Chalcone isomerase in grape vine: gene expression and localization in the developing fruit

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Abstract

Chalcone isomerase (CHI, EC 5.5.1.6) is an entrance enzyme in the flavonoid biosynthesis, which catalyzes the conversion of chalcones to flavanones. In this study, the full-length *CHI* cDNA from grape vine (*Vitis vinifera* L.) was cloned, the recombinant protein was purified and the polyclonal antibody was prepared. Using these tools, the expression and tissue localization of CHI in developing grape berry was analyzed by RT-PCR, gel blot hybridization and immunohistochemical techniques. The expression of CHI was dependent on developmental stage, and CHI protein was mainly distributed in vascular bundles throughout all the stages of berry development, which suggested that flavonoids in the berry might have been partially synthesized *in situ*.

Additional key words: antibody, *Escherichia coli*, flavonoids, immunohistochemical technique, RT-PCR, *Vitis vinifera*.

Introduction

Flavonoids, which include the flavonols, flavones, flavanones, catechins, anthocyanins, isoflavonoids, dihydroflavonols and stilbenes (Haslam 1998), are important secondary metabolites. In plants, the flavonoids are involved in many functions including UV protection, defence against pathogenes, attraction of pollinating insects and initiation of symbiotic relationships (Parr and Bolwell 2000).

In the last stages of the biosynthesis of flavanone, 6-deoxychalcone (4,2,4-trihydroxychalcone) and 4,2′,4′,6′-tetrahydroxychalcone (chalcone), both derived from the upstream enzyme chalcone synthase (CHS, E.C.2.3.1.74), are isomerized into (2S)-naringenin (5,7,4-trihydroxyflavanone) and (2S)-5-deoxyflavanone (7,4-dihydroxyflavanone) by chalcone isomerase (Moustafa and Wong 1967, Hahlbrock *et al*. 1970). Although chalcones spontaneously cyclize in alkaline solution to produce an enantiomeric mixture of flavanones, CHI catalyzes it 10^7-fold more effectively. Suppression of *CHI* gene by RNA interference (RNAi) reduced pigmentation and changed flavonoid composition in flower petals. In contrast, over-expression of *CHI* gene enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* (Zhang *et al*. 2009). It is obvious that CHI plays a major role in the cyclization reaction from chalcone to flavanone, and that spontaneous reactions are scarce, as shown in tobacco plants (Nishihara *et al*. 2005).

So far, *CHI* genes were identified in many plants, such as *Arabidopsis thaliana* (*M86358*), *Callistephus chinensis* (*Z67980*), *Citrus sinensis* (*AB011794*), *Glycine max* (*AY595419*), *Petunia hybrida* (Van Tunen *et al*. 1988), and *Vitis vinifera* (*X75963*). CHI is mostly encoded by a single

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Abbreviations: BSA - bovine serum albumin; CHI - chalcone isomerase; CHS - chalcone synthase; DAF - day after full bloom; EDTA - ethylene diamine tetraacetic acid; IPTG - isopropyl-β-D-thiogalactoside; RT-PCR - reverse transcription-polymerase chain reaction; SDS - sodium dodecyl sulfate; Tris - tris(hydroxymethyl)amino methane.
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gene except for *Petunia* that contains a second gene homologous to *CHI* (Van Tunen *et al*. 1988). Previous studies primarily focused on cloning the gene and/or cDNAs and then studying the regulation mechanism at the transcriptional level (Sparvoli *et al*. 1994, Druka *et al*. 2003, Kim *et al*. 2004, Li *et al*. 2006, Xiao *et al*. 2007). However, little is known about the regulation of this enzyme at protein level and about its biological significance. The flavonoids have been found in the phloem bundles (Gould *et al*. 2000, Gholami 2004), through which they move towards the root tip (Buer *et al*. 2007). The transport mechanisms controlling the distribution of flavonoids were not elucidated. In this paper, we reported the cloning and expression of the grape *CHI* gene in *Escherichia coli*, the purification of fusion proteins, and the generation of polyclonal antibody. Using these tools, the expression and tissue localization of CHI during grape berry development were followed.

Materials and methods

Grape (*Vitis vinifera* L. cv. Cabernet Sauvignon) berries were harvested during the 2007 growing season at 20, 30, 40, 50, 60, 70, 80, 90, 100 and 120 d after full bloom (DAF) from a vineyard in the suburbs of Beijing. The freshly harvested berries were selected on the basis of similar size and were quickly frozen in liquid nitrogen and stored at -80 °C until used.

The full-length coding sequence for the CHI protein was cloned in *pET-30a(+)* (*Novagen*, Gibbstown, USA) using *Eco*RI or *Xho*I restriction sites. All restriction enzymes were purchased from *Takara* (Ostu, Japan). The inserts were sequenced (*Sunbiotech Company*, Beijing, China) and the recombinant plasmids were used to transform *E. coli* strain BL21(DE3). The expression, purification and preparation of antibody of grape CHI were done as described previously (Wang *et al*. 2008).

Grape berries (2 g) at different growth stages were ground in liquid nitrogen and the samples were extracted in 2 % hydrochloric acid in methanol for 24 h in the dark at room temperature. After centrifugation at 12 000 *g* for 20 min at 4 °C, the supernatant was diluted to assay total flavonoids. Total flavonoids were measured according to methods described in Wolfe *et al*. (2003). Absorption was analyzed at 510 nm and the results were expressed as mg(catechin) g^{-1}(f.m.).

Total proteins were isolated from the grape berry at different growth stages, as described by Famiani (2000). Separation of extracted proteins was performed using SDS-PAGE in 10 % polyacrylamide gels as described by Wang *et al.* (2008). The immunoblotting analyses were based on the same amount of loading proteins in each lane (6 μg).

Total RNA was isolated from the berry tissues using the method described by Wen *et al*. (2005) and used to produce the first-strand cDNA with 1.0 unit of AMV reverse transcriptase and *oligo(dT)* (*Promega*, Fitchburg, USA). The mRNA expression patterns of *CHI* were examined by quantitative reverse transcription-polymerase chain reaction. Meanwhile, the RT-PCR amplification for the house-keeping gene (*actin1*) was used as an internal control. 50 ng of total RNA was used to produce first-strand cDNA. For PCR reaction, 1 mm^3 of first-strand solution was used in a total volume of 0.05 cm^3 with 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 5 mM $MgCl_2$, 2.5 mM dNTP, 5 units of Taq DNA polymerase (*TaKaRa*) and 10 pmol of each primer. PCR was carried out with an initial heat action step at 94 °C for 10 min, and amplifications were achieved through 25 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. The amplified products were separated on 1.5 % agarose gel. The primers follow: *CHI* forward, 5′-GCGGATTCG GTTGACTTTTT-3′, and reverse, 5′-CTGGTAGGG ACCCATCTTTG-3′. *ACTIN1* forward, 5′-GATTCTGGT GATGGTGTGAGT-3′, and reverse, 5′-GACAATTTC CCGTTCAGCAGT-3′.

Grape berries collected during their development were immediately post-fixed overnight in a solution containing 4 % paraformaldehyde and 2.5 % glutaraldehyde at 4 °C, under vacuum, for 4 h; dehydrated with a graded ethanol series, embedded in paraffin, and sectioned into 10-μm slices. The slides were spread with polylysine before the fixing of the sections.

Dried sections were deparaffinized with xylene and hydrated in an ethanol-water series. Slides were processed as described previously (Hou and Huang 2005, Wang *et al*. 2010a) with some modifications. After immersion in 10 mM phosphate-buffered saline (PBS, pH 7.0, containing 0.2 g dm^{-3} KCl, 2.19 g dm^{-3} $Na_2HPO_4 \cdot 12\,H_2O$, 0.482 g dm^{-3} KH_2PO_4) for 5 min. Slides were incubated in a blocking solution [BS,10 mM PBS, 0.1 % (v/v) *Tween-20*; 1.5 % (m/v) glycine; 5 % (m/v) bovine serum albumin, BSA] for 45 min at room temperature, then rinsed in a regular salt rinse solution [RSR; 10 mM PBS, 0.1 % (v/v) *Tween-20*, 0.8 % BSA, 0.88 % (m/v) NaCl] for 5 min, and washed briefly with 10 mM PBS solution that contained 0.8 % BSA (PB) to remove the *Tween-20*. A drop of 0.1 cm^3 1:100 (m/v) primary CHI antibody (1 mg cm^{-3}) was added to each slide (*Agdia*, Elkhart, IN, USA) before covering the inner membrane of plastic gloves, then incubated overnight in a humidity chamber at 4 °C. The slides were washed vigorously twice in a high-salt rinse solution (10 mM PBS, 0.1 % *Tween-20*, 0.1 % BSA, 2.9 % NaCl) followed

by a 10-min wash with RSR and a brief rinse with 10 mM PBS. Then, 0.1 cm^3 secondary antibody were added to the slides [1:100, (v/v) dilution of the antirabbit IgG-alkaline phosphatase-conjugate (1 mg dm^{-3}, *Promega*) and incubated overnight in a humidity chamber at room temperature. After rinsing twice in RSR and once in water, the slides were developed for approximately 1 h by adding 0.200 cm^3 of Western Blue stabilized substrate for alkaline phosphatase (*Promega*). When the blue-green colour appeared on the sections, they were rinsed with water, dehydrated and mounted with a cover glass for photographing.

Two controls were carried out to verify the reliability of the immunohistochemical localization of CHI. One substituted normal rabbit serum for the primary CHI antibody, while the other omitted the primary CHI antibody. As before, all other procedures proceeded as usual.

Results and discussion

Using the *CHI* forward and reverse primers for RT-PCR analysis, a 702 bp *CHI* cDNA was obtained from grape berry. The PCR products had 100 % sequence in the activity site (Arg 38, Gly 39, Leu 40, Phe 49, Thr 50, Ile 52, Tyr 108, Lys 111, Val 112, Asn 115, Thr 163, Met 164) (Jez *et al*. 2000) with the grape *CHI* gene (*X75963*).

CHI was over expressed in *E. coli* as a fusion protein with His and exhibited a greater molecular mass (approximately 31 kDa) than expected due to the presence of His. Purification of the pET-*CHI* was shown in Fig. 1*A*. Owing to the His-Tag sequence, the recombinant ANS protein was able to bound to the *Ni-NTA His* bind resin, and to be recovered by imidazole elution. The eluted protein was proved to be a target of the His-Tag monoclonal antibody by immunblotting. Purification of the pET-*CHI* is shown in Fig. 1*B*.

The immunoglobulin fractions (IgG) against CHI were purified from rabbit raw antiserum by $(NH_4)_2SO_4$ precipitation and *HiTrap rProtein A FF* (*GE Healthcare,* UK). The IgG fractions showed two bands, a heavy chain (50 kDa) and a light chain (25 kDa) on SDS-PAGE gel after passing through the *HiTrap rProtein A FF* (data not shown). The titer of the obtained IgG of anti-CHI was determined by protein dot blot and an enzyme-linked immunosorbent analysis (ELISA) (data not shown). The results indicated the anti-CHI antibody had a high degree of detectable sensitivity, a 1/3000 dilution of the antibody (final concentration at 208 μg dm^{-3}) could recognize 1.2 ng of antigen. Moreover, a 1/10 000 dilution (final concentration 62 μg dm^{-3}) of the anti-CHI antibody was capable of detecting 1 μg of the antigen. The pre-immune rabbit serum was used as control and did not recognize the specific antigen. Thus, the anti-CHI antibody had a high degree of detectable sensitivity.

The antibody of CHI in *Arabidopsis* have been prepared (Cain *et al*. 1997), the deduced amino acid sequences from CHI in *Arabidopsis* showed weak homology (60.98 % identity, using *DNAMAN* software) with *Vitis vinifera*, so its antibodies were not well used to perform the detailed analysis of coordinate expression of flavonoid metabolism at the protein level in grape. In this study, the full-length *CHI* cDNA from grape berry was cloned via PCR, the recombinant protein was purified and the grape specific polyclonal antibody was prepared, which provides a substantial basis for the further studies of CHI in grape berry.

Total flavonoid content was 6.3 mg(catechin) g^{-1}(f.m.) at 20 DAF. Afterwards the value decreased sharply until

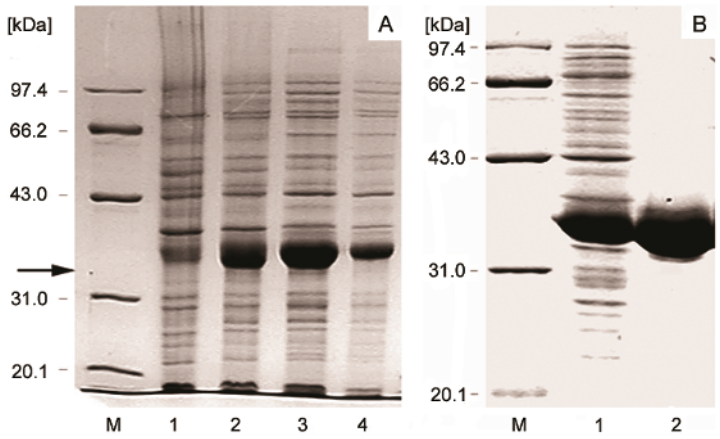

Fig. 1. Expression of CHI at different temperatures (*A*) and purification of recombinant CHI-fusion protein showing staining detection of the purified CHI (*B*). *A*: *lane* 1 - 25 °C, *lane* 2 - 30 °C, *lane* 3 - 37 °C, *lane* 4 - 42 °C, *lane* M - molecular mass markers. *B*: *lane* 1 - lysates of soluble fraction after induction with IPTG for 3 h; *lane* 2 - purified fusion protein; *lane* M - molecular mass standards. The patterns were stained by Coomassie brilliant blue R-250.

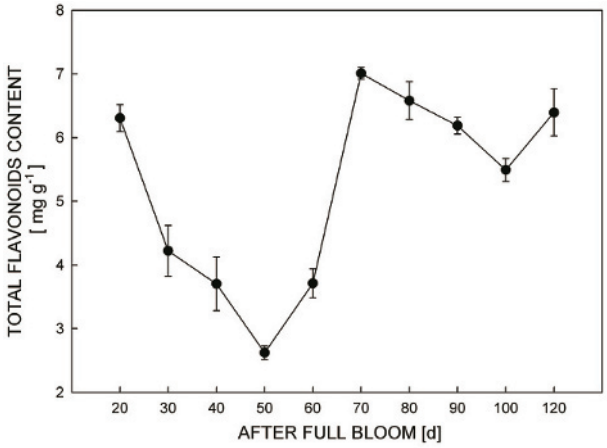

Fig. 2. Changes of total flavonoids content during grape berry development. The data were the means from three separate experiments. *Bars* are standard errors (n = 3).

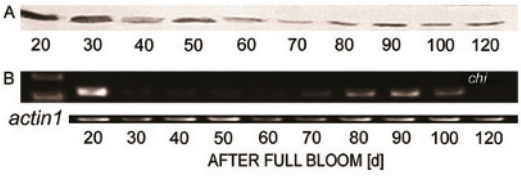

Fig. 3. Immunodetection of chalcone isomerase in the soluble crude extracts during the berry development (*A*) and the expression of *CHI* on the transcriptional level (*B*). Protein extracts from grape berry were separated on 12 % SDS-PAGE (6 μg protein per lane), transferred onto a nitrocellulose membrane, and probed with antibody against CHI followed by alkaline phosphatase detection. During the analysis of RT-PCR total RNA from grape berry fleshes were reverse-transcribed in the presence of an oligo d(T) 15 primer and PCR amplification was performed using *CHI*-specific primers. Meanwhile, *actin1* was amplified as an internal control. The amplified products were separated on 1.5 % agarose gel.

50 DAF reaching the minimum of 2.6 mg g^{-1}, and then rapidly increased toward 70 DAF reaching maximum of 7.0 mg g^{-1}. During the latter stages of berry development, only slight changes were observed (Fig. 2). This result was in agreement with Chen (2006).

CHI expression in grape berry at different fruit development stages was analyzed by protein gel blot hybridization and RT-PCR. Protein gel blot hybridization (Fig. 3*A*) of total protein demonstrated a high CHI content at the early stages from 20 to 30 DAF, and gradual decrease from 30 to 70 DAF. Later, it began to increase and reached the second peak at the ripening stage at 90 DAF. To analyze the accumulation of *CHI* mRNA, ten cDNA clones derived from the developing grape berries were sampled. *actin1* gene, showing a constitutive expression pattern as expected (Wang *et al.* 2008, 2010b), was used as the internal control. RT-PCR analysis (Fig. 3*B*) demonstrated that the *CHI* gene expression pattern was correlated with that of CHI protein. The high contents of *CHI* transcripts at 20 and 70 DAF may be associated with rapid cell division and expansion at the early stage of fruit development. The total flavonoids contents and the *CHI* gene expression exhibited similar patterns during grape berry development except for 70 DAF (the veraison), which represents the time of entry into the second rapid growth phase or ripening period of grape berry. The relevant flavonoids metabolism enzymes activities at this period might contribute to the high content of flavonoids. From then on, the expression of *CHI* also began to increase. In general, we suggest that CHI is regulated at both transcriptional and translational levels, and thus it may contribute to the accumulation of total flavonoids in grape berries.

The blue-green colour is the CHI immune signal, representing the natural distribution of endogenous CHI in the berry (Fig. 4). In young grape berries (20 DAF), strong CHI signals were detected in the vascular bundles and the exocarp, while few signals was also found in fruit parenchyma cells. At 40 DAF, the CHI signal weakened obviously, but it was still detectable in the vascular bundles. In contrast, the CHI signal was little increased at 70 DAF, not only in the vascular bundles but also in the exocarp. There was a strong CHI signal in vascular bundle and clear blue-green signal in exocarp and parenchyma cells in the berries at 90 and 120 DAF.

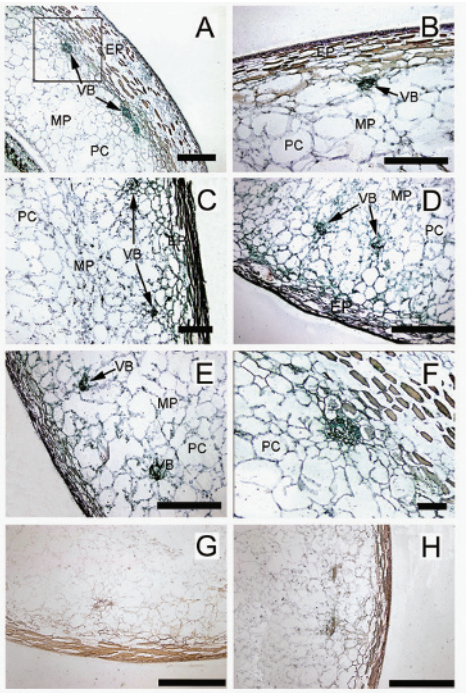

Fig. 4. Immunohistochemical distribution of CHI in grape berry at different developmental stages. Tissue localization of CHI at 20 (*A*), 40 (*B*), 70 (*C*); 90 (*D*) and 120 (*E*) DAF. *F* - enlargement of *A*, showing the CHI signals especially localized in the phloem of vascular bundle. In the control (*G*,*H*) with the antiserum omission no CHI signal was detected. EP - exocarp; MP - mesocarp; VB - vascular bundle; PC - parenchyma cells. *Bar* = 50.0 μm in (*A-E*, *G-H*), 10 μm in (*F*).

In summary, there were both abrupt and gradual changes in the CHI signals in the exocarp and mesocarp cells in the course of grape berry development. Moreover, the changes of CHI signals well corresponded to the temporal accumulation pattern of *CHI* gene transcript and CHI protein during grape berry development. Although the localization of CHI is ubiquitous in grape berry, CHI was mainly distributed in the exocarp and phloem bundles during berry development (Fig. 4*A-E*). Little CHI signal was detected in these sections (Fig. 4*G-H*) of the two controls, indicating the reliability of the method and the specific nature of the antibody used.

Flavonoid association to secondary wall of vascular bundles in pigmented leaves of *Quintinia serrata* had already been demonstrated by fluorescence analysis (Gould *et al*. 2000). Furthermore, it is known that coloured anthocyanins could be detected in phloem bundles of grape with red flesh. The accumulation of flavonoids occurred in transport tissues (Gholami 2004) in response to biotic and abiotic stresses (Dicko *et al*. 2005, Iriti *et al*. 2005). In agreement, recent findings confirmed flavonoid occurrence in vascular bundles, where they moved towards the root tip (Buer *et al*. 2007). In this paper, CHI protein was detected in vascular bundles during all the whole periods of the berry development. Similar to this, flavonoid 3'5'-hydroxylase and anthocyanidin synthase, two enzymes of the flavonoid biosynthetic pathway, were also found to be specifically associated with phloem tissue (Kaltenbach *et al*. 1999, Wang *et al.* 2010a). This suggest that some other flavonoids pathway enzymes are present in the vascular bundles of grapevine. They contribute to the biosynthesis of various flavonoid end-products *in situ*.

Conclusion: In this study, a full-length *CHI* cDNA sequence from grapes was cloned and expressed in *E. coli*, and a highly specific and sensitive antibody against CHI was produced. Vascular bundle localization of CHI has been detected in grape berry. Our results indicated that flavonoids located in the phloem bundles might have been partially synthesized *in situ*. They also documented that regional localization of a biosynthetic pathway may be used to direct the accumulation of metabolites at the diverse berry sites. Continued investigation of the factors responsible for its localizations will shed light on the regulation and alteration of the secondary metabolism in the grape berry.

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