Arabidopsis thaliana* protoplasts. (a) and (e) Figures of protoplasts in bright field. (b) and (f) Red autofluorescence of chloroplasts. (c) and (g) Green fluorescence of green fluorescent protein (GFP) and AhFC1-GFP fusion protein, respectively. (d) and (h) Merged images of a, b, c and e, f, g, respectively. Yellow signal results from superposition of the green (GFP) and red (chloroplast) fluorescence in h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.7. ROS accumulation and antioxidase activities

To assess whether chlorophyll synthesis pathway affected ROS production under salt stress, the contents of $O_2^{\bullet -}$, H_2O_2, and ROS scavenging enzymes activities in *AhHEMA1* transgenic plants and WT were examined (Fig. 9). Compared with WT, overexpression of *AtHEMA1* increased the activities of both SOD (Fig. 9a) and APX (Fig. 9c). Accordingly, the contents of $O_2^{\bullet -}$ (Fig. 9b) and H_2O_2 (Fig. 9d) were lower in the transgenic lines under salt stress. The content of $O_2^{\bullet -}$ in H3, H14, H16 and WT increased by 60.1%,53.3%,54.3% and 89.5% of initial values, respectively, while H_2O_2 content increased by 61.7%,71.9%,51.0% and 130.4% respectively at the end of the 24 h stress. The lower accumulation of $O_2^{\bullet -}$ and H_2O_2 was largely due to the high activity of SOD enzymes in transgenic plants, which catalyzed the production of H_2O_2 and O_2, while high APX activity was conducive to scavenging H_2O_2 in chloroplasts. Likewise, the activity of CAT, POD and APX in the tobacco plants overexpressing AhFC1 gene was significantly higher than that of the wild type under salt stress (Fig. 10a, b, c), but the activity of SOD between WT and the transgenic plants did not exhibit significant differences (Fig. 10d).

3.8. Overexpressing AhHEMA1 and AhFC1 protecting membrane from damage under salt stress

ROS-induced lipid peroxidation reactions can attack biological membranes and accelerate the membrane damage. MDA accumulation and REC, which were currently reported as an index of lipid oxidation were determined in this work. As shown in Fig. 11a, the accumulation of MDA increased after salt stress, and the increases were more obvious in the WT and less in the transgenic lines. The MDA content of H3, H14, and H16, which was 11.53 μmol g^{-1} FW,12.16 μmol g^{-1} FW,11.37 μmol g^{-1} FW, respectively, was lower than that of WT, which was 16.23 μmol g^{-1} FW after 24 h salt stress (Fig. 11a). Similar to MDA, the REC of H3, H14 and H16, which was 60.12%,66.12%,58.68%, respectively, was lower than that of WT, which was 76.73% (Fig. 11b). Overexpressing *AhFC1* lines also had lower MDA accumulation and REC compared with that in WT (Fig. 11c and d). These results illustrated that overexpression of *AhHEMA1* and *FC1* alleviated the peroxidation of membrane.

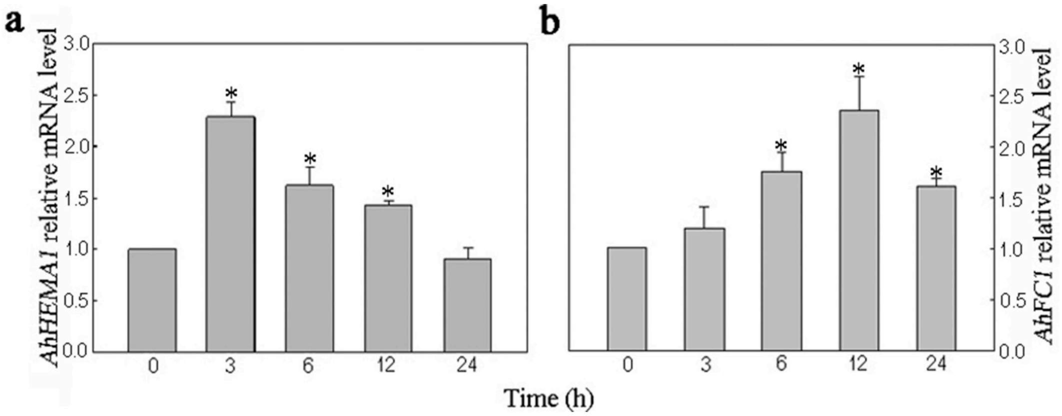

Fig. 4. qRT-PCR analysis of *AhHEMA1* (a) and *AhFC1* (b) expression in peanut leaves. These two genes were induced by salt stress in peanut leaves. The transcript level of *AtTUA5* was used as standard to expression. Error bars represent the SDs of triplicate reactions. Peanut plants were subjected to 200 mM NaCl treatments and the total RNA of leaves was isolated.

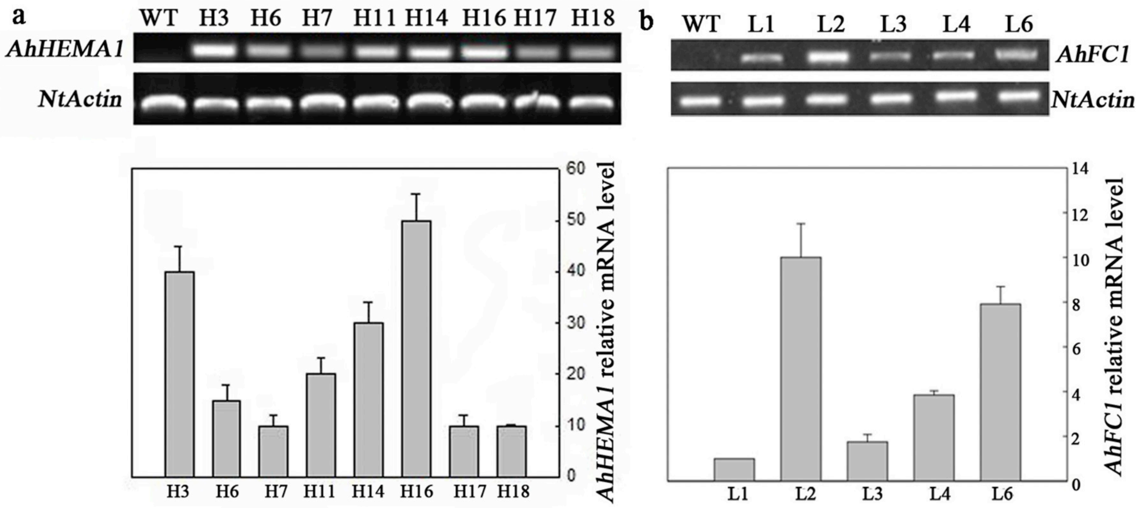

Fig. 5. Analysis of transgenic plants by Fluorescence quantitative PCR and Semiquantitative RT-PCR. Expression of *AhHEMA1* (a) and *AhFC1* (b) in transgenic and WT lines. The transcript level of these two genes was normalized to *Actin* expression. Error bars represent the SDs of triplicate reactions.

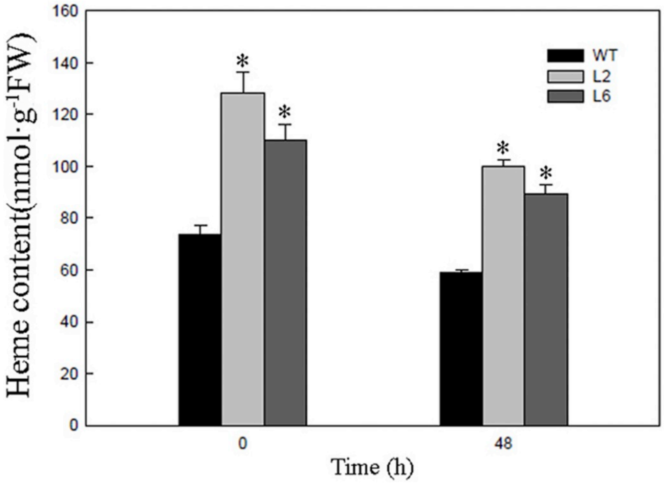

Fig. 6. Effects of salt stress-induced decrease of heme in WT and *AhFC1*-overexpressing tobacco plants. WT and transgenic lines were exposed to 200 mM NaCl for 48 h. Each bar represents the mean from three independent experiments ± SD. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT treatments (*, $P < 0.05$).

4. Discussion

The mechanism of plant response to salinity is a complex and sensitive network controlled by the multiple genes, transcription factors and metabolic pathways. ALA as one of the plant growth regulators was believed to be involved in different biotic and abiotic stresses (Zhang et al., 2012a,b; Sun et al., 2009; Naeem et al., 2011). Similar to the results in this work, the contents of chlorophyll and relative water content as well as CAT activity increased pre-treatment with ALA on soybean, which led to resistance to cold stress (Balestrasse et al., 2010). Tetrapyrroles like hemes and chlorophylls were synthesized from the common tetrapyrrole precursor ALA by successive enzymatic steps. In the present study, *AhHEMA1* and *AhFC1* genes, encoding key enzyme for ALA and heme synthesis, were isolated and characterized. Amino acid sequences analysis revealed that peanut HEMA1 had the highest identity with soybean HEMA1 (Fig. 1) and the results of FC1 protein domain analysis revealed that there was no chlorophyll a/b binding domain, thus it was identified as FC1 rather than FC2. The analysis of amino acid sequence exhibited that AhFC1 was highly similar to other members of this family and its conserved amino acids was clearly showed in Fig. 2. Fig. 3 showed that AhFC1 was localized in chloroplasts and functioned for the production of heme as well as haemoproteins, which were responsible for respiratory cytochromes and defence responses (Scharfenberg et al., 2015). Both *AhHEMA1* and *AhFC1* genes were preferentially induced by salt stress, the difference was that the expression level of *AhHEMA1* gene reached its maximum after 3 h of salt treatment and that of *AhFC1* reached its peak at 12 h (Fig. 4). Whether these two genes in peanut regulate tetrapyrrole biosynthesis and improve the salt resistance need to be discussed.

In order to further confirm the functions of *AhHEMA1* and AhFC1 under salt stress, *AhHEMA1* and *AhFC1* genes were ectopically transformed into tobacco (Fig. 5). Previous study has been showed that overexpression of *Bradyrhizobium japonicum* FC in rice plants resulted in an increasing level of heme and resistance to oxidative stress caused by accumulating porphyrins (Kim et al., 2014). In *AhFC1*-expressing transgenic tobacco, the increased activity of Heme led to enhanced resistance to salt stress, as indicated by alleviation of seedling growth inhibition and germination rate (Figs. 6 and 7). Higher levels of FC activity resulted in high accumulation of heme and Proto IX, but lower chlorophyll content indicated that these excess amounts of Proto IX were shunted away from the Mg branch by FeCh1 (Woodson et al., 2011; Franklin et al., 2003). Treatment of *Chlamydomonas* with exogenous heme can activate the expression of *HEMA* encoding glutamyl-tRNA reductase (Vasileuskaya et al., 2005). In this study, overexpression of *AhHEMA1* in transgenic tobacco could effectively result in a prominent increase in ALA biosynthesis, with higher chlorophyll contents (Fig. 8). It is proposed that a modulated ALA and chlorophyll biosynthesis by *AhHEMA1* expression may influence levels of ROS, which could either improve ROS signaling or scavenging by antioxidants (Fig. 9).

Reactive oxygen species (ROS) are considered as toxic byproducts induced by salt stress. Once excessive ROS can't be scavenged by the scavenging enzymes, excess ROS produced and attack the plasma membrane (Yang et al., 2015). Consequently, membrane leakage occurred. Suitable level of ROS in plant cells can remain harmless. Low concentrations of ROS can activate salt-stress response, however, high concentration of ROS damage DNA, lipids, proteins, and carbohydrates (Miller et al., 2010). ROS scavenging system were involved in maintaining cellular ROS homeostasis under various stress conditions (Yang and Guo, 2018).

ROS was always induced as secondary stresses under salt stress. Accordingly, plants have evolved nonenzymatic and enzymatic defense systems to ease ROS stress (Gill and Tuteja, 2010). Among them,

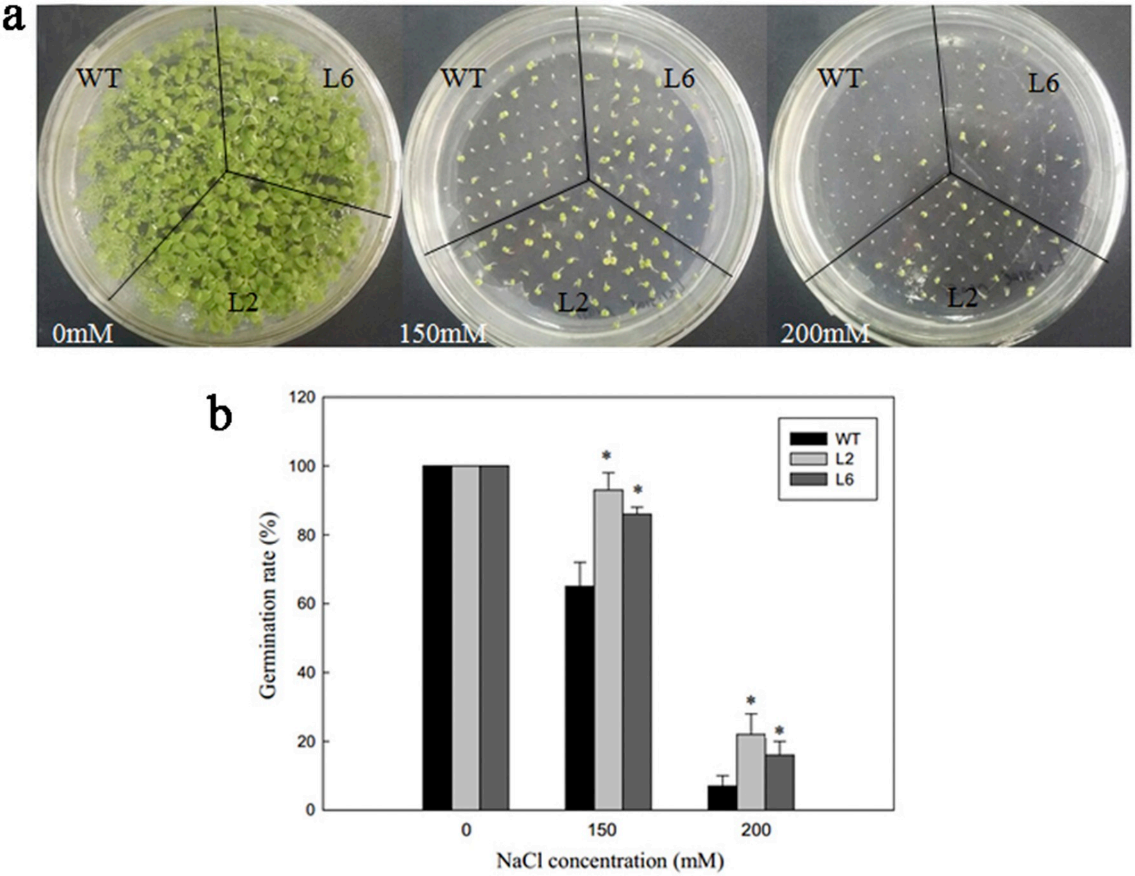

Fig. 7. Germination of the selected 15-day-old WT and *AhFC1*-overexpressing tobacco plants treated with salt stress. A control plant and two transgenic plants (L2 and L6) were grown on the 1/2 MS with different concentration of NaCl (a), respectively. The germination rate was also shown (b) and asterisk marks a significant difference to wild-type ($P < 0.05$).

enzymatic scavengers were mainly comprised of ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), and peroxidase (POD, EC 1.11.1.7) (Begaramorales et al., 2015; Wang et al., 2017). SOD, catalyzes the disproportionation of superoxide radicals $O_2^{\cdot -}$, is the most effective scavenger of reactive oxygen species and plays an important role in the self-protection system under environmental stress (Das and Roychoudhury, 2014). Thus removing ROS either directly or indirectly via accumulating ROS scavengers may be an effective measure to protect plants from salt stress. In our study, whether tobacco plants overexpressing *AhHEMA1* or *AhFC1* transgenic tobacco plants could enhance the adaptability of plants to salt stress probably depends on activating reactive oxygen scavenging enzyme defense system, thus scavenging excessive reactive oxygen species. These results were largely due to that heme, synthesized by FC2 could be used as a prosthetic group to participate in oxidative homeostasis (Khan and Quigley, 2011).

It was interesting that the CAT and APX activities in *AhFC1* transgenic lines L2 and L6 increased significantly compared with wild-type tobacco, but there was no significant difference in SOD enzyme activity between WT and transgenic lines (Fig. 10). One reason for this might be associated with overexpressing *AhFC1* in tobacco induced the increase of heme (Phung et al., 2011). Heme is the cofactor of CAT and APX. It has been reported that overexpression of *OsPPO* increased the content of heme and the expression level of *CatA, CatC, APXb* simultaneously.

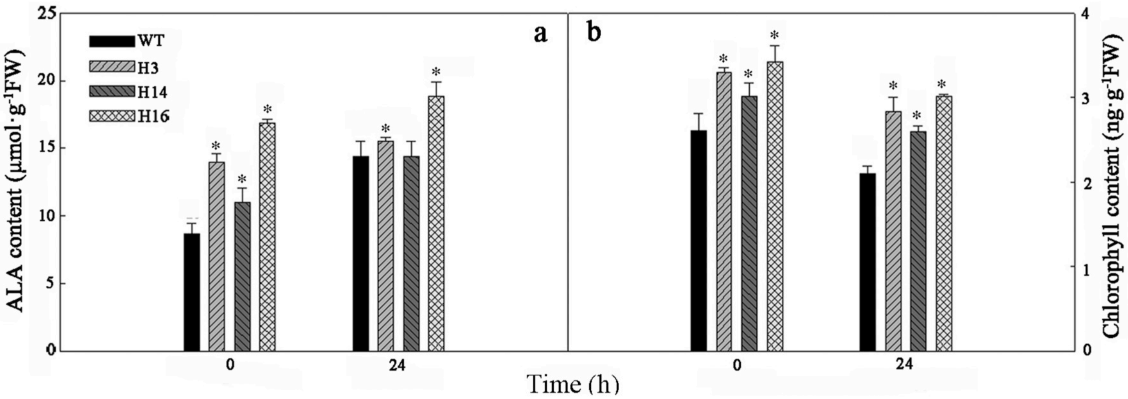

Fig. 8. ALA and chlorophyll (a and b) contents of tobacco wild-type and *AhHEMA1*-overexpressing plants. Asterisk marks a significant difference to wild-type ($P < 0.05$).

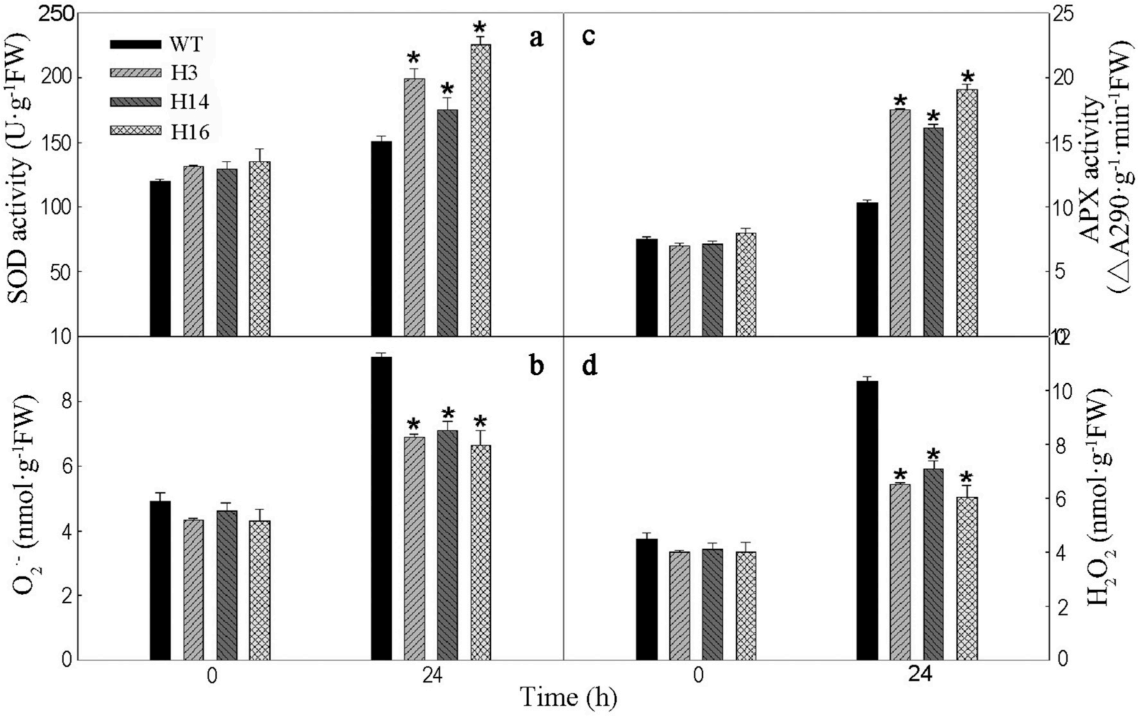

Fig. 9. Variety of the activities of SOD (a) and APX (c), $O_2^{\bullet -}$ (b) and H_2O_2 (d) contents induced by salt stress in WT and *AhHEMA1*-overexpressing tobacco plants. The data presented are the mean values ± SD of three individual experiments. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT treatments (*, $P < 0.05$).

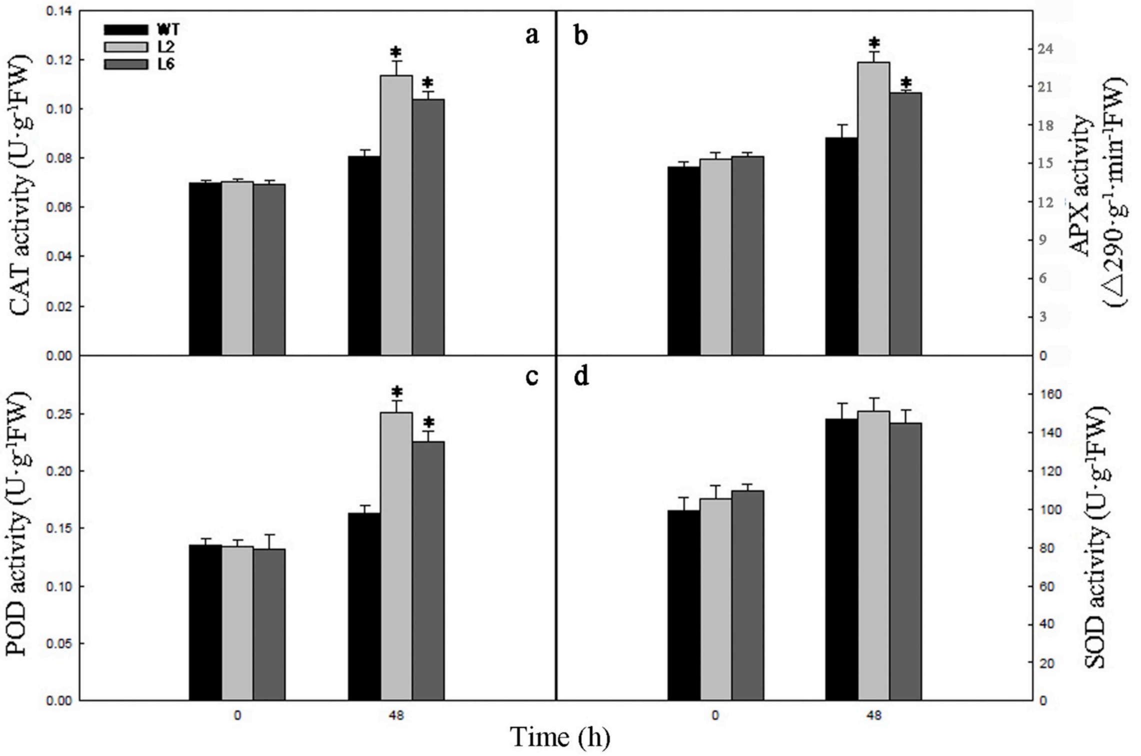

Fig. 10. Effect of salt stress on the activities of (a) CAT, (b) APX, (c) POD and (d) SOD in WT and *AhFC1*-overexpressing tobacco plants. The data presented are the mean values ± SD of three individual experiments. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT treatments (*, $P < 0.05$).

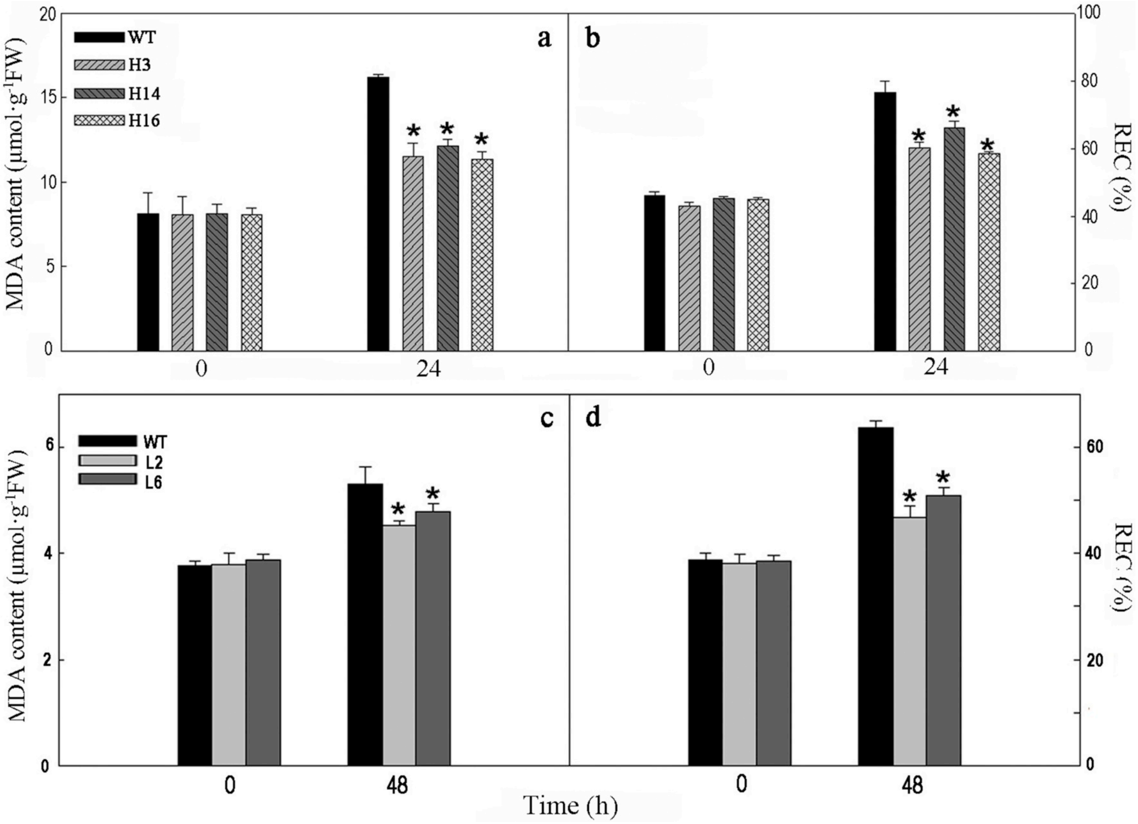

Fig. 11. Analysis of MDA content (a) and REC (b) in the *AhHEMA1*-overexpressing tobacco and WT plants and the content of MDA (c) and REC (d) in the *AhFC1*-overexpressing tobacco and WT plants. Three independent experiments are represented as mean ± SD. Asterisks indicate significant differences from WT of the same treatment (*, $P < 0.05$).

Under salt stress, the increase of heme content might be used as a signal transduction into the nucleus and induced the expression level of these enzymes which can be assembled with heme to form a whole enzyme. However, the cofactor of SOD was Cu-Zn, Mn or Fe, so the enzyme activity was not affected by heme (Phung et al., 2011). In the *AhHEMA1* or *AhFC1* transgenic tobacco, there was fewer ROS accumulation in the cytoplasm, thus, the damage to the plasma membrane was less than that in WT seedlings (Fig. 11). These results suggested that the tetrapyrrole synthesis pathway specifically regulated by *AhHEMA1* and *AhFC1* played an important role in alleviating oxidative stress and could protect cell membrane from damage under salt stress.

Declarations of interest

None.

Author Contributions

Conceived and designed the experiments: SY XL SW. Performed the experiments: LZ JY JZ QW. Analyzed the data: FG YG FY. Wrote the paper: SY XL.

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