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RESEARCH OF IN VITRO AND FLAVONOID 3'5' HYDROXYLASE MANIFESTATION TO ENHANCE FLAVONOID SYNTHETIC IN *Aconitum carmichalii* Debx.

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PUBLIC RESEARCHES RELATED TO THE THESIS

1. Thi Ngoc Lan Nguyen, Thi Thu Hoan Hoang, Huu Quan Nguyen, Quang Tan Tu, Thi Hong Tran, Thi Mai Thu Lo, Thi Thu Thuy Vu, Hoang Mau Chu (2021), "*Agrobacterium tumefaciens*–mediated genetic transformation and overexpression of the flavonoid 3′5′-hydroxylase gene increases the flavonoid content of the transgenic *A. carmichaelii* Debx. plant", *In Vitro Cellular & Developmental Biology – Plant*; https://doi.org/10.1007/s11627-021-10190-4 (SCIE)

2. Yen Thi Hai Nguyen, Hoan Thi Thu Hoang, Anh Thi Hoang Mai, Lan Thi Ngoc Nguyen, Quan Huu Nguyen, Nhan Thi Thanh Pham, Thuong Danh Sy, Mau Hoang Chu (2021), The *A. carmichaelii* F3'5'H gene overexpression increases flavonoid accumulation in transgenic tobacco plants, *Horticulturae*, 7(10), 384, https://doi.org/10.3390/horticulturae7100384 (SCIE)

3. Hoang Thi Thu Hoan, Hoang Thi Phuong, Dang Thi Le, Nguyen Thi Ngoc Lan, Chu Hoang Mau (2017), "Study on morphological, anatomical and molecular characteristics of *A. carmichaelii* (*A. carmichaelii* Debx.)", *Journal of Science & Technology - Thai Nguyen University*, 168(08), pp. 161 - 167.

4. Hoang Thi Thu Hoan, Nguyen Thi Ngoc Lan, Chu Hoang Mau (2020), "Molecular cloning and design of transgenic vectors carrying the flavonoid 3'5' Hydroxylase gene isolated from *A. carmichaelii* Debx.) , *Journal of Science & Technology - Thai Nguyen University*, 225(08), p. 43 - 49.

5. Hoang Thi Thu Hoan, Tran Thi Hong, Nguyen Huu Quan, Nguyen Thi Ngoc Lan, Chu Hoang Mau (2021), "Study on in vitro regeneration system and hairy root induction in *A. carmichaelii* debeaux) ", *Journal of Science & Technology - Thai Nguyen University*, 226(05), pp. 139 - 146.

INTRODUCTION

1. Put the problem

Aconitum carmichaelii Debx, the Radix Aconiti lateralis praeparata contain highly toxic ingredients, but they are still considered a precious medicine, commonly used in traditional oriental medicine. Currently, in the world, there are many studies on *Aconitum* genus in order to develop products towards improving the efficiency of using species of this genus in disease prevention and treatment. In Vietnam, *A. carmichaelii* is found growing wild in the high mountains of Northern Vietnam and later it was planted in Ha Giang, Lao Cai, and Lai Chau. Currently, it is planted a lots in Quan Ba, Dong Van district, Ha Giang province.

Flavonoids are major secondary compounds that play an important role in maintaining redox balance in plant cells. Many types of flavonoids have antibacterial, antioxidant, and anticancer properties. Flavonoids in the *Aconitum* genus are of interest in modern pharmaceutical research, and flavonoid 3'5'-hydroxylase (F3'5'H) is a key enzyme that catalyzes the final reactions in biosynthesis Flavonoid compounds in Aconitum.

F3′5′H, a member of the Cytochrome P450 branch, participates in the conversion of naringenin to flavonoids. Hydrogenation of the 5' position by F3'5'H is an important reaction because it determines the end product of plant flavonoid biosynthesis. Thus, enhancing F3'5'H gene expression would increase the concentration and activity of the enzyme F3'5'H and lead to increased flavonoid accumulation in plants.

Thus, two approaches to increase the content of biologically active substances are selected: application of plant cell culture technology to increase biomass and key gene expression technique to increase accumulation of bioactive compounds. amount of secondary compounds in transgenic plants. Stemming from the above reasons, we have chosen and conducted the thesis: "**Research of *in vitro* and *flavonoid 3'5' hydroxylase* manifestation to enhance flavonoid synthetic in *A. carmichaelii* Debx.**"

2. Research objectives

2.1. Some suitable conditions for *in vitro* culture and induction of hairy root formation have been identified in the *A. carmichaelii* Debx.

2.2. It was demonstrated that the expression of the gene encoding Flavonoid 3'5' hydroxylase of *A. carmichaelii* Debx. increased the accumulation of flavonoids in transgenic plants.

3. Research contents

1) Research on species identification from A. carmichaelii collecting in some localities of Ha Giang province by comparative morphology and DNA barcoding.

2) Research on establishing *in vitro* culture system and hairy root formation in *A. carmichaelii* Debx.

3) Design expression vector of gene encoding Flavonoid 3'5'hydroxylase and create strain *Agrobacterium tumefacience* carrying plant transgenic vector.

4) Study on transformation and expression of *AcF3'5'H* gene in Tobacco plants.

5) Study on transformation and expression of *AcF3'5'H* gene in the *A. carmichaelii* Debx.

4. New contributions of the thesis

The thesis is a systematic research, from identifying the samples of the *A. carmichaelii* to establishing an *in vitro* regeneration system for gene transfer to the induction of hairy root formation and the proliferation of hairy root biomass in the *A. carmichaelii*. From cloning the *AcF3'5'H* gene from the *A. carmichaelii* and designing gene expression vectors in plants to studying genetic transformation and analyzing *AcF3'5'H* gene expression in transgenic plants. The new scientific contributions of the thesis are shown in particular:

1) The *A. carmichaelii* in Quan Ba and Hoang Su Phi districts, Ha Giang province, Vietnam belongs to the same species *A. carmichaelii, Aconitum* genus, Hoang Lien (Ranunculaceae) specie were identified by a combination of comparative morphological methods and DNA barcoding.

2) Determining the suitable conditions for in vitro culture and creating hairy root lines from the roots of *A. carmichaelii in vitro* are materials for selecting hairy root lines with content of high secondary biologically active compounds.

3) For the first time in the world, the *AcF3'5'H* gene from the *A. carmichaelii* was transformed and successfully expressed in Tobacco and the *A. carmichaelii*. Overexpression of *AcF3'5'H* gene increased flavonoid content in transgenic plants.

The research result of the thesis is the first report in the world and in Vietnam on the analysis of *AcF3'5'H* gene expression of the *A. carmichaelii*

5. Scientific and practical significance of the thesis

Scientifically, the results of gene expression analysis on Tobacco and *A. carmichaelii* are the basis for studies on enhancing *AcF3'5'H* gene expression in other medicinal species with the aim of increasing the accumulation of flavonoids in parts of plant. The *AcF3'5'H* gene from *A. carmichaelii* studied in this research can be considered as a candidate to enhance flavonoid accumulation in plants by genetic technology. The results of in vitro culture and hairy root induction are the basis for applying this technology to improve the content and efficiency of obtaining biologically active compounds in the *A. carmichaelii* and some other medical herbs.

6. Thesis structure

The thesis has 152 pages (including appendices), divided into chapters and sections: Introduction (5 pages); Chapter 1: Document overview (37 pages); Chapter 2: Materials and research methods (18 pages); Chapter 3: Results and discussion (51 pages); Conclusion and recommendations (2 pages); Published researches related to the thesis (2 pages); References (23 pages); Appendix (14 pages). The thesis has 11 tables, 40 figures, 14 appendices and 183 references.

Chapter 1. DOCUMENTARY OVERVIEW

The thesis has consulted and summarized 183 documents, including 20 documents in Vietnamese, 162 documents in English on three basic issues, which are (1) The *A. carmichaelii* Debx; (2) *In vitro* culture and induction of hairy roots in medicinal plants by *in vitro* culture; (3) Flavonoids and gene expression study of flavonide 3'5' hydroxylase;

The *A. carmichaelii* has the scientific name *A. carmichaelii* Debx, it belongs to the *Aconitum* L. genus, and contains bioactive compounds such as flavonoids, which have analgesic, immune-enhancing, antioxidant, anti-proliferative cell. Anti-cancer, cardiovascular effects, anti-inflammatory, anti-diarrheal... Currently, there aren't any studies on the application of DNA barcodes (*matK*, *rpoC1*, *rpoB2*) to identify *A. carmichaelii* samples in nature. Therefore, we combined both comparative morphological methods and molecular taxonomy methods to identify *A. carmichaelii* samples collected in some districts of Ha Giang.

Flavonoids are synthesized by the phenylpropanoid in general by the action of a multienzyme complex, including CHS, CHI, F3H, F3'H, F3'5'H, FLS, FST. Hydroxylation of the 5' position by F3'5'H, which is a particularly important step, identifying the ending product is tri-hydroxyl B-circle of flavonoid formation in plants.

In the *A. carmichaelii* Debx, the gene *F3'5'H* isolated from mRNA was published in 2012 by Ma and CS with a size of 1720 bp, coding for 506 amino acids, the code above NCBI is JN635708. There have been studies on the biological function of the *F3'5'H* gene, which show that the *F3'5'H* gene and the F3'5'H enzyme have an important function in the biosynthesis of anthocyanins, delphinidins and determining the biosynthesis of anthocyanins and delphinidins pigment determination in plants. The results of *F3'5'*H gene expression studies confirmed that strong expression of *F3'5'H* gene increased flavonoid content. Thus, affecting the enzyme F3'5'H can increase flavonoid accumulation. However, the study on the expression of genes encoding enzymes involved in flavonoid synthesis, the *F3'5'H*, of *A. carmichaelii* is still very new and so far we have not found any published expression analysis *AcF3'5'H* from the *A. carmichaelii* Debx.

Around the world, there have been studies on induction of hairy rooting to obtain camptothecin in happy trees (Lorence end cs 2004); alkaloids in the hairy roots of poppy (Le and cs, 2004), β-carboline in Tribulus terrestris (Sara and cs, 2014)... In Vietnam, Ha Thi Loan and cs (2014) and Ninh Thi Thao and cs (2015) created hairy roots of Ngoc Linh Ginseng and Dan ginseng to obtained saponins, Vu Thi Nhu Trang and cs (2017) created hairy root of Tho ginseng to increase flavonoid content... However, in the world As well as in Vietnam, there have not been published studies on the regeneration system for gene transfer and hairy root induction in *A. carmichaelii* Debx.

Chapter 2. RESEARCH MATERIALS AND METHODS

2.1. MATERIALS, CHEMICALS, RESEARCH EQUIPMENT

Research materials: the bulbs of *A. carmichaelii* were collected from Ha Giang province, grown at the Experimental Garden of the Department of Biology, University of Education - Thai Nguyen University for species identification for DNA extraction and culture materials in vitro. Tobacco variety K326 (*Nicotiana tabacum*), vectors used in the study include: pBT vector, pRTRA7/3, pCB301 and bacterial strains *E. coli* DH5α, *Agrobacterium tumefaciens* CV58, *Agrobacterium rhizogens* ATTC 15834. PCR primer pairs used in the study is shown in Table 2.1

Table 2.1. Sequences of bait used in the study

Pairs of bait	Sequences nucleotide 5' → 3'	Products (bp)
ITS-F/ITS-R	ACGAATTCATGGTCCGGTGAAGTGTTCG	630
	TAGAATTCCCCGGTTCGCTCGCCGTTAC	
rpoC1-F/ rpoC1-R	GTGGATACACTTCTTGATAATGG	543
	TGAGAAAACATAAGTAAACGGGC	
rpoB2-F/ rpoB2-R	AAGTGCATTGTTGGAACTGG	471
	GATCCCAGCATCACAATTCC	
matK-F/ matK-R	CGATCTATTCATTCAATATTTC	822
	TCTAGCACACGAAAGTCGAAGT	
F3'5'H-NcoI-F /F3'5'H-NotI-R	AGCCATGGATGTTGTCTACCAGAGAACTTG TCGCTGCAGCGATCATTTTTTTCATT	1536
	ATGCGGCCGCGACTACATAAGCAGAGGGTG	
F3'5'H-NcoI-F /F3'H-SacI-R	AGCCATGGATGTTGTCTACCAGAGAACTTG TCGCTGCAGCGATCATTTTTTTCATT	1611
	TAGAGCTCCGCTGATGTATTCGTCTCCCAC	

2.2. RESEARCH METHODS

2.2.1. Group of methods for species identification of the *A. carmichaelii*

The method of identifying samples of the *A. carmichaelii* by a comparative morphology according to Pham Hoang Ho (1999), Do Tat Loi (2004), searching

on the Tropicos website and molecular taxonomy based on a number of DNA barcodes such as *ITS* region, *matK* gene fragment, *rpoC1*, *rpoB2*.

2.2.2. Group of in vitro culture methods and *in vitro* hairy root induction

Studying on *in vitro* regeneration system for gene transfer and hairy root induction method, hairy root multiplication and dry root mass determination

2.2.3. Group of methods of gene cloning and design of transgenic vectors

Gene isolation and molecular cloning:

From the information on the *F3'5'H* gene sequence of the *A. carmichaelii* with code JN635708.1 on GenBank, the pairs of bait *F3'5'H-NcoI-F/F3'5'H-NotI-R* was designed by coding fragment of the gene *AcF3'5'H*. RNA Total was separated by Sigma's GeneElute™ Total RNA Miniprep kit of Sigma label. cDNA was synthesized from RNA total by the SuperScript™ VILO™ cDNA Synthesis kit.

Cloning of the *AcF3'5'H* gene was performed by PCR with the designed pairs of bait *F3'5'H-NcoI-F/F3'5'H-NotI-R.* PCR products were electrophoresed on a 1.0% agarose gel and purified by the GenJET PCR Purification kit of Fermentas label.

Gene cloning technique was carried out according to Sambrook and cs. Extract the plasmid uses the Plasmid Extraction kit according to the manufacturer's instructions. Recombinant plasmids carrying the *AcF3'5'H* gene were identified on an ABI PRISM 3100 Avant Gentic Analyzer automated sequencer. Nucleotide sequences of genes were analyzed and compared on BLAST and BioEdit software.

Gene expression vector design:

The vector design experiments are carried out according to the diagram 2.2.

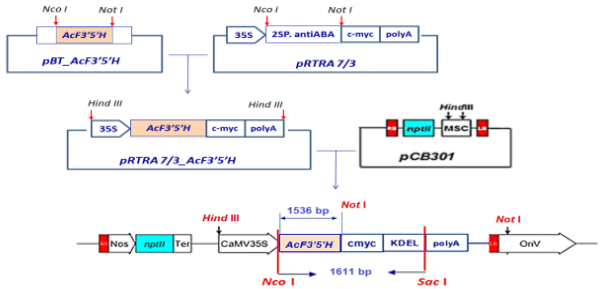

Figure 2.2. Diagram of the experimental design of the transgenic vector *pCB301_AcF3'5'H*

Generating recombinant Agrobacterium tumefaciens:

The vector *pCB301_AcF3'5'H* Transforms into the variable *A. tumefaciens* CV58 and selects the bacterial line carrying the recombinant vector *pCB301_AcF3'5'H* by colony-PCR.

2.2.3. Group of transgenic methods and analysis of transgenic plants

Genetic transformation into Tobacco: Preparation of infectious bacteria, *AcF3'5'H* gene transfer into tobacco through *A. tumefaciens*.

Genetic transformation into the *A. carmichaelii* Debx: Creating genetic transformation materials, setting up a gene transfer experiment through transgenic *uidA* marker gene in the *A. carmichaelii* Debx, Transferring the *CaMV35S_AcF3'5'H_cmyc_polyA* structure into the *A. carmichaelii* Debx.

Analysis of transgenic plants: Transgenic tobacco plants and Greenhouse-grown Transgenic *A. carmichaelii* were used to analyze the presence and expression of transgenes in transgenic plants by PCR, RT-PCR, Western blot and ELISA.

Determining of total flavonoid content in Tobacco plants and *A. carmichaelii* by absorption spectroscopy technique.

2.2.5. Statistical processing

The data are processed by Microsoft Excel software and Statistical Package for the Social Science (SPSS) software to determine statistical values such as mean, variance, standard deviation, sample mean error. . The difference between the mean values was tested by Duncan with $P < 0.05$; 0.01; 0.001.

2.3. RESEARCH LOCATION

Experiments were carried out from August 2016 to December 2020.

Experiments on *in vitro* culture and gene transfer into Tobacco and *A. carmichaelii* were carried out at the Laboratory of Plant Cell Technology, Department of Biology, University of Education - Thai Nguyen University. Experiments to analyze transgenic plants were conducted at the Applied DNA Technology Department, the Plant Cell Technology Department and the Gene Technology main Laboratory of the Institute of Biotechnology - Vietnamese Academy of Science and Technology. Experiments to analyze total flavonoid content in leaves of Tobacco and *A. carmichaelii* were carried out at the Department of Food Technology - National Institute of Food Hygiene and Testing, Ministry of Health.

Chapter 3. RESULTS AND DISCUSSION

3.1. RESULTS OF FINDING SPECIES OF A. CARMICHAELII (*Aconitum carmichaelii*)

3.1.1. Morphological characteristics of *A. carmichaelii* samples collected in Ha Giang

The results of the comparison of the two *A. carmichaelii* samples from Hoang Su Phi and Quan Ba districts, Ha Giang province showed that the samples were similar in morphology, including roots, stems, leaves and flowers. After comparing the morphological characteristics observed in the *A. carmichaelii* samples with those described by Pham Hoang Ho (1999), Do Tat Loi (2004) and at the same time searching on the Tropicos website shows that *A. carmichaelii*

samples in Hoang Su Phi and Quan Ba districts, Ha Giang province belong to the *A. carmichaelii* genus *Aconitum* L, Hoang Lien family (Ranunculaceae), Hoang Lien order (Ranunculales), Hoang Lien subclass (Ranunculidae), class Dicotyledonous (Magnoliopsida), phylum Flowering; Magnolia; Angiosperms (Magnoliophyta).

3.1.2. Nucleotide sequence characterization of the *ITS* region and the *matK, rpoC1, rpoB2* gene fragments

3.1.2.1. ITS region sequence characterization

The testing results of PCR products on 0.8 % agarose gel showed that in both lanes, there was only a single band with the size of more than 600 bp, corresponding to the expected size of the *ITS* region.

The results of nucleotide sequencing have identified the *ITS* region with the size of 630 bp. Using BLAST software in NCBI showed that the ITS region isolated from 2 study *A. carmichaelii* samples in Ha Giang, Vietnam *(ITS-QB, ITS-HSP*) had the similarity rate of 98.41%, 98.25% (for with *ITS* isolated from QB sample), and 97.94%, 97.78% (for *ITS* isolated from HSP sample) with two *ITS* sequences of *A. carmichaelii* (GenBank codes are AY571352 and MH922985). This result confirmed that the isolated nucleotide sequence was the *ITS* region belonging to *A. carmichaelii* species. The sequence of the *ITS* region of the two samples (*ITS-HSP, ITS-QB*) was accepted and published by GenBank with codes MH410519.1, MH410520.1.

3.1.2.2. Sequence characterization of the matK gene

The test results of PCR products on 0.8 % agarose gel showed that in both lanes, there was only a single band with the size of about 800 bp corresponding to the expected size of the *matK* gene fragment. The results of nucleotide sequencing have identified the *matK* gene fragment with the size of 822 bp. The results of BLAST analysis based on nucleotide sequences have the similarity ratio of *matK* sequences isolated from *A. carmichaelii* in Ha Giang, Vietnam are of 98.30% compared with two *matK* sequences of *A. carmichaelii* (codes on GenBank are KY407560 and KX347251). Therefore, these results demonstrated that the DNA fragment isolated from samples in Hoang Su Phi and Quan Ba, Ha Giang, Vietnam are the *matK* gene fragments of *A. carmichaelii* species. The *matK* gene sequences of two research samples *(matK-QB, matK-HSP*) were accepted and published by GenBank with codes LS398143.1, LS398144.1 respectively.

Thus, the sequence of the *ITS* region and the *matK* gene segment allow the identification of the *A. carmichaelii* in Hoang Su Phi and Quan Ba belonging to the species *A. carmichaelii* Debx.

3.1.2.3. Sequence characterization of rpoC1 and rpoB2 gene fragments

The results of amplifying the *rpoC1* gene fragment by PCR with the pairs of bait *rpoC1-F/rpoC1-R* obtained a DNA fragment with a size of more than 0.55

kb. The results of nucleotide sequencing obtained the *rpoC1* gene fragment isolated from *A. carmichaelii* in Ha Giang with the size of 543 nucleotides. The results of BLAST analysis in NCBI showed that the *rpoC1* gene sequence isolated from *A. carmichaelii* in Ha Giang has high similarity, 99% compared with the *rpoC1* gene sequence in the chloroplast genome of the *A. carmichaelii* in Ha Giang with KX347251, KT820663, KT820666, KT820667, KT820668, KT820669, KT820670 on GenBank. Thus, it can be confirmed that the gene fragment isolated from Ha Giang's *A. carmichaelii* is the *rpoC1* gene fragment of the *A. carmichaelii*.

The results of amplifying the *rpoB2* gene fragment by PCR with the pairs of bait *rpoB2-F/rpoB2-R* obtained a DNA fragment with a size of nearly 0.5 kb (Figure 3.5B). The size of the cloned DNA fragment was exactly the same as the expected size of the *rpoB2* gene fragment. The results of nucleotide sequencing showed that the *rpoB2* gene fragment isolated from *A. carmichaelii* in Ha Giang has a size of 471 nucleotides. BLAST analysis from NCBI showed that the sequence of *rpoB2* gene fragment isolated from Ha Giang tree has 99% similarity with the sequence of *rpoB2* gene in the chloroplast genome of KX347251 on GenBank. . The results of BLAST analysis confirmed that the gene fragment isolated from Ha Giang's *A. carmichaelii* was the *rpoB2* gene of the *A. carmichaelii* Debx.

3.1.3. Discussion of sample identification results of *A. carmichaelii*

The *A. carmichaelii* (*A. carmichaelii* Debx.) is an herb that grows naturally or is grown for medicinal purposes. By comparative morphological method, the samples of the *A. carmichaelii* were determined to have the same nutritional and reproductive characteristics and similar to the descriptive characteristics of the *A. carmichaelii* according to Pham Hoang Ho (1999), Do Tat Loi (2004). However, a number of features of the *A. carmichaelii* have many similarities with other species of the same genus *Aconitum*, so it is not possible to identify these *A. carmichaelii* belonging to the same or different species. Therefore, the combination of comparative morphology method with DNA barcoding (*ITS* region, *matK* gene fragment, *rpoC1* and *rpoB2*) helps to identify at the species level for medicinal plants in general and *A. carmichaelii* in particular. It is difficult to distinguish two species that are close to each other based only on morphology or deformed plant samples... From the *A. carmichaelii* sample genome, the isolated *ITS* region has a size of 630 bp; the three gene fragments *matK*, *rpoC1* and *rpoB2* have sizes of 822 bp, 543 bp and 471 bp respectively. By BLAST in NCBI, the *matK*, *rpoC1* and *rpoB2* gene sequences of the studied samples have the similarity rate of 98%, 99%, 99%, respectively, with the chloroplast gene sequences of *A. carmichaelii* species by Jihai Gao and cs (2016) sequenced, bearing code KY006977 on GenBank. At the same time, the sequence of the *ITS* region of the two samples has the similarity rate of 97% with the sequence of the *ITS* region in *A. carmichaelii* species sequenced by Luo Y and

cs. (2005) has the above code on GenBank as AY571352. This result serves as a basis to confirm that the *A. carmichaelii* in Ha Giang province, Vietnam belong to species *A. carmichaelii*, genus *Aconitum* L, Hoang Lien family (Ranunculaceae). This study focuses on analyzing two bar codes *mat*K and *ITS* to identify reliable markers in species identification of *A. carmichaelii*. In Vietnam, Vu Duc Loi and cs (2014) initially used the *ITS* gene sequence to identify the *A. carmichaelii* species, however, our research results show that the *ITS* barcode has degree of accuracy confidence is not high in species identification of *A. carmichaelii*, therefore, we believe that *matK* sequence is a better candidate for identification of the *A. carmichaelii* species (*A. carmichaelii*), and it is the solution in molecular evolutionary and phylogenetic analysis of the *Aconitum* genus.

3.2. RESULTS OF ESTABLISHING *IN VITRO* CULTURE AND HAIRY GENERATION ROOT IN *A. CARMICHAELII* DEBX

3.2.1. Establishment of *in vitro* culture system for gene transfer in *A. carmichaelii*

3.2.1.1. Generating starting material for in vitro culture from stem segment

The results of the study on the effect of the sterilization time with 0.1% $HgCl_2$ on the process of creating starting materials for in vitro culture from the stem of the *A.carmichaelii* showed that the sterilization time of 9 minutes was appropriate at the highest percentage of uninfected budding samples and better quality shoots than the other treatments.

3.2.1.2. Multi-bud induction and in vitro A. carmichaelii progeny

Effect of BAP and kinetin on shoot growth of the A. carmichaelii Debx

Concentrations of BAP 1.5 mg/l and kinetin 1.0 mg/l were suitable for *in vitro* multi-shoot regeneration from explants that were segmented with nodules ($p < 0.05$) (Table 3.2).

Table 3.2. Effect of BAP and kinetin on the induction of shoot formation from the *A. carmichaelii* stem segment. N=30; P<0.05

Concentration (mg/l)	BAP			kinetin		
	Number of buds/sample	Bud height (cm)	Bud quality	Number of buds/sample	Bud quality (cm)	Bud quality
After 8 weeks						
1,0	$3,13^{c} \pm 0,10$	$2,58^{c} \pm 0,13$	++	$3,80^{d} \pm 0,14$	$3,38^{d} \pm 0,18$	+++
1,5	$4,57^{d} \pm 0,14$	$3,50^{d} \pm 0,16$	+++	$3,20^{c} \pm 0,09$	$2,35^{c} \pm 0,15$	++

MS medium supplemented with sucrose 30 g/l and agar 9 g/l and BAP 1.5 mg/l gave the highest number of buds/explant (4.57 ± 0.14; after 8 weeks of culture transplant). Among the two tested cytokinins, BAP was more effective than kinetin in inducing multiple shoots and the number of shoots produced was higher than kinetin ($P < 0.05$).

The effect of α-NAA and IBA on in vitro rooting in A. carmichaelii Debx

Table 3.3 shows that α-NAA and IBA differentially affect rooting induction results of plants in vitro. After 4, 6, 8 weeks of culture with concentrations of α-NAA 0.7 mg/l and IBA 0.5 mg/l both gave the highest number of roots/buds. Among the different concentrations of IBA tested, the maximum *in vitro* rooting capacity of each shoot with MS medium supplemented with 0.5 mg/l IBA and the highest was 3.6 ± 0.16 roots/ well developed buds and roots, long fat roots; meanwhile, the maximum *in vitro* rooting ability of each shoot in MS medium supplemented with α-NAA 0.7 mg/l was 3.07 ± 0.07 (after 8 weeks of culture).

Table 3.3. Effect of α-NAA and IBA on rooting of *A. carmichaelii* buds in MS medium

Concentration (mg/l)	**α-NAA**			**IBA**		
	Number of roots/buds	Root length (cm)	Root quality	Number of roots/buds	Root length (cm)	Root quality
After 8 weeks						
0,5	$2,73^{c} \pm 0,12$	$2,31^{c} \pm 0,12$	++	$3,60^{d} \pm 0,16$	$3,72^{d} \pm 0,20$	+++
0,7	$3,07^{d} \pm 0,07$	$2,98^{d} \pm 0,15$	+++	$2,27^{b} \pm 0,15$	$2,32^{c} \pm 0,14$	++

Effect of media on the survival rate of in vitro plants and the growth of seedlings outside the nursery

The percentage of plants living on the media after 4 weeks on alluvial soil + rice husk + coir (2:1:2), the survival rate reached 93.56%, the plants grew and developed better than other plants comparing with other formulas, the tree grows quickly, the tree forms new leaves with dark green color, and there is not the phenomenon of dropping old leaves.

3.2.2. Cultivation to create hairy roots in *A. carmichaelii*

Induction of hairy roots in vitro in A. carmichaelii

The suitable conditions for induction of hairy rooting in the *A. carmichaelii* from root segments in vitro through infection with *R. rhizogenes* strain ATTC 15834 was OD_{600} = 0,6, infection time was 15 minutes and then co-cultured in 3 days in MS + sucrose 30 g/l + cefotaxime 500 mg/l supplemented with AS 100 µmol/l.

Survey on culture medium to increase *A. carmichaelii* hairy root

Conducting a survey on three conditions of the media for growing *A. carmichaelii* hairy root, namely solid, semi-liquid and liquid in shaking condition after 6 weeks, showed that the mass of hairy roots produced in the medium liquid, semi-liquid and solid increased 5,85 times, 4,11 times and 2,98 times respectively. The mass of dry hairy roots in liquid shaking medium was 0.39 g, semi-liquid shaking medium was 0,213 g and solid medium was 0,151 g.

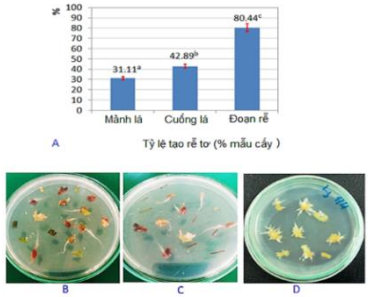

Figure 3.12. Image of results of surveying materials that induce *A. carmichaelii* hairy roots.

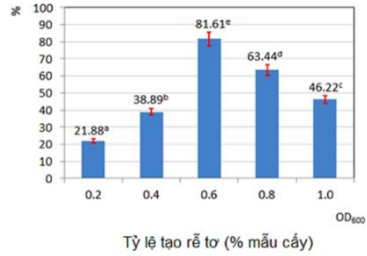

Figure 3.13. Induction of hairy roots from *in vitro* root segments infected

3.2.3. Discussing the results of establishing *in vitro* culture system and creating hairy roots in *A. carmichaelii*

In the process of genetic transformation mediated by *A. tumefaciens*, the *in vitro* regeneration system plays a particularly important role in determining the success of the gene transfer into tissues and regeneration of transgenic plants and in research. In this case, the segment of the stem bearing the node of the first tree was selected as the explant for shoot regeneration. This selection method is consistent with studies in some other plants, because the explants contain meristems or callus regenerative materials. For the *A. carmichaelii* genus, reported by Rawat et al. (2013)) suggested that the *in vitro* regeneration system from *A. carmichaelii* was performed from explants of the segmented gills. Multi-bud induction was achieved on MS medium supplemented with BAP 0,5 mg/l and naphthalene acetic acid (NAA) 0,1 mg/l, with a successful regeneration rate of about 85,43%. Singh and cs (2020) reported on *in vitro* propagation from the root segment of *Aconitum ferox*, an endangered Himalayan medicinal plant. *Aconitum ferox* root tips were used for callus formation and then transferred to MS medium supplemented with BAP for shoot regeneration. In this study, the suitable medium for creating regenerative multi-buds from stem segments of the tree was basal MS supplemented with BAP 1,5 mg/l or kinetin 1,0 mg/l. After 8 weeks, in MS basal medium supplemented with BAP 1,5 mg/l produced 4,57 ± 0,14 (buds/sample), while in MS basal medium supplemented with kinetin 1,0 mg/ l produces 3,80 ± 0,14 (buds/sample). Therefore, MS medium supplemented with 1,5 mg/l BAP was selected for the regeneration system of *A. carmichaelii* for gene transfer.

To increase biomass and produce bioactive substances in hairy root cultures of medicinal plants, studies on infecting *R. rhizogens* into medicinal plant tissues to produce hairy roots have been interested and successfully studied with many different medicinal plants. An efficient hairy root induction system for

Dracocephalum kotschy, an endangered medicinal plant, has been developed mediated by *R. rhizogens*. This report shows that the frequency of transformation was increased when the MS medium lacked NH_4NO_3, KH_2PO_4, KNO_3 and $CaCl_2$, leading to an increase in the frequency of hairy root induction from 52,3% to 80,0%. For Turkish ginseng, hairy roots were created from leaf fragments infected with *R. rhizogens* strain LB510. Silky roots were grown in liquid MS medium without growth regulators and different sucrose concentrations affected the biomass accumulation of hairy roots and the maximum biomass reached the yield of MS medium supplemented with 6% sucrose, was about 3 times higher than that of the control. An optimized procedure for soybean *R. rhizogens*-mediated transformation and induction of hairy root development *in vitro* from cotyledons has been described by Chen and cs. (2018). After 10-12 days of inoculation and co-culture, hairy roots were produced in the culture medium and 90-99% of inoculated explants of 5 different cultivars produced hairy roots within one month. For *A. heterozygous*, a species of the *A. carmichaelii* genus induction of hairy roots from callus was investigated. Embryo callus was infected with *R. rhizogens* to induce hairy roots and the best growth of hairy roots on ¼MS medium with sucrose 30 g/l. Recently, there have been many published studies on the results of hairy root induction from different medicinal plants, however, research on hairy root induction in *Aconitum* species is very limited and has not been studied. There are no published studies on hairy root induction from *A. carmichaelii*. In this study, hairy roots were induced from root segments of *A. carmichaelii* in vitro by infection with *R. rhizogens*. Root segments in vitro were infected with *R. rhizogens* with OD_{600} = 0,6 in 15 min, cultured in 3 days in MS medium (MS + sucrose 30 g/l + cefotaxime 500 mg/l) supplemented with AS 100 µmol /l. After 6 weeks of culture, hairy roots in liquid medium, under shaking conditions increased the highest biomass of hairy roots (3,18 g fresh weight/jar), hairy root weight increased by 5,85 times compared to initial fresh root mass. The active substances in the roots and tubers of the plant have many important medicinal and medicinal values, therefore, research on growth of hairy root biomass is a promising research direction to increase the acquisition of compounds with biological activity from *A. carmichaelii* Debx.

3.3. RESULTS OF GENE ISOLATION, VECTOR DESIGN AND EXPRESSION OF FLAVONOID 3'5' HYDROXYLASE GENERATOR AND CREATION OF VECTOR *A. tumefacinens* PLANT TRANSFORMED

3.3.1. Isolation of *AcF3'5'H* gene from *A. carmichaelii*.

The results of testing the *AcF3'5'H* genome PCR product obtained a specific DNA band with the size of about 1,5 kb, exactly as calculated according to the theory (Figure 3.15A). The results of colony-PCR product electrophoresis in Figure 3.15B show that a DNA band about 1,5 kb in size is the size of the *AcF3'5'H* gene.

The results of sequencing the DNA fragment from the recombinant plasmid *pBT_AcF3'5'H* on the automated device and analysis obtained a nucleotide sequence

of 1536 bp in size. The results of BLAST analysis in NCBI showed that the main DNA fragment is the nucleotide sequence of the coding segment of the *AcF3'5'H* gene isolated from the *A. carmichaelii* Debx, with 99,47% similarity compared with the carrier gene sequence. code JN635708.1 on GenBank. The results of BLAST analysis confirmed that the gene fragment isolated from the *A. carmichaelii* in Quan Ba district, Ha Giang province is the *AcF3'5'H* mRNA gene of the *A. carmichaelii* Debx.

Compared with the amino acid sequence inferred from the *F3'5'H* gene with the code JN635708.1 on GenBank, the protein derived from the coding segment of the *AcF3'5'H* gene isolated from *A. carmichaelii* in Quan Ba, Ha Giang has difference in 7 amino acids at positions 246, 280, 317, 325, 459, 481, 505

3.3.2. Design of plant transgenic vectors carrying *AcF3'5'H* gene

3.3.2.1. Generating independent structures carrying the AcF3'5'H transgene

Carrying out the parallel treatment of the recombinant vector *pBT_AcF3'5'H* and vector pRTRA7/3 with the enzyme pair *NcoI/NotI* to receive the *AcF3'5'H* gene and the pRTRA7/3 loop opener vector (Figure 3.18). In Figure 3.18, in electrophoresis lane 1, there are two electrophoresis bands in which the 1,5 kb DNA segment is the *AcF3'5'H* gene to be acquired. The product that cuts the pRTRA7/3 vector with the *NcoI/NotI* enzyme pair in Figure 3.18 (electrophoresis lane 2) shows that there are 2 DNA bands in which, the 3,3 kb segment is the pPTRA7/3 open loop. The results of electrophoresis test in Figure 3.19 showed that all 3 colony lines appeared a DNA band with the size of about 1,5 kb corresponding to the size of the *AcF3'5'H* gene.

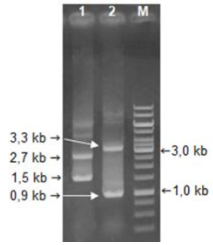

Figure 3.18. Electrophoresis of the *pBT_AcF3'5'H* circle opener and pRTRA7/3 cut with the restriction of pairs of enzyme *NcoI/NotI*. M: Marker 1 kb; 1: Recombinant vector cutting product *pBT_AcF3'5'H* by *NcoI/NotI*; 2: The product of pRTRA7/3 vector cutting by *NcoI/NotI*.

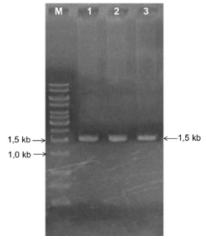

Figure 3.19. Electrophoresis of colony-PCR products cloned *AcF3'5'H* gene from *E. coli* DH5α colony lines. M: Marker 1 kb; 1-3: Colony-PCR from *pRTRA7/3_AcF3'5'H*-transformed colony lines

Thus, three bacterial strains containing the recombinant plasmid carrying the *AcF3'5'H* gene were selected. The recombinant plasmid *pRTRA_ AcF3'5'H* extracted and purified from PCR-positive colonies was used to obtain structures carrying the *AcF3'5'H* transgene for the creation of transgenic vectors in plants.

3.3.2.2. Generating vector pCB301_AcF3'5'H

Using *Hind*III, cut vector *pRTRA7/3_AcF3'5'H* to obtain structures carrying transgene *CaMV35S_AcF3'5'H_cmyc_KDEL_polyA* (2,4 kb) and open-loop transgenic vector pCB301 (5,5 kb) (Figure 3.20). Using T4 ligase to attach the *CaMV35S_AcF3'5'H_cmyc_KDEL_polyA* structure to the pCB301 vector to create the transgenic vector *pCB301_AcF3'5'H*.

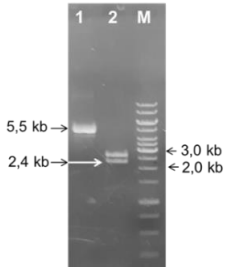

Figure 3.20. Electrophoresis of plasmid cleavage products using *Hind*III. M: Marker 1 kb; 1: plasmid pCB301 is cleaved by *Hind*III; 2: Plasmid *pRTRA7/3_AcF3'5'H* is cleaved by *Hind*III.

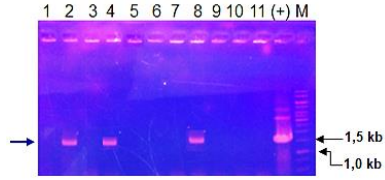

Figure 3.22. Electrophoresis of the colony-PCR cloned *AcF3'5'H* gene from recombinant *E. coli* colonies. M: Marker 1 kb; 1-11: colony strains; (+): plasmid *pBT_AcF3'5'H*

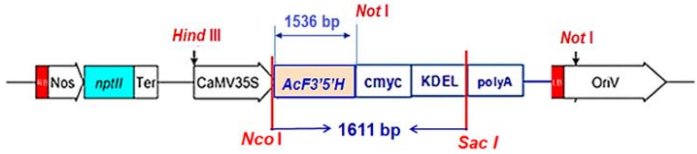

Figure 3.21. Structure diagram of the transgenic vector *pCB301_AcF3'5'H*. RB: Right bank; *Nos*: Synthetic nopalin; *nptII*: kanamycin resistance gene; *Ter*: terminator; *CaMV35S*: CaMV35S promoter; *AcF3'5'H*: Flavonoid 3'5'hydroxylase gene (*AcF3'5'H*) isolated from the *A. carmichaelii* Debx; cmyc: nucleotide sequence encoding the c-myc peptide; *KDEL*: nucleotide sequence encoding the KDEL peptide; *poly A*: Chain polyA; LB: Left bank; *OriV*: *A. tumefaciens* replication promoter region; The cleavage sites of restriction enzymes *Hind*III, *Not*I, *Nco*I, *Sac*I are shown in the diagram.

The primer pair *F3'5'H-NcoI-F/F3'H-SacI-R* was designed for PCR to clone the *AcF3'5'H* gene with a size of 1536 bp. In the vector *pCB301_ AcF3'5'H*, the *AcF3'5'H* gene has 1536 bp, adding the nucleotide sequence encoding the cmyc peptide (33 bp), KDEL (12 bp) and the DNA fragment containing the *Sac*I enzyme cleavage site (30 bp), so the *AcF3'5'H_cmyc_KDEL* DNA fragment in the *pCB301_ AcF3'5'H* vector has 1611 bp (Figure 3.21).

The results of electrophoresis of the colony-PCR product in Figure 3.22 confirmed that the bacterial lines contained the recombinant vector *pCB301_ AcF3'5'H*. The results of cloning showed that, out of 11 tested colony lines, 3 were positive for colony-PCR.

3.3.3. Generating *Agrobacterium tumefaciens* CV58 containing transgenic vector *pCB301_AcF3'5'H*

The electrophoresis image of colony-PCR products in Figure 3.23 shows that in 3 tested colony lines 2 have positive results, on gel electrophoresis appears a single DNA band with size about 1 ,5 kb, corresponding to the size of the AcF3'5'H transgene.

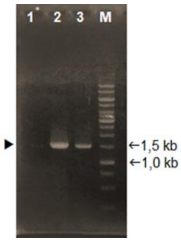

Figure 3.23. Electrophoresis of colony-PCR products with primer pairs F3'5'H-NcoI-F/F3'5H-NotI-R from A. tumefaciens CV58 colony lines. M: Marker 1 kb; 1, 2, 3: colony lines of A. tumefaciens CV58 containing vector pCB301_AcF3'5'H

Thus, the plant transgenic vector *pCB301_AcF3'5'H* was successfully designed and created two recombinant *A. tumefaciens* lines carrying transgenic vector *pCB301_AcF3'5'H*.

3.3.4. Discuss the results of designing *AcF3'5'H* gene expression vector and creating *A. tumefacience* strain carrying plant transgenic vector

Flavonoid biosynthesis is an important secondary metabolic pathway involving the involvement of many enzymes, such as CHS, IFS, F3'H, F3′5′H, FLS and FST. Several studies on the expression of genes encoding these enzymes that increase flavonoid accumulation have been performed. The report of Hu and cs (2019) suggested that *CHS* overexpression increases flavonoid accumulation in tobacco and that *CHS* is a candidate gene for genetic engineering to enhance drought tolerance in plants, animals and improve their response to oxidative stress. Vu Thi Nhu Trang and cs. (2018) demonstrated that the expression of the *GmCHI* gene from soybean allowed Tho ginseng to improve the total flavonoid content. Among the important enzymes of the flavonoid biosynthetic F3'5'H is the main enzyme that catalyzes flavonoid formation reactions. The increased activity of F3'5'H allows the synthesis of anthocyanins and other flavonoids. Therefore, enhancing *F3'5'H* gene expression will increase the concentration and activity of F3'5'H enzyme and will increase the accumulation of flavonoid content in *A. carmichaelii* Debx. Therefore, the gene *A. carmichaelii F3'5'H* (*AcF3'5'H*) was selected as the target and the transgenic technique was applied to increase the flavonoid content in the strategy of creating a high-quality *A. carmichaelii* with high medicinal value. In this study, we have identified the coding region of the *AcF3'5'H* gene with 1521 nucleotides, coding for 506 amino acids isolated from *A. carmichaelii* collected in Ha Giang

province, Vietnam, which is consistent with the announcement of Ma et al (2012). The *AcF3'5'H* gene of the *A. carmichaelii* encodes the enzyme protein AcF3'5'H which is one of the two main enzymes involved in the synthesis of flavonoids and isoflavonoids in the phenylpropanoid. In the *A. carmichaelii* Debx, the activity of the *AcF3'5'H* gene was the strongest in leaf tissues, therefore, the selection of a promoter that controls the activity of the *AcF3'5'H* transgene which is very important. Promoter CaMV35S (35S) is a strong promoter derived from the cauliflower mosaic virus (Cauliflower Mosaic Virus - CaMV). Promoter 35S can initiate gene transcription in all types of plant tissues. In our study, the 35S promoter was selected and is a component of the transgene carrier structure (*35S_ AcF3'5'H _cmyc*) in the transgenic vector pCB301. In the *pCB301_AcF3'5'H* structure, the CaMV35S promoter initiates transcription of the *AcF3'5'H* transgene, increasing the synthesis of the AcF3'5'H enzyme in the transgenic plants. In the transgenic vector, the *nptII* gene was selected as a selective marker at the *in vitro* regeneration stage. The *nptII* gene encodes a protein that exhibits resistance to the antibiotic kanamycin. Kanamycin was added during both shoot regeneration, shoot elongation and rooting of the transgenic A. carmichaelii Debx. At this time, the transgenic structure has an additional DNA fragment encoding the c-myc antigen to serve as the basis for detection and quantification of the recombinant protein rAcF3'5'H in transgenic plants by Western blot and ELISA. Thus, it can be seen that the design of a complete and suitable structure for the host cell in the design of plant gene expression vectors is the initial basis for determining the success of genetically engineered plants.

3.4. ANALYSIS OF *ACF3'5'H* GENE EXPRESSION IN TOBACCO

3.4.1. Genetic transformation and expression of the recombinant F3'5'H protein in *AcF3'5'H* transgenic tobacco

Carrying out structural transformation carrying the *AcF3'5'H* transgene through infection by *A. tumefaciens* into Tobacco leaf tissue (Figure 3.24).

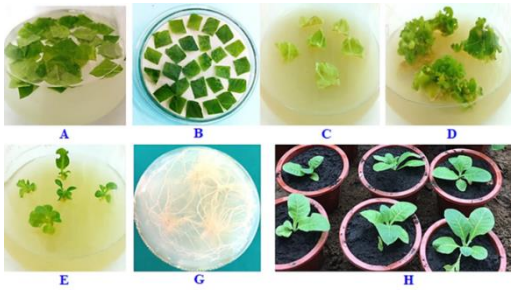

Figure 3.24. Image of transformation and regeneration of *AcF3'5'H* transgenic tobacco plants. A: Tobacco leaf fragments in bacterial and infectious diseases; B: Co-culture in CCM medium; C, D: Multi-bud regeneration in selective medium containing kanamycin; E: Shoot elongation; G: Rooting on RM medium; Q: Transgenic tobacco plants grown on substrates.

The results of the transformation experiment presented in Table 3.8 show that, after 3 times of transformation in the experimental lot, 28 plants survived under net house conditions.

Among 28 *AcF3'5'H* transgenic tobacco plants, 7 were positive by PCR, corresponding to a DNA band approximately 1.5 kb in size appearing in electrophoresis lanes 1, 3, 5, 14, 17, 19, and 22 (Figure 3.25 A). Transgenic plants positive for PCR $T_0$1, $T_0$3, $T_0$5, $T_0$14, $T_0$17, $T_0$19 and $T_0$22 were further analyzed by RT-PCR and the analysis results showed that only new transgenic plants $T_0$1, $T_0$3, $T_0$5 and $T_0$14 have RT-PCR products (Figure 3.25 B).

Table 3.8. Results of structural transformation carrying *AcF3'5'H* transgene into tobacco

Control Experiment	Number of Model	Number of live samples forming bud clusters	Total number of buds	Number of buds that survived rooting	Number of trees in the soil	Number of survived trees
Total of 3 experiments	90	81	268	174	96	28
Control 0 (ĐC0)	30	30	95	57	41	10
Control 1 (ĐC1)	30	0	0	0	0	0

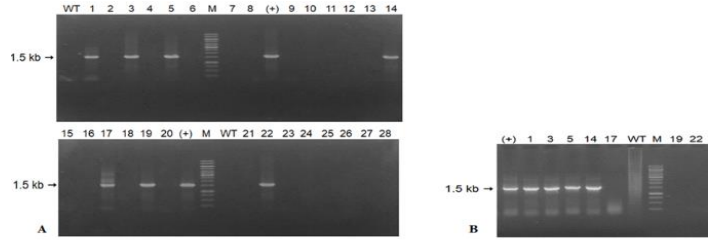

Figure 3.25. Electrophoresis examined the presence and transcription of *AcF3'5'H* transgene in transgenic plants. A: Electrophoresis image of PCR product amplifying the *AcF3'5'H* transgene. (+): plasmid *pCB301_AcF3'5'H*; WT: Non-transgenic Tobacco; M: Marker 1 kb; 1-28: Transgenic Tobacco. B: RT-PCR product electrophoresis image, confirming the transcription of *AcF3'5'H* transgene in transgenic tobacco plants. M: Marker 1 kb; (+): plasmid *pCB301_AcF3'5'H*; WT: Non-transgenic Tobacco; Electrophoresis lanes 1, 3, 5, 14, 17, 19 and 22 are PCR-positive transgenic plants, corresponding to $T_0$1, $T_0$3, $T_0$5, $T_0$14, $T_0$17, $T_0$19 and $T_0$22.

Western blot analysis in Figure 3.26A shows that all T_0 transgenic lines have a colored band with a size of approximately 57 kDa, corresponding to the molecular weight of the rAcF3'5'H protein.

The analysis results of rAcF3'5'H protein content in Figure 3.26 B showed that rAcF3'5'H protein content of transgenic plants ranged from 0,2033 µg/µl to 0,3250 µg/µl ($P < 0,001$).). Therefore, when adding endogenous F3'5'H protein, the F3'5'H protein content in transgenic plants increased from 20,33% to 32,50% compared with WT plants. These results demonstrate that the *AcF3'5'H* transgene was incorporated into the transgenic Tobacco genome and expressed recombinant protein production.

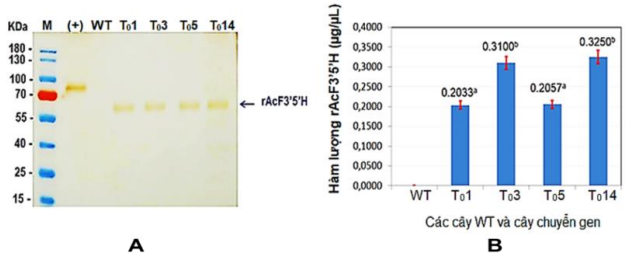

Figure 3.26. Expression of rAcF3'5'H protein in T_0-transgenic tobacco plants. A: Expression analysis of rAcF3'5'H protein by western blot. M: Standard protein scale; (+): The H5 protein of influenza A/H5N1 virus with c-myc binding was a positive control; WT: Non-transformed Tobacco; $T_0$1, $T_0$3, $T_0$5 and $T_0$14: transgenic tobacco plants. B: Protein content rAcF3'5'H (µg/µl) in T_0 transgenic tobacco. WT: Non-transgenic Tobacco; $T_0$1, $T_0$3, $T_0$5, $T_0$14: GM tobacco.

3.4.2. Total flavonoid content in leaves of transgenic tobacco lines

Observation results of transgenic tobacco plants and WT plants showed that the transgenic plants had normal morphology and growth. However, the transgenic lines had lower plant height and slower growth rate than the WT plants. In addition, the petals of the transgenic lines were darker purple than those of the WT plants (Figure 3.27).

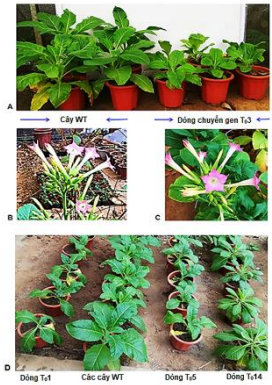

Figure 3.27. Morphology of WT Tobacco plants and transgenic lines. A- WT plants and transgenic lines $T_0$3; B- Flowers of WT plants; C- Flower of the transgenic line $T_0$3; D- WT plants and transgenic lines $T_0$1, $T_0$5, $T_0$14

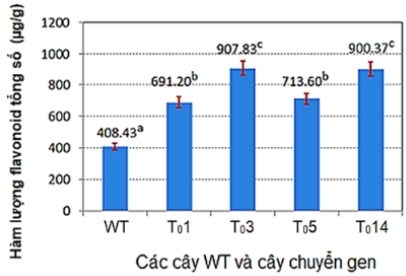

Figure 3.28. Total favonoid content (µg/g) of 4 transgenic tobacco lines, $T_0$1, $T_0$3, $T_0$5, $T_0$14, and WT plants. WT: Non-transformed Tobacco; $T_0$1, $T_0$3, $T_0$5, $T_0$14: T_0 transgenic tobacco. The vertical bars represent the standard error.

From the analysis results on Tobacco plants, it can be concluded that the transgenic vector *pCB301_AcF3'5'H* works well in transgenic tobacco plants and can be used to transfer into the *A. carmichaelii* and other crops.

3.4.3. Discussing the results of *AcF3'5'H* transformation and expression in Tobacco plants

Studies on the expression of *F3'5'H* gene derived from different plant species or functional gene overexpression analysis were performed. Following the analysis of the overexpression of the *F3'5'H* gene, Wu et al (2020) showed that the *GbF3'5'H1* gene has a function in the biosynthesis of flavonoid-related metabolites and flavonoid-related metabolites. Overexpression of the *GbF3'5'H1* gene improves epicatechin and gallocatechin content in Ginkgo biloba. In our study on *A. carmichaelii* Debx, the stem and leaves of the plant were identified as a new source of medicinal herbs. The leaves and flowers of the *A. carmichaelii* contain carotenoids, sterols and flavonoids. Thus, analysis of the association between the expression of *F3'5'H* gene from the *A. carmichaelii* and flavonoid accumulation in different plant species is needed. Tobacco is used as a model plant to study the function of plant genes and test applications to improve some plant properties grow. The gene encoding *F3'5'H* of petunia expressed in tobacco changed the synthesis of anthocyanin pigments. The tea plant *CsF3'5'H* gene is strongly expressed in the shoots and expressed at low levels in the roots. The results of *CsF3'5'H* gene expression analysis in Tobacco plants showed that overexpression of *CsF3'5'H* gene created new delphinidin derivatives and increased cyanidin derivatives content of tobacco plants. gene. In this study, the *AcF3'5'H* gene isolated from the *A. carmichaelii* designed to create the structure *CaMV35S_AcF3'5'H_cmyc_KDEL_polyA* was transferred into Tobacco tissues and created *AcF3'5'H* transgenic Tobacco plants and the results showed that the recombinant protein rAcF3'5'H was expressed in four transgenic Tobacco lines $T_0$1, $T_0$3, $T_0$5 and $T_0$14. The flavonoid content in leaves of the $T_0$1, $T_0$3, $T_0$5, $T_0$14 transgenic tobacco lines reached 691,20 ± 2,02, 907,83 ± 5,14, 713,60 ± 4,21 and 900,37 ± 0,81 respectively. Compared with non-transgenic plants, the flavonoid content in leaves of the transgenic tobacco lines increased from 69,23% to 222,27% (Figure 3.28). Within the scope of this study, flavonoid content and recombinant protein rAcF3'5'H content of 4 genetically modified tobacco lines were positively correlated, with correlation coefficient (R) = 99,09%; The regression equation is Y = 1766,72X + 342,14, Y is the flavonoid content (µg/g) and X is the protein content of rAcF3'5'H (µg/µL) ($P < 0,05$) . Our study is the first report on the expression results of *AcF3'5'H* gene isolated from the *A. carmichaelii* in Tobacco. Expression of the recombinant protein rAcF3'5'H increases flavonoid content in transgenic tobacco plants. Therefore, the *AcF3'5'H* gene from *A. carmichaelii* studied in this work is considered as a candidate gene for genetic engineering to enhance flavonoid accumulation in plants.

3.5. GENETIC TRANSFER AND ANALYSIS OF *ACF3'5'H* GENE CHARACTERISTICS IN *A. CARMICHAELII* DEBX

3.5.1. Transduction of *uidA* gene into the *A. carmichaelii* Debx

The experiment was conducted with 90 infected samples and repeated three times. After 4 weeks, the number of surviving shoots was 26 shoots that were transferred to rooting medium, of which 15 were rooted and 8 transgenic plants were selected to survive under greenhouse conditions. Figure 3.29 shows that the transformation of uidA gene through infection with A. tumefaciens in the *A. carmichaelii* was highly effective at bacterial density corresponding to OD_{600} = 0,8; with AS concentration 100 µmol/l; The infection time is 30 minutes, the concentration of bactericidal antibiotic is 50 mg/l.

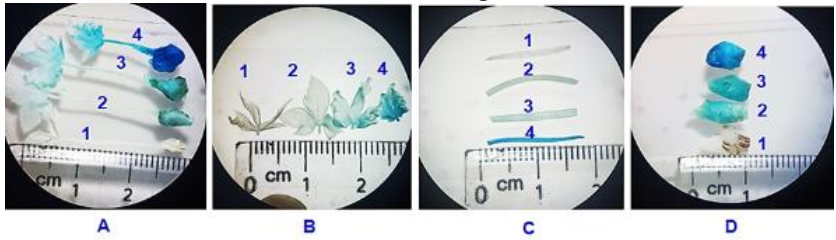

Figure 3.29. Results of in vitro GUS histochemical staining analysis in *A. carmichaelii* with different density of *A. tumefaciens* (OD). 1: *A. carmichaelii* is not transformed; 2: OD_{600} = 0,4; 3: OD_{600} = 0,6; 4: OD_{600} = 0,8. (A) GUS-transformed *A. carmichaelii* Debx; (B) GUS Transformation *A. carmichaelii* Tile; (C) GUS-transformed petioles; (D) GUS *A. carmichaelii* transformed stem cell.

3.5.2. Transformation and expression of *AcF3'5'H* gene in the *A. carmichaelii* Debx

3.5.2.1. Genetic transformation of the 35S_AcF3'5'H_cmyc structure into the A. carmichaelii Debx

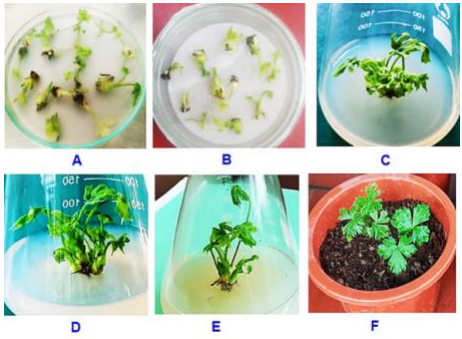

Figure 3.30. Image of *AcF3'5'H* gene transformation and *in vitro* regeneration of the *A. carmichaelii* Debx. (A) Individual shoots were immersed in *A. tumefaciens* carrying the vector *pCB301_AcF3'5'H*. (B) Co-culture on CCM. (C) Multi-bud induction on SIM1 and SIM2. (D) The shoots were cultured on SEM shoot elongation medium; (E) Rooting on RM medium. (F) Transgenic *A. carmichaelii* are grown in media.

The *AcF3'5'H* transgene-carrying construct was transformed into the *A. carmichaelii* shoot *in vitro* through infection with recombinant *A. tumefaciens* (Figure 3.30).

On Table 3.10, after three transformations, with 180 samples, 24 plants survived under greenhouse conditions. In the control plot ĐC0, 30 non-

transformed shoot explants *A. carmichaelii* obtained 10 plants with normal growth under greenhouse conditions. In plot ĐC1, the shoots did not form clusters of buds.

Table 3.10. The results of structural transformation carrying the *AcF3'5'H* transgene into the *A. carmichaelii*

Control Experiment	Model number	Number of survival samples forming bud clusters	Total number buds	Number of shoots that survived rooting	Number of trees growing in the soil	Number of survived trees that
Total of 3 times of experiments	180	86	215	95	54	24
Control 0 (ĐC0)	30	30	116	92	30	10
Control 1 (ĐC1)	30	0	0	0	0	0

Note: *ĐC1: Non-transformed samples were cultured on regeneration medium supplemented with antibiotics. ĐC0: Non-transformed samples cultured on regenerating medium without adding antibiotics*

3.5.2.2. Expression analysis of AcF3'5'H transgene in T_0 transgenic head cells

Fifteen well-developed transgenic plants were selected for PCR analysis to confirm the presence of the *AcF3'5'H* transgene in the transformed A. carmichaelii Debx. Trees in the T_0 generation are designated T0-1 through T_0-15. PCR analysis results with pais of baits *F3'5'H-NcoI-F/F3'H-SacI-R* showed that 8 plants were positive for transgene; namely, trees T_0-3, T_0-4, T_0-5, T_0-6, T_0-7, T_0-9, T_0-13, and T_0-15 (Figure 3.32A).

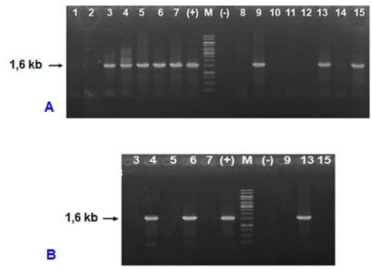

Figure 3.32. Electrophoresis confirmed the presence and transcription of the *AcF3'5'H* transgene. (A): PCR detected the presence of *AcF3'5'H* transgene in transgenic and non-transgenic *A. carmichaelii* Debx; (B): RT-PCR confirms the transcription of the *AcF3'5'H* transgene in the *A. carmichaelii* Debx

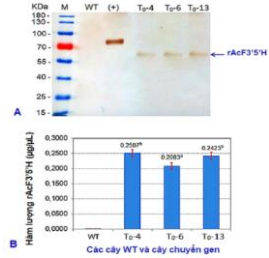

Figure 3.33. Overexpression of rAcF3'5'H protein in transgenic head cells in the T_0 generation. (A): The results of Western blot analysis to confirm the expression of rAcF3'5'H protein in transgenic *A. carmichaelii* Debx. (B): Comparison of rAcF3'5'H protein content (µg/µl) in T_0 transgenic *A. carmichaelii* Debx.

Eight T_0 plants identified as PCR-positive were further analyzed by RT-PCR to confirm transgene transcription. The results are shown in Figure 3.32 B. The RT-PCR results showed that only 3 out of the 8 plants (T_0-4, T_0-6 and T_0-13) had DNA bands with approximately 1,6 kb in size, respectively. with the size of *AcF3'5'H*.

Plants T_0-4, T_0-6 and T_0-13, were used to analyze the expression of rAcF3'5'H protein by SDS-PAGE electrophoresis and Western blot method. In Figure 3.31A, plants T_0-4, T_0-6 and T_0-13 all have colored bands with size > 55 kDa corresponding to the molecular weight of rAcF3'5'H protein. Thus, the results of Western blot analysis showed that the *AcF3'5'H* transgene was translated into recombinant *AcF3'5'H* protein in three transgenic *A. carmichaelii* lines in the T_0 generation. The analysis results of rAcF3'5'H protein content from three transgenic plants by ELISA are shown in Figure 3.31B. The rAcF3'5'H protein content of transgenic plants ranged from 0,2083 to 0,2507 µg/µl. These results demonstrate that the *AcF3'5'H* transgene was incorporated into the transgene, transcription and expression of the recombinant protein.

3.5.2.3. Determination of total flavonoid content from leaves of the transgenic A. carmichaelii lines

Transgenic *A. carmichaelii* lines in the T_0 generation and WT plants had not morphological differences. The three lines T_0-4, T_0-6 and T_0-13 had high flavonoid content of 773,50 ± 12,87, 661,73 ± 2,85 and 761,61 ± 9,10 (µg/g), respectively. This result is 139,13 to 162,63% higher than that of WT plants (475,62 ± 10,16 µg/g). These results demonstrated that overexpression of the *AcF3'5'H* gene in the three transgenic *A. carmichaelii* lines (T_0-4, T_0-6 and T_0-13) increased the flavonoid content in the transgenic lines. genes in the T_0 generation.

3.5.3. Discuss the results of transformation and overexpression of *AcF3'5'H* gene in the *A. carmichaelii* Debx

Currently, research on enhancing the synthesis of biologically active substances in medicinal plants by strongly expressing the gene encoding the key enzyme in the biosynthesis of secondary compounds is of interest. The *A. carmichaelii* medicinal plants contains many biologically active compounds, such as alkaloids, flavonoids and polysaccharides. The alkaloids of this medicinal plant have antibacterial, antiviral and antioxidant activities. Flavonoids are found in all parts of stems, flowers, roots and rhizomes, but in low concentrations. In order to improve the flavonoid content in *A. carmichaelii* Debx, this study chose to overexpress the gene encoding an important enzyme involved in the flavonoid biosynthetic pathway in the *A. carmichaelii* Debx, F3'5'H.

The experimental results of transforming *uidA* marker gene into the *A. carmichaelii* from in vitro shoot explants through infection with *Agrobacterium tumefaciens* showed that the appropriate *A. tumefaciens* density was OD_{600} = 0,8, AS concentration was 100 µmol /l and the infection time was 30 minutes. Select

the antibiotic with kanamycin 50 mg/l, and the gene transfer efficiency was 8.88%. This result is the basis for the success of genetic transformation and creation of transgenic *A. carmichaelii* Debx.

Among the enzymes involved in catalyzing the reactions of the flavonoid synthesis in *A. carmichaelii* Debx, F3'H and F3'5'H participate in the final reactions to form flavonoid compounds. Therefore, the gene encoding the enzyme F3'5'H was selected for overexpression analysis in *A. tumefaciens*-mediated transformation. In the past years, studies on isolation and analysis of *F3'5'H* expression in plants have been carried out to determine gene function. Ishiguro and cs. (2012) isolated *F3'H* and *F3'5'H* from the cDNA library of *A. kelloggii*, and expression analysis in transgenic petunia showed that expression of these genes increased levels of cyanidin and delphinidin as well as anthocyanidin. Increased accumulation of anthocyanidins also changed flower color in transgenic plants. A study in carnations (*Dianthus caryophyllus*), transgenic plants carrying herbicide resistance genes (mutant gene encoding acetolactate synthase -ALS) and gene encoding F3′5′H showed increased accumulation of delphinidin and anthocyanins compared with wild plants, but not harmful to human or animal health. In this study, the *AcF3'5'H* gene was isolated from the mRNA of the tree with the coding segment *AcF3'5'H* 1521 bp, encoding 506 amino acids. The structure carrying the *AcF3'5'H* transgene in the transgenic vector pCB301 containing the *35S*, *c-myc* and *KDEL* promoter sequences was transferred to the shoots by *A. tumefaciens in vitro*, and produced the transgenic *A. carmichaelii* Debx. The rAcF3'5'H protein was expressed and the rAcF3'5'H protein content increased from 20,83% to 25,07% compared with that in the non-transgenic plants. The increase in rAcF3'5'H protein content increased the total flavonoid content in the transgenic lines T_0-4, T_0-6 and T_0-13 from 39,13% to 62,63% compared with WT plants. These results demonstrated that overexpression of *AcF3'5'H* in three transgenic *A. carmichaelii* lines (T_0-4, T_0-6, and T_0-13) increased flavonoid content in transgenic plants. gene. This is the first report on gene transformation and analysis of the overexpression of the F3'5'H gene in *A. carmichaelii* Debx.

CONCLUSIONS AND RECOMMENDATIONS

Conclude

1. By comparative morphology method combined with DNA barcoding analysis based on *ITS* region sequence, *matK*, *rpoC1*, *rpoB2* gene fragments have identified the *A. carmichaelii* samples collected in Quan Ba and Hoang Su Phi districts, Ha Giang province, Vietnam, which belongs to the same species *A. carmichaelii*, genus *Aconitum*, Hoang Lien family (Ranunculaceae). The *matK* sequence is a good DNA barcode candidate for identification *A. carmichaelii* species and is the solution in molecular evolutionary and phylogenetic analysis of the genus *Aconitum*.

2. The suitable medium to induce multi-shooting in the *A. carmichaelii* was basic MS + sucrose 30 g/l + agar 9 g/l + BAP 1.5 mg/l. Root tissue is a suitable material for induction of hairy root formation in *A. carmichaelii* Debx. Root tissue infection by *R. rhizogens* with OD_{600} = 0,6; AS 100 µmol/l; infection time 15 minutes; co-cultivation period of 3 days; The concentration of cefotaxime 500 mg/l were suitable conditions for the induction of hairy rooting in the *A. carmichaelii* Debx. MS medium in liquid state without adding growth regulators, shaking culture is suitable for growth of hairy roots in *A. carmichaelii* Debx.

3. The coding region of the *AcF3'5'H* gene isolated from the mRNA of the tree has a size of 1521 nucleotides, encoding 506 amino acids. The plant transgenic vector *pCB301_AcF3'5'H* with the control of the 35S promoter, containing the *AcF3'5'H* gene isolated from the *A. carmichaelii* Debx, adding the c-myc and KDEL constructs has been successfully designed and created. two lines of *A. tumefaciens* carrying transgenic vector *pCB301_AcF3'5'H*.

4. The transgenic vector *pCB301_AcF3'5'H* was successfully transformed into Tobacco leaf tissues and produced *AcF3'5'H* transgenic Tobacco plants. The *AcF3'5'H* transgene expressed rAcF3'5'H recombinant protein and increased flavonoid content in transgenic tobacco from 691,20 ± 2,02 to 907,83 ± 5,14 (µg) /g) and from 69,23 to 222,27 (%) higher than WT plants (408,43 ± 5,11 µg/g).

5. The transformed sample for infection with *Agrobacterium* was in vitro shoot, suitable *A. tumefaciens* density was OD_{600} = 0,8, AS concentration was 100 µmol/l and incubation time was 30 minutes. The *AcF3'5'H* transgene was successfully transformed into *A. tumefaciens* by infecting recombinant *A. tumefaciens* into shoots in vitro, and three transgenic *A. carmichaelii* lines were generated in the T_0 generation. Overexpression of the *AcF3'5'H* transgene increased the enzyme F3'5'H and total flavonoid content in leaves of the T_0-4, T_0-6 and T_0-13 transgenic lines. from 39,13% to 62,63% compared to WT plants.

Suggestions

1. Continue to analyze and evaluate the 3 transgenic *A. carmichaelii* lines in order to select the transgenic *A. carmichaelii* lines with high and stable flavonoid content.

2. Continue to analyze the chemical composition, search for compounds with pharmacological value and compare the medicinal content of hairy roots, in vitro roots and roots and tubers of *A. carmichaelii* to use as a basis for selecting a hairy root line with high quality of content of secondary compounds with high biological activity.