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Molecular cloning of dihydroflavonol 4-reductase gene from grape berry and preparation of an anti-DFR polyclonal antibody

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Summary

Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219) is a key enzyme of the flavonoid pathway, which synthesizes numerous secondary metabolites to determine the quality of grape berry and wine. The full-length *dfr* cDNA with 1014 bp was cloned from grape berry, and then introduced into an expressed plasmid pET-30a (+) vector at the *Eco*R I and *Xho* I restriction sites. With induction of the isopropyl-β-D-thiogalactoside (IPTG), the pET-*dfr* was highly expressed in *Escherichia coli* BL21 (DE3) pLysS cells. A fusion protein with the His-Tag was purified through Ni-NTA His Bind Resin and then used as the antigen to immunize a New Zealand rabbit. The resulting antiserum was further purified precipitated by 50 % saturated ammonium sulfate and DEAE-Sepharose FF chromatography to obtain the immunoglobulin G (IgG) fraction. The resulting polyclonal antibody was found capable of immuno-recognizing the DFR of the crude protein extracts from grape berry. This work undoubtedly provides the possibility for further studies on biological regulation of DFR activity in grape berry.

K e y w o r d s : DFR, *Escherichia coli* expression, antibody, purification, grape berry.

Introduction

Flavonoids are important plant metabolites, and widely spread in plant kingdom. To date, more than 6,400 different compounds have been described (Martens *et al*. 2003). Flavonoids represent a large group of plant secondary metabolites, with diverse biological activities, and the biochemical and genetic investigations of flavonoid biosynthesis have been well documented (Dixon *et al*. 1999; Winkel-Shirley 2001, Dixon *et al*. 2005). Flavonoids are divided into several structural classes, including anthocyanins, which provide flower and leaf colors, catechins, and proanthocyanidins (PAs, also called condensed tannin, CTs), which contribute to resistance to microbes and other derivatives with diverse roles in plant development and interactions with the environment, such as UV protection, legume nodulation and pollen viability (Sparvoli *et al*. 1994, Cain *et al*. 1997, Dixon 1986, Lamb *et al*. 1989, Dixon *et al*. 1995, Paiva 2000). Since several flavonoids appear to confer health benefits to humans when consumed regularly, especially the catechins and proanthocyanidins have been paid more attention to because of their antioxidant activities and their interactions with proteins (Dixon *et al*. 2005, Prior *et al*. 2005, Rasmussen *et al*. 2005, Skerget *et al*. 2005).

The biosynthesis pathway for flavonoid is well established and several enzymes of this pathway have been identified, including chalcone synthase (CHS), chalcone flavanone isomerase (CHI), flavanone 3-hydroxylase(F3H), dihydroflavonol 4-reductase(DFR), leucoanthocyanidin dioxygenase (LDOX, namely anthocyanidin synthase, ANS), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), and flavonoid 3-O-glucosyltransferase (UFGT), and the gene have been isolated from various plant species, such as grape, apple, *Arabidopsis thaliana*, etc. Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219), which catalyzes the NADPH-dependent conversion of stereospecific dihydroflavonols such as dihydrokaempferol, dihydromyricetin and dihydroquercetin into unstable corresponding leucoanthocyanidins (or flavan-3,4-diols), is a "later" key enzyme controlling metabolic flux into biosynthetic pathway branches leading to anthocyanins and proanthocyanidins (Xie *et al*. 2004). Owing to the crucial role of this enzyme in flavonoid pathways, DFR genes have been isolated from several higher plants (Belt *et al*. 1989, Sparvoli *et al*. 1994, Liew *et al*. 1998, Fischer *et al*. 2003, Xie *et al*. 2004, Piero *et al*. 2006) and their regulation mechanism at transcriptional level has been extensively studied (Liew *et al*. 1998, Boss *et al*. 1996, Honda *et al*. 2002, Piero *et al*. 2006) However, it remains unclear about the biological regulation of the enzyme at the protein level.

In this paper, we reported on the cloning and expression of the grape (*Vitis vinifera* L.) *dfr* gene in *Escherichia coli*, and preparation of polyclonal antibody. The expression profiles of *dfr* were also investigated during grape berry development. Our work enables further investigation of the roles *DFR* genes play in the determination of above-mentioned important traits, and will undoubtedly provide the possibility to improve the quality of grape berry and wine through regulating the expression levels of *DFR* genes.

Material and Methods

G e n e r a l : The pET-30a (+) vector plasmid was purchased from Novagen (USA), while the pGEM-T easy

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vector was purchased from Promega (USA). Ni-NTA His Bind Resin was purchased from Novagen (USA). Reverse Transcription System Kit (A3500) was purchased from Promega (USA). All restriction enzymes and *Taq* DNA polymerase were purchased from Takara (Japan). All the chemicals were purchased from Sigma (USA) unless otherwise noted. The oligonucleotide primers were synthesized by Shanghai Biological Company (Shanghai, China), and all PCR products used for cloning were confirmed by sequencing at the Bioasia Biological Company (Shanghai, China).

Plant material: Grape berries (*Vitis vinifera* L. 'Cabernet Sauvignon') were harvested from a vineyard in the suburbs of Beijing at the stage of veraision (about 70th d after full bloom). The freshly harvested berries were selected on the basis of similar size and the absence of physical injuries or infections. After washing with distilled water, the berries were immediately frozen in liquid nitrogen and stored at -80 °C until further use.

Cloning the full-length *dfr* gene: The total RNA was isolated according to the method of WEN *et al.* (2005), and the cDNA was synthesized according to the manufacturer's instruction. A pair of specific primers, *DFR* (S) 5'-cggaattcatgggttcacaaagt-3' (the start codon was boxed), *DFR* (A) 5'-ccggtcgacctaggtcttgccatct-3' (the stop codon was boxed) were designed according to the grape mRNA sequence desposited in GenBank (GenBank Accession No. X75964). The PCR was carried out in a 50 µl total volume reaction containing 160 ng·µl^{-1} cDNA, 2.5 mM dNTPs, 20 µM of each primer, and 5 U Taq DNA polymerase. The template was pre-denatured at 94 °C for 5 min followed by 37 cycles of amplification (94 °C for 30 s, 45 °C for 60 s, 72 °C for 1 min and 30 s), a final extension at 72 °C for 10 min, and cooled to 4 °C in a GeneAmp PTC-100 cycler. The PCR products were isolated using 1 % agarose gel electrophoresis, and a strong band was clearly detected on agarose gel at 1014 bp area (Fig. 1). The PCR product was purified by DNA gel extraction kit according to the operating instructions. The purified product was ligated into pGEM-T easy vector, and then transferred into *E. coli* strain DH5α. The pGEM-*dfr* was sequenced from both sides. BLAST analysis indicated that the PCR product had 100 % identity in the activity site with the reported grape *dfr* gene (GenBank accession No. X75964, Data not shown), which confirmed that the obtained gene is grape *dfr*.

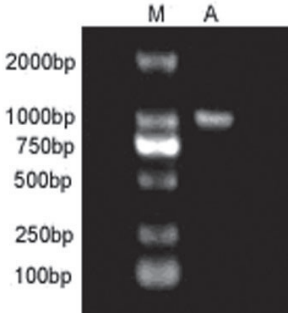

Fig. 1: Agarose gel electrophoretic analysis of PCR product of *dfr* gene M indicates DNA marker DL2000; A indicates PCR product of grape *dfr* gene

Construction of the expression vector pET-*dfr*: A pair of primers, DFR (S) 5'- cggaattcatgggttcacaaagt-3'(the start codon was boxed and the *Eco*R I restriction enzyme site was underlined), DFR (A) 5'-ccgctcgagctaggtcttgccatct -3' (the stop codon was boxed and the *Xho* I restriction enzyme site was underlined) were designed and synthesized to amplify the coding region of *dfr* cDNA. The plasmid pGEM-dfr was used as template. The PCR was carried out in a 50 µl total volume reaction containing 10 ng pGEM-dfr plasmid, 2.5 mM dNTPs, 20 µM of each primers, and 5 U Taq DNA polymerase. The template was pre-denatured at 94 °C for 5 min followed by 39 cycles of amplification (94 °C for 30 s, 55 °C for 60 s, 72 °C for 1 min and 30 s), a final extension at 72 °C for 10 min, and cooled to 4 °C in a GeneAmp PTC-100 cycler. The PCR products were isolated by 1% agarose gel electrophoresis, and recycled by DNA gel extraction kit. After double-digestion with two restriction enzymes *Eco*R I and *Xho* I, the full length *dfr* gene was sub-cloned into the pET-30a (+) vector that had been digested by the same enzymes overnight at 16 °C. Before being transferred into *E. coli* BL21 (DE3) pLysS cells, the recombinant plasmid pET-*dfr* was digested by *Eco*R I and *Xho* I (Fig. 2), and then was sequenced from both sides to confirm the nucleotide sequence of pET-*dfr* was correct.

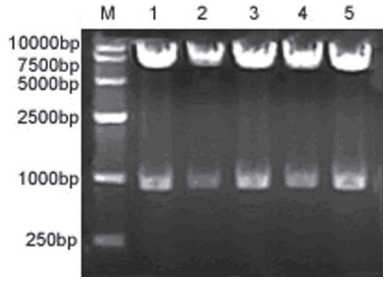

Fig. 2: Restriction enzyme digestion analysis of pET-*dfr* M indicates DNA marker, DL10000; Lanes 1-5 indicate the fragments with EcoR I and Xho I digestion. The arrow indicates the position of the fragments with *EcoR* I + *Xho* I digestion (1014 bp).

Expression of the recombinant protein from *E. coli*: Expression of the fusion protein was performed according to the methods described by SAMBROOK *et al.* (2001) and TIAN *et al.* (2006) with slight modification. A single colony of an *E. coli* strain BL21 (DE3) pLysS cells harboring the pET-*dfr* was induced and cultured overnight at 37 °C in Luria-Bertani (LB) liquid medium containing Kanamycin (60 µg·ml^{-1}), then transferred to fresh medium and incubated for another 3 h until the optical density (OD_{600}) of the cultured cells reached 0.8. Isopropyl-β-D-thiogalactoside (IPTG) was then added to the medium to induce the protein expression. The resulting fractions were assessed using SDS-PAGE. At the same time, a BL21 strain and BL21 strain harboring the pET-30A (+) empty vector were induced and analyzed as controls.

Purification procedures: Purification procedures were performed according to the manufacturer's instruction for Ni-NTA His Bind Resin with some modifications. The cells were harvested from liquid culture by

centrifugation at 10,000 ×g for 10 min, and the precipitates were dissolved with the binding buffer containing 300 mM NaCl, 50 mM sodium phosphate buffer, and 10 mM imidazole at pH 8.0. Lysozyme was added to the suspension at 1 mg·ml^{-1} and incubated on ice for 30 min. Following sonication, the supernatants were collected by centrifugation at 10,000 ×g for 10 min. The sample was applied to the resin column equilibrated with the binding buffer and washed with a 10 bed volumes binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0). The fusion protein was then eluted with a 5 bed volumes eluting buffer containing 300 mM NaCl, 50 mM sodium phosphate buffer, and 250 mM imidazole at pH 8.0.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: A Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) to determine the subunit molecular mass and purity of the protein.

Antibody production: Initially, 500 µg of the purified DFR protein was injected (subcutaneous) into a New Zealand rabbit after being emulsified with Freund's Complete adjuvant. Four booster injections were given at a 10 d interval, and the antiserum was collected at 10 d after the last injection. Purification of the rabbit IgG was performed according to methods described by Pan *et al.* (2005) with some slightly modification. Firstly, the IgG fraction was purified by precipitation with 50 % saturated $(NH_4)_2SO_4$, and then purified by passing through DEAE-Sephadex A-50 column chromatography.

Antibody titer: Antibody titer was determined according to the method described by Pan *et al.* (2005) with some modification. Briefly, the purified antigen, which was dissolved to 10 µg·ml^{-1} in 50 mM carbonate salt buffer (pH 9.6), was coated on immunoplates at 100 µl aliquot per well at 37 °C for 2 h and then at 4 °C for 48 h. Different concentrations of antibodies diluted from 1,000-fold to 512,000-fold were used to react with the DFR antigen on the immunoplates. The goat antibody against a rabbit IgG, conjugated with peroxidase (1:2000), was used as the second antibody. Peroxidase activity on the immunoplate was detected using o-phenylenediamine and H_2O_2 as enzyme substrate. The reaction was stopped with 2 M of H_2SO_4, and the optical density (OD_{492}) was determined by a Microplate Reader (Bio-Rad, USA). The antibody titer was defined as the dilution times of the antibody corresponding to the absorbance above 1.0 at 492 nm.

Results and Discussion

In order to improve the amount of expression, a range of different temperatures was tested for pET-*dfr* induction. The results were shown in Fig. 3. A protein band with a molecular mass of 43 KD was detected on SDS-PAGE gel from a crude lysates. The molecular weight of the expressed recombinant protein was estimated to be about 43 KD fused with His-Tag, therefore the size of expressed pET-*dfr* protein was in good agreement with that deduced from the amino acid sequence of pGEM-*dfr*. Meanwhile,

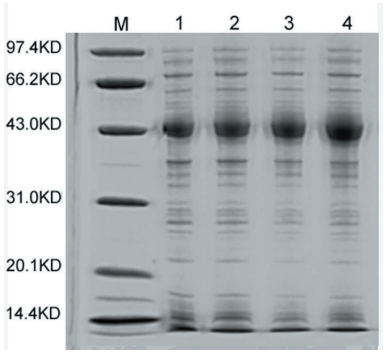

Fig. 3: Induced expression results of *dfr* gene under different temperatures M indicates low molecular protein marker; Lane 1-4 indicate the expressed DFR proteins at 25 °C, 28 °C 30 °C and 37 °C, respectively.

the amount of the expressed protein was different under different induction temperature. The amount of pET-*dfr* protein was lower at 25 °C and 28 °C compared to that at 30 °C and 37 °C. At 30 °C, the expression amount of the protein increased significantly. The amount reached the maximum when the induced temperature increased to 37 °C. The quantitative thin layer scanning indicated that the pET-*dfr* protein expression was 24 % and 36 % of total cellular protein at 30 °C and 37 °C respectively

The expression of pET-*dfr* with different IPTG concentration was shown in Fig. 4. With the increasing concentration of IPTG, the expression of pET-*dfr* protein increased. The expression reached maximum when the IPTG concentration increased to 0.5 mmol·l^{-1}. The quantitative result of thin layer scanning showed that the expression of the protein was 52 % of total cellular protein. There is no significant increase of pET-*dfr* protein expression when the IPTG concentration was higher than 0.5 mmol·l^{-1}.

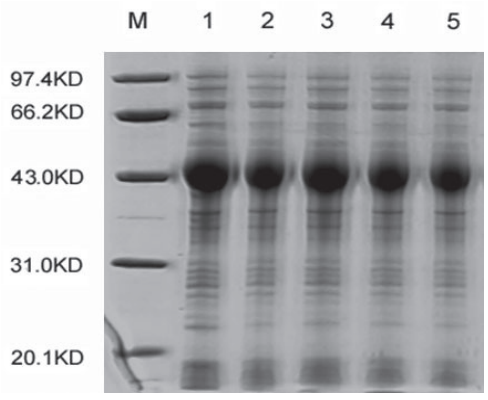

Fig. 4: Induced expression results of *dfr* gene for different IPTG concentrations. M indicates low molecular protein marker; lane 1-5 indicate the expressed pET-dfr BL21 with IPTG concentration of 0.8, 0.6, 0.5, 0.2 and 0.05 mmol·l^{-1}, respectively.

The effect of induction time on pET-*dfr* protein expression was shown in Fig. 5. With the increase of induction time, the amount of expression increased. The maximum expression of pET-*dfr* protein was obtained at 3 h of the induction, and it is estimated that the maximum yield of the protein was 52 % of total cellular protein by quantitative thin layer scanning analysis.

Purification of the pET-*dfr* was shown in Fig. 6. Owning to the His-Tag sequence, the recombinant DFR protein

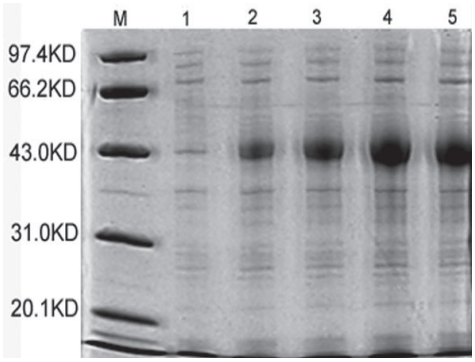

Fig. 5: Induced expression results of *dfr* gene for different time. M indicates low molecular protein marker; lane 1-5 indicate the expressed pET-dfr BL21 for 0, 1, 2, 3 and 4 r, respectively.

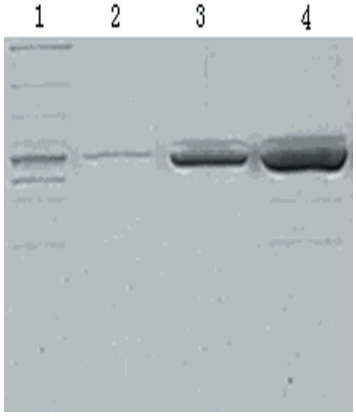

Fig. 6: Coomassie brilliant blue R-250- stained SDS polyacrylamide gels of the purified dihydroflavonol 4-reductase fusion proteins. Lane 1 indicates non-induced; lane 2-4 indicate the purified DFR proteins

could bind to the Ni-NTA His Bind Resin, and eluted with imidazole. In the un-induced stain, the expression amount of pET-*dfr* protein was very low. However, the expressed protein accumulated highly in the 0.5 mmol·l^{-1} IPTG-induced stain. The analysis of the thin layer scanning indicated that the expression amount of pET-*dfr* protein was 53 and 45 % of insoluble and soluble total protein, respectively.

The titer of anti-DFR serum was determined 5 d after last booster injection, and the result was shown in the Table. The anti-DFR serum has a high degree of detectable sensitivity. The OD_{492nm} of 1/4000 dilution of the polyclonal antibody was above 1.0, whereas the OD_{492nm} of control (pre-immune) was below 0.1, which indicated that the titer of polyclonal antibody obtained in this research was above 4,000.

The immunoglobulin fractions (IgG) against grape berry DFR were purified from rabbit raw antiserum by $(NH4)_2SO_4$ precipitation and DEAE-Sephadex A-50 chromatography. The sensitivity of anti-DFR polyclonal antibody was detected by Western Blotting (Fig. 7). The anti-

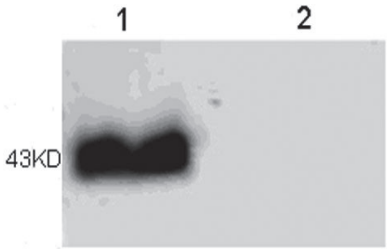

Fig. 7: Western blotting of dihydroflavonol 4-reductase from grape berry (lane 1) and bovine serum albumin (BSA, lane 2) with anti-DFR serum.

body could immuno-react with the expressed protein with a mass weight of about 43 KD, but not with BSA, which suggested that the anti-DFR polyclonal antibody was capable of specifically detecting the DFR protein.

The changes of DFR protein amount during the development of grape berry was investigated using the obtained DFR antibody. As shown in Fig. 8, the antibody against grape berry DFR recognized a single peptide with a mass weight of approximately 43 KD in the fractions that were extracted from the berries of different developmental stage. Immunoblots of DFR at the same enzyme concentration, with the same experimental procedures, showed different immunoreaction intensities. Higher immunoreaction intensities were detected at the beginning of berry development (20 d after full bloom) and at the late developmental stage (after veraison, which is characterized by softening and coloring of the berry). These results indicated that DFR

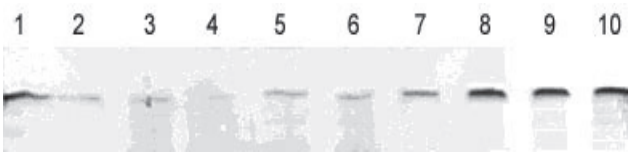

Fig. 8: Western blotting analysis of diflavonol 4-reductases from grape berries at the indicated d. Lane 1-10 indicate grape berry from 20, 30, 40, 50, 60, 70, 80, 90, 100 and 120 d after full bloom, respectively. The identical amount of protein (10 μg) was loaded per lane.

may be involved in berry growth and coloring. What role and how does DFR plays in berry growth and coloring, or what factor is DFR activity regulated by are of interest to be studied in the future.

Table

Titer analysis of anti-DFR serum

Dilution - fold	256,000	128,000	64,000	32,000	16,000	8,000	4,000	2,000	1,000	500
OD of sample	0.04	0.05	0.16	0.25	0.45	0.78	1.21	1.52	1.87	>20
OD of control	0.00	0.00	0.02	0.03	0.05	0.06	0.09	0.12	0.18	0.24

OD indicats optical density. Pre-immune rabbit serum was used as the control.

Acknowledgements

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