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MOLECULAR AND FUNCTIONAL ANALYSIS OF THE FLAVANONE-3-HYDROXYLASE (*F3H*) GENE IN WELSH ONION (*ALLIUM FISTULOSUM* L.)

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Abstract

Secondary metabolites contribute to the biological activities and medicinal attributes of plants. Flavonoids are major metabolites in *Allium fistulosum*. Flavanone-3-hydroxylase is a key enzyme in the flavonoid biosynthesis pathway. However, the molecular basis of *F3H* in *A. fistulosum* is still unknown. In the present study, *F3H was* cloned *from A. fistulosum* and named *AfF3H*; this gene contains 1269 bp and encodes 368 amino acids. Subcellular localization results showed that *AfF3H* was located in the cytosol. The catalytic activity analysis of the *AfF3H* recombinant protein showed that the protein catalyses the formation of dihydroquercetin and dihydrokaempferol from eriodyctiol and naringenin. Furthermore, the expression of *AfF3H* was specific and closely related to flavonoid content. The increased expression of *F3H* was induced by cold stress. Simultaneously, the content of total flavonoids in Welsh onion also increased with the expression of *AfF3H*. These results showed that cold stress induced the expression of *AfF3H* and increased the metabolic flux of flavonoids. The results of this study suggest that *AfF3H* plays an essential role in flavonoid biosynthesis in *A. fistulosum*.

Key words: Flavonoids, Flavanone 3-hydroxylase, *Allium fistulosum* L., Cold stress.

Introduction

Flavonoids are important metabolites with multifarious physiological effects in plants (Hassan & Mathesius, 2012; Li *et al*., 2012; Agati *et al*., 2013). Furthermore, flavonoids play important roles in abiotic stress resistance and plant colouring. Flavanone 3-hydroxylase belongs to the 2-ODD family (Aguadé, 2001). The protease encoded by this gene can further catalyse the synthesis of dihydroflavanol from flavanone. Dihydroflavanols are important intermediates of flavonols and anthocyanins(Jia *et al*., 2016; Gutiérrez-Albanchez *et al*., 2020). The dihydroflavanol synthesized in this step is a key branch point of anthocyanins and flavonoids (Han *et al*., 2017). Therefore, the study of *F3H* is of great importance for understanding the regulation of flavonoids and anthocyanins. The expression of *F3H* is closely related to the content of flavonoids in plants (Zuker *et al*., 2002; Liu *et al*., 2013; Xiong *et al*., 2016). In *Camellia sinensis*, *CsF3H* can increase most flavonoid glycosides and proanthocyanidins through overexpression in *Arabidopsis* (Han *et al*., 2017). The expression of *AaF3H* was correlated with flavonoid contents in *Artemisia annua* (Xiong *et al*., 2016). *RsF3H* in *Reaumuria soongorica* was used as an intermediate for flavonol synthesis (Liu *et al*., 2013). The anthocyanin content of *tt6*, an Arabidopsis *f3h* mutant, was lower than that of the wild type (Preuß *et al*., 2009). Flavonoids and other phenylpropanoid pathway compounds have been considered to be involved in abiotic stress resistance. In *Arabidopsis*, the concentration of kaempferol and other flavonoids increased with the intensity of UV radiation (Ferreyra *et al*., 2010). Drought, salt, and cold stress induced the expression of *CsF3H* genes (Han *et al*., 2017). In spruce, the expression of *F3H* can enhance resistance to damage caused by fungi (Hammerbacher *et al*., 2019). Overexpression of *PnF3H* in *Arabidopsis* inhibits the effect of naringin on plant growth by increasing metabolic flux and enhancing tolerance (Li *et al*., 2017).

A. fistulosum is a biennial herb of Liliaceae. It is deeply loved by people in Southeast Asia because of the special flavour of this plant (Aoyama & Yamamoto, 2007; Sun *et al*., 2019; Gao *et al*., 2021). *A. fistulosum* is rich in various bioactive substances, such as sulfur compounds, flavonoids, steroids, dietary fibre, and polysaccharides (Kothari *et al*., 2020; Chernukha *et al*., 2021; Liu *et al*., 2021). Flavonoids are the main bioactive substances in *A. fistulosum* and have various physiological effects, including antioxidant, antitumor, anti-inflammatory, anti-platelet aggregation, immunosuppression, and cardiovascular protection effects (Miean & Mohamed, 2001; Sun *et al*., 2019; Marefati *et al*., 2021). However, *F3H* in *A. fistulosum* has not been characterized.

The function of *F3H* has been clarified in *A. fistulosum* in this study. The mechanism of the *F3H* gene in the synthesis of flavonoids was preliminarily elucidated. A comprehensive analysis of *AfF3H* was conducted, including bioinformatics analysis, subcellular localization, catalytic activity, and response to cold stress, which laid a foundation for further study on the synthesis of flavonoids.

Materials and Methods

Plant materials: *A. fistulosum* was obtained from Beijing, China. For cold stress, *A. fistulosum* was transferred to an incubator at 4°C for 0, 12, 24, 48, and 72 h. The last leaf of *A. fistulosum* was taken as the test material, and the samples were stored at -80°C.

Clone and basic analysis of AfF3H: The samples were collected and stored at -80°C. Total RNA was extracted immediately with the RNA Extraction Kit from Beijing Tiangen Biotechnology Co., Ltd. The quality of the RNA samples was determined by a NanoDrop 2000, noting the ratio of 260 nm/280 nm. cDNA was synthesized following the instructions in the PrimeScript RT Reagent Kit (Takara).

The *AfF3H* sequence was obtained from the transcriptome database. The PCR system included 1 µl cDNA, 10 µ1 mol/L of each primer, 2 × Easy Taq ® PCR Super Mix, and 10 µl water to make the total 20 µl. The PCR amplification procedure was as described in the Product description. Products were then purified and recovered into pEASY-Blune-Zero. The sequence of *AfF3H* was compared to others on NCBI.

The basic characteristics of *AfF3H* were analysed with the ExPASy online tools. Phylogenetic analysis was carried out by MEGA 6.0 (Tamura *et al*., 2013). Cluster analysis of *F3H*s was performed using the neighbour-joining (NJ) method. Protein domain prediction was performed using the NCBI Conserved Domains online search tools.

Subcellular localization assay: The subcellular localization of *AfF3H* was analysed. The plasmid PYBA-1332 was digested with *Sac*I and *Kpn*I, and the coding sequence of *AfF3H* was ligated to the subcellular localization vector with GFP by homologous recombination. The fusion expression vector 35S-*AfF3H*-GFP was obtained. The constructed fusion plasmid was transformed into competent GV3101 cells, and the Agrobacterium strain with 35S-GFP was used as the control. After mixing P19 with the target bacteria in the same proportion, the mixture was injected into the back leaves of *Nicotiana tabacum* that grew normally for 4-5 weeks. After 1 day of dark culture, the cells were cultured normally for 2 days. The GFP signal was observed under a 488 nm laser confocal microscope.

Enzymatic activity assay: The pET-MBP vector was digested with a single enzyme, *Ssp*I, and the digested products were recovered. The PCR product was ligated to the vector. The plasmid was named as pET-*AfF3H* and was transformed into *Escherichia coli* BL21 (DE3). The cells were cultured in medium and induced to express protein by IPTG at different concentrations. The cells were induced overnight at 28°C. Naringenin and eriodyctiol were dissolved in DMSO, which was added to the crude enzyme solution. The final concentration of naringenin and eriodyctiol was 100 µM. After incubation at 28°C for 3 h, the culture was treated with ultrasonication, and an equal volume of ethyl acetate was added. The ethyl acetate extract was evaporated with nitrogen and dissolved in 100 µL methanol for HPLC analysis. SDS–PAGE analysis showed that the recombinant MBP-*AfF3H*-His protein was expressed.

Conditions of HPLC analysis: HPLC analysis followed the methods provided by Park (Park *et al*., 2020). The details are as follows: Agilent 1260 liquid chromatograph equipped with Agilent C18 column (5 µm, 250 × 4.6 mm; Agilent). The chromatographic separation adopted 0.1% formic acid aqueous (solution A) and 0.1% formic acid methanol (solution B), and the gradient conditions were as follows: 0 min, 95%A/10%B; 30 min, 45%A/55%B; 45 min, 35%A/65%B; 50 min, 0%A/100%B; 52 min, 95%A/5%B; and 60 min, 95%A/5%B. The flow rate was 1 ml · min^{-1} and the column temperature was 40°C. UV/Vis. Was used to detect compounds. The spectra of the compounds were recorded in the range of 362 nm through the retention time of the standard. The corresponding peaks of each compound were determined by comparison with UV spectra.

Expression analysis: Leaf tissue was collected and immediately placed in liquid nitrogen to prevent RNA degradation; samples were stored at −80°C until extraction. These steps were consistent with those provided in the rapid extraction and in the first chain synthesis kit. The samples were then diluted and placed at −20°C for storage. The Ct value was determined by real-time PCR, and the expression was calculated by $2^{-\Delta\Delta CT}$(Schmittgen *et al*., 2001).

Analysis of flavonoid content: The flavonoid content in *A. fistulosum* was determined by a Standard Curve for Plant Flavonoids Content Assay Kit from Beijing Solarbio (www.solarbio.com). The method was conducted according to Wang (Wang *et al*., 2020). Briefly, the absorbance of the extract was measured at 470 nm, and a standard curve was drawn to analyse the content of total flavonoids.

Results

Characterization analysis of *AfF3H:* *AfF3H was screened from a full-length transcriptome sequencing database and had a length of 1269 and an open reading frame encoding* 367 amino acids. The basic features of *AfF3H* are described below (Table 1).

The conserved domains found on NCBI were used to analyse the conserved domains of *AfF3H*. The results showed that the protein contained a PcbC conserved domain located in at aa 40-321 and a 2OG-Fell-Oxy domain located at aa 197-297 (Fig. 1).

Multiple alignment analysis showed that the structures of *F3H*s were quite conserved across species. The amino acid sequence encoded by *AfF3H* shared the highest similarity with that of *AcF3H*. The 2-oxoglutarate binding sites and ferrous binding sites found in *F3H*s were highly conserved in *A. fistulosum* and other plants (Fig. 1).

The phylogenetic analysis showed that the 2-ODD family members were divided into multiple categories. *F3H*s and flavone synthases (*FNS*s) shared the same evolutionary branch suggesting that *F3H*s and *FNS*s had the same evolutionary relationship (Fig. 2). The other category included anthocyanin synthases and flavonol synthases. *F3H*s and *FNS*s share the same evolutionary branch. These results supported previous studies suggesting that *F3H* evolved from *FNS* by gene replication (Lukačina *et al*., 2003; Gebhardt *et al*., 2005; Agati *et al*., 2013; Han *et al*., 2017).

Table 1. The basic information of *AfF3H genes.*

Gene name	Mw (kD)	CDNA length (bp)	ORF length (bp)	5'-UTR (bp)	3'-UTR (bp)	Size (aa)	pI
AfF3H	41.14	1269	1104	44	121	367	5.48

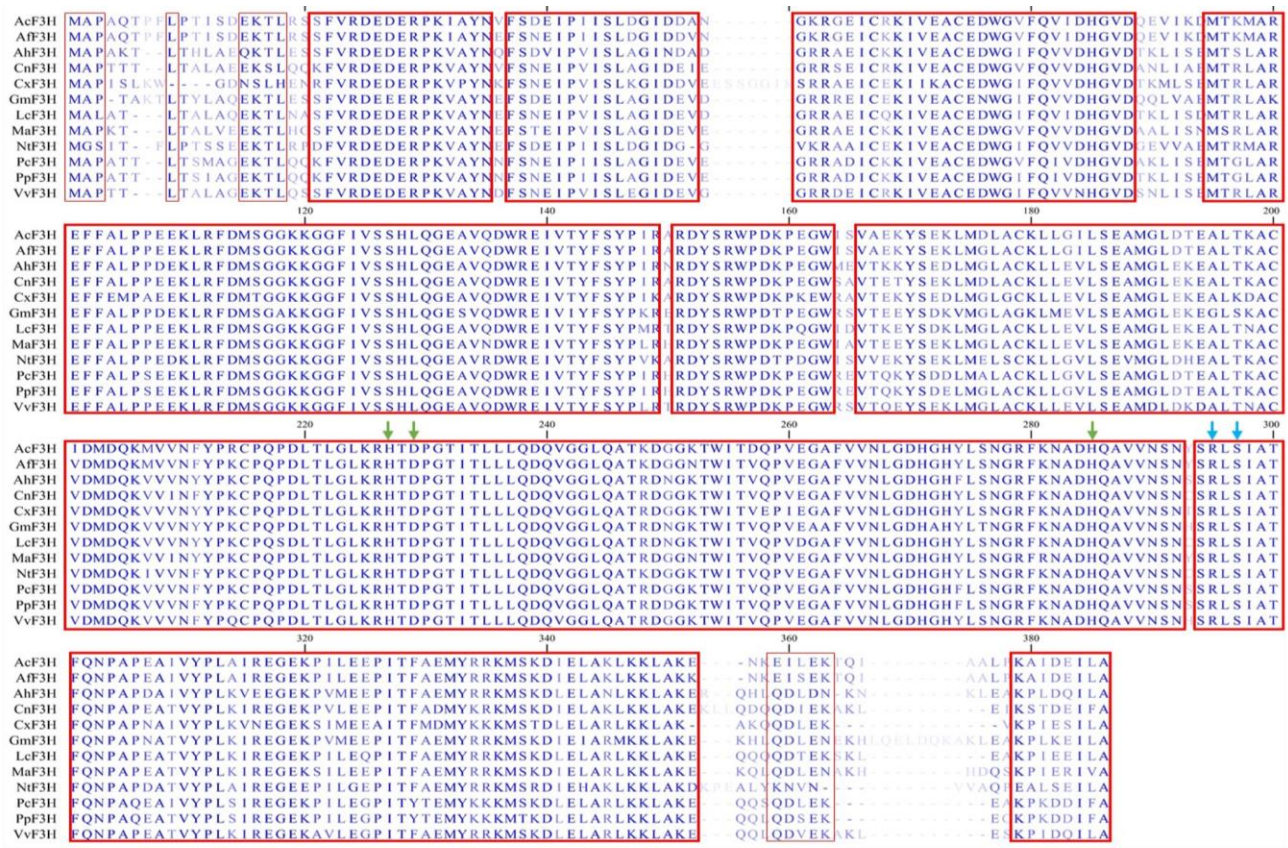

Fig. 1. Multiple alignment of *AfF3H* with F3Hs from other plants. The blue and green symbols represent the iron binding sites and 2 –oxoglutarate –dependent dioxygenase (2 –ODD) family.

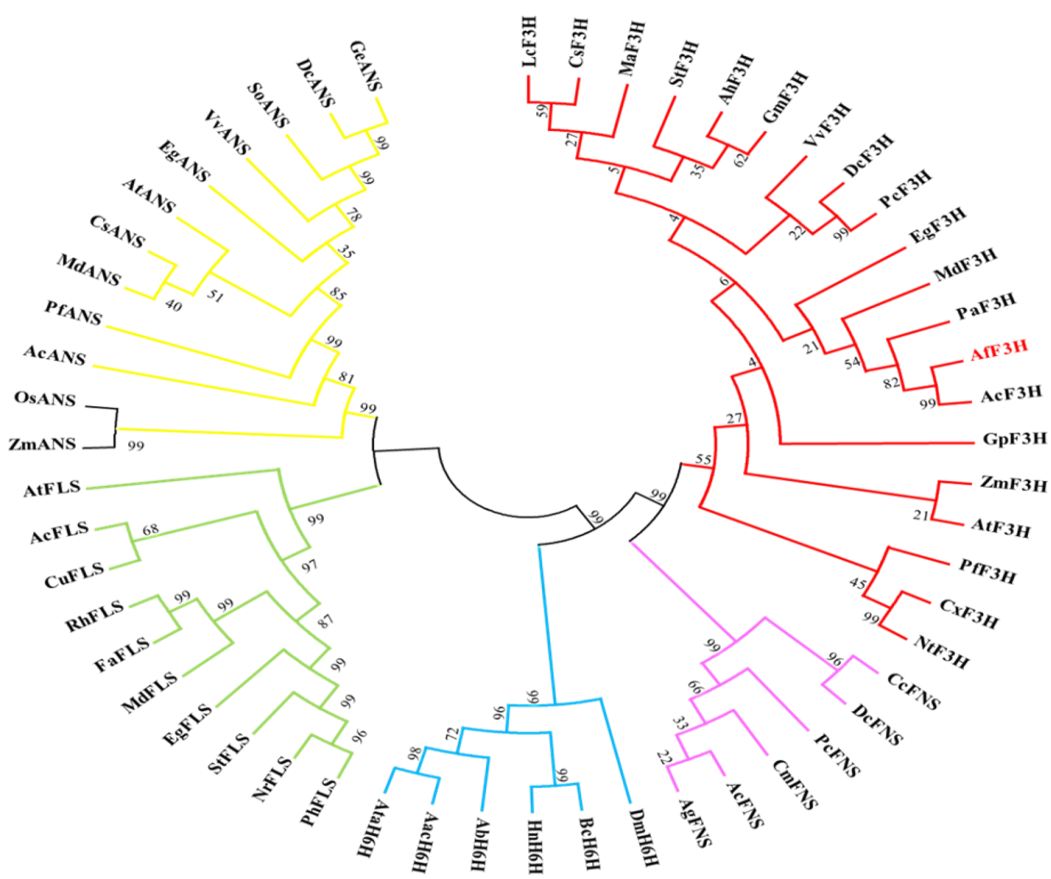

Fig. 2. Phylogenetic relationship of *AfF3H* with 2 –ODD family memebers from other plants. *AfF3H* indicated by red color.

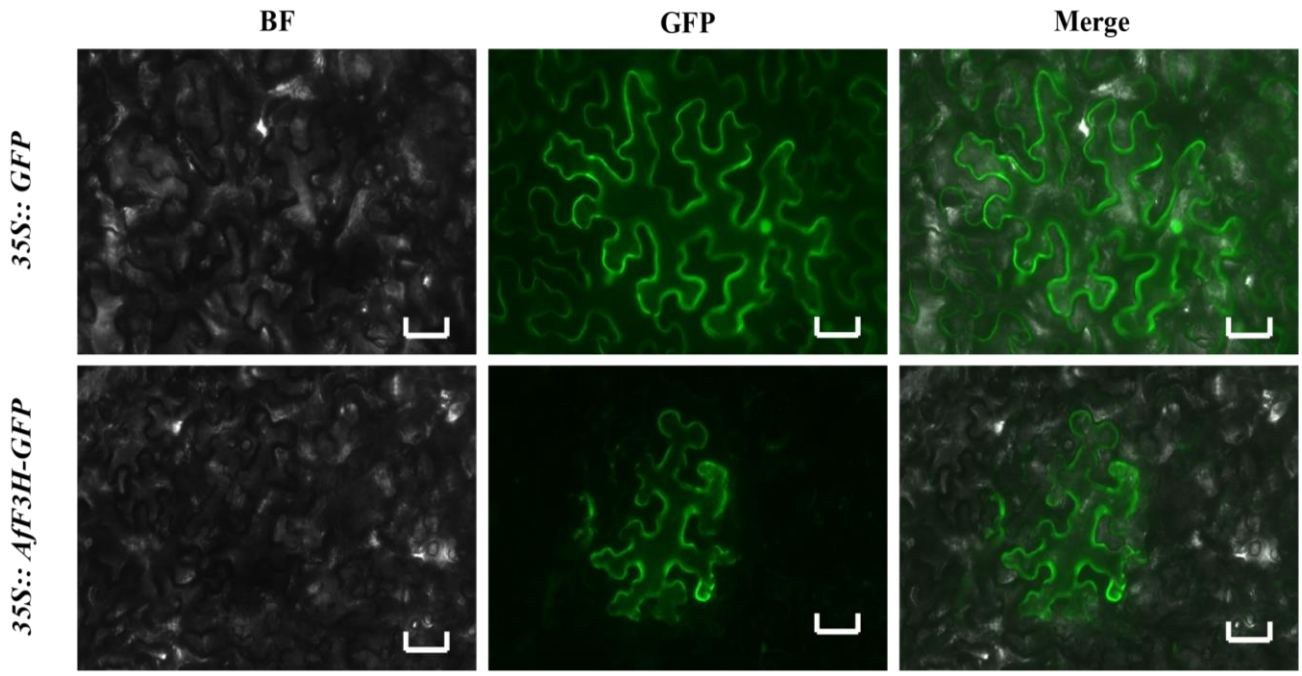

Fig. 3. Subcellular localication of *AfF3H* proteins in *Nicotiana benthamiana* cells. *35S:: GFP* was used as a positive control.

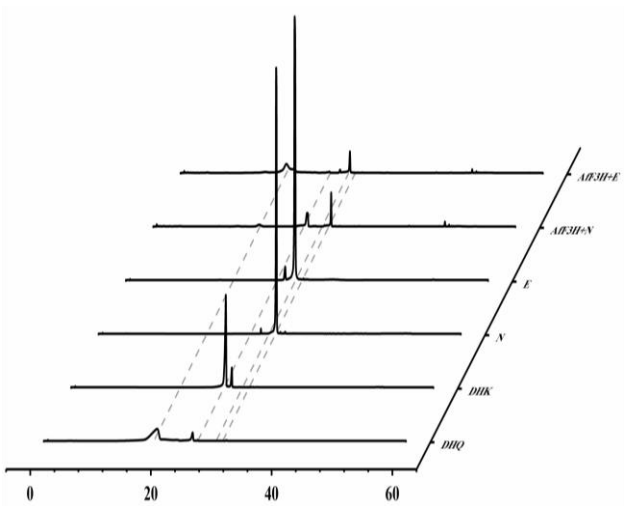

Fig. 4. Enzymatic reaction products analysis of recombinant *AfF3H* proteins. Standard including NA, ER, DHK, and DHQ.

Subcellular localization of AfF3H: The online tool ProtComp was used to forecast the localization of *AfF3H*, which was most likely extracellular. To determine the subcellular localization of *AfF3H*, Promoter$_{CaMV35S}$-*AfF3H*-GFP was constructed and transformed into tobacco using Agrobacterium. Fluorescence was clearly observed. In contrast, the results of transient expression showed that *AfF3H* had a strong fluorescence signal in the cytosol. This finding indicates that *AfF3H* is a cytosol-localized protein (Fig. 3).

Induction of the recombinant protein and its catalytic activity *In vitro*: To determine the catalytic activity of *AfF3H* on different substrates, an expression vector of *AfF3H* was constructed, and the target protein was successfully induced (Fig. S1). The contents of DHK and DHQ were determined by HPLC by adding NA and ER directly. The catalytic activity of *AfF3H* towards different substrates was indicated according to the amount of DHK and DHQ. The results showed that *AfF3H* could catalyse the production of dihydroflavonol by catalysing both NA and ER (Fig. 4).

Analysis of tissue expression specificity and flavonoid content: The expression of *AfF3H* in different tissues of *A. fistulosum* was determined by qPCR. The results suggested that the expression of *AfF3H* in flowers was much higher than that in other tissues, such as the roots, and almost no expression was observed in the leaves (Fig. 5a). To determine the impact of *AfF3H* on the accumulation of flavonoids in *A. fistulosum*, the content of flavonoids in different tissues was analysed. The results showed that the content of flavonoids in leaves was followed by that in leaf sheaths and roots. stems, the flavonoid content was lowest in stems and flowers (Fig. 5b). The expression level of *AfF3H* in leaves was consistent with flavonoid accumulation patterns. These results indicated that *F3H* in *A. fistulosum* most likely plays important roles in flavonoid biosynthesis.

The effect of cold stress on the expression of *AfF3H* and the flavonoid content: The expression of AfF3H under cold stress was detected using real-time qPCR. The results showed that the expression of *AfF3H* in the first leaf increased significantly within 48 hours after 4°C treatment and decreased at 72 hours (Fig. 6a). Simultaneously, the flavonoid content in the first leaf of *A. fistulosum* reached its highest level at 12 h after 4°C treatment and gradually decreased to the 0 h level from 24 h to 72 h (Fig. 6b). These results indicated that flavonoid biosynthesis might be induced by cold stress in *A. fistulosum*, suggesting that there were transcription factor binding sites regulated by cold stress in the promoter of the *F3H* gene.

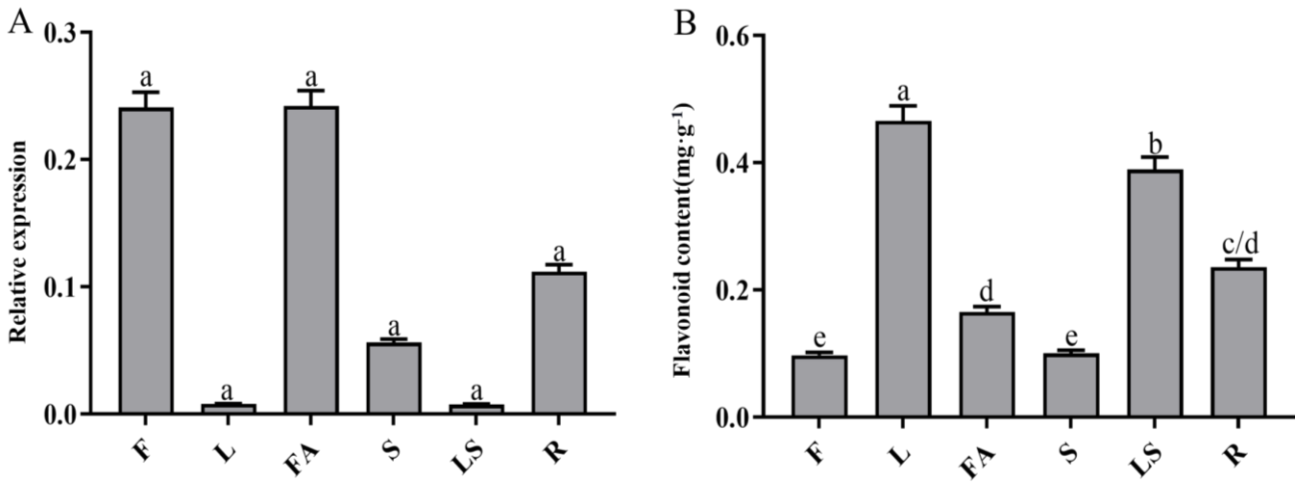

Fig. 5. Relative expression of *AfF3H* and flavonoid content analysis in different tissue. (A) Relative expression of of *AfF3H* in different tissue. (B) Flavonoid content analysis in different tissue. F: Flower, L: Leaf, FA: Floral asix, S: Stem, LS: Leaf sheath, R: Root (p<0.05).

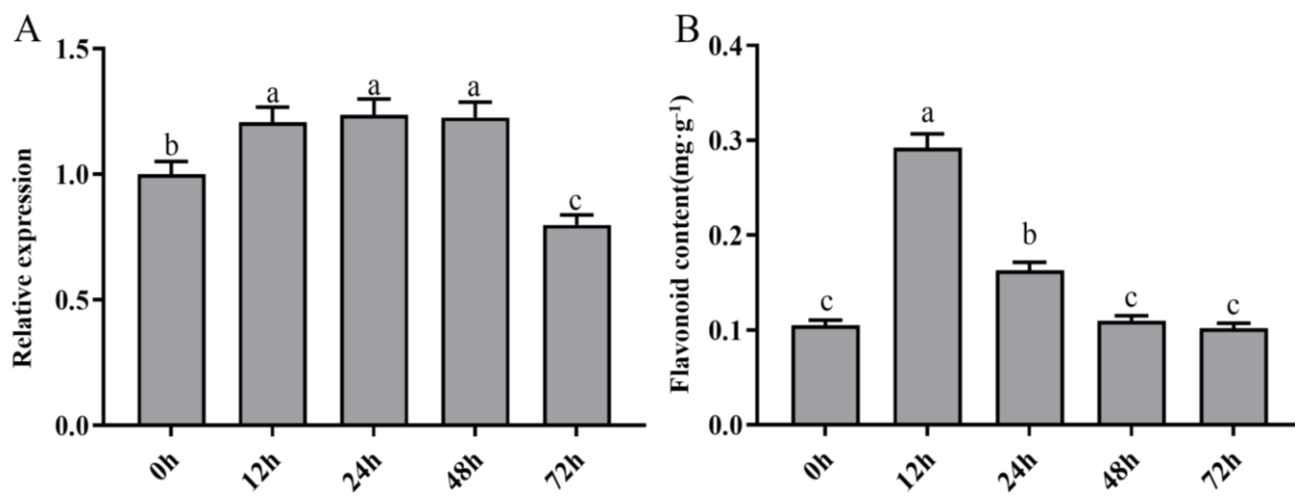

Fig. 6. The expression of *AfF3H* and the content of flavonoids under low-temperature stress from 0 h to 72 h. (A). Relative expression of *AfF3H* under low-temperature stress. (B) Flavoniod content analysis in leaves under low –temperature stress (p<0.05).

Discussion

Dihydroflavonol is the precursor of flavonols and leucoanthocyanidin. Flavanone-3-hydroxylase can catalyse flavanone to produce dihydroflavonol, which is the key enzyme that regulates flavonoid metabolism and accumulation (Owens *et al*., 2008; Khumkarjorn *et al*., 2017a; b). In this study, we isolated the gene encoding *F3H* from *A. fistulosum* and expressed *AfF3H* protein in *E. coli* by a prokaryotic expression system. The recombinant *AfF3H* in *E. coli* could catalyse naringenin to DHK and eriodyctiol to DHQ. *AfF3H* in *A. fistulosum* has a highly conserved ferrous binding site HxDxNH and 2-oxoglutarate binding site RXS, which is consistent with other *F3H* proteins (Koehntop *et al*., 2005; Clifton *et al*., 2006; Xiong *et al*., 2016), indicating that the *AfF3H* protein belongs to the 2-ODD family. Three conserved prolines (Pro148, Pro204, and Pro207) were proposed to play an important role in protein folding (Xiong *et al*., 2016). These three prolines are also conserved in *AfF3H*, which may have helped maintain the function of F3H during the evolution of *A. fistulosum*.

The expression of *AfF3H* leads to an increase in downstream products, such as flavonols, catechin, and anthocyanin (Shirley *et al*., 1995; Koehntop *et al*., 2005; Owens *et al*., 2008; Flachowsky *et al*., 2012; Tu *et al*., 2016). Different substrate specificities and tissue expression specificities of the *F3H* enzyme in different plants lead to differences in anthocyanins, flavonols, and other flavonoids in plant species. The expression of the *CsF3H* gene in tea plants is specific and regulated by light. The expression level of *CsF3H* was the highest in mature leaves, and it was speculated to be related to the increase in flavanol glycosides in tea leaves (Han *et al*., 2017). In this study, RT–PCR was used to analyse the expression of the *F3H* gene and the content of flavonoids in Welsh onion. The results suggested that the expression of *AfF3H* was specific to *A. fistulosum* and highly expressed in flowers. The increase in flavonoids in *A. fistulosum* is inconsistent with the expression of *AfF3H*, which may be because *AfF3H* is an early gene in the flavonoid synthesis pathway.

When plants are under abiotic stress, flavonoids can protect the photosystem by inhibiting the level of reactive oxygen species. Previous studies have shown that cold

stress increases the accumulation of flavonoids. Overexpression of *SlHY5* in tomato showed that *HY5* could induce the accumulation of flavonoids and alleviate chilling stress, and the early flavonoid genes *CHS* and *F3H* were also upregulated (Han *et al*., 2020). In strawberry research, *F3H* was also found to respond to cold stress. By analysing the *F3H* response of cold-tolerant and cold-sensitive strawberries under cold stress, it was found that the expression of *F3H* in low-temperature-treated strawberries increased, while the expression of *F3H* in cold-tolerant strawberries was significantly higher than that in cold-sensitive strawberries (Badek *et al*., 2014). In the present study, the expression of *AfF3H* was caused by cold stress, and flavonoids were also accumulated. These results support that *F3H* can participate in the resistance to cold stress by increasing the flavonoid metabolic flux through upregulation of expression.

Conclusion

In this study, the flavonone-3-hydroxylase gene was identified in *A. fistulosum*. The subcellular localization results showed that *AfF3H* was located in the cytosol. Prokaryotic expression analysis confirmed that *F3H* could catalyse dihydroquercetin and dihydrokaempferol from eriodyctiol and naringenin, respectively. The expression of *AfF3H* was specific. The flavonoid content in the leaves of *A. fistulosum* was the highest. Cold stress increased the expression of *AfF3H* and the accumulation of flavonoids. This study laid a foundation for further study of the biosynthesis pathway of flavonoids in *A. fistulosum* and provided theoretical data for the postharvest treatment of *A. fistulosum*.

References

Agati, G., C. Brunetti, M. Di Ferdinando, F. Ferrini, S. Pollastri and M. Tattini. 2013. Functional roles of flavonoids in photoprotection: New evidence, lessons from the past. *Plant Physiol. Biochem.*, 72: 35-45.

Aguadé, M. 2001. Nucleotide sequence variation at two genes of the phenylpropanoid pathway, the *FAH1* and *F3H* genes, in *Arabidopsis thaliana*. *Mol. Biol. Evol*., 1: 1-9.

Aoyama, S. and Y. Yamamoto. 2007. Antioxidant activity and flavonoid content of welsh onion (*Allium fistulosum*) and the effect of thermal treatment. *Food Sci. Technol. Res.*, 13: 67-72.

Badek, B., B. Napiórkowska, A. Masny and M. Korbin. 2014. Changes in the expression of three cold-regulated genes in "Elsanta" and "Selvik" strawberry (*Fragaria* × *ananassa*) plants exposed to freezing. *J. Hort. Res.*, 22(2): 53-61.

Chernukha, I., L. Fedulova, E. Vasilevskaya, A. Kulikovskii, N. Kupaeva and E. Kotenkova. 2021. Antioxidant effect of ethanolic onion (*Allium cepa*) husk extract in ageing rats. *Saudi J. Biol.*, 28(10): 2877-2885.

Clifton, I.J., M.A. McDonough, D. Ehrismann, N.J. Kershaw, N. Granatino and C.J. Schofield. 2006. Structural studies on 2-oxoglutarate oxygenases and related double-stranded beta-helix fold proteins. *J. Inorg. Biochem.*, 100: 644-669.

Ferreyra, M.L.F., S. Rius, J. Emiliani, L. Pourcel, A. Feller, K. Morohashi, P. Casati and E. Grotewold. 2010. Cloning and characterization of a UV-B-inducible maize flavonol synthase. *Plant J*., 62: 77-91.

Flachowsky, H., H. Halbwirth, D. Treutter, K. Richter, M.V. Hanke, I. Szankowski, C. Gosch, K. Stich and T.C. Fischer. 2012. Silencing of flavanone-3-hydroxylase in apple (*Malus* × *domestica* Borkh.) leads to accumulation of flavanones, but not to reduced fire blight susceptibility. *Plant Physiol. Biochem.*, 51: 18-25.

Gao, S., X. Liu, Y. Liu, B. Cao, Z. Chen and K. Xu. 2021. Comparison of the effects of LED light quality combination on growth and nutrient accumulation in green onion (*Allium fistulosum* L.). *Protoplasma*, 258: 753-763.

Gebhardt, Y., S. Witte, G. Forkmann, R. Lukačin, U. Matern and S. Martens. 2005. Molecular evolution of flavonoid dioxygenases in the family Apiaceae. *Phytochem*., 66: 1273-1284.

Gutiérrez-Albanchez, E., A. Gradillas, A. García, A. García-Villaraco, F.J. Gutierrez-Mañero and B. Ramos-Solano. 2020. Elicitation with Bacillus QV15 reveals a pivotal role of F3H on flavonoid metabolism improving adaptation to biotic stress in blackberry. *PLoS One*, 15: 1-22.

Hammerbacher, A., D. Kandasamy, C. Ullah, A. Schmidt, L.P. Wright and J. Gershenzon. 2019. Flavanone-3-hydroxylase plays an important role in the biosynthesis of spruce phenolic defenses against bark beetles and their fungal associates. *Front. Plant Sci.*, 10: 208.

Han, N., S. Fan, T. Zhang, H. Sun, Y. Zhu, H. Gong and J. Guo. 2020. *SlHY5* is a necessary regulator of the cold acclimation response in tomato. *Plant Growth Regul.*, 91: 1-12.

Han, Y., K. Huang, Y. Liu, T. Jiao, G. Ma, Y. Qian, P. Wang, X. Dai, L. Gao and T. Xia. 2017. Functional analysis of two flavanone-3-hydroxylase genes from *Camellia sinensis*: A critical role in flavonoid accumulation. *Genes (Basel)*, 8(11): 300.

Hassan, S. and U. Mathesius. 2012. The role of flavonoids in root-rhizosphere signalling: Opportunities and challenges for improving plant-microbe interactions. *J. Exp. Bot*., 63: 3429-3444.

Jia, H., S. Jiu, C. Zhang, C. Wang, P. Tariq, Z. Liu, B. Wang, L. Cui and J. Fang. 2016. Abscisic acid and sucrose regulate tomato and strawberry fruit ripening through the abscisic acid-stress-ripening transcription factor. *Plant Biotechnol. J*., 14: 2045-2065.

Khumkarjorn, N., S. and P. Thanonkeo. 2017b. Cloning and expression analysis of a flavanone 3-hydroxylase gene in *Ascocenda orchid*. *J. Plant Biochem. Biotechnol*., 26: 1-12.

Khumkarjorn, N., S. Thanonkeo, M. Yamada, P. Klanrit and P. Thanonkeo. 2017a. Agrobacterium-mediated transformation of dendrobium orchid with the flavanone 3-hydroxylase gene. *Turk. J. Bot.*, 41: 442-454.

Koehntop, K.D., J.P. Emerson and L.J. Que. 2005. The 2-His-1-carboxylate facial triad: a versatile platform for dioxygen activation by mononuclear non-heme iron(II) enzymes. *J. Biol. Inorg. Chem.*, 10: 87-93.

Kothari, D., W. Do Lee and S.K. Kim. 2020. *Allium* flavonols: Health benefits, molecular targets, and bioavailability. *Antioxidants*., 9:888.

Li, C., S. Liu, X. Yao, J. Wang, T. Wang, Z. Zhang, P. Zhang and K. Chen. 2017. PnF3H, a flavanone 3-hydroxylase from the *Antarctic moss* Pohlia nutans , confers tolerance to salt stress and ABA treatment in transgenic *Arabidopsis*. *Plant Growth Regul.*, 83: 489-500.

Li, H., Y. Dong, J. Yang, X. Liu, Y. Wang, N. Yao, L. Guan, N. Wang, J. Wu and X. Li. 2012. De novo transcriptome of safflower and the identification of putative genes for oleosin and the biosynthesis of flavonoids. *PLoS One*, 7: e30987.

Liu, M., X. Li, Y. Liu and B. Cao. 2013. Regulation of flavanone 3-hydroxylase gene involved in the flavonoid biosynthesis pathway in response to UV-B radiation and drought stress in the desert plant, *Reaumuria soongorica*. *Plant Physiol. Biochem.*, 73: 161-167.

Liu, Q., F. Wu, Y. Chen, S.T. Alrashood and S.A. Alharbi. 2021. Anti-human colon cancer properties of a novel chemotherapeutic supplement formulated by gold nanoparticles containing *Allium sativum* L., leaf aqueous extract and investigation of its cytotoxicity and antioxidant activities. *Arab. J. Chem.*, 14: 103039.

Lukačina, R., F. Wellmanna, L. Britsch, S. Martens and U. Matern. 2003. Flavonol synthase from Citrus unshiu is a bifunctional dioxygenase. *Phytochem.*, 62: 287-292.

Marefati, N., V. Ghorani, F. Shakeri, M. Boskabady, F. Kianian, R. Rezaee and M.H. Boskabady. 2021. A review of anti-inflammatory, antioxidant, and immunomodulatory effects of *Allium cepa* and its main constituents. *Pharm. Biol.*, 59: 287-302.

Miean, K.H. and S. Mohamed. 2001. Flavonoid (myricetin, quercetin, kaemferol, luteolin, and aapigenin) content of edible tropical plants. *J. Agric. Food Chem.*, 49: 3106-3112.

Owens, D.K., A.B. Alerding, K.C. Crosby, A.B. Bandara, J.H. Westwood and B.S.J. Winkel. 2008. Functional analysis of a predicted flavonol synthase gene family in *Arabidopsis*. *Plant Physiol.*, 147: 1046-1061.

Park, S., D.H. Kim, J.H. Yang, J.Y. Lee and S.H. Lim. 2020. Increased flavonol levels in tobacco expressing AcFLS affect flower color and root growth. *Int. J. Mol. Sci.*, 21(3): 1011.

Preuß, A., R. Stracke, B. Weisshaar, A. Hillebrecht, U. Matern and S. Martens. 2009. *Arabidopsis thaliana* expresses a second functional flavonol synthase. *FEBS Lett.*, 583: 1981-1986.

Schmittgen, T.D. and K.J. Livak. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.*, 25: 402-408.

Shirley, B.W., W.L. Kubasek, G. Storz, E. Bruggemann, M. Koornneef, F.M. Ausubel and H.M. Goodman. 1995. Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J.*, 8: 659-671.

Sun, Y., J. Hu, W. Wang, B. Zhang and Y. Shen. 2019. Characterization of γ-glutamyltranspeptidases from dormant garlic and onion bulbs. *Food Sci. Nutr.*, 7: 499-505.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.*, 30: 2725-2729.

Tu, Y.H., F. Liu, D.D. Guo, L.J. Fan, Z.X. Zhu, Y.R. Xue, Y. Gao and M.L. Guo. 2016. Molecular characterization of flavanone 3-hydroxylase gene and flavonoid accumulation in two chemotyped safflower lines in response to methyl jasmonate stimulation. *B.M.C. Plant Biol.*, 16: 132.

Wang, Y., S. Gao, X. He, Y. Li, Y. Zhang and W. Chen. 2020. Response of total phenols, flavonoids, minerals, and amino acids of four edible fern species to four shading treatments. *Peer J.*, 8:e8354.

Xiong, S., N. Tian, J. Long, Y. Chen, Y. Qin, J. Feng, W. Xiao and S. Liu. 2016. Molecular cloning and characterization of a flavanone 3-Hydroxylase gene from *Artemisia annua* L. *Plant Physiol. Biochem.*, 105: 29-36.

Zuker, A., T. Tzfira, H. Ben-Meir, M. Ovadis, E. Shklarman, H. Itzhaki, G. Forkmann, S. Martens, I. Neta-Sharir, D. Weiss and A. Vainstein. 2002. Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. *Mol. Breed.*, 9: 33-41.

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