Molecular Cloning, Characterization, and Expression Analysis of Flavanone 3-Hydroxylase (F3H) Gene during Muscadine Grape Berry Development

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Abstract

Flavonoids are natural antioxidants that include the groups of notable pigments such as anthocyanins and proanthocyanidins. Flavanone 3-Hydroxylase (F3H) is a key enzyme needed for the biosynthesis of flavonoids, the main ingredients of muscadine grape extracts. This study reports the first successful isolation, cloning and characterization of F3H gene from Vitis rotundifolia Michx. The full length cDNA of V. rotundifolia F3H gene (designated as VrF3H) had an open reading frame (ORF) of 1081 bp encoding 364 amino acids with a calculated molecular mass of 40.8kDa as well as an isoelectric point of 5.60. Comparative and in silico analyses revealed that the cloned VrF3H from muscadine grapes has high identity with F3H from other plant species. The deduced VrF3H protein showed similarities with other available plant F3H proteins, and the conserved amino acids ligating ferrous iron and residues participating in 2-oxoglutarate binding were found in similar positions comparable to other F3Hs. Furthermore, three-dimensional structure modeling showed that F3H protein had the enzyme core consisting of β-sheet, a typical structure shared by all 2-oxo-glutarate-dependent dioxygenases including F3Hs. Phylogenetic tree analysis indicated that VrF3H belongs to the Vitis F3H cluster. VrF3H transcripts were found to be abundantly expressed in the in-vitro red cells, véraison and physiologically mature red berries, but not expressed in the skins of the green berries. The isolation and characterization of VrF3H gene will enable further study in the role of VrF3H gene in the biosynthesis of flavonoids in V. rotundifolia.

Keywords: Flavanone 3-hydroxylase; MVrF3H; Muscadine; Muscadinia rotundifolia (Michx.) Small

Introduction

Flavonoids are secondary metabolites present throughout the plant kingdom. They have important functions in the plant’s adaptations to specific ecological niches or its responses to biotic and abiotic stresses. However, some of the secondary compounds are known to be beneficial for humans as pharmaceuticals. Over the last decades, the pharmacological industry had slowly slowed down on the use of natural products and shifted more towards the combinatorial chemistry approach. But recently there has been a renewed interest from the industry to look for active natural products with putative pharmaceutical importance in plants [1]. Success has been limited in the combinatorial-chemistry to deliver novel drugs and consumer demand for natural products have increased, because of their high antioxidant activities while flavonoids are extensively used in human nutrition [2-4] as anticancer, antimutagenic, antimicrobial, anti-inflammatory, and anti-atherosclerotic agents [4,5]. Current molecular tools are allowing scientists to clone and characterized structural genes from the flavonoid biosynthesis pathway in many plant species to enable the determination of their specific roles.

The structural genes of anthocyanin biosynthesis pathway in Vitis vinifera grapes have been greatly investigated at the molecular level [6,7]. Among them is the flavanone 3-hydroxylase (F3H), which is an important enzyme in the anthocyanin biosynthesis pathway. It catalyzes the stereospecific hydroxylation of (2S) -eriodictyol and (2S)-naringenin to form (2R, 3R)-dihydroquercetin and (2R, 3R)-dihydrokaempferol [8-10]. In other words, it acts at the bifurcation of the anthocyanin and flavonoid branches. The function of F3H was first described from crude extracts of Matthiola incana and illuminated through parsley cell cultures [9,10]. Subsequently, the F3H gene was cloned from Petunia hybrida and functionally expressed in Escherichia coli with high activity [11]. Afterwards, more F3H genes have been cloned and characterized from a variety of plant species: such as Hordeum vulgare [12], Malus [13], Medicago sativa [14], Zea mays [15], Arabidopsis thaliana [16] and Perilla frutescens [17]. However, there have been no published reports on the molecular cloning and characterization of F3H in muscadine grapes. Nucleotide sequences of anthocyanin biosynthesis genes in muscadine grapes, are lacking in the databases except chalcone synthase (CHS) and dihydroflavonoid 4-reductase (DFR) genes that were reported as cloned by our lab, [18, 19].

In the present study, we report for the first time: (1) the isolation of a full-length cDNA clone of the gene encoding F3H from muscadine grapes (designated VrF3H), (2) we compare the deduced amino acid sequence of muscadine VrF3H to other published F3H sequences from other plants, and (3) characterize the expression pattern of VrF3H gene in the in vitro grown cell culture and field grown green, véraison and
physiologically mature muscadine berries using RT-PCR. In order to unveil the overall biosynthetic pathway of flavonoids in muscadine grapes, it is necessary to identify and characterize each gene involved in the pathway. Together with our previous work on CHS expression [20], these studies add to the molecular understanding of the biosynthesis of flavonoids and anthocyanins in muscadines, with significant practical implications for enhancing the nutraceutical contents in muscadine using genetic engineering.

Materials and Methods

Plant materials

_Muscadinia rotundifolia_ (Michx.) Small "Noble" var. berry skins were harvested from the Florida A&M University vineyard at three different development stages (green, véraison and physiologically mature stage). Berries that were free of physical injuries and uniform in size were used. They were washed with distilled water; the skins were peeled and immediately frozen with liquid nitrogen and stored at -80°C until use. _In vitro_ red cell culture was established from sub-epidermal cells of the same vine [21] (patent publication no. US2011/0045195 A1). These cells were grown in a growth chamber at 23°C under a white light (150 µE m⁻² s⁻¹) with a 16 h light/8h dark cycle. The developed callus produces anthocyanins.

Sample preparation for phytochemical assays

Two grams fresh tissue (callus and/or skins) were kept frozen at -80°C, then homogenized (5000 rpm, 5 min, Bio Homogenizer, Switzerland) in 10 ml extraction solvent (Methanol: 1% HCL - 1:1) and centrifuged at 11000 rpm for 15 min at 4°C (Eppendorf 5804R, USA). The supernatant was collected and the residue was homogenized and re-extracted two more times. The combined extract was filtrated by 0.45 µm syringe filter and used for total anthocyanins assay.

Total anthocyanins assay

Total anthocyanins content was measured by using the pH differential spectrophotometric method [22]. Two portions of anthocyanin extracts were diluted (by using pre-determined dilution factor) with potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5), respectively. After 15 min, the absorbance of both dilutions was measured at 520 nm and 700 nm against water. Total anthocyanins content was measured by using the pH differential spectrophotometric method [22]. Two portions of anthocyanin extracts were diluted (by using pre-determined dilution factor) with potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5), respectively. After 15 min, the absorbance of both dilutions was measured at 520 nm and 700 nm against water. Total anthocyanins content was measured by using the pH differential spectrophotometric method [22]. Two portions of anthocyanin extracts were diluted (by using pre-determined dilution factor) with potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5), respectively. After 15 min, the absorbance of both dilutions was measured at 520 nm and 700 nm against water.

RNA extraction, gel electrophoresis and cDNA synthesis

Samples were prepared from different tissues of 'Noble' grape as mentioned above. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, CA) according to the manufacturer's protocol. RNA was quantified using Nano drop, and the inactivity was inspected by formaldehyde agarose gel electrophoresis. Purified RNA was treated with RNase-free DNase I, and immediately frozen to -20°C. Formaldehyde gel electrophoresis (1% agarose) was used to evaluate the RNA quality. The gel apparatus (including the gel tray and comb) was treated with RNase Away and rinsed with RNase-free distilled water. Total RNA was used in primary gene expression profiling. The SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) was used to synthesize cDNA in a 20 µL reaction containing 5 µg of DNase I-treated total RNA, 20mM Tris-HCl (pH 8.4), 50mM KCI, 2.5mM MgCl₂, 10mM dithiothreitol, 0.5 mg oligo (dt), 0.5mM each of dATP, dGTP, dCTP, and dTTP, and 200U SuperScript II Reverse Transcriptase. RNA, dNTPs, and oligo (dt) were mixed first, heated to 65°C for 5 min, and placed on ice until the addition of the remaining reaction components. The reaction was incubated at 50°C for 50 min, and terminated by heat inactivation at 85°C for 5 min. The cDNA product was treated with 1ml of RNase H (Invitrogen) for 20 min at 37°C. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA (no-RT control). The cDNA was stored at -20°C until it was ready for use.

Cloning of VrF3H full-length cDNA by RACE

After RNaseH treatment, the single-strand cDNA mixtures were used as templates for PCR amplification of the core fragment of F3H from _V. rotundifolia_. Two primers, F3H1 (5’-GCCCTACGACACTGAC-3’) and RF3H (5’-TAGGCACATTCTGGTC-3’) were designed based on the conserved regions of F3H and used for the amplification of the core cDNA fragment of VrF3H. This core fragment was amplified at the annealing temperature of 56°C, and sub-cloned into pMD18-T vector (TaKaRa, Japan), transformed into _Escherichia coli_ strain DH5α followed by sequencing. The core fragment was subsequently used to design the primers for the cloning of the full-length cDNA of _VrF3H_ by RACE.

SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used to amplify the 3’-end and 5’-end of _VrF3H_ cDNA. The first-strand 3’-RACE-ready and 5’-RACE-ready cDNA samples from _V. rotundifolia_ were prepared according to the manufacturer’s protocol and used as templates for 3’-RACE and 5’-RACE, respectively. Two 3’-gene-specific primers were designed for 3’-RACE. For the first cycle of amplification of 3’-end of _VrF3H_ cDNA, _VrF3H3-1_ (5’-CACATGCGCTAGCAGACTG-3’) and UPM (Universal Primer A, 5’-AAGCAGTGGTATCAACGCAGAGT-3’) were used as templates for PCR amplification of the core fragment of F3H from _V. rotundifolia_ and used as templates for the first amplification as templates. Two 5’-gene-specific primers were designed for 5’-RACE. For the first cycle of amplification of 5’-RACE, _VrF3H5-1_ (5’-TTAGGCCTACGCAGACTGAGC-3’) and UPM were used with the products of the first amplification as templates.
Relative-quantitative real-time PCR using SYBR green assay

Relative-quantitative real-time PCR reactions were performed in a 96-well plate with an iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad; [23]), using an iQ SYBR Green Supermix (Bio-Rad) to monitor cDNA amplification, according to the manufacturer's protocol. Three independent experiments were performed for each sample. After the real-time PCR had been performed, the absence of unwanted byproducts was confirmed by an automated melting curve analysis and agarose gel electrophoresis of the PCR products. In all the experiments, three replicates for each RNA sample were included; averages were calculated, and differences in the threshold cycle (Ct) were evaluated. The comparative Ct method was used, which mathematically transforms the Ct data into the relative transcription-level genes. When comparing the expression of VrF3H in different tissues, the relative quantification of VrF3H expression was achieved by calibrating its transcription level to that of the reference gene, Actin. The expression level calculated by the formula $2^{-\Delta\Delta Ct}$ represents the x-fold difference from the calibrator.

Search for muscadine F3H-related sequences

F3H sequence was retrieved through Basic Local Alignment Tool (BLAST), homology, and domain searches in public domains, namely GenBank (www.ncbi.nlm.nih.gov). The GenBank submitted F3H protein sequence of muscadine (Accession no. KF040970) was used for BLAST and homology searches against other plants.

Multiple sequence alignments and phylogenetic tree construction

Multiple alignment of the putative amino acid sequence of muscadine F3H was performed using the T-Coffee program [24]. The alignment of 20 F3H proteins was summarized using the Pplotcon sequence similarity graph (http://bioweb2.pasteur.fr/docs/EMBOSS/plotcon.html), which represents the similarity along the set of aligned sequences. The molecular phylogenetic tree for F3H was built with Neighbor Joining, using p-distance as a substitution model, and Maximum Parsimony methods in MEGA Version 5.0, with 5000 iterations for calculating bootstrap confidence levels [25]. The phylogenetic tree construction included the sequences for the muscadine F3H protein and/or putative F3H proteins reported in the NCBI database for 20 plants.

Protein three-dimensional structure prediction

The Muscadine F3H structural model was obtained from its amino-acid sequence by using the SWISS MODEL (http://swissmodel.expasy.org) [26] and Protein Homology/analogy Recognition Engine (PHRYE) (www.sbg.bio.ic.ac.uk/phyre) prediction servers [27,28]. The model obtained was classified according to identity percentage.

Results

Cloning of full-length VrF3H cDNA and sequence analysis

Following PCR amplification, an approximately 985 bp product was obtained and sequenced. A BLAST search revealed that this 985 bp cDNA fragment had high homology to F3H genes from Vitis species (99% identity to Vitis vinifera). Thus, this fragment was used to design gene specific primers for both 5’-RACE and 3’-RACE.

Using 3’-RACE and 5’-RACE, two PCR products sized about 500 and 315 bp were obtained, respectively. The products were sequenced and confirmed to be the 364 bp 3’-end and 255 bp 5’-end. By aligning and assembling the sequences of 3’-RACE, 5’-RACE and the core fragment on Contig Express (Vector NTI Suite 8.0), the full-length cDNA sequence of VrF3H with 1081 bp (Figure 1) was deduced and subsequently confirmed by sequencing (Figure 2). ORF Finder program analysis on NCBI showed that the VrF3H contained a 1081 bp ORF encoding a protein of 364 amino acids (Figure 2) with a calculated molecular mass of 40.8 kDa and an isoelectric point of 5.60.

The deduced amino acid sequence of VrF3H was submitted to the NCBI for PSI-BLAST search and the result showed that VrF3H was homologous to F3H sequences from other plant species, with 97% identity to F3H from V. vinifera. VrF3H was also similar to F3H from P. lactiflora (96% identities), P. suffrutii (95% identities), which suggests that VrF3H belongs to the F3H family. The search for a conserved domain database in the GenBank revealed that VrF3H belongs to the 2OG-FeII_Oxy superfamily. This family contains other genes like flavonol synthase (FLS) and anthocyanidin synthase (ANS). Our analysis indicated that VrF3H contains conserved domains from 2-ODD superfamily similar to other members of the 2OG-Fe (II) dioxygenase family. A comparative modeling of 3-D structure of VrF3H (Figure 3) was performed using SWISS-MODEL software. This was done to enhance an understanding of VrF3H. The 3-D model showed the presence of a conserved undisturbed helix on the surface containing a noticeable motif with amino acids resembling a putative leucine zipper.

Phylogenetic relationships of F3Hs

Phylogenetic tree was constructed using the predicted amino sequence of the putative F3H protein from muscadine as well as other plant species (Figure 4). The F3Hs from different plant species were divided into four subgroups: I, II, III, and IV (Figure 5). Our cloned VrF3H belongs to I subgroup, close to V. vinifera. In fact, part of the branch forming subgroup I are members of the Vitis spp. Phylogenetic
tree confirmed that there is a close relationship between muscadine grapes F3H and *V. vinifera* F3H. Our results concur with those by [15], which suggest that F3H is well conserved among plants of different classifications and has distinct species specificity.

**Expression profile analysis of VrF3H gene in berry skin and in vitro cell lines and total anthocyanin assay**

The expression of *VrF3H* was investigated in samples taken throughout the development of muscadine grape berry skin tissues (Figure 5B). Real-time PCR analysis indicated that *VrF3H* gene expression occurred in two phases. It was expressed at veraison, the start of the berry ripening and the expression continued to physiological maturity stage. *VrF3H* was highly expressed in in vitro cell lines starting from the 12th day of cultivation. There was no expression of *VrF3H* at the green stage of berry development. Following this period of little or no expression (green berries), there was a coordinated increase in expression of *VrF3H* gene. Expression of *VrF3H* gene then continued throughout the remainder of berry development. Thus, the expression of *VrF3H* gene coincided with other genes previously investigated in the anthocyanin biosynthetic pathway.

Figure 2: The complete cDNA sequence and amino acid sequence of the protein encoded by VrF3H (GenBank accession number: KF040970).
pathway [7,17,29]. The expression of VrF3H coincided precisely with the accumulation of anthocyanin pigments in the berry skin as indicated in Fig. 5A. The accumulation of total anthocyanin was monitored in all the samples, and the results showed that in vitro red cells had the highest accumulation followed by mature red skins (Figure 5A) with significant difference between the in vitro red cell cultures and physiologically mature skins.

Discussion

Results from this study further support that, functions of different plant species and their relationship in genome structure can be determined by comparative genomics [30]. Previous research indicates that F3H proteins from different species are highly conserved [13-15,17], and that the genes encoding F3H protein have been characterized at the genetic, chemical, and proteomic levels [16,29,31-34]. However, cloning of an F3H gene from muscadine grapes has not yet been reported. In this study, VrF3H gene from muscadine grapes was isolated, cloned and characterized. The isolated VrF3H cDNA was verified to have 1081bp encoding 364 amino acids. Comparison of its amino acid sequence showed high homologies (>90%) with the proteins of seven species: V. vinifera, P. lactiflora, P. suffruticosa, E. grandiflorum, N. tabacum, P. persica, and A. chinensis. Our results indicate that VrF3H is highly conserved and this is in agreement with [16]. From this study we determined that VrF3H gene is highly expressed in in vitro red cell cultures, as compared to véraison or physiologically mature berry skins (Figure 5B). The expression of VrF3H is controlled by transcriptional regulators, which have been studied in other plants including for the regulation of F3H. Quattrochio et al., [35] reported that F3H gene is independently expressed in Petunia, however, according to Martin et al., [36], F3H expression in Antirrhinum is coordinately controlled with downstream genes such as dihydroflavonol reductase. In Arabidopsis, F3H gene is coordinately controlled with the upstream genes for chalcone isomerase and chalcone synthase [16]. In another study, Quattrochio et al., [37] demonstrated deferential control of F3H expression in Z. mays and P. hybrid, which indicated that regulatory anthocyanin genes were conserved between species and that divergent evolution of the target gene promoters were responsible for the species-specific differences in regulatory networks.

However, the appearance of anthocyanins in muscadine grape berry skins at the onset of ripening coincides with increased expression of VrF3H gene encoding F3H enzyme in anthocyanin biosynthetic pathway. This means that VrF3H is one of the rate-determining enzymes in anthocyanin biosynthesis pathway of V. rotundifolia. But, further investigations are required to characterize the F3H enzyme activity and fully understand its structural role in anthocyanin biosynthesis. The tissue-specific expression analysis revealed higher levels of F3H expression in in vitro cell lines than in véraison and physiologically mature berry skins. This indicates that in vitro cultivation of pigment-specific tissues can be an improved model of increasing the production of anthocyanins for pharmaceuticals. According to Jeong et al., [38], the expression of F3H gene can also be induced by abiotic and biotic stresses.

Figure 3: The three-dimensional model of VrF3H. The α-helices are indicated in red and green and β-sheets are indicated by patches in blue. Turns and loops are indicated by lines.

Figure 4: A phylogenetic tree showing the relationship of amino acid sequence of WF3H from muscadine grapes and various F3H proteins from other plants. The muscadine F3H protein falls within the Vitis family.

Figure 5: (A)-Analysis of anthocyanin accumulation in in vitro cell cultures (Callus), as well as in different development stages of muscadine berry skins (Mature, Veriason, and green skins). Mean values with different small letters are significantly different at (p<0.01). The accumulation pattern of anthocyanins follows the pattern of expression of VrF3H. (B) The expression analysis of VrF3H in different tissues using real-time PCR.
For example, Shinozaki and Yamaguchi-Shinozaki [39] demonstrated that F3H genes not only showed increased expression under drought stress, but also under salt, cold, and hormonal stresses. Since different hormones were used in our in vitro cultivation processes in this study, the cells likely underwent a certain level of stress, which could have led to increased expression of the VfF3H gene (Figure 5). However, studies have also shown that plants that contain gene families from F3H show different transcription patterns [40]. According to Klesskin et al. [40], the transcript level of RfF3H2 was significantly higher than that of BrF3H1 in the root of R. trimyna, however, both genes were highly expressed in the stem and not in the leaves. But in a separate study, F3H was highly expressed in the roots and stems of alfalfa seedlings [41]. Therefore, the expression of VfF3H suggests tissue specificity, which allows anthocyanin accumulation in the skins, as well as in the in vitro cell lines of muscadine grapes.

In Shiraz grape berries, anthocyanins have been determined to accumulate in the skin but not in the flesh [42]. The pattern of expression seen in the muscadin berry skin was similar to that in the berry skin of Shiraz grape, where anthocyanins begin to accumulate at about veraison, and this coincides with the increase in expression of F3H. This suggests that there is coordinated regulation of F3H gene during the development of grape berry skin. According to Sparvoli et al. [7], as anthocyanins accumulate in dark-grown grape seedlings subsequently exposed to light, there is a coordinated induction of the genes from the committed steps of the anthocyanin biosynthetic pathway. This is similar to the control of the anthocyanin biosynthetic pathway in maize aleurone, which is regulated by the R and CI gene families [43].

Due to the nutritional and physiological roles of flavonoids, it is essential to understand the flavonoid pathway in muscadine grapes. Therefore, cloning and characterization of genes encoding key enzymes or transcriptional factors is the first step to understanding the regulatory mechanisms controlling flavonoid biosynthesis in muscadine grapes. It is clear that much more work is required before the regulation of flavonoid biosynthesis pathway and its biological significance are fully understood in muscadine grapes. We have isolated a muscadin F3H gene and performed some necessary analysis. But further investigations are required to characterize enzymatic activities of VfF3H to fully understand and deduce its regulatory role in anthocyanin biosynthesis.

Acknowledgements

The research has been done with the financial support of USDA/NIFA/AFRI Plant Biochemistry Program Grant # 2009-03127 and USDA/NIFA/1890 Capacity Building Grant #2010-02388.

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