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The *Aconitum carmichaelii F3′5′H* Gene Overexpression Increases Flavonoid Accumulation in Transgenic Tobacco Plants

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Abstract: *Aconitum carmichaelii* Debx. is a herbal species that contains many precious bioactive substances, which are alkaloids, flavonoids, steroids, and glycosides. Flavonoids, which are major secondary compounds, play an important role in maintaining redox balance in the cells of the plant body. Many flavonoids have antibacterial, antioxidant, and anticancer properties. However, studies have mainly focused on aconitine, which is a highly toxic group A poison belonging to the alkaloid group, but with little mention of flavonoids. The flavonoids in *A. carmichaelii* are a group of substances with high content, concentrated in leaves and flowers, including quercetin and kaempferol. F3′5′H (Flavonoid 3′5′-hydroxylase) has been identified as the key enzyme involved in the final steps of flavonoid biosynthesis in plants in general and in *A. carmichaelii* specifically. This study offers the first report, and demonstrates that the overexpression of the *F3′5′H* gene from a herbal plant, *A. carmichaelii*, increases flavonoid content in genetically modified tobacco plants. The *A. carmichaelii* gene was transformed into tobacco leaf tissue to create transgenic tobacco plants. The *AcF3′5′H* gene was incorporated into the tobacco genome and was expressed in four transgenic tobacco lines ($T_0$1, $T_0$3, $T_0$5, and $T_0$14). The *F3′5′H* content increased from 20.33% to 32.00% compared with that in non-transformed plants ($P < 0.001$). Therefore, the flavonoid content of four transgenic tobacco lines increased compared to the WT, from 69.23% to 122.23% ($P < 0.001$). The results of the successful expression of the *AcF3′5′H* gene in model tobacco plants are the basis for using the *AcF3′5′H* gene for improving flavonoid content in other medicinal plants. Thus, the *AcF3′5′H* gene considered in this work could be a candidate for gene technology to enhance flavonoid accumulation in plants.

Keywords: *AcF3′5′H* gene overexpression; flavonoid; recombinant flavonoid 3′5′-hydroxylase; genetic transformation; genetically modified tobacco

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1. Introduction

Flavonoids are major secondary compounds with important functions in maintaining redox balance in plant cells. Many flavonoid types have antibacterial, antioxidant, and anticancer properties. *Aconitum carmichaelii* contains aconitine, which is a highly toxic group A poison, but *A. carmichaelii* is still believed to be a precious medicine and is widely used in traditional medicine [1], such that flavonoids from medicinal plants, including *Aconitum* species, are of interest in the development of modern pharmaceuticals. Flavonoid biosynthesis (Figure S1) is a metabolic process with the participation of many important enzymes, such as chalcone synthase, chalcone isomerase, flavone 3-hydroxylase, flavonoid 3′-hydroxylase, flavonoid 3′5′-hydroxylase (F3′5′H), flavonol synthase enzyme, flavonol

4-sulfotransferase . . . , and F3′5′H is key enzyme for the final reactions in the biosynthesis of these secondary compounds [2–4].

F3′5′H, a member of the cytochrome P450 family, is associated with the membrane part of the endoplasmic reticulum, dependent on NADPH and O_2, and is sensitive to inhibitors, such as plant growth regulators (1-aminobenzotriazole and tetcyclacis) and CO, N-ethylmaleimide, diethyldithiocarbamate, and cytochrome [5,6]. *F3′5′H* participates in the reaction converting naringenin to flavonoids [2,7–9]. The final product of flavonoid biosynthesis in plants was determined by hydrogenation of the 5′ position with the participation of the *F3′5′H* enzyme [2,3,8]. Therefore, the overexpression of the *F3′5′H* gene should increase the *F3′5′H* enzyme concentration and result in an increment in flavonoid accumulation.

Tobacco is used as a model for research on the function of plant genes and testing for applications to improve some characteristics of crops using gene transfer. Several reports on the results of *F3′5′H* gene expression from a few different plants that changed the flavonoid content in transgenic tobacco have been found in several publications. The *F3′5′H* gene from petunia plants expressed in tobacco changed the synthesis of anthocyanin pigment [3], while the overexpression of the *CsF3′5′H* gene from the *C. sinensis* plant produced new delphinidin derivatives and increased the cyanidin derivative content of transgenic tobacco plants [8]. Research by Okinaka et al. (2003) [10] showed that the expression of the *F3′5′H* gene from *Campanula medium* plants increases the accumulation of delphinidin derivatives in transgenic tobacco.

Several studies using genes encoding several other enzymes involved in flavonoid synthesis were also investigated for expression. Hu et al. (2019) reported that the overexpression of *CHS* increased flavonoid accumulation in tobacco and that *CHS* is a candidate gene for gene transfer to improve the abiotic stress tolerance of plants [11]. Vu et al. (2018) reported that *GmCHI* expression in soybeans enabled *Talinum paniculatum* plants to improve their total flavonoid content [12]. The results of the gene expression analyses in these studies showed that the *F3′5′H* transgene derived from different plant species has different effects on altering the accumulation of flavonoid content in transgenic plants.

F3′5′H (EC 1.14.14.81) is the key enzyme in flavonoid biosynthesis, and is commonly encoded by the *F3′5′H* gene in higher plants. The diversity and different levels of *F3′5′H* gene expression among species have resulted in the accumulation levels of flavonoids between species being highly variable. The leaves of *A. carmichaelii* were reported to contain flavonoids at a relatively high content (1.6% dry weight) [13]. The genome of *A. carmichaelii*, a medicinal plant, contains the *F3′5′H* enzyme-encoding gene that is 1521 bp in length, and which encodes 506 amino acids [14]. The Basic Local Alignment Search Tool (BLAST) was used to find regions of similarity between the *AcF3′5′H* (of *A. carmichaelii*) and *F3′5′H* sequences in GenBank. The evolutionary analysis of the deduced proteins of these *F3′5′H* sequences by the maximum likelihood method shows that the *F3′5′H* of the *Aconitum* family is split from the other plant families (details are given in the discussion section). The above research results were the basis for us to select and clarify the role of the *AcF3′5′H* gene in the flavonoids synthesis and to answer the question of whether the *AcF3′5′H* gene from *A. carmichaelii* could be considered a candidate gene to enhance the accumulation of flavonoids in herbal plants.

The aim of this study was to demonstrate the expression of the gene encoding the key enzyme *F3′5′H* in the flavonoid biosynthetic pathway isolated from *A. carmichaelii*, which contains a high flavonoid content and is a candidate gene for improving the flavonoid content of medicinal herbs. Therefore, the results of the successful testing of the *AcF3′5′H* transgene in model plants will be the basis for using this gene to improve the flavonoid contents of other herbs.

2. Materials and Methods

2.1. Materials

The K326 tobacco (*Nicotiana tabacum*) cultivar stored at the Plant Cell Biotechnology Laboratory, Faculty of Biology, TNU University of Education, Vietnam, was used for gene transfer and other analyses.

The pCB301_Ac*F3′5′H* transgenic construct as described in Figure 1 was introduced into *Agrobacterium tumefaciens*. The *AcF3′5′H* gene with a size of 1536 bp includes nucleotides containing cut-off points of *Nco*I and *Not*I, and was appended to cmyc and KDEL segments, so the *AcF3′5′H-cmy-KDEL* segment was 1581 bp in size [14].

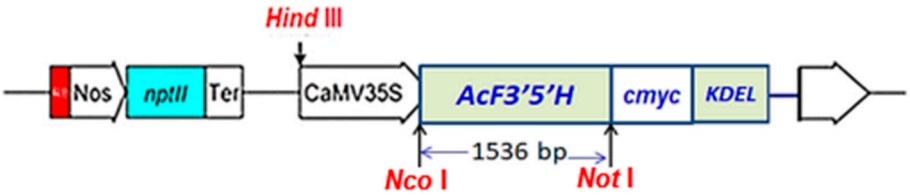

Figure 1. Schematic of the pCB301 transgenic vector carrying the *35S_AcF3′5′H_cmyc_KDEL* structure. *nptII*: neomycin phosphotransferase II gene. *CaMV35S*: *cauliflower mosaic virus* 35S promoter; *AcF3′5′H:* the *A. carmichaelii flavonoid 3′5′-hydroxylase* gene containing the cut-off points *Nco*I and *Not*I; *cmyc:* the DNA segment encodes the cmyc antigen; *KDEL:* the DNA segment encodes the KDEL peptide.

PCR and RT-PCR were used in the genetic analysis of the transformed plants with a primer pair, including *Fla_NcoI-F:* 5′-AGccatggATGTTGTCTACCAGAGAACTTGTCGCTGCAGCGATCATTTTTTTCATT-3′ and *Fla_NotI-R:* 5′-ATgcggccgcGACTACATAAGCAGAGGGTG-3′, and the clone *AcF3′5′H* gene segment had an expected size of 1536 bp (Figure 1).

2.2. Transferring the A. Carmichaelii F3′5′H Gene into Tobacco through A. Tumefaciens

The pCB301_*AcF3′5′H* structure was transferred into tobacco leaf tissue through *A. tumefaciens*, and the shoot regeneration induction from the transformed samples was performed according to Topping et al. (1998) [15]. The transformed samples were regenerated on MS 1X medium added with BAP and 50 mg L^{-1} kanamycin. The leaf pieces were submerged in the *A. tumefaciens* suspension for 30 min; then, explants were transferred to CCM (co-cultivation medium) for 3 days in the dark. The transformed samples were washed with cefotaxime (500 mg L^{-1}) and multi-bud regenerated in SIM (shoot induction medium). The SIM contained basic MS 1X, 1.0 mg L^{-1} BAP, 30 g L^{-1} sucrose, 9.0 g L^{-1} agar. We added cefotaxime (500 mg L^{-1}) and kanamycin (50 mg L^{-1}) for 35 days of shoot regeneration. The shoots were separated from the explants and transplanted into a shoot-growing medium supplemented with kanamycin for 28 days. Then the shoots induced for rooting roots on RM (rooting medium) included basic MS 1X, MES (1.0 g L^{-1}), sucrose (30 g L^{-1}), agar (9.0 g L^{-1}), and coconut water (100 mL L^{-1}). We added IBA (0.5 mg L^{-1}) and kanamycin (50 mg L^{-1}) for 35 days, which formed plants with sufficient roots, stems, and leaves. The plantlets with good growth qualities were transferred to pots containing a mixture that included soil, rice husk biochar, and coir with a ratio of 2:1:2, and the surviving plants were cared for in greenhouse conditions.

2.3. Confirmation of the Transgene in Transformed Tobacco

Total DNA was isolated from young leaves based on the method of Murray and Thompson (1980) [16]. A Genelute Total RNA Miniprep kit (Sigma) was used for total RNA extraction and the First-Strand cDNA synthesis kit (Fermentas) was used for cDNA synthesis.

PCR was performed to confirm the presence of the *AcF3′5′H* transgene in the transgenic tobacco plants. Expression analysis of the *AcF3′5′H* gene at the transcriptional level

was performed by RT-PCR. The composition of PCR and RT-PCR included the Dream Taq 2X master mix, primers, template DNA or cDNA, and deionized water at a total volume of 25 µL. The thermal cycling of PCR consisted of 94 °C/3 min, and 30 cycles with temperatures and durations of each cycle of 94 °C/30 s, 58 °C/45 s, 72 °C/1 min 30 s, and 7 min at 72 °C, before storage at 4 °C.

2.4. Confirmation of the Recombinant AcF3′5′H Protein Expression in Genetically Modified (GM) Tobacco Plants

RT-PCR-positive transgenic tobacco plants were used for the expression analysis of recombinant *A. carmichaelii* flavonoid 3′5′-hydroxylase (r*AcF3′5′H*) using Western blotting and enzyme-linked immunosorbent assays (ELISA). The monoclonal anti-c-myc antibody produced in mice was used to detect the *F3′5′H* recombinant protein in the transgenic tobacco plants. The protein of WT and transgenic tobacco leaves was analyzed using 10% SDS–polyacrylamide gel electrophoresis [17] and Western blot. The recombinant *A. carmichaelii* flavonoid 3′5′-hydroxylase content was determined by ELISA [18]. Total protein concentration was diluted at a level of 200 µg mL^{-1}. The cmyc-tagged H5 protein was used as a positive control. The standard curve for the quantification of the rAcF3′5′H protein was constructed based on the concentration of the diluted H5 protein. The r*AcF3′5′H* protein content (µg μL^{-1}) was calculated according to the formula: $Y = 0.0019\ X + 0.056$, with R as 0.9993; Y is the recombinant *A. carmichaelii* flavonoid 3′5′-hydroxylase protein content and R is correlation coefficient.

2.5. Content Analysis of total Flavonoid in GM Tobacco and Non-Transformed Plants

The total flavonoid content in GM and non-transformed tobacco plants (wild-type: WT) was determined by absorption spectroscopy [19]. Flavonoids from tobacco leaves were extracted with methanol and flavonoids in the methanolic extract were reacted with aluminum chloride. The absorbance of flavonoids was measured at the 415 nm wavelength using a UV-VIS device (UV2401 Shimadzu). The flavonoid quantitative calibration curve was built on a quercetin standard as Y (quercetin content) = $0.0061\ X - 0.0029$, and with an R of 0.9999. The content of flavonoids (Cm) was determined according to the formula Cm (µg $g^{-}1$) = (Am/Ac) Cc k (V/m), where Cc—the quercetin (µg mL^{-1}); Am—the absorbances; Ac—the standard solutions; V—the final volume; m (g)—sample weight and k—the dilution factor.

2.6. Statistical Analysis

The statistical data of protein r*AcF3′5′H* and flavonoid content were analyzed using the Statistical Package for the Social Sciences (SPSS) software and we used Duncan's test to determine the difference at $P < 0.001$.

Evolutionary analysis of the *F3′5′H* gene and *F3′5′H* protein by the maximum likelihood method was conducted in MEGA X [20]. The evolutionary history was inferred using the maximum likelihood method and the JTT matrix-based model [21]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [22].

3. Results

3.1. Results of Expression of the A. Carmichaelii Flavonoid 3′5′-Hydroxylase Gene in GM Tobacco Plants

Ninety leaf pieces from in vitro tobacco plants were infected using the transformed *A. tumefaciens* containing the construction *35S_AcF3′5′H_cmyc_KDEL* (Figure S2). The results yielded 81 explants inducing multiple shoots and proceed to select 268 well-developed shoots for culturing on RM. The transformed tobacco plants were transferred to pots and maintained in a greenhouse. Twenty-eight normal growing transformed plants were selected for molecular biology analysis. As a control setup, 30 non-transformed tobacco leaf

pieces were cultured in an antibiotic-free medium, and 10 WT plants were to be grown in pots in the greenhouse.

The transformed tobacco plants were analyzed by PCR; the PCR results show that 7 out of 28 were found to be positive for the *AcF3′5′H* gene (Figure 2A). In Figure 2A, a DNA band of approximately 1.5 kb in size appeared in electrophoresis lanes 1, 3, 5, 14, 19, and 22. The PCR-positive transgenic plants $T_0$1, $T_0$3, $T_0$5, $T_0$14, $T_0$19, and $T_0$22 were further analyzed by RT-PCR, and the analysis results show that only transgenic plants $T_0$1, $T_0$3, $T_0$5, and $T_0$14 had RT-PCR products (Figure 2B).

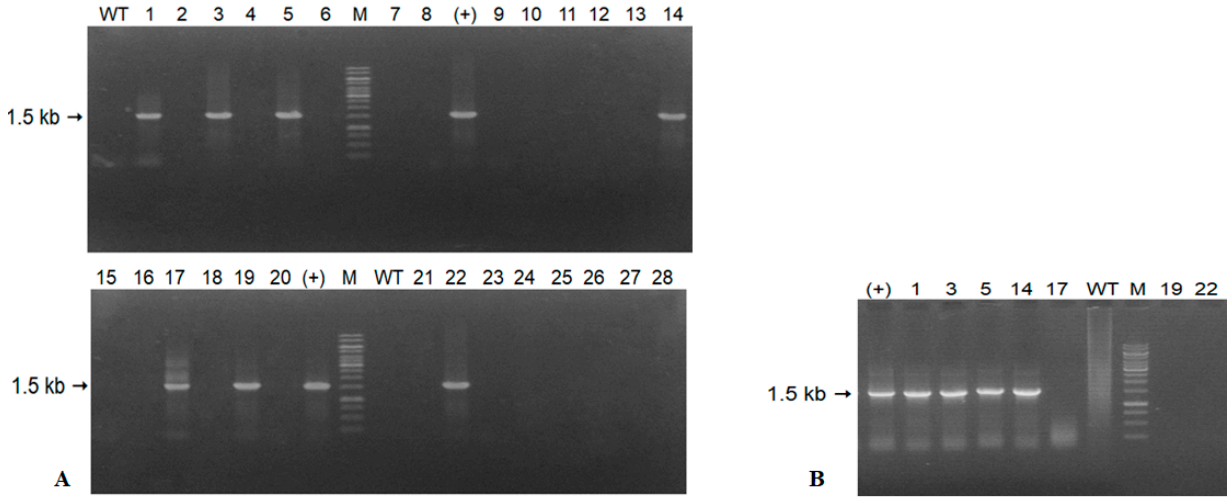

Figure 2. Electrophoresis results confirming the presence and transcription of the *AcF3′5′H* gene in the transformed plants. (**A**) Results of electrophoresis analysis of PCR products that amplified the *AcF3′5′H* transgene. M: 1.0 kb DNA ladder; (+): pCB301_*AcF3′5′H* vector; WT: non-transformed tobacco. Electrophoresis lanes from 1 to 28: transgenic tobacco plants. (**B**) Results of electrophoretic analysis of RT-PCR products, confirming the transcription of the *A. tumefaciens F3′5′H* gene in the transformed plants. M: 1.0 kb DNA ladder; (+): pCB301_AcF3′5′H vector; WT: non-transformed tobacco; 1, 3, 5, 14, 17, 19, and 22 electrophoresis lanes show the RT-PCR analysis results of 7 the PCR-positive transformed plants designated as T01, T03, T05, T014, T017, T019 and T022.

The recombinant *A. carmichaelii F3′5′H* protein expressed in $T_0$1, $T_0$3, $T_0$5, and $T_0$14 lines was confirmed using the analysis of SDS-PAGE electrophoresis and Western blot using the monoclonal anti-c-myc antibody produced in mice, and the results obtained are shown in Figure 3A. The Western blot analysis in Figure 3A shows that all T_0 transgenic lines had a color band with a 57 kDa in size as the molecular weight of the recombinant *A. carmichaelii F3′5′H* protein, including both the cmyc and KDEL amino acid sequences. The recombinant *A. carmichaelii F3′5′H* protein content in the GM lines ranged from 0.2033 μg μL^{-1} to 0.3250 μg μL^{-1} ($P < 0.001$) (Figure 3B). Thus, if the endogenous *F3′5′H* and recombinant *F3′5′H* proteins are included, the total *F3′5′H* protein content in the GM lines was increased, and is more than the *F3′5′H* protein content in WT plants. These results demonstrate that the *AcF3′5′H* was incorporated into the transgenic tobacco plant genome and expressed the recombinant protein.

3.2. Content of Flavonoid in Leaves of GM Tobacco Lines

The results of observations of the transgenic and WT plants confirm that the GM tobacco lines presented normal morphological characteristics as the WT plants. However, the GM lines had lower plant heights and slower growth rates in comparison to WT plants. In addition, the petals of the transgenic lines were darker than those of WT (Figure 4).

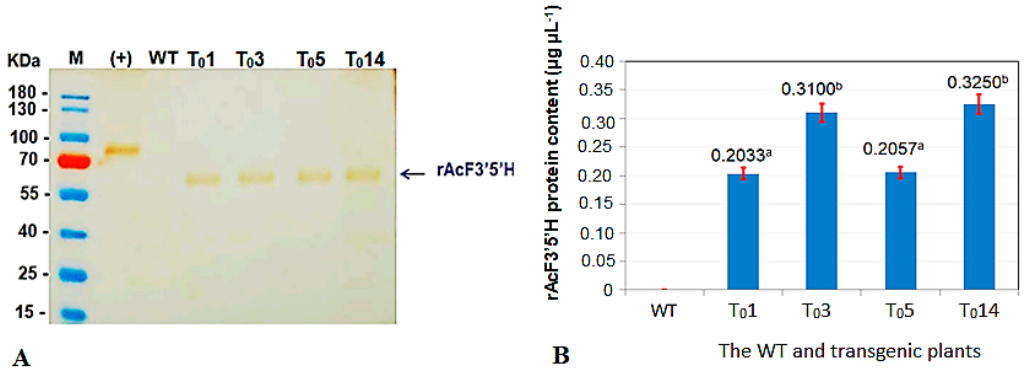

Figure 3. Expression of the r*AcF3′5′H* protein in T_0 transgenic tobacco plants. (**A**) Analysis of the r*AcF3′5′H* protein's expression using Western blot. M: standard protein ladder; (+): the cmyc-tagged H5 protein was used as a positive control; WT: non-transformed plants; $T_0$1, $T_0$3, $T_0$5, and $T_0$14: transgenic tobacco plants. (**B**) Recombinant *A. carmichaelii F3′5′H* content (μg μL^{-1}) in the T_0 transgenic tobacco plants. WT: non-transformed plants; $T_0$1, $T_0$3, $T_0$5, $T_0$14: T_0 transgenic tobacco plants. The letters a and b on the columns represent differences with $P < 0.001$; $n = 3$.

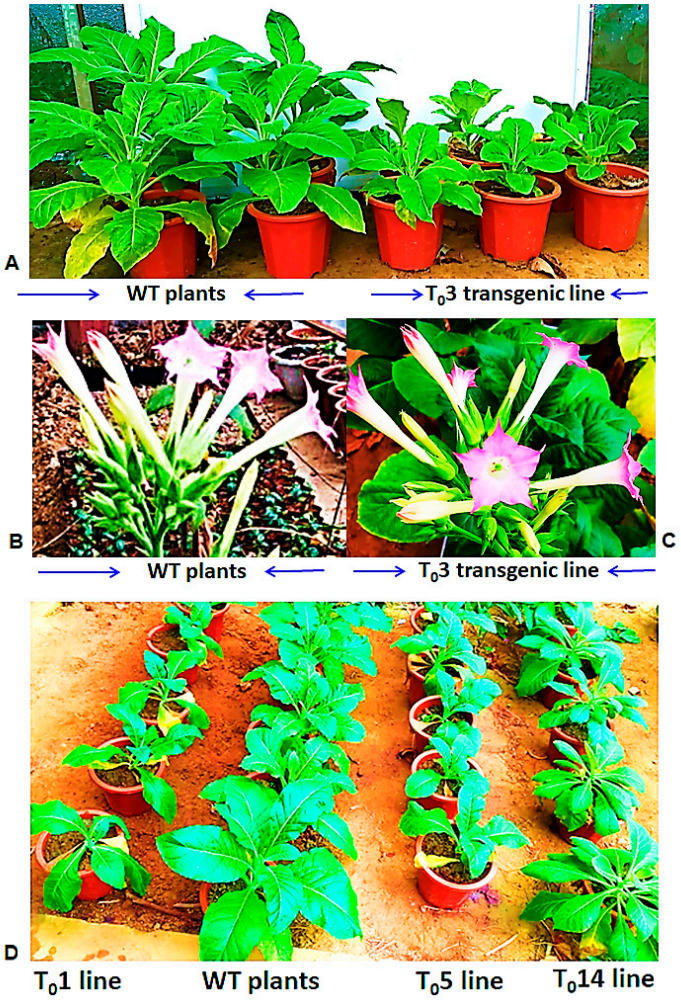

Figure 4. Morphology of the WT plants and GM tobacco lines. (**A**) WT plants and $T_0$3 transgenic lines; (**B**) flowers of WT plants; (**C**) flowers of $T_0$3 transgenic line; (**D**) WT plants and $T_0$1, $T_0$5, $T_0$14 transgenic lines.

The contents of total flavonoids in the leaves of the four GM tobacco lines and WT plants were determined (Table 1). In Table 1 and Figure S3, four transgenic lines, $T_0$1, $T_0$3, $T_0$5, and $T_0$14, had flavonoid contents from 691.20 ± 2.02 to 907.83 ± 5.14 (μg g^{-1}) and 69.23 to 122.23 (%) higher than those of WT plants (408.43 ± 5.11 μg g^{-1}) ($P < 0.001$). These analytical results demonstrate that the *AcF3′5′H* gene's overexpression in four transgenic tobacco lines, $T_0$1, $T_0$3, $T_0$5, and $T_0$14, increases flavonoids in GM tobacco plants.

Table 1. Comparison of total flavonoid content between GM tobacco lines, $T_0$1, $T_0$3, $T_0$5, $T_0$14, and WT plants.

Samples	Content of Flavonoids * (μg g^{-1})	The Increase Compared to WT (%)
WT	408.43 ^{a} ± 5.11	0
$T_0$1	691.20 ^{b} ± 2.02	69.23
$T_0$3	907.83 ^{c} ± 5.14	122.23
$T_0$5	713.60 ^{b} ± 4.21	74.72
$T_0$14	900.37 ^{c} ± 0.81	120.45

* $\overline{X} \pm S_E$; the letters a, b, c represents difference with $P < 0.001$; $n = 3$.

Within the scope of this study, the flavonoid content and r*AcF3′5′H* recombinant protein content of the four genetically modified tobacco lines were positively correlated, with a correlation coefficient (R) = 99.09%; the regression equation is Y =1 766.72 X + 342.14, where Y is the flavonoid content (μg/g) and X is the protein content of r*AcF3′5′H* (μg/μL) ($P < 0.05$).

4. Discussion

Flavonoid biosynthesis is an important secondary metabolic pathway involving the participation of many important enzymes, such as CHS, CHI [23], IFS, F3′H, F3′5′H, FLS, and FST [2–4,24]. Flavonoid biosynthetic pathways provide various anthocyanins, flavonoids, and isoflavonoids to medicinal plants, and they have the effects of preventing cancer, inhibiting mitosis, and increasing estrogen as well as antioxidants [24]. Of the important enzymes of the flavonoid biosynthetic pathway, *F3′5′H* is a key enzyme that catalyzes reactions in the formation of flavonoids [2–4]. Studies about the expression of *F3′5′H* genes derived from different plant species or overexpression analyses to investigate gene function have been performed [25–27]. According to the *F3′5′H* gene overexpression research approach, Wu et al. (2020) pointed out that the *GbF3′5′H1* gene plays an important role in the biosynthesis of flavonoid-related metabolites in *Ginkgo biloba* plants, and that the *GbF3′5′H1* gene's overexpression increased the content of epicatechin and gallocatechin in this species [28]. For the *A. carmichaelii* plant, the stems and leaves were identified as a new medicinal resource. Leaves and flowers of *A. carmichaelii* accumulate carotenoids, sterols, and flavonoids [13]. The results of these studies have suggested the search for an association between the expression of the *A. carmichaelii F3′5′H* gene and the increase in flavonoid content in other plant species. In a previous study, the *A. carmichaelii F3′5′H* gene was cloned and included in the expression vector in plants (Figure 1) [14], and it has been demonstrated that *A. carmichaelii F3′5′H* gene overexpression increased the flavonoid content of genetically modified (GM) *A. carmichaelii* plants from 39.13 to 63.63% compared with WT plants [29].

In this study, the *AcF3′5′H* gene was transformed into tobacco tissue to produce GM tobacco plants, and the recombinant *A. carmichaelii F3′5′H* protein was expressed in four GM tobacco lines, namely, $T_0$1, $T_0$3, $T_0$5, and $T_0$14. The flavonoid contents in the leaves of transgenic tobacco lines $T_0$1, $T_0$3, $T_0$5, and $T_0$14 reached 691.20 ± 2.02, 907.83 ± 5.14, 713.60 ± 4.21, and 900.37 ± 0.81, respectively. Compared to that in the non-transformed plants (408.43 ± 5.11), the flavonoid content in the leaves of transgenic tobacco lines increased from 69.23% to 122.23% (Table 1 and Figure S3).

To clarify this research idea, we would like to further discuss why the *AcF3′5′H* gene of *A. carmichaelii* [14], a herb with high flavonoid content [13], was used to enhance the flavonoid content in transgenic plants. From the data on NCBI, the results of comparing 15 *F3′5′H* sequences via BLAST show that the *F3′5′H* sequence cloned from *A. carmichaelii* was close to species of the *Aconitum* genus, and accounted for 99 to 100% of the query coverage and the relatedness to the *Aconitum* species, with a total blast score of 2501 to 2765 and 96.38 to 99.47% sequence identity (Table S1). There were fifteen plants in the top 100 blast hits of *F3′5′H* (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on July 28 2021).

Compared with species in the same family, Ranunculaceae, *AcF3′5′H* was closer to species of the *Delphinium* and *Clematis* genus, and provided for 93 to 94% of query coverage and relatedness to the *Aconitum* species, with a total blast score of 876 to 1701 and 86.52 to 88.12% sequence identity. However, when compared with species belonging to other families, the query coverage and total blast score were much lower, at 2 to 91% and 54.7 to 512, respectively. The diversity of the *F3′5′H* gene sequence was also inferred from the results of the evolutionary analysis. The *AcF3′5′H* gene of the *A. carmichaelii* species was located far from that of the other plant families (Figure 5A), and the *F3′5′H* protein of the *Aconitum* species showed similar differences (Figure 5B).

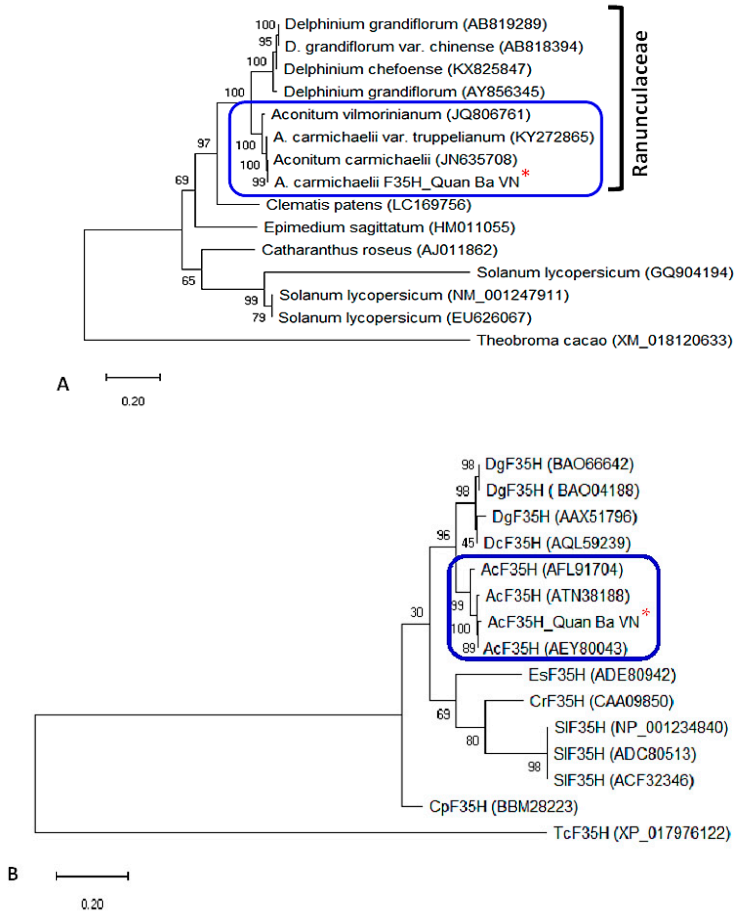

Figure 5. Evolutionary analysis of the *F3′5′H* gene (**A**) and F3′5′H protein (**B**) by the maximum likelihood method. A: The analysis results involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 1482 positions in the final dataset. B: Analysis results involved 15 amino acid sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 488 positions in the final dataset. **AcF3′5′H* gene and *F3′5′H* protein of *A. carmichaelii.* The blue frames are the nucleotide sequences of the *AcF3′5′H* gene and the amino acid sequences of the *F3′5′H* protein of *A. carmichaelii*.

Together with the above analyses, the *AcF3′5′H* gene encoding the key enzyme of the flavonoid biosynthetic pathway was isolated from *A. carmichaelii*, a herbal species containing relatively high flavonoid content, which is the basis for explaining why the *F3′5′H* gene was selected for testing to improve the content of flavonoid in medicinal plants. The flavonoid pathway, which is a complex metabolic network in plants, begins with general phenylpropanoid metabolism and leads to a multitude of end-products, including flavonoids. Recombinant *F3′5′H*, together with enzymes of flavonoid biosynthesis, act as multi-enzyme complexes to increase flavonoid accumulation efficiency in transgenic plants.

In gene expression engineering, tobacco is a model plant, and the successful expression of *F3′5′H* in the *A. carmichaelii* from transformed tobacco lines is the basis for us to conclude that the *AcF3′5′H* gene studied in this work can be considered a candidate gene for genetic engineering to enhance flavonoid accumulation in plants.

Thus, it can be seen that a candidate gene used for the purpose of improving flavonoid content in plants must satisfy the following two criteria. First, the gene must be a gene encoding an important enzyme involved in flavonoid biosynthesis. Second, it must be cloned from a plant with a high flavonoid content.

5. Conclusions

In this study, the *AcF3′5′H* gene was successfully expressed in tobacco plants, and the flavonoid accumulation in the leaves of the four transgenic tobacco lines increased from 1.69- to 2.22-fold compared with that of WT. The flavonoid content and recombinant *F3′5′H* enzyme content in transgenic tobacco lines were positively correlated with a correlation coefficient of 99.09%. It is possible to generate medicinal plant lines with high flavonoid contents using *AcF3′5′H* as a transgene. These plant lines can be propagated to become plant sources for extracting flavonoids for medicinal purposes. Therefore, the gene encoding a vital enzyme in flavonoid biosynthesis isolated from a high-flavonoid content-species is the first criteria to be selected as a candidate gene in improving the flavonoid content in plants.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7100384/s1, Figure S1: Flavonoid biosynthesis in plants; Figure S2. *AcF3′5′H* transgene transformation via *A. tumefaciens* and in vitro generation of the transgenic tobacco plants; Figure S3. Total flavonoid content (μg g^{-1}) of four transgenic tobacco lines, Table S1: Fifteen plants in the top 100 blast hits of F3′5′H.

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Abbreviations

AcF3′5′H: *Aconitum carmichaelii F3′5′H* gene; AS: acetosyringone; BAP: 6-benzylaminopurine; CCM: co-cultivation medium; ELISA: enzyme-linked immunosorbent assay; F3′5′H: flavonoid 3′5′-hydroxylase; GM: genetically modified; IBA: idolbutylic acid; MS: plant cell culture medium according to Murashige và Skoog (1962); MES: 2-(Nmorpholino) ethanesulfonic acid; RM: rooting medium; rAcF3′5′H: recombinant AcF3′5′H; SIM: shoot induction medium; T_0: generation of in vitro transgenic plants transferred from culture vessel to pot; $T_0$1, $T_0$3, $T_0$5, and $T_0$14: transgenic lines in T_0 generation; WT: wild type, non-transformed.

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