Relationship between HrANR Gene, Flavonoid Accumulation, and Drought Resistance in Chinese Seabuckthorn (Postprint)

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Abstract

Anthocyanidin reductase (ANR) is one of the key enzymes involved in flavonoid synthesis. To elucidate the structure of its encoding gene, its expression pattern under drought stress, flavonoid content, and the correlation between these factors, this study identified an ANR gene from the transcriptome data of Chinese seabuckthorn, designated as the HrANR gene. Bioinformatics software was employed to analyze the gene sequence and encoded protein, and correlation analysis was conducted between HrANR gene expression levels in various tissues under different stress conditions and flavonoid compound content in leaves.

The results indicated: (1) The ORF of the Chinese seabuckthorn HrANR gene is 1,017 bp, encoding 338 amino acids, representing a stable hydrophilic protein, with its ANR homologous proteins displaying distinct family- and genus-specific characteristics. (2) Under drought stress, the HrANR gene was expressed in roots, stems, and leaves of Chinese seabuckthorn, but with divergent expression patterns: expression in roots exhibited an initial increase followed by a decrease and then another increase; in stems, a continuous decline; and in leaves, an initial increase followed by a sustained decrease. (3) The flavonoid content in Chinese seabuckthorn leaves under different stress levels was determined using a rutin standard curve, revealing that flavonoid content first increased continuously, then decreased slightly, and rose to its maximum after rewatering, indicating that leaf flavonoid content was positively correlated with drought stress in the early stage, but negatively correlated under severe stress conditions. (4) The expression levels of the HrANR gene in leaves and stems were negatively correlated with flavonoid content (P_{leaf} = -0.75143, P_{stem} = -0.934), while positively correlated in roots (P_{root} = 0.444).

These results demonstrate that the expression of the Chinese seabuckthorn HrANR gene and the variation in flavonoid content are closely associated with

its drought resistance, and these findings can provide a foundation for elucidating the drought resistance mechanisms of Chinese seabuckthorn.

Full Text

Preamble

Correlation of HrANR Genes and Flavonoid Accumulation with Drought Resistance in Sea Buckthorn (*Hippophae rhamnoides* subsp. *sinensis*)

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Abstract: Anthocyanidin reductase (ANR) is one of the key enzymes involved in flavonoid synthesis. To elucidate the gene structure, expression patterns under drought stress, and their correlation with flavonoid content, we identified an ANR gene from the transcriptome data of sea buckthorn, designated as *HrANR*. Bioinformatics tools were employed to analyze the gene sequence and encoded protein, and the expression levels of *HrANR* in various tissues under different stress conditions were correlated with flavonoid content in leaves. The results showed: (1) The *HrANR* open reading frame (ORF) is 1,017 bp, encoding 338 amino acids. The protein is stable and hydrophilic, with ANR homologs exhibiting distinct family- and genus-specific characteristics. (2) Under drought stress, *HrANR* was expressed in roots, stems, and leaves of sea buckthorn, but with different trends: expression in roots first increased, then decreased, and increased again; in stems it continuously decreased; and in leaves it first increased then continuously decreased. (3) Flavonoid content in leaves under different stress levels was determined using a rutin standard curve, showing an initial continuous increase, followed by a slight decrease, and then rising to the highest point after rehydration. This indicates that leaf flavonoid content was positively correlated with drought stress in the early stage, but negatively correlated under severe stress. (4) *HrANR* expression in leaves and stems was negatively correlated with flavonoid content (P_{leaf} = -0.75143, P_{stem} = -0.934), while a positive correlation was observed in roots (P_{root} = 0.444). These results demonstrate that *HrANR* expression and flavonoid content changes are closely associated with drought resistance in sea buckthorn, providing a basis for elucidating its drought resistance mechanisms.

Keywords: *Hippophae rhamnoides* subsp. *sinensis*, anthocyanidin reductase, drought stress, expression pattern, flavonoids

Plants frequently encounter various biotic and abiotic stresses during growth and development, including drought, low temperature, salinity, and pests. Among these, drought is the most damaging and persistently affects global ecological environments (Zhang, 2021; Wang, 2017). Under drought stress, plant leaves re-

spond first by closing stomata to reduce transpiration and accumulating osmotic regulators to maintain turgor pressure and prevent organelle damage. Additionally, leaves accumulate secondary metabolites such as phenolics, terpenoids, and alkaloids to adapt to environmental changes (Xie et al., 2020). Chinese sea buckthorn (*Hippophae rhamnoides* subsp. *sinensis*) is a unique subspecies endemic to China with the longest cultivation history in the country (Wang et al., 2014). It exhibits strong drought resistance and soil conservation properties, and is widely distributed in the arid Loess Plateau and Qinghai-Tibet Plateau regions.

Over fifty flavonoid compounds have been identified in sea buckthorn (Zhou et al., 2020), which possess antioxidant, hypoglycemic, hypotensive, and immune-enhancing functions (Chen, 2012; Zhang and Wu, 2019; Qi and Dong, 2020). Flavonoids are primarily found in fruits, roots, stems, and leaves. Domestic researchers have isolated and identified flavonoids from sea buckthorn pomace, leaves, and fruits (Kang et al., 2017; Hong et al., 2017; Wei et al., 2020), finding that active substances are consistent between leaves and fruits, with the highest content in leaves (Wang et al., 1997). Flavonoid synthesis includes pathways for anthocyanins, flavonols, and proanthocyanidins. The ANR gene encodes anthocyanidin reductase, a key enzyme in proanthocyanidin synthesis from phenylalanine that acts on anthocyanidins to form epicatechin. The *ANR* gene has been cloned in woody plants such as tea (*Camellia sinensis*; Senguttuvan et al., 2014), bog bilberry (*Vaccinium uliginosum*; Song et al., 2017), mango (*Mangifera indica*; Li et al., 2017), and grape (*Vitis* spp.; Zhu et al., 2014), and its expression has been shown to correlate with plant adaptability in Chinese toon (*Toona sinensis*; Sui et al., 2021), alfalfa (*Medicago truncatula*; Xie et al., 2004), and mulberry (*Morus* spp.; Li et al., 2016). Gao et al. (2018) found through transcriptome sequencing that Chinese sea buckthorn responds to drought stress primarily through ABA-dependent signaling pathways and reactive oxygen species scavenging pathways dominated by flavonoid synthesis. These studies demonstrate the importance of flavonoids in sea buckthorn's response to drought stress. However, current research on flavonoids in sea buckthorn has focused on extraction, purification, and activity analysis, with no reports on the relationship between flavonoid synthesis-related genes and drought stress or their correlation with flavonoid content changes.

This study focused on the differentially expressed *HrANR* gene screened from Chinese sea buckthorn transcriptome data. Specific primers were designed for PCR amplification to obtain the full-length *HrANR* gene sequence for sequencing. Bioinformatics software was used to analyze sequence information and evolutionary relationships. Total flavonoids were extracted from Chinese sea buckthorn leaves using the reflux method, and content was measured by UV spectrophotometry. Statistical analysis was performed to address: (1) sequence information and homology of the Chinese sea buckthorn *HrANR* gene; (2) temporal expression characteristics of *HrANR* under drought stress; and (3) whether a direct relationship exists between *HrANR* spatiotemporal expression and total flavonoid content. The aim is to provide a basis for elucidating the relationship

between flavonoids and drought resistance in Chinese sea buckthorn.

Materials and Methods

1.1 Experimental Materials

Chinese sea buckthorn seeds were collected from Huangyuan County, Xining, Qinghai Province (36°46 24 N, 101°14 11 E, altitude 3,010 m) and sown at the Makehe Seedling Base in Qinghai Province. Seedlings were cultivated using the method of Zhang et al. (2021). When seedlings reached approximately 20 cm in height, healthy, pest-free seedlings were selected. Leaves were collected from some seedlings for *HrANR* gene amplification, while others were subjected to drought stress treatment.

1.2 Drought Stress Treatment

Uniform, healthy, pest-free seedlings were selected and divided into a control group (CK) and a treatment group (drought stress, DS). The CK group received normal irrigation, while the DS group was deprived of water until seedlings showed wilting. Before treatment, all seedlings were thoroughly watered. The experiment began 24 h later. Rehydration was performed on day 9 of stress (preliminary experiments showed that seedlings rehydrated on day 10 could not recover and died). Samples were collected daily at 9:00 AM, with three biological replicates per treatment group. Samples were collected at 0 d (CK), 3 d (DS1), 6 d (DS2), 9 d (DS3), and 48 h after rehydration (RW). For gene expression analysis, root, stem, and leaf tissues were snap-frozen in liquid nitrogen and stored at -80 °C. For flavonoid content analysis, leaves were collected and dried. Each tissue and treatment had three biological replicates.

1.3 Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from Chinese sea buckthorn tissues using the RN38 Plant RNA Kit (Aidlab Biotechnologies, Dalian, 50 reactions). RNA qualified by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and 2% agarose gel electrophoresis was reverse-transcribed into cDNA using the PrimeScript® II 1st Strand cDNA Synthesis Kit (TaKaRa, 6210A, 50 reactions).

1.4 HrANR Gene Amplification

Based on the *HrANR* gene sequence obtained from previous full-length transcriptome sequencing of Chinese sea buckthorn (PLOS ONE, https://doi.org/10.1371/journal.pone.0202213), specific primers were designed: *HrANR*-F: 5 -ATAAATCGTCAACGAACC-3 and *HrANR*-R: 5 -TTCATACCCTAACTTCTA-3 , with an expected product length of 1,017 bp. PCR amplification was performed using rTaq polymerase (TaKaRa, DR100A, 250 U) with leaf cDNA as template. Products were examined by gel electrophoresis, and bands of the expected size were sent to Sangon Biotech (Shanghai) for bidirectional sequencing.

1.5 Bioinformatics Analysis

Nucleotide and amino acid sequence characteristics were analyzed using EditSeq. The relative molecular mass, isoelectric point, and stability of the HrANR-encoded protein were analyzed using ProtParam (https://web.expasy.org/protparam/). Hydrophobicity was analyzed by the Kyte-Doolittle method. Secondary and tertiary structures were predicted using PredictProtein (http://www.predictprotein.org/) and Swiss Model (https://swissmodel.expasy.org/), respectively. Signal peptide prediction was performed using SignalP 5.0 (SignalP-5.0-Services-DTU Health Tech). Transmembrane regions were predicted using TMHMM (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). Subcellular localization was predicted using the online tool PredictProtein (https://www.predictprotein.org/). Homologous protein sequences were analyzed using DNAMAN. Homologous sequences were searched using NCBI Blastp, and an NJ phylogenetic tree was constructed using MEGA 6.0. Homology was calculated using Megalin.

1.6 Quantitative Real-Time PCR (qRT-PCR) Analysis

Fluorescent quantitative primers were designed using online software: *HrANR*-DL-F: 5 -AATTCACTTACAGGCACAGGGTTGG-3 and *HrANR*-DL-R: 5 -AGCTAGTGTCTTGGAGGCAGGATAG-3 . Reference genes stably expressed under different stresses were selected: *TATA* for roots (F: 5 -AAGTTGGCAGCACGAAAGTATG-3 , R: 5 -GGGGAATTTAACATCACAAGAACC-3), *HIS3* for leaves (F: 5 -CCGTAAATCAGCCCCAACC-3 , R: 5 -GAACAAGCCTCTGGAATGGAA-3), and *PEPC* for stems (F: 5 -GTCGTCCATCAAAACGCAAG-3 , R: 5 -AAGCCAAGCCACACAGGTAAA-3). qRT-PCR was performed on a Q2000 B instrument (Hangzhou Langke) with three biological replicates.

1.7 Flavonoid Extraction and Content Determination from Chinese Sea Buckthorn Leaves

Leaves from drought-stressed Chinese sea buckthorn were dried using a freeze dryer (Shanghai BILON-FD80A), pulverized using a tissue homogenizer (Changzhou JTLIANGYHOU-JJ-2), and passed through an 80-mesh sieve. After petroleum ether defatting, flavonoids were extracted twice using the method of Wang (2008). The combined extracts were prepared by refluxing with 50% ethanol at 70 °C for 2.0 h (material-to-solvent ratio 1:10) for the first extraction and 1.5 h (ratio 1:8) for the second. Flavonoid content was measured using a UV spectrophotometer (DR6000, Hach, USA) at 510 nm. Although proanthocyanidin content is typically measured at 280 nm, this method is only suitable for high-purity solutions; since catechin also has maximum absorption at this wavelength, total flavonoid content was measured at 510 nm. A standard curve was prepared using rutin standard (National Institute for Food and Drug Control) following the method of Feng et al. (2017).

1.8 Statistical Analysis

Relative expression levels of *HrANR* were calculated using the 2^(-ΔΔCt) method. A standard curve for flavonoid extraction was established using Excel. Statistical analysis was performed using Microsoft Excel 2010 and SPSS 22.0, and figures were generated using SigmaPlot 14.0.

Results

2.1 Cloning and Sequence Analysis of HrANR Gene

Using leaf total RNA reverse-transcribed cDNA as template and *HrANR*-F/*HrANR*-R primers, PCR amplification yielded a single band [Figure 1: see original paper]. Sequencing of the amplified product revealed the *HrANR* gene sequence. DNAMAN analysis showed an ORF length of 1,017 bp.

2.2 Amino Acid Sequence Analysis and Protein Bioinformatics

The *HrANR* gene encodes 338 amino acids, with leucine being most abundant (10.1%) and tryptophan least abundant (0.9%). The protein contains 38 negatively charged residues (Asp + Glu) and 35 positively charged residues (Arg + Lys). The encoded protein has a molecular weight of 36,660.19 Da and a theoretical isoelectric point (pI) of 6.03, classifying it as a hydrophilic protein [Figure 2: see original paper]. Isoleucine at position 194 showed the strongest hydrophobicity (2.178), while glutamic acid at position 43 showed the strongest hydrophilicity (-2.633). PredictProtein analysis indicated cytoplasmic subcellular localization. Secondary structure prediction revealed α-helices (40.83%), random coils (37.57%), extended strands (14.20%), and β-sheets (7.40%) [Figure 3: see original paper], indicating that α-helices and random coils are the main structural components. The tertiary structure model predicted by Swiss Model [Figure 4: see original paper] was consistent with the secondary structure prediction. SignalP 5.0 predicted a signal peptide probability of 0.15%, indicating HrANR is a non-secretory protein without a signal peptide. TMHMM prediction showed no transmembrane structures, consistent with cytoplasmic localization.

2.3 Homology and Phylogenetic Analysis of HrANR

Eleven homologous sequences to HrANR were identified from NCBI: Chinese toon (*Toona sinensis*, QWB49502.1), lychee (*Litchi chinensis*, QRV61380.1), sea island cotton (*Gossypium barbadense*, ALF38091.1), Ussuri poplar (*Populus ussuriensis*, UNN46810.1), marshmallow (*Althaea officinalis*, UOI87834.1), longan (*Dimocarpus longan*, QRV61373.1), cacao (*Theobroma cacao*, ADD51354.1), mango (*Mangifera indica*, AXN94092.1), highbush blueberry (*Vaccinium corymbosum*, AYC35398.1), grape (*Vitis bellula*, AFG28175.1), and ginkgo (*Ginkgo biloba*, AAU95082.1). Multiple sequence alignment [Figure 5: see original paper] and homology analysis [Figure 6: see original paper] were performed, and

an NJ phylogenetic tree was constructed [Figure 7: see original paper]. Quick alignment using DNAMAN showed 83.36% sequence identity. Megalin analysis revealed single-chain identity between multiple sequences ranging from 57.7% to 97.0%, with Chinese toon showing the highest identity (84.2%) to HrANR and ginkgo showing the largest difference (54.1%). The phylogenetic tree clustered the 12 species into two major groups with four branches: Chinese sea buckthorn grouped with angiosperms, while gymnosperm ginkgo formed a separate cluster, consistent with the divergence relationships. Lychee and longan (Sapindaceae) clustered together, and marshmallow and sea island cotton (Malvaceae) formed another group, demonstrating clear family- and genus-specific characteristics of ANR proteins.

2.4 Expression Analysis of HrANR Gene

qRT-PCR was performed on roots, stems, and leaves of Chinese sea buckthorn under different drought stress levels. As shown in [Figure 8: see original paper], *HrANR* expression in roots first increased, then decreased to a minimum at DS2, and subsequently increased again, reaching maximum after rehydration. In stems, expression continuously decreased with intensifying drought and failed to recover after rehydration. In leaves, expression initially increased then continuously decreased under severe stress and after rehydration. ANOVA revealed extremely significant differences in *HrANR* expression among roots, stems, and leaves under different drought treatments ($P < 0.01$).

2.5 Flavonoid Content in Chinese Sea Buckthorn Leaves Under Different Stresses

After combining the two leaf extracts from different stress levels, 5 mL of the combined extract was diluted 5-fold and compared with rutin standard solution (standard curve: y = 0.4108x - 0.0033, $R^2 = 0.9986$) using UV spectrophotometry at 510 nm. Results are shown in [Figure 9: see original paper]. Flavonoid content in leaves gradually increased during early stress, peaked at DS2 (3.38 $mg \cdot mL^{-1}$), slightly decreased at DS3, and rose to maximum after rehydration (3.975 $mg \cdot mL^{-1}$). Changes in leaf flavonoid content under different stress levels were extremely significant ($P < 0.01$). Comparison of [Figure 8: see original paper] and [Figure 9: see original paper] shows inconsistent trends between *HrANR* expression in leaves and flavonoid accumulation. SPSS analysis revealed negative correlations between flavonoid content and *HrANR* expression in leaves and stems (Pearson correlation: P_{leaf} = -0.75143, P_{stem} = -0.934), but a positive correlation in roots (P_{root} = 0.444).

Discussion and Conclusion

Drought stress affects plant growth and development, triggering a series of physiological and biochemical responses to prevent organelle damage. Studies have shown that plant defense, signal transduction, and growth under stress are asso-

ciated with secondary metabolites (Shao et al., 2009). The *ANR* gene directly affects total flavonoid content by encoding an enzyme in the proanthocyanidin biosynthesis pathway, thereby responding to stress.

Chinese sea buckthorn, a primary source of medicinal sea buckthorn, contains abundant flavonoids in its leaves, which not only have high medicinal value but also play important roles in coping with environmental stresses such as drought (Li et al., 2022). Investigating *ANR* expression under drought stress and its relationship with flavonoid accumulation can help elucidate the molecular mechanisms of drought resistance and has practical applications. Environmental regulation could be used to manipulate *ANR* expression to control flavonoid yield in sea buckthorn leaves, providing a new approach for directed breeding of high-flavonoid sea buckthorn cultivars.

Protein structure determines function. This study found that Chinese sea buckthorn HrANR protein, like that of Chinese fir (Wang et al., 2019), is a hydrophilic protein lacking signal peptides and transmembrane structures, localized in the cytoplasm. Its secondary structure, dominated by α-helices and random coils, is similar to that of safflower (Lu et al., 2022) and bog bilberry (Song et al., 2017). Homology analysis revealed highest similarity with Chinese toon, indicating the closest phylogenetic relationship and presumably similar protein structure and function. The NJ phylogenetic tree showed clear species-specific characteristics among the 12 species, with parallel evolution within branches consistent with morphological classification (Flora of China Editorial Committee, 1983).

Ma et al. (2012) found differential *ANR* expression in peanut tissues. Lu et al. (2022) reported low *ANR* expression in safflower roots and stems. Zhu et al. (2010) observed fluctuating but overall increasing *ANR* expression in ginkgo under prolonged drought. Lu (2012) suggested that plant antioxidant capacity is proportional to *ANR* expression within a certain range. In this study, *HrANR* expression showed different trends in roots, stems, and leaves of drought-stressed Chinese sea buckthorn: leaves showed an initial increase followed by decrease; stems showed continuous decrease; and roots showed a fluctuating pattern of increase-decrease-increase. This indicates that *HrANR* expression is a dynamic adaptive process during drought stress, closely related to Chinese sea buckthorn's drought response.

Flavonoids include flavanones, catechins, and anthocyanins (Leonard et al., 2006). Flavonoids possess antioxidant properties, and drought can induce flavonoid accumulation to enhance drought tolerance (Foyer et al., 2010), demonstrating their importance in plant stress responses. Studies have shown that drought resistance in potato (Watkinson et al., 2006) and *Epimedium* (Shi et al., 2004) correlates with flavonoid content. Anthocyanin antioxidant activity is reportedly much higher than that of vitamin C, vitamin E, and β-carotene (Lu, 2012). Li et al. (2022) found that *ANR* overexpression promotes anthocyanin accumulation and enhances drought resistance. In this study, leaf flavonoid content was positively correlated with drought stress in

the early stage, corresponding with increased *HrANR* expression. Under severe stress, both were negatively correlated, suggesting that moderate drought stress induces *HrANR* expression to accumulate flavonoids for enhanced ROS scavenging, consistent with Meng et al. (2012). However, after prolonged severe stress exceeding the plant's tolerance, toxic substances accumulated, severely affecting growth and inhibiting synthesis of protective compounds.

Notably, *HrANR* expression in leaves began decreasing on day 6, while flavonoid content decreased later (on day 9). After rehydration, leaf flavonoid content increased sharply, contrary to the continued decrease in leaf *HrANR* expression. Conversely, root *HrANR* expression increased sharply after rehydration. We hypothesize that due to the short rehydration period, water absorbed by roots first met root needs while aboveground parts remained water-deficient. Massive *HrANR* expression in roots synthesized flavonoids that were transported to leaves, increasing leaf flavonoid content. These results demonstrate that *HrANR* expression and flavonoid content changes are closely related to drought resistance in Chinese sea buckthorn.

Gene expression reflects transcription levels, while RNA is unstable, especially under severe stress (days 6 and 9 in this study) when it has a shorter half-life and is prone to degradation. In contrast, gene expression products are more stable (Yang, 2017). Therefore, expression products may continue accumulating and exerting drought resistance functions even when gene expression declines, possibly explaining the inconsistency between flavonoid content and *ANR* expression. Additionally, plants transport organic compounds downward through phloem. In this study, root *HrANR* expression increased sharply on day 9 and continued rising after rehydration, accumulating flavonoids in roots that were transported to leaves via phloem, which may be another major reason for the inconsistency between leaf flavonoid content and *HrANR* expression after rehydration.

Current research on drought stress-related genes in sea buckthorn has focused on ABA signaling, Ca^{2+} signaling, and MAPK kinase pathways, with only Sun et al. (2015) investigating the relationship between secondary metabolites and drought resistance in *Hippophae neurocarpa*. To further clarify the relationship between *HrANR* and drought resistance in Chinese sea buckthorn, comprehensive evaluation of other flavonoid synthesis-related genes and flavonoid content under drought stress is necessary. Additionally, generating overexpression lines through transgenic approaches to study *HrANR* drought function would provide further insights into the drought resistance mechanisms of Chinese sea buckthorn.

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Note: Figure translations are in progress. See original paper for figures.