Genetics and Molecular Research

Cloning of three genes involved in the flavonoid metabolic pathway and their expression during insect resistance in *Pinus massoniana* Lamb.

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ABSTRACT. *Pinus massoniana* Lamb. is an important timber and turpentine-producing tree species in China. *Dendrolimus punctatus* and *Dasychira axutha* are leaf-eating pests that have harmful effects on *P. massoniana* production. Few studies have focused on the molecular mechanisms underlying pest resistance in *P. massoniana*. Based on sequencing analysis of the transcriptomes of insect-resistant *P. massoniana*, three key genes involved in the flavonoid metabolic pathway were identified in the present study (PmF3H, PmF3'5'H, and PmC4H). Structural domain analysis showed that the PmF3H gene contains typical binding sites for the 2OG-Fe (II) oxygenase superfamily, while PmF3'5'H and PmC4H both contain the cytochrome P450 structural domain, which is specific for P450 enzymes. Phylogenetic analysis showed that each of the three *P. massoniana* genes, and the homologous

genes in gymnosperms, clustered into a group. Expression of these three genes was highest in the stems, and was higher in the insect-resistant *P. massoniana* varieties than in the controls. The extent of the increased expression in the insect-resistant *P. massoniana* varieties indicated that these three genes are involved in defense mechanisms against pests in this species. In the insect-resistant varieties, rapid induction of PmF3H increased the levels of PmF3'5'H and PmC4H expression. The enhanced anti-pest capability of the insect-resistant varieties could be related to temperature and humidity. In addition, these results suggest that these three genes maycontribute to the change in flower color during female cone development.

Key words: *Pinus massoniana* Lamb; Flavonoid metabolism; Pest resistance genes; Gene expression analysis

INTRODUCTION

When plants encounter biotic stress imposed by herbivores, pathogens, and viruses, they produce small-molecule compounds, polypeptides, or proteins. These have various anti-insect or bacteriostatic effects that help to defend the plants against such stress (Kamphuis et al., 2013; Meihls et al., 2013). In the natural world, plants undergo morphological, biochemical, and molecular evolution in order to defend against the biotic stress conditions they encounter (Hu et al., 2011; Consales et al., 2012). Resistance of plants to biotic stress is a defense process involving various enzymes and secondary metabolites, including phenylalanine ammonia lyase, flavonoids, terpenoids, phenolic aldehydes, tannin, and alkaloids (Lamb et al., 1989; Long, 1989; Gulsen et al., 2010).

Flavonoids are important secondary metabolites in plants and are essential pigments in flowers, fruits, and seeds. In addition, flavonoids play important roles in disease resistance, stress resistance, plant growth and development, defense against insect and herbivore attack, plant reproductive capacity, and pollen germination. Flavonoids may also function as phytoalexins and scavengers of reactive oxygen species (Treutter, 2006). Flavonoid and anthocyanin biosynthesis pathways have been thoroughly studied in plants (Forkmann, 1991). To date, genes encoding phenylalanine ammonialyase, cinnamate-4-hydroxylase (*C4H*), flavanone 3-hydroxylase (*F3H*), and flavonoid 3',5'-hydroxylase (*F3'5'H*) (Schwinn et al., 2014; Zhai et al., 2014), which are main catalytic enzymes (structural genes) in flavonoid biosynthesis, have been discovered. Regulatory transcription factors (regulatory genes) include MYB protein, MYC-type basic helix-loop-helix (bHLH) protein, WRKY transcription factors, and zinc finger protein (Sweeney et al., 2006; Xu et al., 2015).

Studies have demonstrated that flavonoids possess anti-insect effects. For example, flavonoids isolated from cotton inhibit the growth of the cotton bollworm, *Helicoverpa armigera* Hübner, and flavonoids extracted from wheat significantly inhibit the growth and reproduction of the wheat aphid, *Macrosiphum avenae* (Mahajan and Yadav, 2014).

There are approximately 600 conifer species on Earth, occupying 80% of the Earth's available area. *Pinus massoniana* Lamb. is a native coniferous species typically grown in the eastern humid subtropical climate zones of China. The characteristics of *P. massoniana* include strong adaptability, rapid growth, and wide distribution, which render the plant an

important timber species suitable for wide-spreadutilization. A previous study showed that *P. massoniana* accounts for 16% of the forest volume in South China (Guan et al., 2015). However, *P. massoniana* can be attacked by many harmful pests. Therefore, as the cultivation area of *P. massoniana* continuously expands, the incidence of pest and disease damage gradually increases. Consequently, the amount of pine-related forest products produced is greatly reduced, which restricts the development of the pine industry and poses a great threat to the forest economy. Pine caterpillar (*Dendrolimus punctatus*) and pine tussock moth (*Dasychira axutha*) are the main pests of *P. massoniana*. In China, about 1,330,000 ha of pine forest are damaged by pine caterpillars every year, causing a substantial loss of *P. massoniana* timber and turpentine (Zhang and Li, 2008). Studies have shown that the direct annual economic loss caused by the pine caterpillar reaches tens of millions yuan in Guangxi Province alone, and the ecological function and value loss exceeds 2.6 billion yuan. Therefore, preventing pest damage to *P. massoniana*, and treating damaged plants, are important for *P. massoniana* production. Governance of production, from the aspect of comprehensive environmental protection, is a new task facing workers involved in *P. massoniana* protection. Pest prevention and management practices used for other plants have shown that planting insect-resistant species is the most safe and cost-effective way to prevent pest damage, leading to persistent and cumulative economic benefits. Therefore, planting insect-resistant species has become the preferred measure to comprehensively prevent pest damage. In addition, this does not conflict with other prevention measures. Large-scale cultivation of insect-resistant plants may reduce the number of pest populations throughout the entire region.

Our research group has cultivated an insect-resistant variety of *P. massoniana*, named Songyun. Using this insect-resistant variety, we attempt to further understand and study the mechanisms underlying pest resistance in *P. massoniana,* and to identify potential insect-resistant substances and resistance genes in *P. massoniana*. The results will provide theoretical and practical guidance for the selective breeding of suitable insect-resistant *P. massoniana* varieties in order to achieve sustainable control of pest damage to *P. massoniana*. In the present study, three differentially expressed genes in the flavonoid pathway were identified based on previous sequencing analysis of the transcriptome of insect-resistant materials. These three genes were subsequently subjected to bioinformatics analysis. The mechanism of action of these three genes in defense against pests was explored in *P. massoniana* through the expressional analysis of tissues exhibiting different levels of pest resistance.

MATERIAL AND METHODS

Plant materials

All materials used in the present study were provided by the GenBank of *P. massoniana* Germplasm Resources, Guangxi Academy of Forestry, and various tissue samples were collected in February 2015. Samples were collected from various tissues of the insect-resistant material Songyun (IR) and the non-resistant material (control, CK) GC101 in May 2015 at different times of the day. All of the materials were placed in liquid nitrogen immediately after collection and then stored in a -80°C freezer for future use.

Reagents

The RNA extraction kit for polyphenol- and polysaccharide-rich plants was purchased

from Tiangen Biochemical Technology Co., Ltd. The pMD18-T vector, Gel Extraction Kit, dNTP, M-MLV reverse transcriptase, Taq DNA polymerase, and SYBR Premix Ex Taq II Kit were purchased from Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). All primers used in the present study were synthesized and sequenced by SangonBiotech (Shanghai) Co., Ltd.

RNA extraction and cloning of the full-length genes

RNA was isolated from all plant materials using an RNA extraction kit for polyphenol- and polysaccharide-rich plants (Tiangen Biochemical Technology Co., Ltd.) following the manufacturer instructions. After extraction, all RNA samples were diluted to a consistent concentration. Subsequently, complementary DNA (cDNA) was synthesized for all samples using oligo$(dT)_{18}$ reverse transcription primers following the M-MLV reverse transcriptase instruction manual. After verification, the synthesized cDNA was diluted to the same concentration and stored for future use.

Based on a sequencing analysis conducted previously by our research group investigating the transcriptome of insect-resistant *P. massoniana* materials, we obtained fragments of three significantly differentially expressed genes in the flavonoid biosynthetic pathway: *F3H*, *C4H*, and *F3'5'H*. Based on sequence alignment results obtained using Gen Bank of the National Center for Biotechnology Information (NCBI),and the high degree of homology between *P. massoniana*, spruce, and radiata pine (*Pinus radiata*), upstream and downstream primers specific for the open reading frames (ORFs) of the three genes were designed (**Table S1**). The templates used for gene cloning included an equivalent mixture of cDNA obtained from the stems and leaves of various insect-resistant materials. The volume of the polymerase chain reaction (PCR) amplification system was 25 μL. PCR was performed according to the instruction manual of the Taq DNA polymerase. The PCR amplification parameters were as follows: denaturation at 95°C for 5 min; denaturation at 95°C for 1 min; annealing at 60°C for 1.5 min; elongation at 72°C for 2 min; a total of 35 cycles; and storage after a final elongation at 72°C for 10 min. The PCR amplification products were examined by agarose gel electrophoresis. The target bands were recovered using a gel extraction kit, purified, ligated into the pMD18-T vector, and heat-shock transformed into *Escherichia coli* DH5α competent cells. Well-isolated colonies were selected, cultured, PCR verified, and sent to SangonBiotech (Shanghai) Co., Ltd., for sequencing analysis.

Sequence analysis

The ORFs and aminoacid sequences of the three genes were analyzed using the BioXM2.6 software. Based on the obtained full-length sequences, NCBI's online retrieval system was used to identify the amino acid sequence homology between the three genes from *P. massoniana* and other plants. Amino acid sequences from different plants were aligned using the ClustalX software. A homology tree was constructed using ClustalX and DNAMAN. The isoelectric point, signal peptide, secondary structure, subcellular localization, hydrophilicity, and transmembrane structure of the proteins encoded by the three genes were analyzed using the Proteomics Server, SignalP 4.1 Server, SOPMA, TargetP 1.1 Server, and DAS software provided by the SIB Bioinformatics Resource Portal, ExPASy (http://www.expasy.org/). The functional domains of the genes were analyzed using NCBI and the Motif Scan software.

Gene expression analysis

Primers for real-time, fluorescence-based, quantitative PCR were designed based on the obtained sequences of the three genes. Based on reference gene screening results (unpublished data), the polyubiquitin (*UBI4*) gene was selected as the reference gene for various tissues, while the cytochrome P450 (*CYP*) gene was used as the reference gene for materials with different levels of insect resistance (**Table S1**). The cDNA obtained from various *P. massoniana* tissues, and from male and female flowers at different developmental stages, was used as a template. The PCR system was prepared according to the instruction manual of the SYBR Premix Ex Taq II (Perfect real-time) Kit (Takara Biotechnology Co., Ltd). PCR amplification was conducted on a Light Cycle 480II Real-Time PCR Instrument (F. Hoffmann-La Roche, Ltd.). Each sample was tested in three biological replicates and each reaction was repeated technically three times. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the relative expression levels were determined and plotted using Excel 2007.

RESULTS

Obtaining the full length gene sequences

Previously, we used the stems and needles of insect-resistant and insect-susceptible *P. massoniana* (CK) as research materials, and the transcriptomes of materials with different levels of insect resistance were sequenced. The study yielded information on 523 differentially expressed genes (unpublished data). These differentially expressed genes were analyzed, and only three, which were part of the flavonoid biosynthesis pathway, were found to be significantly differentially expressed (Figure 1).

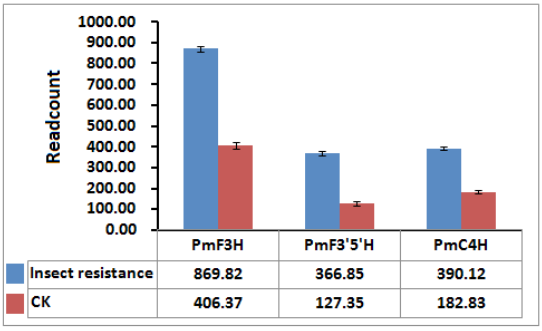

Figure 1. Read-count data of differentially expressed genes identified from transcriptome sequencing analysis in materials with different resistance to insects. The three genes are clearly upregulated in insect-resistant materials.

Based on the acquired fragments of the three genes, specific primers were designed for the full-length genes. cDNA derived from various tissues of the insect-resistant *P. massoniana* was mixed and served as the template for PCR amplification. The PCR products were subjected to agarose gel electrophoresis, and the results showed that amplification of the *PmF3H*, *PmF3'5'H*, and *PmC4H* genes produced sharp bands approximately 950, 1450, and 1500 bp

in length, respectively (**Figure S1**). The target bands of the three genes were recovered and sequenced. The full-length sequences of the three genes were determined through sequence alignment. The three genes were 1089, 1527, and 1521 bp in length, respectively, and encoded 362, 508, and 506 amino acids (**Figure S2**). The *PmF3'5'H* and *PmC4H* genes belonged to the CYP75A and CYP73A gene families, respectively.

Bioinformatic analysis

The amino acid sequences of the *PmF3H*, *PmF3'5'H*, and *PmC4H* genes were analyzed using relevant software. The results are shown in **Table S2**. The isoelectric points of the PmF3H, PmF3'5'H, and PmC4H proteins were 5.52, 8.22, and 8.49, respectively. The molecular weights of the PmF3H, PmF3'5'H, and PmC4H proteins were 40.62, 57.26, and 57.62 kDa, respectively. PmF3H is predicted to be localized to the cytoplasm, whereas PmF3'5'H and PmC4H are predicted to be localized to the endoplasmic reticulum. None of the three proteins contained a signal peptide. Only PmF3'5'H contained a single glycosylation site, G. There are differences in the phosphorylation sites and the composition of secondary structures between the three genes (**Table S2**).

The three genes each contain one amidation site and one N-glycosylation site. Marked differences existed in the number of casein kinase II phosphorylation sites, N-myristoylation sites, and protein kinase C phosphorylation sites between the three genes, especially between *PmF3H* and *PmF3'5'H*, and between *PmF3H* and *PmC4H.* The numbers of casein kinase II phosphorylation sites in the *PmF3H, PmF3'5'H*, and *PmC4H* genes were one, five, and five, respectively; the numbers of N-myristoylation sites were two, three, and five, respectively; and the numbers of protein kinase C phosphorylation sites were three, six, and seven, respectively. PmF3'5'H and PmC4H each contained a conserved cytochrome P450 structural domain. In addition, PmF3H contained two cAMP- and cGMP-dependent protein kinase phosphorylation sites, a 2OG-Fe (II) oxygenase superfamily domain, and a phage tail fiber repeat domain. PmF3'5'H contained a bacterial Ig-like domain 1 (Big-1) domain, bipartite nuclear localization profile, and tetratricopeptide repeat (TPR) profile, which were not detected in PmC4H. In contrast, PmC4H contained an aldehyde dehydrogenase glutamic acid active site, which was not present in PmF3'5'H (**Table S3**).

Homology analysis

The amino acid sequences encoded by the *PmF3H*, *Pm F3'5'H*, and *PmC4H* genes were subjected to sequence alignment analysis using NCBI online software. It was found that *PmF3H*, *PmF3'5'H*, and *PmC4H* of *P. massoniana* shared 68-99, 64-95%, and 76-98% amino acid sequence homology, respectively, with genes from other plants. All three genes showed high homology with gymnosperms, including radiata pine, spruce, and ginkgo (*Ginkgo biloba*). The three genes exhibited a low degree of evolution among coniferous plants (Figure 2).

Gene expression

To further analyze the expression patterns of the three genes, real-time fluorescence-based quantitative PCR was performed. The polyubiquitin 4 (*UBI4*) and cyclophilin (*CYP*) genes of *P. massoniana* were used as internal controls. The spatial expression of the three

genes was examined in various tissues of *P. massoniana*, and their temporal expression was examined in the needles and stems of materials exhibiting different levels of insect resistance. The expression patterns of the three genes in various tissues are presented in Figure 3. All three genes were expressed at the highest levels in tender stems, followed by the old stems.

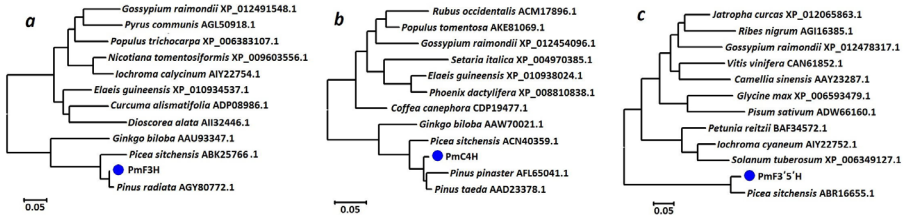

Figure 2. Analysis of the phylogenetic relationships of the three genes in Masson pine and other plants based on amino acid sequences. The bootstrap value shows the robustness of the phylogeny.

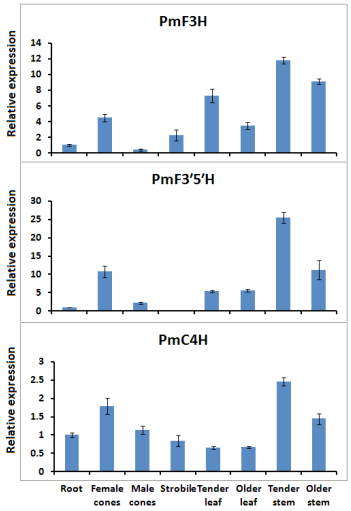

Figure 3. Expression analysis of the three genes in various tissues using fluorescence-based quantitative PCR. Relative expression levels of the genes *PmF3H* (a), *PmF3'5'H* (b), and *PmC4H* (c), were investigated the in root, female cones, male cones, strobile, tender leaf, older leaf, tender stem, and older stem.

However, differential expression patterns were observed in other tissues. Expression of the *PmF3H* gene was high in tender leaves and low in male cones. Expression of the *PmF3H* gene in tender leaves reached 7.3-fold, which was 18.2-fold higher than in male cones. The *Pm F3'5'H* gene showed a high level of expression in stems and in female cones. Expression of the *Pm F3'5'H* gene in female cones was similar to that in old stems. The *Pm F3'5'H* gene was not substantially expressed in cones. The *PmC4H* gene was also expressed at a high level in female cones.

As shown in Figure 4, the expression of the three genes in insect-resistant materials and controls followed the same overall trends, especially in samples obtained in the morning. The expression and changes in expression of the three genes were higher in the tender stems, old stems, tender leaves, and old leaves of the insect-resistant materials, compared with the controls. The increase was particularly dramatic in tender leaves and tender stems. In terms of

trends in tissue-specific gene expression, the expression of *PmF3H* exhibited a rapid upward trend between morning and noon, whereas the expression of *PmF3'5'H* and *PmC4H* increased between the afternoon and evening. In addition, significant differences were detected in the expression trends of these genes between 4:00 pm and 7:00 pm between the insect-resistant materials and controls. The expression of the three genes in the insect-resistant materials followed a rapid upward trend. In contrast, the expression of the three genes increased slowly in the controls and exhibited a downward trend.

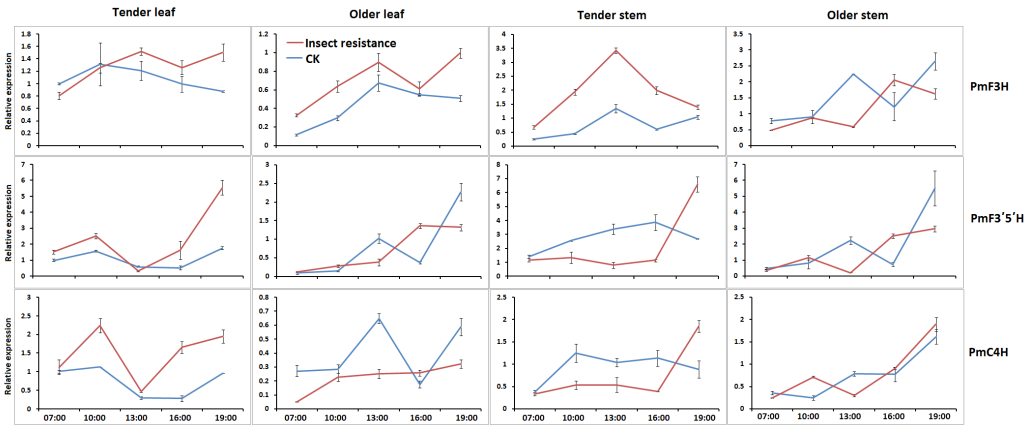

Figure 4. Variation in the daily expression patterns of the three genes in the stems and needles of materials with different resistance to insects.

We recorded the temperature and humidity on days when the samples were collected (Figure 5). We found there was a negative correlation between temperature and humidity throughout the day; the higher the temperature, the lower the humidity. We combined the information on temperature and humidity with the expression of the three genes during the day (Figure 4) and found that gene expression remained consistent with the temperature variation trend until the evening (7:00 pm). Gene expression increased as the temperature increased. However, the expression of the three genes continued to increase when the temperature fell at 7:00 pm. This phenomenon was most evident in insect-resistant materials.

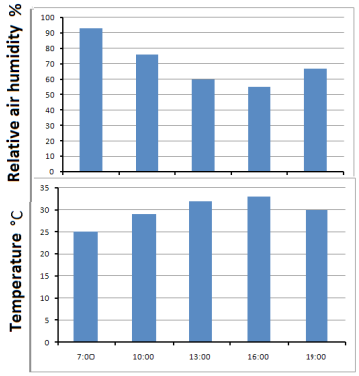

Figure 5. *The changes in air te*mperature and humidity during the day when the samples were collected.

DISCUSSION

In the present study, experimental data on the transcriptomes of insect-resistant *P. massoniana* were screened, and three key genes of the flavonoid biosynthetic pathway were found to be significantly differentially expressed. After cloning, the full-length sequences of the three genes were obtained. Bioinformatic analysis revealed that all three genes contained one or two nuclear binding sites. In particular, two nuclear binding sites were present in *PmF3H*. We speculate that transcription factors bind to these sites via the nuclear localization signal-containing functional domain in the enzyme gene and subsequently promote the expression of downstream genes. This is consistent with results obtained in a study of flavonoid metabolic pathway genes in snapdragons and petunias (Spelt et al., 2000; Nakatsuka et al., 2008; Schwinn et al., 2014). In the F3H protein, the amino acid residues H217, H275, and D219 serve as binding sites for Fe^{2+}andplay important roles in F3H enzymatic activity

All three genes are constitutively expressed in various tissues of *P. massoniana*. The relative expression levels of the three genes were highest in needles and stems, followed by the female cones. In alfalfa, the *F3H* gene is expressed in flowers and roots but not in leaves. The level of *F3H* expression gradually decreased as the flowers reached full bloom (Charrier et al., 1995). In phalaenopsis orchids, *F3'5'H* is specifically expressed in the petals; no *F3'5'H* expression is detected in the leaves and roots (Wang et al., 2006). In strawberry, expression of the flavonol synthase gene is highest in the flowers, followed by the leaves (Salvatierra et al., 2010). In *Dendrobium candidum*, the *F3'5'H* gene is expressed at the highest level in blooming flowers. However, the *F3'5'H* gene is not expressed in non-colored flowers (Whang et al., 2011). These findings indicate that the expression of flavonoid synthesis-related genes is species- and tissue-dependent, and involves multiple regulatory mechanisms. Therefore, it has been speculated that the three genes can be classified as early genes, which play roles in the early stages of plant development, as is the case in Arabidopsis and Satsuma mandarin. It is also possible that the three genes are related to the flavonol glycosides that accumulate in the needles and stems of *P. massoniana*. In addition, it has been found that the three genes are expressed at high levels in insect-resistant *P. massoniana* materials. Further investigation is required to determine whether high expression leads to differences in the turpentine composition between insect-resistant and insect-susceptible varieties.

The present study showed that the expression levels of the three genes were higher in insect-resistant *P. massoniana* varieties than in the insect-susceptible varieties. In addition, expression increased over time. The increase was greater in insect-resistant varieties compared with insect-susceptible varieties. Therefore, it is believed that the activities of the three genes are related to insect resistance. Moreover, expression of *F3H* increased more rapidly over time when compared with the *PmF3'5'H* and *PmC4H* genes. The flavonoid metabolic pathway is a branch of the phenylalanine metabolic pathway (Tanaka et al., 1998; Schwinn et al., 2014). Phenylalanine metabolism can be divided into three phases. In the first phase, phenylalanine is converted to coumaroyl-CoA. This phase is common to many secondary metabolites. In the second phase, coumaroyl-CoA is converted to dihydroflavonol, which is the key reaction in flavonoid metabolism. *F3H* is the primary enzyme mediating the reaction. In the third phase, various anthocyanins are synthesized. *F3'5'H* is the most critical enzyme in the third phase, which determines the type of anthocyanin synthesized and color formation (Schwinn et al., 2014; Wang et al., 2014; Zhai et al., 2014). The results of our study also showed that *F3H* is a key upstream enzyme in the flavonoid biosynthetic pathway (Li, 2014; Xu et al., 2015). Increased

expression of *F3H* promotes *F3'5'H* activity in the third phase, thereby enhancing the levels of secondary products such as dihydrotricetin, dihydromyricetin, myricetin, quercetin, and eriodictyol. Consequently, the ability of *P. massoniana* to resist pests is improved (Shimada et al., 1999; Boase et al., 2010; Wang et al., 2014).

C4H is the second enzyme in the plant phenylpropane metabolic pathway. The level of C4H in plant cells may affect multiple branches of the metabolic pathways responsible for lignin and flavonoid biosynthesis (Bell-Lelong et al., 1997). The present study showed that increased expression of the *PmC4H* gene provided abundant precursors for the flavonoid metabolic pathway through four metabolic branches, thereby enhancing the amount of secondary metabolites. These results are consistent with the findings of several studies conducted on blackberries, petunias, and tea trees. Those studies demonstrate that *C4H* activity at the transcriptional level, affects the amount of plant flavonoid synthesized (Ryan et al., 2002; Baek et al., 2008; Singh et al., 2009).

The present investigation revealed that diurnal expression patterns of the three genes were related to air temperature and humidity. A number of studies have also shown that the activities of genes encoding enzymes of the flavonoid metabolic pathway are affected by a variety of factors, including environmental stress, temperature, light, and damage (Schijlen et al., 2004; Bellés et al., 2008; Mahajan and Yadav, 2014; Zoratti et al., 2014). Under adverse conditions, plants modulate the synthesis and accumulation of flavonoids through the transcriptional regulation of a series of enzyme genes related to flavonoid biosynthesis, including *C4H*. For example, in petunia subjected to UV-B stress, the expression of *C4H* and other genes increased, leading to an increase in the overall flavonoid content in plant tissues (Ryan et al., 2002).

To date, the majority of in-depth studies on the *F3H*, *F3'5'H*, and *C4H* genes have focused on their functions in pigment biosynthesis. Studies on the role of the flavonoid metabolic pathway in the resistance of plants to environmental stress, particularly resistance to pests, are rare. Coniferous gymnosperms such as *P. massoniana* are unique in that pines possess unique secondary metabolic pathways to synthesize turpentine. In addition, conifer-related genes share poor homology with monocotyledons and dicotyledons. It is likely that conifers such as *P. massoniana* have developed unique pathways to defend against pests. Therefore, it is particularly important to further strengthen research on the anti-pest effects of the *PmF3H*, *Pm F3'5'H*, and *PmC4H* genes.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Baek MH, Chung BY, Kim JH, Kim JS, et al. (2008). cDNA cloning and expression pattern of cinnamate-4-hydroxylase in the Korean black raspberry. *BMB Rep.* 41: 529-536. http://dx.doi.org/10.5483/BMBRep.2008.41.7.529

Bell-Lelong DA, Cusumano JC, Meyer K and Chapple C (1997). Cinnamate-4-hydroxylase expression in *Arabidopsis*. Regulation in response to development and the environment. *Plant Physiol.* 113: 729-738. http://dx.doi.org/10.1104/pp.113.3.729

Bellés JM, López-Gresa MP, Fayos J, Pallás V, et al. (2008). Induction of cinnamate 4-hydroxylase and phenylpropanoids in virus-infected cucumber and melon plants. *Plant Sci.* 174: 524-533. http://dx.doi.org/10.1016/j.plantsci.2008.02.008

Boase MR, Lewis DH, Davies KM, Marshall GB, et al. (2010). Isolation and antisense suppression of flavonoid 3', 5'-hydroxylase modifies flower pigments and colour in cyclamen. *BMC Plant Biol.* 10: 107. http://dx.doi.org/10.1186/1471-2229-10-107

Charrier B, Coronado C, Kondorosi A and Ratet P (1995). Molecular characterization and expression of alfalfa (*Medicago sativa* L.) flavanone-3-hydroxylase and dihydroflavonol-4-reductase encoding genes. *Plant Mol. Biol.* 29: 773-786. http://dx.doi.org/10.1007/BF00041167

Consales F, Schweizer F, Erb M, Gouhier-Darimont C, et al. (2012). Insect oral secretions suppress wound-induced responses in *Arabidopsis*. *J. Exp. Bot.* 63: 727-737. http://dx.doi.org/10.1093/jxb/err308

Forkmann G (1991). Flavonoids as flower Pigments: The formation of the natural spectrum and its extension by genetic engineering. *Plant Breed.* 106: l-26. http://dx.doi.org/10.1111/j.1439-0523.1991.tb00474.x

Guan H, Xiao HY and Liu CQ (2015). Sulfur isotopic signatures of water-soluble sulfate in needles of *Pinus Massoniana* Lamb in two Chinese areas. *Environ. Earth Sci.* 73: 1805-1811. http://dx.doi.org/10.1007/s12665-014-3532-y

Gulsen O, Eickhoff T, Heng-Moss T, Shearman R, et al. (2010). Characterization of peroxidase changes in resistant and susceptible warm-season turfgrasses challenged by *Blissus occiduus*. *Arthropod-Plant Interact.* 4: 45-55.

Hu J, Zhou J, Peng X, Xu H, et al. (2011). The Bphi008a gene interacts with the ethylene pathway and transcriptionally regulates MAPK genes in the response of rice to brown planthopper feeding. *Plant Physiol.* 156: 856-872. http://dx.doi.org/10.1104/pp.111.174334

Kamphuis LG, Lichtenzveig J, Peng K, Guo SM, et al. (2013). Characterization and genetic dissection of resistance to spotted alfalfa aphid (*Therioaphis trifolii*) in *Medicago truncatula*. *J. Exp. Bot.* 64: 5157-5172. http://dx.doi.org/10.1093/jxb/ert305

Lamb CJ, Lawton MA, Dron M and Dixon RA (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* 56: 215-224. http://dx.doi.org/10.1016/0092-8674(89)90894-5

Li S (2014). Transcriptional control of flavonoid biosynthesis: fine-tuning of the MYB-bHLH-WD40 (MBW) complex. *Plant Signal. Behav.* 9: e27522. http://dx.doi.org/10.4161/psb.27522

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ Method. *Methods* 25: 402-408. http://dx.doi.org/10.1006/meth.2001.1262

Long SR (1989). *Rhizobium*-legume nodulation: life together in the underground. *Cell* 56: 203-214. http://dx.doi.org/10.1016/0092-8674(89)90893-3

Mahajan M and Yadav SK (2014). Overexpression of a tea flavanone 3-hydroxylase gene confers tolerance to salt stress and *Alternaria solani* in transgenic tobacco. *Plant Mol. Biol.* 85: 551-573. http://dx.doi.org/10.1007/s11103-014-0203-z

Meihls LN, Handrick V, Glauser G, Barbier H, et al. (2013). Natural variation in maize aphid resistance is associated with 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside methyltransferase activity. *Plant Cell* 25: 2341-2355. http://dx.doi.org/10.1105/tpc.113.112409

Nakatsuka T, Haruta KS, Pitaksutheepong C, Abe Y, et al. (2008). Identification and characterization of R2R3-MYB and bHLH transcription factors regulating anthocyanin biosynthesis in gentian flowers. *Plant Cell Physiol.* 49: 1818-1829. http://dx.doi.org/10.1093/pcp/pcn163

Ryan KG, Swinny EE, Markham KR and Winefield C (2002). Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. *Phytochemistry* 59: 23-32. http://dx.doi.org/10.1016/S0031-9422(01)00404-6

Salvatierra A, Pimentel P, Moya-Leon MA, Caligari PD, et al. (2010). Comparison of transcriptional profiles of flavonoid genes and anthocyanin contents during fruit development of two botanical forms of *Fragaria chiloensis* ssp. *chiloensis*. *Phytochemistry* 71: 1839-1847. http://dx.doi.org/10.1016/j.phytochem.2010.08.005

Schijlen EG, Ric de Vos CH, van Tunen AJ and Bovy AG (2004). Modification of flavonoid biosynthesis in crop plants. *Phytochemistry* 65: 2631-2648. http://dx.doi.org/10.1016/j.phytochem.2004.07.028

Schwinn K, Miosic S, Davies K, Thill J, et al. (2014). The B-ring hydroxylation pattern of anthocyanins can be determined through activity of the flavonoid 3'-hydroxylase on leucoanthocyanidins. *Planta* 240: 1003-1010. http://dx.doi.org/10.1007/s00425-014-2166-3

Shimada Y, Nakano-Shimada R, Ohbayashi M, Okinaka Y, et al. (1999). Expression of chimeric P450 genes encoding flavonoid-3', 5'-hydroxylase in transgenic tobacco and petunia plants(1). *FEBS Lett.* 461: 241-245. http://dx.doi.org/10.1016/S0014-5793(99)01425-8

Singh K, Kumar S, Rani A, Gulati A, et al. (2009). Phenylalanine ammonia-lyase (*PAL*) and cinnamate 4-hydroxylase (*C4H*) and catechins (flavan-3-ols) accumulation in tea. *Funct. Integr. Genomics* 9: 125-134. http://dx.doi.org/10.1007/s10142-008-0092-9

Spelt C, Quattrocchio F, Mol JN and Koes R (2000). anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. *Plant Cell* 12: 1619-1632. http://dx.doi.org/10.1105/tpc.12.9.1619

Sweeney MT, Thomson MJ, Pfeil BE and McCouch S (2006). Caught red-handed: Rc encodes a basic helix-loop-helix protein conditioning red pericarp in rice. *Plant Cell* 18: 283-294. http://dx.doi.org/10.1105/tpc.105.038430

Tanaka Y, Tsuda S and Kusumi T (1998). Metabolic engineering to modify flower color. *Plant Cell Physiol.* 39: 1119-1126. http://dx.doi.org/10.1093/oxfordjournals.pcp.a029312

Treutter D (2006). Significance of flavonoids in plant resistance: A review. *Environ. Chem. Lett.* 4: 147-157. http://dx.doi.org/10.1007/s10311-006-0068-8

Wang J, Ming F, Han Y and Shen D (2006). Flavonoid-3',5'-hydroxylase from Phalaenopsis: a novel member of cytochrome P450s, its cDNA cloning, endogenous expression and molecular modeling. *Biotechnol. Lett.* 28: 327-334. http://dx.doi.org/10.1007/s10529-005-5718-6

Wang YS, Xu YJ, Gao LP, Yu O, et al. (2014). Functional analysis of flavonoid 3',5'-hydroxylase from tea plant (*Camellia sinensis*): critical role in the accumulation of catechins. *BMC Plant Biol.* 14: 347. http://dx.doi.org/10.1186/s12870-014-0347-7

Whang SS, Um WS, Song IJ, Lim PO, et al. (2011). Molecular analysis of anthocyanin biosynthetic genes and control of flower coloration by flavonoid-3',5'-hydroxylase(*F3'5'H*) in *Dendrobium moniliforme*. *J. Plant Biol.* 54: 209-218. http://dx.doi.org/10.1007/s12374-011-9158-7

Xu W, Dubos C and Lepiniec L (2015). Transcriptional control of flavonoid biosynthesis by MYB-bHLH-WDR complexes. *Trends Plant Sci.* 20: 176-185. http://dx.doi.org/10.1016/j.tplants.2014.12.001

Zhai R, Liu XT, Feng WT, Chen SS, et al. (2014). Different biosynthesis patterns among flavonoid 3-glycosides with distinct effects on accumulation of other flavonoid metabolites in pears (*Pyrus bretschneideri* Rehd.). *PLoS One* 9: e91945. http://dx.doi.org/10.1371/journal.pone.0091945

Zhang Z and Li DM (2008). Approach to outbreak mechanism of *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae). *Sci. Sil. Sin.* 44: 140-150.

Zoratti L, Karppinen K, Luengo Escobar A, Häggman H, et al. (2014). Light-controlled flavonoid biosynthesis in fruits. *Front. Plant Sci.* 5: 534-534. http://dx.doi.org/10.3389/fpls.2014.00534

Supplementary material

Table S1. Primers used in the experiment.

Table S2. Bioinformatics analysis of the three genes.

Table S3. Analysis of the structural domains of the three genes.

Figure S1. Gel electrophoresis analysis of the three genes M. DL2 000 marker; **a.** PCR product of the full-length cDNA of the *PmF3H* gene, **b.** PCR product of the full-length cDNA of the *PmF3'5'H* gene, **c.** PCR product of the full-length cDNA of the *PmC4H* gene.

Figure S2. cDNA and amino acid sequences of the coding regions of the three genes. **a.** *PmF3H* gene, **b.** *PmF3'5'H* gene, **c.** *PmC4H* gene.