Journal of Cell and Molecular Research (2010) 2 (2), 61-66

Irrigation effects on *pds* and *bch* genes expression of the Iranian Saffron

Nasrin Moshtaghi*, Robab Ghahremanzadeh and Seyyed Hasan Marashi

Department of Biotechnology, Faculty of Agricultural, Ferdowsi University of Mashhad, Mashhad, Iran.

Received 21 November 2010 Accepted 18 December 2010

Abstract

Saffron (*Crocus sativus* L.) is an indigenous and the most valuable and marginal plant in Iran. However, limited knowledge exists on its molecular biology. The importance of this plant is due to the color, flavor and medicinal properties of its red stigmas. Saffron stigmas contain a high amount of carotenoids such as crocetin and crocin. Two genes, *bch* and *pds*, have essential role in carotenoid production. In this study, the effect of four different irrigation regimes was evaluated on the expression of *bch* and *pds* genes. Semi-quantitative RT-PCR showed no significant difference in the expression levels of genes of interest related to the internal standard (18S rRNA). Results of Real-Time PCR assays showed that the expression of *bch* and *pds* genes were affected by irrigation treatments as their expression decreased in irrigated plants in comparison to non irrigated ones, exept for one irrigation treatment (one irrigation in September) where the pds gene expression showed higher level. However, the expression profile of the genes was almost the same in all treatments. The comparison between results of two techniques indicated that the Real-Time PCR is more accurate for determination of the level of transcript in the Iranian saffron. It was interesting that by decreasing of irrigation, the expression level of these two genes increased indicating that abiotic stress and drought can affect on the gene expression relating to the saffron color.

***Keywords*:** gene expression, irrigation, Real-Time PCR, Saffron

Introduction

Saffron (*Crocus sativus*) is considered as one of the most expensive spice in the world (Negbie, 1999). Saffron is a perennial crop well adapted to arid and semi-arid lands which produce stigmas annually (Lage and Cantrell, 2009). The shoots remain underground during the hot summer, to pierce the soil and flower when the temperature falls below 15 °C (Molina et al., 2005). The light purple flowers of *Crocus sativus* have thread-like reddish colored stigma that is valued both as a spice and a natural colorant (Negbie, 1999). Saffron is hand-harvested in the autumn, and the stigmas are laboriously separated to yield the reddish colored spice. About 70,000 flowers are needed to yield one pound (0.45 kilo) of saffron spice (Himeno and Sano, 1987). Saffron is a triploid sterile plant which produces and stores significant quantities of the apocarotenoids, crocetin and crocin, formed from the oxidative cleavage of zeaxanthin (Castillo et al., 2005). Carotenoids are ubiquitous terpenoid compounds in nature (Horton et al., 1996). All isoprenoids, including carotenoids, are derived from the ubiquitous C5 building blocks isopentenyl diphosphate and dimethylallyl diphosphate (Castillo et al., 2005). The first step of carotenoid biosynthesis is a head-to-head coupling of two molecules of geranylgeranyl pyrophosphate to yield colorless phytoene by phytoene synthase (PSY). Subsequently, four additional double bonds are introduced by phytoene desaturase (PDS) and zeaxanthin desaturase (ZDS) producing the colored carotenes phytofluene, ζ-carotene, neurosporene, and lycopene (Rodríguez-Concepción and Boronat, 2002). Carotenoid isomerase functions as the isomerization of poly-cis-carotenoids to all-trans-carotenoids. Two β-ring hydroxylations of β-carotene yield zeaxanthin in a reaction catalyzed by beta-carotene hydroxylase (BCH). These carotenoids can be further modified and processed to generate, among others, apocarotenoids that vary considerably among species (Britton, 1998; Jie et al., 2004).

Fernandez et al. (2004) indicated that BCH and PDS show basic roles in production of crocetin and crocin in saffron. BCH and PDS are key enzymes during the chain reaction for carotenoid production. PDS is a key enzyme in the carotenogenic pathway because it competes with PBS for lycopene and

∗Corresponding author E-mail: moshtaghi@um.ac.ir

directs the flux towards astaxanthin synthesis (Verdoes et al., 2003)

It seems that some abiotic stresses affect the expression of genes encoding these enzymes. The most important abiotic stress conditions are light, water availability, high temperature, salinity, cold and frost conditions (Wallis et al., 1997). Plants have developed a variety of defense systems against stresses by exhibiting qualitative and quantitative changes in patterns of gene transcripts and proteins (Nicot et al., 2005). In the research carried out by Rossel et al. (2002), the expression level of some genes such as *bch* II in Arabidopsis was increased under high light stress. The reverse transcription polymerase chain reaction (RT-PCR) has become a commonly used method for analyzing mRNA levels from interesting genes (Horton et al., 1996; Fernandez, 2004). Theoretically, there is a quantitative relationship between the amount of starting target sample and the amount of PCR production at any given cycle number. Beside, Real-Time PCR is also able to detect the accumulation of amplicon during the reaction more exact that semi-quantitative RT-PCR (Wilkening and Bader, 2004; Zaros et al., 2007; Logan et al., 2009). The objective of this study was to determine effects of different irrigation treatments on the rate of transcripts of *bch* and *pds* genes coding key enzymes by employing two techniques of semi-quantitative RT-PCR versus Real Time RT-PCR.

Material and Methods

Field experiment

Saffron corms had already been cultivated (3 years before doing this design- 2006) at the research farm of Ferdowsi University of Mashhad, in North Eastern of Iran. The saffron accession was used, is a native accession of Mashhad. This experiment was done at Sep-Oct of 2009 on the saffron field before flowering. Four irrigation treatments were applied in a completely randomized block design with three replications. The irrigation treatments were (I) two irrigations with an interval of one month (Sep and Oct), (II) two irrigations with an interval of 15 days (Sep), (III) one irrigation in September and (IV) no irrigation as control treatment. The water volume for each irrigation was 400 liters to each plot (4×4m).

RNA extraction

Saffron stigmas were collected and used for extracting RNA by RNX-Plus (Cinnagen Institute, Iran) according to the manufacturer's protocol. The extracted RNA was treated by DNase (Fermentas, USA) to remove the DNA contamination in RNA samples. The integrity of total RNA was estimated by 1% agarose gel. The purity and concentration of total RNA was determined by spectrophotometer, if the OD was between 1.8-2, the RNA samples were used in next experiments. Also the total extracted RNAs were used for PCR by the primers targeting of 18S rRNA as an internal control.

cDNA synthesis and semi-quantitative RT-PCR reaction

1 µg of the extracted RNA was used for cDNA synthesis in 20 µl reaction containing, 0.5 µg of oligo-dT, 200 units MMULV Reverse Transcriptase, 750 µM dNTPs, reaction Buffer, 10 units Ribolock. All the materials were provided by Fermentas Inc. For semi-quantitative RT-PCR, the introduced cDNAs per reaction were equilibrated through the balanced band intensity of 18S rRNA. PCR reaction was done using specific primers for *pds* (GenBank accession no. AY183118.1), *bch* (GenBank accession no. AJ416711.2) and *18S rRNA* (as housekeeping gene AJ489273.1) genes (table 1) in 25 µl volume. 30 cycles of PCR for all reactions were good for discrimination of band intensity. The PCR product was loaded on 1.2% agarose gel and the products were observed in the expected size. The intensity of each band was compared with that corresponding to the 18S rRNA. For quantification of each band, Lab Works software (version 3.0.2) was used. The primers were designed to produce product sizes between 100-200bp for specificity also in Real-Time PCR by Primer3 software (http://frodo.wi.mit.edu/primer3/). The RT-PCR conditions produced expected amplicon sizes and a single-band amplicon for all genes, indicating that the primers and conditions were specific for the target genes.

Real-Time PCR

Real-time PCR was performed by ABI Prism 7300 Real-Time PCR, and SYBR Green I (SYBR® Premix Ex Taq™ , Takara Bio Inc, Japan) according to the manufacturer's instructions. The real time PCR reactions contained: 2 µl of cDNA, 10 µl SYBR® Premix Ex Taq™ (2X), 0.4 µl of each primer (10 µM), 0.4 µl ROX™ Reference Dye (50X), 6.8 µl dH_2O for 20 µl final volume. The denaturation step was the same for all primers, i.e.: 95 °C for 10s in first cycle and 95°C for 5s in other cycles. The annealing temperature was 57°C for 1 min and that of the extension step was 60°C for 35s. This cycle was repeated for 40 cycles. The fluorescence acquisition temperature was 60 °C for

all genes.

Data acquisition and analysis method

Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (CT) (Walker, 2002; Nicot et al., 2005). We have used a relative quantification relating the PCR signal of the target transcript in a treatment group to that of another sample e.g., untreated control. The $2^{-\Delta\Delta CT}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen, 2001).

Data analysis using the $2^{-\Delta\Delta CT}$ method

The *C*T values were provided by real-time PCR instrumentation, ABI PRISM 7300 Sequence Detection Systems (Applied Biosystems, Foster City, USA) were easily imported into a spread sheet program such as Microsoft Excel. The mean *C*T values were determined for both the target and internal control genes. The changes in the expression of the *bch* and *pds* target genes were normalized to *18S rRNA* over 4 treatments. Duplicate samples were collected for all treatments. The data were analyzed using Eq (1).

$$\text{Amount of target} = 2^{-\Delta\Delta C_T} \quad (1)$$

Where $\Delta\Delta CT$ is ($CT_{,\text{Target}}$ - $CT_{,\ 18S\ rRNA}$) in certain treatment - ($CT_{,\text{Target}}$ - $CT_{,\ 18S\ rRNA}$) in the control treatment. The fold change in the target gene was normalized to *18S rRNA* and relative to the control treatment (treatment IV). The mean of CT and standard deviation (SD) were determined from samples for each treatment. ΔCT, ΔΔCT, $2^{-\Delta\Delta CT}$ and the SDs of ΔCTs were calculated as well. Using this analysis, the value of the mean fold change in the control treatment should be very close to one. A value calculated for different treatments shows the fold changes of gene expression relative to internal standard and control treatment (Livak and Schmittgen, 2001). Also, the analysis of variance was carried out for the fold of expression of these genes by duplicate samples.

Results

Semi-quantitative RT-PCR

RT-PCR reaction resulted in amplification of two bands, 108bp and 148 bp for *bch* and *pds* genes, respectively. The intensity of each band after 30 cycles was compared with *18S rRNA* in all treatments (figure 1). The results showed that treatments I, II and IV have no effects on the expression of these genes but in the treatment III, the band intensity was decreased in comparison with 18S rRNA. It seemed that twice irrigation and no irrigation had no effects on carotenoid production but once irrigation could decrease the expression of these enzymes.

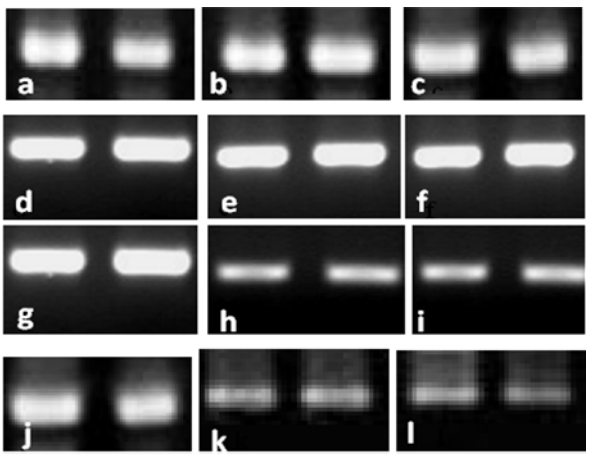

Figure 1. (a-b-c) *18S rRNA*, *bch*, and *pds* genes respectively in treatment I (two bands for two replications), (d-e-f) *18S rRNA, bch*, and *pds* genes respectively in treatment II, (g-h-i) are*18S rRNA*, *bch*, and *pds* genes respectively in treatment III, (j-k-l) are*18S rRNA*, *bch*, and *pds* genes respectively in treatment IV.

Real-Time PCR

The results of Real-Time PCR were a little different from RT-RCR and were summarized in table 2. As shown, the CTs for *bch* and *pds* genes were higher than the internal standard gene (table 2). PCR efficiency was good in real time PCR reactions. Amplification plots of *bch* and *pds* genes in Real Time PCR were shown in figure 2.

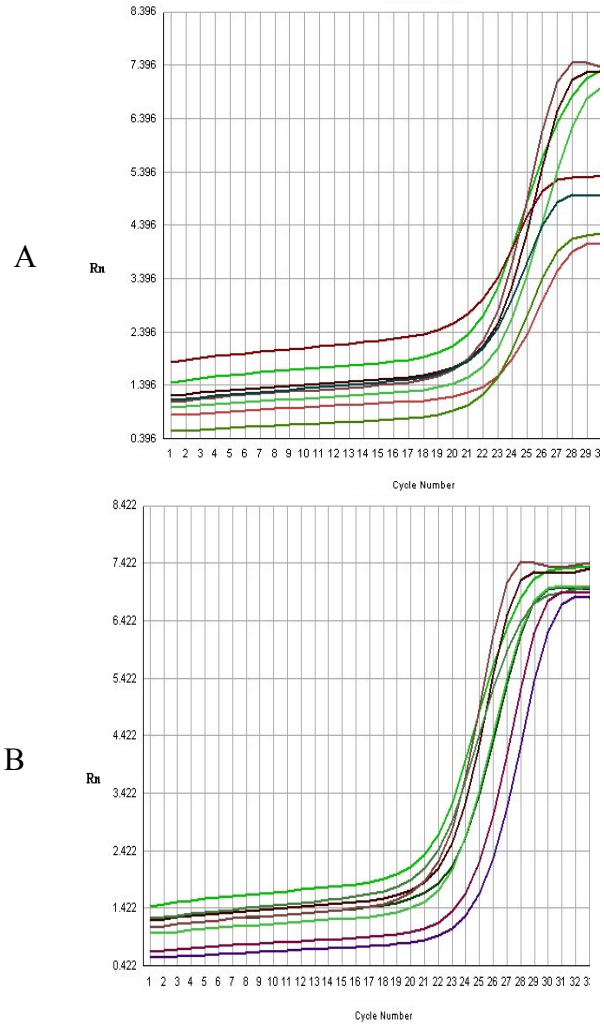

Figure 2. Amplification plots of *bch* (A) and *pds* (B) genes in Real Time PCR.

Discussion

In spite of the RT-PCR, the results of Real-Time PCR showed that these two genes (*bch* and *pds* genes) had different expressions in the treatments. In treatments I and II, both genes had lower expression relative to no irrigation (treatment IV) while in treatment III, the *bch* gene and the *pds* gene had respectively lower and higher expression compared to the control treatment (table 3).

The analysis of variance showed that the expression level of these two genes was significantly different ($p<0.05$) among treatments (figure 3). The expression level of *pds* and *bch* genes in treatments I and II were close while it was different for treatments III. The highest expression of *bch* gene was observed in treatment IV and the highest expression of *pds* gene was observed in treatment III.

Therefore, two irrigations during one month (Sep) or during two months (Sep and Oct) may decrease the expression of these two enzymes compared with control (no irrigation) or one irrigation. However, single irrigation in September and no irrigation had positive effects on the expression of these enzymes. As these enzymes have basic roles in production of apocarotenoids in saffron, so decreasing the expression of these genes may reduce carotenoid production. As a result, irrigation may not have any significant effect on carotenoid production and increasing the color intensity of red stigmas in saffron.

Castillo *et al* (2005) studied on carotenoid biosynthetic genes in apocarotenoid formation during the stigma development of *Crocus sativus* and observed that only the relative levels of zeaxanthin in the stigma of each cultivar were correlated with the levels of BCH transcripts. By contrast, the expression levels of ZCD (Crocus zeaxanthin 7,8-cleavage dioxygenase enzyme) were not affected by the apocarotenoid content suggesting that the reaction catalyzed by the BCH

enzyme could be the limiting step in the formation of saffron apocarotenoids in the stigma tissue.

Therefore, the *bch* gene and its expression directly affect the carotenoid production. In our research, *bch* gene had lower expression in the irrigation treatments in comparison to no irrigation, so irrigation does not have any positive direct relation with carotenoid production in saffron because with the decreasing of irrigation, the flower yield will decrease in the saffron field and this phenomenon is undesirable.

No irrigation and one irrigation act as an abiotic stress at the growth season of saffron so this tension can increase the expression of some genes in the flowering process of plant to guarantee the plant survival. In the study of Rossel et al. (2002), the effect of high light conditions were examined on the gene expression profiling of Arabidopsis and they determined that the abiotic stress such as high light can lead to oxidative stress; thus, a prompt and effective response to oxidative stress is crucial for the survival of plants. In their study, the expression level of *BCH* II gene (in this plant, *BCH* II is one of two genes encoding the BCH that catalyzes the synthesis of zeaxanthin and lutein) were up-regulated under high light conditions (Rossel et al., 2002). Also, in our study, the water tension, like high light tension, could slightly increase the expression level of *bch* gene. In addition, semi-quantitative RT-PCR doesn't have the sensitivity of Real-Time PCR to show the slight change in the expression level of these genes.

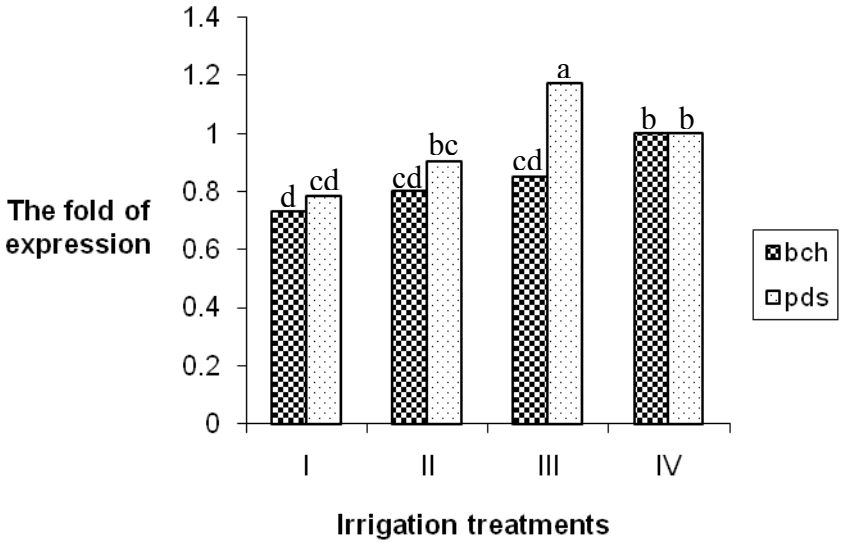

Figure 3. Diagram of the folds of gene expression ($p<0.05$). The same alphabets show no significant difference in gene expression.

Table1. Sequences of specific primers for genes in RT- PCR and Real-Time PCR reactions.

Gene	Primer Sequence		Product size (bp)	Tm(°C)
pds	Forward	5′-TTCAGCCGTTTTGATTTTCC-3′	148	57
	Reverse	5′-CAAGTCAGCATTTCATTGTTCC-3′		
bch	Forward	5′-GCATCATCCTCCTCTCTTCG-3′	108	57
	Reverse	5′-CGAGAACGAAAAACACTGTCG-3′		
18S rRNA	Forward	5′-TGTTATTGCCTCAGCCTTCC-3′	133	57
	Reverse	5′-GCGGTTTCTCTGGTTAATTCC-3′		

Tm: Melting temperature

Table2. Means of CTs and their SDs for different genes and treatments.

	Treatment			
Gene	**I**	**II**	**III**	**IV(control)**
bch	22.52±0.03	23.12±0.03	22.95±0.07	23.18±0.04
pds	22.76±0.08	23.28±0.05	22.83±0.06	23.52±0.18
18S rRNA	8.34±0.06	9.07±0.04	8.99±0.05	9.45±0.21

Table3. The folds change of gene expression based on 2-ΔΔCT method.

	Treatment			
Gene	**I**	**II**	**III**	**IV (control)**
bch	0.73	0.8	0.85	1
pds	0.78	0.9	1.17	1

Acknowledgment

We gratefully acknowledge the Ferdowsi University of Mashhad for funding and their support. We also wish to thank Dr. Heravi Mousavi for his help at some stages of this work.

References

1- Britton, G. 1998. Overview of carotenoid biosynthesis, p. 13-147. *In* G. Britton, S. Liaaen-Jensen, and H. Pfander (ed.), Carotenoids, vol. 3. Birkhauser Verlag, Basel, Switzerland.

2- Castillo R., Fernandez J. A. and Gomez-Gomez L., (2005) Implications of carotenoid biosynthetic genes in apocarotenoid formation during the stigma development of *Crocus sativus* and its closer relatives. Plant Physiology 139: 74–689.

3- Fernandez J. A. (2004). Biology, biotechnology and biomedicine of saffron. Recent Research Development of Plant Science 2: 127–159.

4- Himeno H. and Sano K. (1987) Synthesis of crocin, picrocrocin and safranal by saffron stigma-like structures proliferated in vitro. Agricultural and Biological Chemistry 51: 2395- 2400.

5- Horton P., Ruban A. V. and Walters R. G. (1996) Regulation of light harvesting in green plants. Annual Review of Plant Physiology and Plant Molecular Biology 47: 654–684.

6- Jie B., Chen M., Ying X., Lin T., Zhi-tao W. and Fang C. (2004) Cloning, expression of *Crocus sativus* phytoene desaturase gene and preparation of antiserum against it. Wuhan University Journal of Natural Science 9: 252-258.

7- Lage M. and Cantrell C. L. (2009) Quantification of saffron (*Crocus sativus* L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions. Scientia Horticulturae 121:366–373.

8- Livak K. J. and Schmittgen T. D. (2001) Analysis of relative gene expression data using Real-Time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402–408.

9- Logan J., Edwards K. and Saunders N. (2009). Real-Time PCR: Current Technology and Applications. Caister Academic Press.

10- Molina R. V., Valero1 M., Navarro Y., Guardiola J. L. and Garcı´a-Luis A. (2005) Temperature effects on flower formation in saffron (*Crocus sativus* L.). Scientia Horticulturae 103: 361–379.

11- Negbie M. (1999) Saffron: Crocus sativus L. Medicinal and Aromatic Plants Industrial Profiles. Amsterdam, Harwood Academic Publishers.

12- Nicot N., Hausman J. F., Hoffmann L. and LeEvers D. (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of Experimental Botany 56:2907–2914.

13- Rodríguez-Concepción M. and Boronat A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids: a metabolic milestone achieved through genomics. Plant Physiology 130:1079–1089 .

14- Rossel J. B., Wilson I. W. and Pogson B. J. (2002) Global Changes in Gene Expression in Response to High Light in Arabidopsis. Plant Physiology 130: 1109-1120.

15- Serazin-Leroy V., Denis-Henriot D., Morot M., Mazancourt P. D. and Giudicelli Y. (1998) Semi-quantitative RT-PCR for comparison of mRNAs in cells with different amounts of housekeeping gene transcripts. Molecular and Cellular Probes 12: 283–291.

16- Verdoes J. C., Sandmann G., Visser H., Diaz M., Van Mossel M. and Van Ooyen A. J. (2003) Metabolic engineering of the carotenoid biosynthetic pathway in the yeast Xanthophyllomyces dendrorhous (*Phaffia rhodozyma*). Applied and Environmental Microbogy 69: 3728–3738.

17- Walker N. J. (2002) A technique whose time has come. Science 296: 557–559.

18- Wallis J. G., Wang H. and Guerra D. J. (1997) Expression of a synthetic antifreeze protein in potato reduces electrolyte release at freezing temperatures. Plant Molecular Biology 35: 323–330.

19- Wilkening S. and Bader A. (2004) Quantitative real-time polymerase chain reaction: Methodical analysis and mathematical model. Journal of Biomolecular Techniques 15: 107-111.

20- Zaros L. G., Bricarello P. A., Amarante A. F. T. and Coutinho L. L. (2007) Quantification of bovine cytokine gene expression using real-time RT-PCR methodology. Genetics and Molecular Biology 30: 575-579.