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CHALCONE ISOMERASE GENE FROM *AVICENNIA MARINA* UNDER SALT STRESS

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Abstract

Flavonoid accumulation in *Avicennia marina* under salt stress and the structural and transcriptional characteristics of its chalcone isomerase gene (*AmCHI*) were investigated. Seedlings were exposed to 0, 200, and 400 mmol/l NaCl. Leaf flavonoid content increased significantly with salinity, concomitant with a concentration-dependent upregulation of *AmCHI* expression, as confirmed by qRT-PCR. The full-length *AmCHI* cDNA was cloned and bioinformatically analyzed, revealing a conserved chalcone_3 domain, cytoplasmic localization, and absence of signal peptides. Protein-protein interaction network analysis indicated that AmCHI interacts with core flavonoid biosynthetic enzymes (F3H, F3'H, DFR, ANS, FLS), modification/transport proteins (GSTF12, UGT78D2), and the transcription factor MYB75. These findings suggest that AmCHI-enhances flavonoid production under salt stress to alleviate oxidative damage and may coordinate with the MYB regulatory network via protein interactions to bolster salt tolerance in A. marina.

Introduction

Mangrove forests are characteristic wetland ecosystems of tropical and subtropical intertidal zones, playing an irreplaceable role in maintaining coastal ecological balance. To cope with periodic tidal flooding, soil anoxia, and high salinity, mangroves have evolved specialized adaptations: *Avicennia marina* develops aerial pneumatophores, while *Bruguiera gymnorrhiza* forms knee roots to mitigate oxygen deficiency (Srikanth *et al.* 2016), several other species have developed different level of leaf anatomical adaptation (Rashid *et al.* 2020). Salt management strategies further diverge-salt-excreting species expel excess ions via salt glands, whereas salt-excluding species rely on root ultrafiltration to restrict ion uptake. This salt tolerance is tightly regulated at the transcriptional level. In *A. marina*, salt stress strongly induces betaine/proline transporter genes *AmT1*, *AmT2*, and *AmT3* in both roots and leaves (Waditee *et al.* 2002), and NaCl treatment modulates expression of genes encoding H^+-ATPase, Na^+/H^+ antiporters, and K^+ channels, enhancing salt gland-mediated ion excretion (Wei *et al.* 2022).

Concurrently, abiotic stresses such as salinity, drought, or biotic challenges trigger flavonoid biosynthesis (Shomali *et al.* 2022). As non-enzymatic antioxidants, flavonoids scavenge reactive oxygen species (ROS) to alleviate oxidative damage and enhance plant stress tolerance and are regulated by stress-responsive networks (Shen *et al.* 2022). Notably, *A. marina* has a long history of medicinal use, with flavonoids among its major bioactive constituents, alongside phenolic glycosides, terpenoids, and alkaloids (Cerri *et al.* 2022). Yet, whether its intertidal adaptation is linked to flavonoid pathway gene expression remains unclear.

Chalcone isomerase (CHI) is a pivotal rate-limiting enzyme in flavonoid biosynthesis, catalyzing chalcone cyclization to form flavanone skeletons and contributing critically to abiotic stress responses. *AcCHI* was significantly induced in the roots or leaves of *Atriplex canescens* by 100 or 300 mM NaCl, suggesting its potential to enhance stress tolerance in forages and crops

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(Feng *et al.* 2024), but *CHI*'s function in mangroves is uncharacterized. Here, we investigate salt-induced flavonoid accumulation and characterize the *AmCHI* in *A. marina* through molecular cloning and comprehensive bioinformatic analysis. Our findings provide new insights into flavonoid-mediated salt tolerance in mangroves and highlight *AmCHI* as a potential target for improving stress resilience in coastal vegetation.

Materials and Methods

Fruits of *A. marina* were collected from the Zhangjiangkou National Mangrove Nature Reserve, Fujian, China (23º55'N, 117º26'E). Healthy fruits were sown in trays (40 × 30 × 30 cm) filled with washed river sand and grown in a greenhouse under controlled conditions: 25°C, 65% relative humidity, 12 hrs photoperiod, and 800-1000 μmol m^{-2} s^{-1} photosynthetically active radiation, with daily watering and biweekly application of full-strength Hoagland's solution. After two months, uniform seedlings were subjected to 0, 200, or 400 mM NaCl in Hoagland's solution-representing control (no NaCl), normal seawater salinity, and high-salt stress, respectively. Salt concentration was increased incrementally by 100 mM every three days (Wei *et al.* 2022). Solutions were refreshed every three days, with three biological replicates (18 seedlings each). After 30 days, the second leaf pair from the apex was harvested for analysis.

Fresh leaves (0.2 g) were reflux-extracted for 8 h in a Soxhlet apparatus using 100 ml of 70% (v/v) ethanol containing a trace of $CaCO_3$. The extract was concentrated under reduced pressure, defatted with three equal-volume diethyl ether extractions in a separatory funnel, and diluted to 100 ml with 70% ethanol. A rutin standard curve (0-50 µg/ml) was prepared. For colorimetric quantification, 1 ml of sample or standard was mixed with 1 ml 70% ethanol, followed sequentially by 0.3 ml 5% $NaNO_2$ (6 min), 0.3 ml 10% Al $(NO_3)_3$ (6 min), and 2 ml 4% NaOH. Absorbance was measured at 510 nm after 10 min. Total flavonoid content was calculated from the rutin-based calibration curve and expressed as rutin equivalents (Ref).

Approximately 0.1 g of tissue was ground in liquid nitrogen. Total RNA was extracted using the BioTeke Rapid Total RNA Extraction Kit (BioTeke, China). First-strand cDNA was synthesized with the Zhejiang Eastar cDNA Synthesis Kit (China), diluted 3-fold with 40 µl ddH_2O, and stored at -20°C.

The full-length of *AmCHI* coding sequence was amplified using primers AmCHI-F (5'-ATGTCTGCGTCACCGTTGGT-3') and AmCHI-R (5'-TCAAGTCGTGGAGGATTCTTGTC-3') with 2×Rapid Taq Master Mix (Vazyme, China).

DNAMAN was used for primer design, sequence assembly, and multiple amino acid alignment. Physicochemical properties, signal peptide, transmembrane domains, secondary and tertiary structures, conserved domains, and subcellular localization were predicted using ExPASy ProtParam, SignalP 6.0, TMHMM 2.0, PSIPRED, SWISS-MODEL, NCBI CDD, and DeepLoc 2.0, respectively. The phylogenetic tree was built with MEGA 11 (neighbor-joining), and the PPI network was obtained from STRING and visualized in Cytoscape.

qRT-PCR was performed using the TaKaRa SYBR PrimeScript RT-PCR Kit. Gene-specific primers qAmCHI-F (5'-CAGAGAAGGTGGTGGAGAACT-3') and qAmCHI-R (5'-GTGATT GAGCGAAGAGGATGG-3') were used, with *18S rRNA* (GenBank: AY289641) as the internal reference (Wei *et al.* 2022). Amplification and melting curves confirmed specificity, and relative expression was calculated using the $2^{-\triangle\triangle}$CT method (Livak and Schmittgen 2001).

Data were analyzed by one-way ANOVA in SPSS 21 ($p < 0.05$), presented as mean ± SE, and visualized using GraphPad Prism 9.0.0.

Results and Discussion

Flavonoids-key bioactive secondary metabolites in *A. marina*-function as core non-enzymatic antioxidants by scavenging ROS and mitigating oxidative damage from UV radiation, drought, or salinity (Gao *et al.* 2024, Yunusoglu *et al.* 2025). Recent spatial metabolomics in *Kandelia obovata* revealed that flavonoids and three condensed tannin monomers are positively correlated with salt stress in mesophyll cells. Consistently, 200 and 400 mM NaCl significantly elevated flavonoid levels in *A. marina* leaves (Fig. 1), likely as a defense against salt-induced oxidative stress.

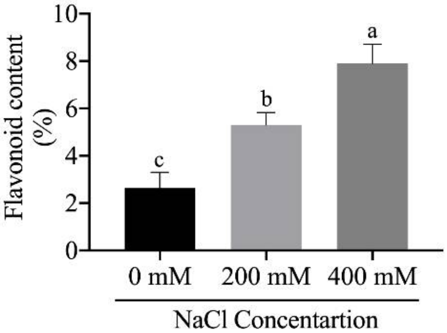


Fig. 1. Flavonoid content in *Avicennia marina* leaves under different NaCl concentration.

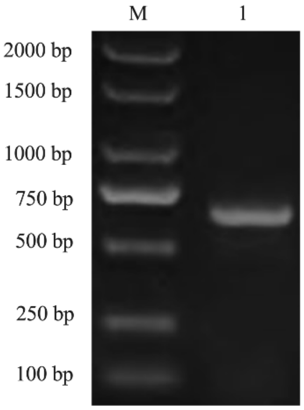


Fig. 2. Agarose gel electrophoresis of *AmCHI*. M: DL2000 DNA Marker; 1: ORF of *AmCHI*.

PCR amplification from leaf cDNA yielded a ~0.7 kb fragment (Fig. 2), sequencing of which confirmed a complete 672 bp open reading frame (ORF) encoding a 223 amino acid protein. ExPASy ProtParam predicted a molecular weight of 23.85 kDa (Table 1), with acidic (pI=5.08), hydrophilic (GRAVY=-0.099), and moderately unstable (instability index=29.88) properties. No signal peptide or transmembrane domain was detected. Secondary structure prediction indicated α-helices as the dominant element, followed by random coils and extended strands (Fig. 3a). SWISS-MODEL generated two 3D models; Model 1 shared 74.21% identity with *Agastache rugosa* CHI and comprised primarily α-helices and random coils, consistent with secondary structure predictions. Ramachandran plot analysis confirmed model reliability, with 98.64% of residues in favored regions and negligible outliers (Fig. 3b).

Table 1. Physicochemical properties of AmCHI.

Predicted value	AmCHI
Protein molecular weight（kDa）	23.85
Isoelectric point (pI)	5.08
Molecular formula	$C_{1076}H_{1680}N_{270}O_{335}S_3$
GRAVY value	-0.099
Instability index (II)	42.15
Asp+Glu	27
Arg+Lys	21

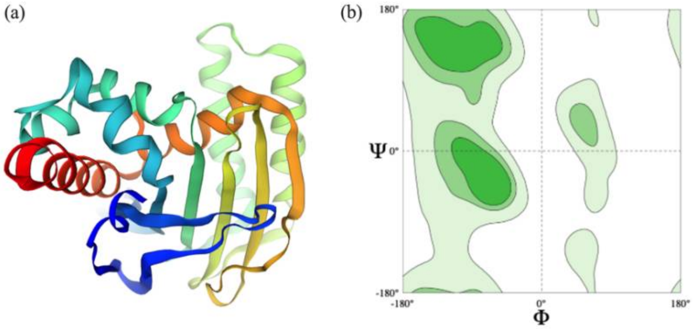
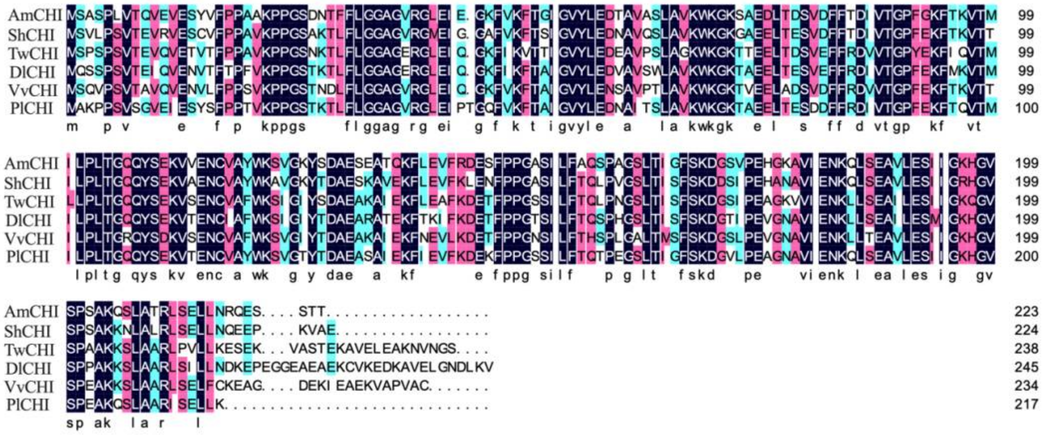


Fig. 3. Predicted tertiary structure of the AmCHI protein. (a) Homology-modeled 3D structure and (b) Ramachandran plot for model validation.

Further analysis identified a conserved Chalcone_3 superfamily domain (residues 5-215) and predicted cytoplasmic localization. Homology alignment (Fig. 4) showed AmCHI shares 78.13, 75, 67.52, 66.81 and 64.63% identity with CHIs from *Striga hermonthica* (ShCHI, CAA0832052.1), *Paeonia lactiflora* (PlCHI, AEK32592.1), *Vitis vinifera* (VvCHI, NP_001268033.1), *Tripterygium wilfordii* (TwCHI, XP_038688926.1), and *Dimocarpus longan* (DlCHI, AEO36980.1), respectively. Phylogenetic analysis placed AmCHI in a clade with CHIs from *S. hermonthica*, *P. lactiflora*, and *Malania oleifer*a, but distantly from *Handroanthus impetiginosus* (Fig. 5).



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AmCHI MSASPLVTQVEVESYVFPPAAKPPGSDNTFFLGGAGVRGLEI E. GKFVKFTGI GVYLEDTAVASLAVKWKGKSAEDLTDSVDFFTDI VTGPFGKFTKVTM   99
ShCHI MSVLPSVTEVRVESCVFPPAVKPPGSAKTLFLGGAGVRGMEI G. GAFVKFTSI GVYLEDNAVQSLAVKWKGKGAEELTESVDFFTDI VTGPFEKFTKVTT   99
TwCHI MSPSPSVTEVQVETVTFPPAVKPPGSNKTLFLGGAGERGLEI Q. GKFI KVTTI GVYLEDEAVPSLAGKWKGKTTEELTDSVEFFRDVVTGPYEKFI QVTM   99
DlCHI MQSSPSVTEI QVENVTFTPFVKPPGSTKTLFLGGAGERGLEI Q. GKFI KFTAI GVYLEDVAVSWLAVKWKGKTAEELTESVEFFRDI VTGPFEKFMKVTM   99
VvCHI MSQVPSVTAVQVENVLFPPSVKPPGSTNDLFLGGAGVRGLEI Q. GKFVKFTAI GVYLENSAVPTLAVKWKGKTVEELADSVDFFRDVVTGPFEKFTKVTT   99
PlCHI MAKPPSVSGVEI ESYSFPPTVKPPGSTKTLFLGGAGVRGLEI PTGQFVKFTAI GVYLEDNAI TSLAVKWKGKTAEELTESDDFFRDI VTGPFEKFTQVTM  100
      m  p v    e  f p  kppgs    flggag rg ei   g f k t igvyle a   la kwkgk  e l  s  ff d vtgp  kf  vt

AmCHI I LPLTGCQYSEKVVENCVAYWKSVGKYSDAESEATQKFLEVFRDESFPPGASI LFAQSPAGSLTI GFSKDGSVPEHGKAVI ENKQLSEAVLESI I GKHGV  199
ShCHI I LPLTGCQYSEKVAENCVAYWKAVGKYTDAESKAVEKFLEVFKLENFPPGASI LFTQLPVGSLTI SFSKDDSI PEHANAVI ENKQLSEAVLESI I GRHGV  199
TwCHI LLPLTGCQYSEKVSENCVAFWKSI GI YSDAEAKAI EKFLEAFKDETFPPGSSI LFTQLPNGSLTI SFSKDGSI PEAGKVVI ENKLLSEAI LESI I GKCGV  199
DlCHI I LPLTGCQYSEKVTENCI AFWKSI GI YTDAEARATEKFTKI FKDETFPPGTSI LFTQSPHGSLTI SFSKDGTI PEVGNAVI ENKLLSEAI LESM GKHGV  199
VvCHI I LPLTGRQYSDKVSENCVAFWKSVGI YTDAEAKAI EKFNEVLKDETFPPGNSI LFTHSPLGALTMSFSKDGSLPEVGNAVI ENKLLTEAVLESI I GKHGV  199
PlCHI I LPLTGCQYSEKVTENCVAYWKSVGTYTDAEASAI EKFI EVFKDEKFPPGSSI LFTQTPEGSLTI GFSKDGVLPEAGNAVI ENKQLSEAVLESI I GKHGV  200
       lpltg qys kv enc a wk  g y dae  a  kf        e fppg silf  p g lt  fskd   pe    vi enk l ea les ig  gv

AmCHI SPSAKQSLATRLSELLNRQES. . . . STT. . . . . . . . . . . . . . . . .                           223
ShCHI SPSAKKNLALRLSELLNQEEP. . . . KVAE. . . . . . . . . . . . . . . .                           224
TwCHI SPAAKKSLAARLPVLLKESEK. . . VASTEKAVELEAKNVNGS. . . .                                      238
DlCHI SPPAKKSLAARLSI LLNDKEPEGGEAEAEKCVKEDKAVELGNDLKV                                          245
VvCHI SPEAKKSLAARLSELFCKEAG. . . . DEKI EAEKVAPVAC. . . . . . .                                 234
PlCHI SPEAKQSLAARI SELLK. . . . . . . . . . . . . . . . . . . . . . . . . . . .                 217
      sp ak  la r   l
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Fig. 4. AmCHI amino acid sequence alignment.

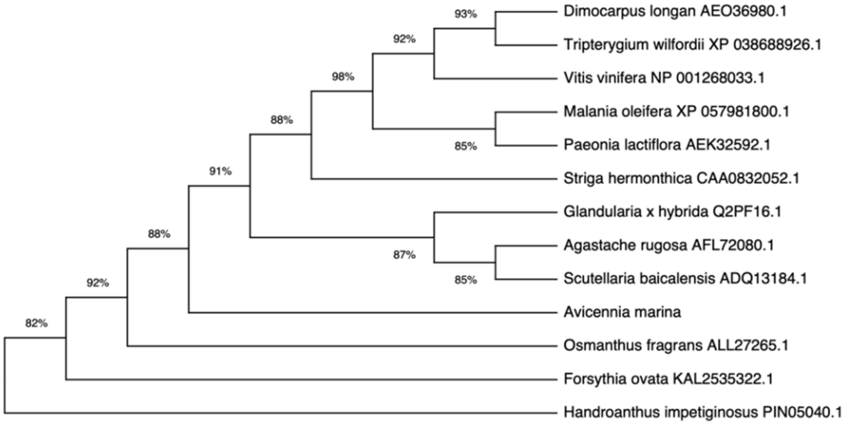

Fig. 5. Phylogenetic analysis of AmCHI.

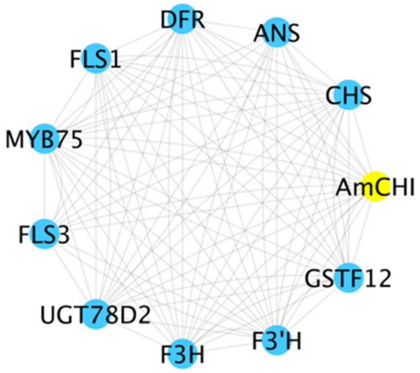

Fig. 6. Protein–protein interaction network analysis of AmCHI.

Protein–protein interaction analysis revealed that AmCHI interacts with key flavonoid biosynthetic enzymes-F3H, F3’H, DFR, ANS, and FLS-forming the chalcone-to-anthocyanin/flavonol branch pathway (Fig. 6). The network also includes GSTF12 and UGT78D2, which mediate anthocyanin vacuolar transport and glycosylation, respectively (Cui *et al.* 2021, Luo *et al.* 2025), suggesting AmCHI may coordinate end-product stabilization beyond catalysis. Notably, AmCHI interacts with the transcription factor MYB75. Given that ScMYB3 regulates ScGST3 to facilitate cyanidin-3-O-glucoside and delphinidin-3-O-glucoside transport in *Senecio cruentus* (Cui *et al*. 2021), this interaction implies AmCHI is likely embedded in an upstream transcriptional regulatory network.

CHI is a key enzyme in flavonoid biosynthesis, catalyzing the cyclization of chalcone to naringenin-the precursor for flavones, flavonols, and anthocyanins. It plays a well-established role in abiotic stress tolerance: overexpression of soybean *GmCHI4A/4B* enhances salt tolerance in hairy roots (Zhang *et al*. 2024), and heterologous expression of *Millettia pinnata CHI* improves salt resistance in yeast mutants (Wang *et al*. 2013). Here, *AmCHI* transcription was significantly upregulated under 200 and 400 mM NaCl (Fig. 7), confirming its involvement in *A. marina*’s salt response-a pattern mirrored in *K. obovata*, where *CHI* expression increases with 200-600 mM NaCl (Xing and Chen 2021).

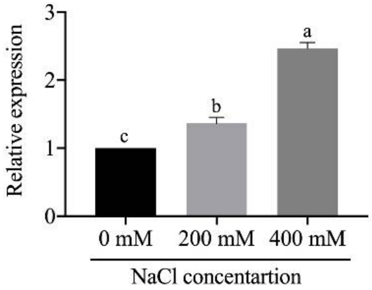

Fig. 7. Relative transcription levels of *AmCHI* under different NaCl treatment.

Collectively, these results demonstrate that *AmCHI* plays a pivotal role in both flavonoid biosynthesis and salt stress adaptation in *A. marina*. Its structural features, interaction network, and stress-responsive expression provide a strong foundation for future functional validation and molecular breeding aimed at enhancing mangrove resilience in saline coastal environments.

Acknowledgements

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