Bioinformatics and differential expression analysis of chalcone synthase genes (*CHS1, 2, 3*) under gamma rays elicitation in *Silybum marianum* L.

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ABSTRACT

Silymarin is a constitutive secondary metabolite extracted from *Silybum marianum* seeds with wide therapeutic and medical effects. Gamma irradiation could be a powerful tool for obtaining promising mutated genotypes of *S. marianum* with high contents of flavonolignans. The transcript levels of the chalcone synthase genes (*CHS1, CHS2, and CHS3*), encoded for silymarin, in response to two different gamma irradiation doses (200 and 600GY) were determined using qRT-PCR. Also, Molecular isolation and characterization of chalcone synthase genes and its encoded protein were investigated via bioinformatics tools. Results of qRT-PCR confirmed that exposure of *S. marianum* seeds to gamma irradiation up-regulated the expression of the *CHS1, CHS2* and *CHS3* genes which could be positively correlated with increasing of silymarin content in the fruits. In conclusion, the increasing of flavonolignans content conflicts a great contribution in the field of medicine.

Keywords: *S. marianum*, Gamma radiation, qRT-PCR, Sequencing, Chalcone synthase genes, Bioinformatics

Introduction

Silybum marianum **L.** (milk thistle) is an annual and biennial medicinal plant belonging to the Asteraceae family. Originally a native of the Mediterranean basin, *S. marianum* is now found throughout the world **(Lv *et al.*, 2017a).** The most important secondary metabolite of the plant is a type of flavonoid compound called silymarin, which is an old and known medicine drug **(Hidalgo *et al.*, 2017).** The impact of this medicinal plant has established on different liver diseases, hepatitis, and cancers **(Lv et al., 2017b).**

Silymarin has gained the attraction of the scientific community as it introduces itself strongly as a medical compound. It is a constitutive secondary metabolite of *S. marianum* (L.). Silymarin is composed of an isomeric mixture of the flavonolignans, silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin and a flavonoid taxifolin**, (Lee and Liu 2003; Davis Searle et al., 2005; Biedermann et al., 2014; Lv *et al.*, 2017a, b).** Interestingly, it has many pharmacological activities such as anti-fibrotic **(El-Lakkany *et al.*, 2012)**, anticancer **(Cheung *et al.*, 2010),** antiviral, antibacterial, anti-inflammatory, and antiallergic **(Köksal *et al.*, 2009; Kandemir *et al.*, 2017).** Further, Silymarin is clinically used for its hepatoprotective, cardioprotective, neuroprotective, UV-protective and hypocholesterolemic effects **(Roubalová *et al.*, 2017).** Hence, Silymarin has been accepted as a safe plant-based product for its therapeutic effects **(Köksal *et al.*, 2009; Kandemir *et al.*, 2017).**

Chalcone synthase is an allosteric enzyme playing the key role in flavonolignan biosynthesis (CHS, EC 2.3.1.74), which catalyzes naringenin chalcone formation **(Sanjari *et al.*, 2015).** Many genes encoding *CHS* have been cloned, sequenced and characterized from *S. marianum* and various plant species **(Jiang *et al.*, 2006; Lu *et al.*, 2009; Pitakdantham *et al.*, 2010; El-Garhy *et al.*, 2016).** Chalcone synthases provide the starting materials for a diverse set of metabolites such as flavonoids (Feng *et al.*, 2015). The Chalcone synthase (CHS) is a key enzyme in the biosynthesis of flavonoids hence protect the plants from harmful effects **(Almatar, 2013)** thereby, the identification of CHS encoding genes in milk thistle plant can be of great importance **(Sanjari *et al.*,2016).**

The previous study results confirmed that chalcone synthase (*CHS*) genes are elicitor-responsive **(El-Garhy *et al.*, 2016).** Results of using qRT-PCR showed that *SmCHS1*, *SmCHS2* and *SmCHS3* genes from *S. marianum* are involved in the silymarin biosynthetic pathway **(Sanjari *et al.*, 2015; El-Garhy *et al.*, 2016).** Also, the relationship between the expression of silymarin (Sm) pathway genes (*SmCHS*) and the production of these metabolites in *S. marianum* was found (**Torres and Corchete, 2016**). Molecular pathways involved in radiation-induced stress response were first discovered using traditional biochemical approaches that monitored the activation of a single gene. Different physiological mechanisms are activated in living cells after exposure to radiation through gene modifications, which can be determined by qRT-PCR **(Roy *et al.*, 2009; El-Garhy *et al.*, 2016)**. Gamma irradiation, as a physical mutagen, has an effective role in improving silymarin yield. Where, it could be a promising approach to enhance genetic variation for the selection of stable mutated genotypes with a high content of silymarin **(El-Garhy *et al.*, 2016)**.

The introduction of new techniques, such as the use of different elicitors, and the study of their effect on the expression and regulation of biosynthetic pathways is a significant and applicable step towards the commercial production of useful secondary metabolites. Therefore, the aims of the present study

are: 1) characterization and analysis of *CHS1*, *CHS2* and *CHS3* genes and its encoded protein in S. marianum from the *de novo* assembled DNA contigs of high throughput sequencing data, 2) Comparing the structure of the obtained deduced amino acid sequences to other plant species and 3) evaluate the transcript levels of the studied genes in response to 200 and 600GY irradiation treatments using quantitative real-time (qRT-PCR).

Materials and Methods

Plant materials

This study was partially funded by the EU Commission, joint master's degree tempus project n.543865. It had been done in laboratories of Genetics and Genetic engineering dept., Faculty of Agriculture, Benha University, Egypt, and Plant Physiology dept., Faculty of Pharmacy, Barcelona University, Spain. Seeds of *S. marianum* were obtained from Genetics and genetic engineering dept., Faculty of Agriculture, Benha University. Seeds were divided into two groups: the first group was control seeds, non-treated, and the second group in which seeds were elicited before by exposing to 200 and 600 GY doses of gamma irradiation.

DNA extraction and PCR amplification

Total genomic DNA from plant tissues of all the studied groups was extracted according to the manufactures of QIAGEN DNeasy Plant DNA extraction Mini Kit (Cat. #:69104). The primers were tested by *in silico* PCR tool (http://insilico.ehu.eus/PCR/). The expected PCR amplicons were: 622bp for both *Chs1* and *Chs2* genes as well as 605bp for *Chs3* gene. The DNA fragments were amplified using the primers listed in Table 1 according to **(El-Garhy *et al.*, 2016).** PCR reaction was performed in a 50 µl mixture containing 0.4 µM of each primer with concentration of 10 pmol, 400 µM of dNTPs mix, 5 µl of 10x PCR reaction buffer, 2 µM MgCl2, 2.5 units of TAKARA Taq DNA polymerase (Cat. #:R001AM), 1 µl of template DNA and the final volume was adjusted with sterilized doubled distilled water (d.dH2O). A PCR thermocycler (BioRAD) was used to amplify the reactions consisting of 95 °C for 3 min followed by 40 cycles at 95 °C for 50 sec, 58.9°C as annealing temperature for 1 min with an extension of 72^0 C for 1 min followed by final extension temperature at 72°C for 10 min. Amplified PCR products were stored at -200C for further purification and downstream application. About 5 µl of PCR amplified product was loaded on 1.2% agarose gel electrophoresis stained with Ethidium bromide using GeneRuler™ 1kb DNA ladder (Cat. #: SM0313), then visualized under UV Transilluminator (Bio RAD).

Table 1. Oligonucleotide name and sequence of qRT-PCR and sequencing primers

Gene	Oligonucleotide name and sequence of qRT-PCR primers	Amplicon length (bp)	Reference
Chalcone synthase1 (CHS1)	CHS1F 5-TCTTGATTCCCTCGTTGGTC-3 CHS1R 5- TCTCAAACAACGGCCTCTCT-3	101	**GenBank: JN182805.1**
Chalcone synthase2 (CHS2)	CHS2F 5- AGGACATTGCGGAAAACAAC-3 CHS2R 5- AACGGCCTCTCTGTCTTCAA-3	184	**GenBank: JN182806.1**
Chalcone synthase3 (CHS3)	CHS3F 5- ACCCACCTCATCTTTTGCAC-3 CHS3R 5- CATCATGAGGCGTTTGATTG-3	105	**GenBank: JN182807.1**
NADH	ndhchs_L 5-TTCCGCATTTTGGAAATACC-3 ndhchs_R 5-CCCGTCTTGATTGAAAGGAA-3	134	**GenBank: KC589999.1**
Oligonucleotide name and sequence of sequencing primers			
CHS1, CHS2	CHS2F 5- AGGACATTGCGGAAAACAAC-3 qCHS2_R2 5-ACCGTCTCCACTGTCAAACC-3	622	**GenBank: JN182805.1, JN182806.1**
CHS3	CHS3F 5- ACCCACCTCATCTTTTGCAC-3 chs3_R1 5- GCCCTCAATTTTCCCTTCTC-3	605	**GenBank: JN182807.1**

Cloning and Sequencing

The resulted DNA amplicons, almost 622 and 605 bp, were eluted from agarose gel and purified using QIAquick Gel Extraction Kit (Cat. #: 28704). The purified PCR fragments were ligated into pGEMR-T Easy Vector Systems (Cat. #: A1360) according to its manufacturer. The competent cells of *E. coli* top 10 strain were prepared and transformed as described by **(Inoue et al., 1990)**. From LB/Amp/Xgal plates, white colonies were selected and inoculated on LB/Amp broth media. Then it was incubated overnight at 33 ºC with shaking for stabilizing the plasmid inside the transformed cells. The alkaline method of Birnboim and Doly **(Bimboim and Doly, 1979)** was used to isolate the plasmid. To confirm the recombinant plasmids the purified plasmids were examined by electrophoresis on 1.5% agarose gel using GeneRuler^{TM} 1 kb DNA Ladder (Cat. #: SM0313). Macrogen Company (South Korea) sequenced the obtained recombinant plasmids. The obtained sequence for *CHS1, 2* and *3* genes were examined for vector contamination using the VecScreen tool (http:// www.ncbi.nlm.nih.gov/tools/vecscreen).While Jalview software (http://www.jalview.org) was used to show single nucleotide polymorphisms (SNPs) and consensus resulted from the alignment of our obtained sequences and the nearest strains in NCBI database (Waterhouse, 2009). Construction of the phylogenetic tree was done using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA7 software (Kumar, et al., 2016). The obtained sequences were deposited at NCBI database (http://www.ncbi.nlm.nih.gov) under accession numbers MG751175.1, MG751178.1, and MG751181.1 for *Chs1*, *Chs2*, and *Chs3*, respectively.

Multi-sequence alignment (MSA) and phylogenetic analysis

To identify sequence Similarities with homologous proteins from other organisms, DELTA-BLAST tools were performed to the obtained CHS1, 2 and3 deduced amino acid sequences of *S. marianum*. Clustal omega (http://www.ebi.ac.uk/tools/msa/clustalo/) was used to generate multiple sequence alignment for the obtained transcripts. The maximum likelihood method was used to build phylogeny tree **(Saitou and Nei, 1987).** A bootstrap value of compared algorithms was attached to each branch to indicate the confidence level.

RNA extraction and relative expression of *CHS1, 2* and *3 genes*

Total RNA was isolated from *S. marianum* young leaves of irradiation treatments as well as control using SV Total RNA Isolation System (Cat. #Z3100) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from RNA using High Capacity cDNA (Thermo Fisher Scientific, Cat. No .436, 8814) reverse transcriptase kit. Conventional PCR was performed to confirm the amplicon lengths and primer specificity of target genes according to El-Garhy *et Al.*, 2016. Triplicate PCR was performed for each analyzed sample in addition to non-template control (NTC) and cDNA template negative. Each PCR reaction consisted of 500ng/reaction of cDNA (except for NTC and cDNA control), 12.5 µl SYBR Green PCR MasterMix (Maxima SYBR Green qPCR, Thermo Fisher Scientific, Cat. No # k0251), 0.3 µM of each forward and reverse primer, 10 nM/ 100 Nm ROX Solution, Nucleases free water to a final volume of 25 µl. Reactions were then analyzed on an AriaMx Real-Time PCR System (Agilent technologies), Two-step cycling protocol, under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s. The expression levels of *Chs1, 2 and 3* Genes were normalized to that of the housekeeping gene *NADH* gene. All experimentally induced changes in the expression of the studied genes are presented as n-fold changes relative to the corresponding controls. Relative gene expression ratios (RQ) between treated and control groups were calculated using the formula: RQ $=2^{-\Delta\Delta Ct}$ **(Livak and Schmittgen, 2001).** Specific primers used for qPCR are listed in Table (1).

Results and discussion

This work focused on eliciting and sequencing chalcone synthase genes (*CHSs*) encoding for silymarin in *S. marianum* plants. Silymarin is currently used for treating different types of liver disorders and has been applied as a medicine for more than 2000 years **(Crocenzi and Roma, 2006)**. Changing the secondary metabolite profile through eliciting molecular pathways in medicinal plants to enhance the production level of beneficial components is of great importance **(Gomez-Galera *et al.*, 2007; El-Garhy *et al.*, 2016).** Chalcone synthase, an allosteric enzyme, is playing an important role in the biosynthesis of flavonolignans in several parts of *S. marianum* as well as other medicinal plants **(Doa *et al.*, 2011; Sanjari *et al.*, 2015; El-Garhy *et al.*, 2016).**

Bioinformatics analysis of CHS1, CHS2, and CHS3 sequences

To confirm that the *chalcone synthase* genes were amplified and not others, the PCR products of *CHS1*, *CHS2* (622 bp) and *CHS3* (605 bp) genes (Fig.1) were partially sequenced and registered in the NCBI database under accession numbers MG751175.1, MG751178.1 and MG751181.1 for *CHS1*, *CHS2*, and *CHS3*, respectively.

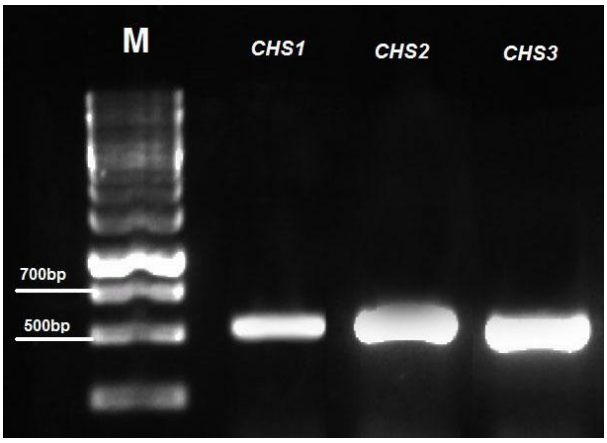

Fig.1. PCR products of chalcone synthesase, *CHS1*, *CHS2* and *CHS3* genes in Silybum marianum genomic DNA. M refers to Gene Ruler^{TM} 1 kb DNA ladder (Cat. #:SM0313)

First, these DNA fragments were examined via the VecScreen database, which revealed no contamination with vector sequences. The obtained DNA sequences were aligned with other sequences of *S. marianum* and other plant species available in the NCBI database using the BLAST alignment algorithm (http://www.ncbi.nlm.nih.gov/BLAST) and related available sequences are presented in Tables (2, 3, and 4, respectively). BLAST results indicated that CHS1 (MG751175.1), CHS2 (MG751178.1) and CHS3 (MG751181.1) from the current study are similar to CHS1 (JN182805.1), CHS2 (JN182806.1) and CHS3 (JN182807.1) in the NCBI from *S. marianum* with 99% max identity, respectively. Phylogenetic analysis also showed that all the studied *CHS* genes (*CHS1*, *2* and *3*) were related to each other with variable distances which confirmed the same identity ratios on the roots of clades and reflects the close similarity in accordance with the relatively high identity 99% (Fig.2A, B, and C).

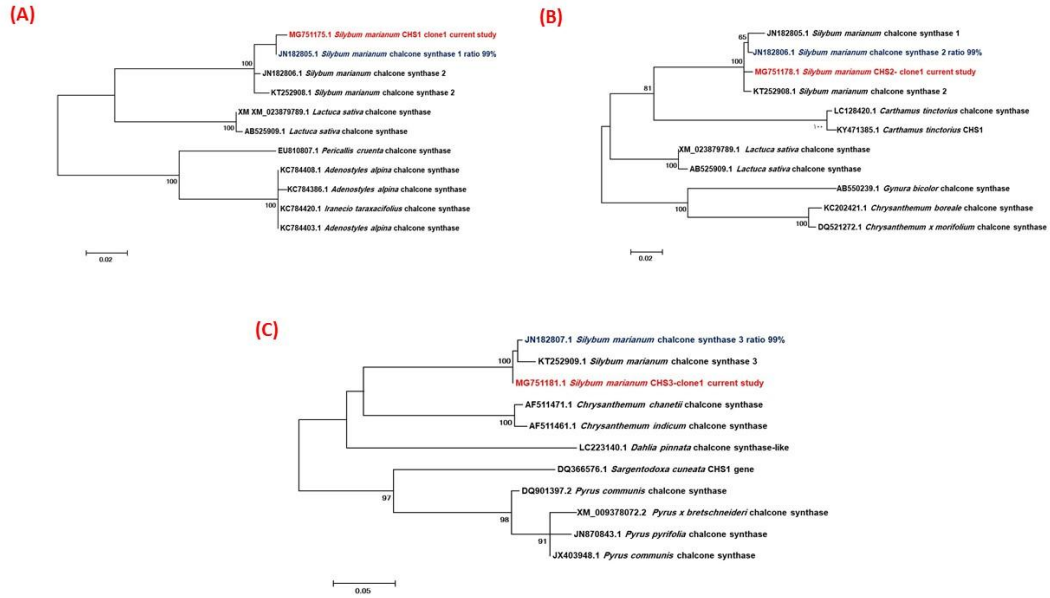

Fig.2. Phylogenetic tree showing the relation between, A: the *CHS1* (MG751175.1), B: *CHS2* (MG751178.1) and C: *CHS3* (MG751181.1) partial sequences from *Silybum marianum*, and the chalcone synthase (*CHS*) genes from the NCBI data base

Results of open reading frame (ORF) analysis showed partial-length ORFs, where three ORFs were exhibited for *CHS1*, two for *CHS2* and six ORFs for *CHS3* partial sequence with start and stop codons (Figure 3).

Fig.3. Open reading frame (ORF) analysis of the partial sequences of *Silybum marianum* A: *CHS1* (MG751175.1), B: *CHS2* (MG751178.1) and C: *CHS3* (MG751181.1) genes obtained in this study and chalcone synthase (*CHS*) sequences from the NCBI.

By calculating pairwise alignment, analysis data exhibited only two SNPs and one gap between the *CHS1* (MG751175.1) and *CHS1* (JN182805.1), registered in the NCBI database (Fig. 4A). Meanwhile, there were two SNPs between *CHS2*

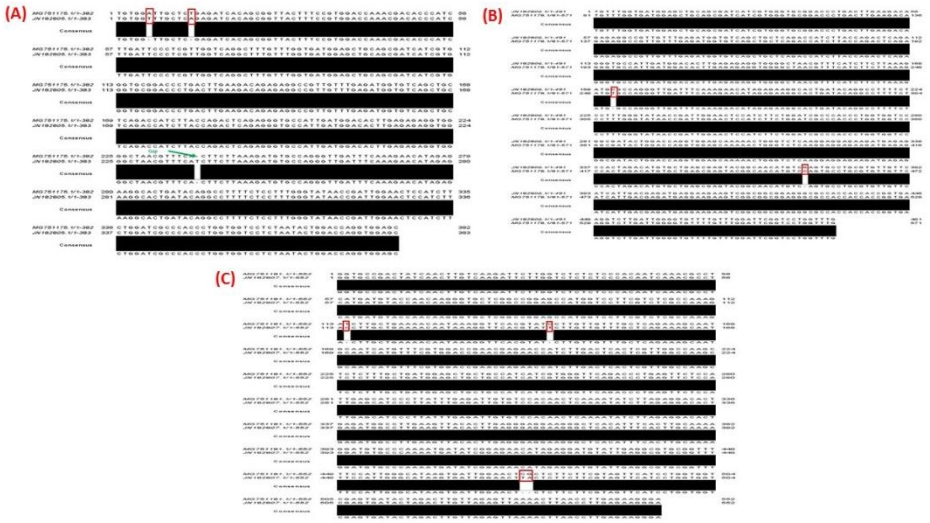

Fig.4. Pairwise alignment analysis of the partial sequences of *Silybum marianum CHS1*, *CHS2* and *CHS3* showed (A): two SNPs and one gap between the partially sequenced *CHS1*gene (MG751175.1) and *CHS1* (JN182805.1) from the NCBI. (B): two SNPs between the partially sequenced *CHS2* gene (MG751178.1) and *CHS2* (JN182806.1) from the NCBI. (C): four SNPs between the partially sequenced *CHS3* gene (MG751181.1) and the *CHS3* (JN182807.1) in the NCBI.

Table 2. Accession numbers and of the genes and organisms with highest similarities to chalcone synthase1 gene, *CHS1,* (accession no. MG751175.1) of *Silybum marianum*

Accession No.	Description/ Organism	Score	E value	Identity
JN182805.1	*Silybum marianum* chalcone synthase 1 (chs1) gene, partial cds	689	0.0	99%
JN182806.1	*Silybum marianum* chalcone synthase 2 (chs2) mRNA, complete cds	662	0.0	98%
KT252908.1	*Silybum marianum* chalcone synthase 2 (CHS2) mRNA, partial cds	475	4e-130	97%
XM_023879789.1	PREDICTED: *Lactuca sativa* chalcone synthase (LOC111883451), mRNA	435	6e-118	87%
AB525909.1	*Lactuca sativa* CHS mRNA for chalcone synthase, partial cds	429	3e-116	87%
EU810807.1	*Pericallis cruenta* chalcone synthase mRNA, partial cds	333	2e-87	83%
KC784408.1	*Adenostyles alpina* subsp. briquetii isolate B1 chalcone synthase (CHS) gene, partial cds	296	3e-76	83%
KC784386.1	*Adenostyles alpina* subsp. macrocephala isolate M chalcone synthase (CHS) gene, partial cds	296	3e-76	83%
KC784403.1	*Adenostyles alpina* subsp. alpina isolate Ac14 chalcone synthase (CHS) gene, partial cds	292	4e-75	82%
KC784420.1	*Iranecio taraxacifolius* isolate IT chalcone synthase (CHS) gene, partial cds	291	1e-74	82%

Table 3. Accession numbers and of the genes and organisms with highest similarities to chalcone synthase2gene, *CHS2,* (accession no. MG751178.1) of *Silybum marianum*

Accession No.	Description/ Organism	Score	E value	Identity
JN182806.1	*Silybum marianum* chalcone synthase 2 (*chs2*) mRNA, complete cds	1022	0.0	99%
KT252908.1	*Silybum marianum* chalcone synthase 2 (*CHS2*) mRNA, partial cds	846	0.0	99%
XM_023879789.1	PREDICTED: *Lactuca sativa* chalcone synthase (LOC111883451), mRNA	684	0.0	88%
JN182805.1	*Silybum marianum* chalcone synthase 1 (*chs1*) gene, partial cds	664	0.0	98%
AB525909.1	*Lactuca sativa CHS* mRNA for chalcone synthase, partial cds	621	7e-174	88%
LC128420.1	*Carthamus tinctorius CtCHS1* mRNA for chalcone synthase, complete cds	584	9e-163	85%
KY471385.1	*Carthamus tinctorius CHS1* mRNA, complete cds	573	2e-159	85%
KC202421.1	*Chrysanthemum boreale* chalcone synthase mRNA, complete cds	520	3e-143	83%
DQ521272.1	*Chrysanthemum x morifolium* chalcone synthase (*CHS*) mRNA, complete cds	508	6e-140	83%
AB550239.1	*Gynura bicolor GbCHS* mRNA for chalcone synthase, complete cds	492	6e-135	83%

Table 4. Accession numbers and of the genes and organisms with highest similarities to chalcone synthase3 gene, *CHS3*, (accession no. MG751181.1) of *Silybum marianum*

Accession No.	Description/ Organism	Score	E value	Identity
JN182807.1	*Silybum marianum* chalcone synthase 3 (*chs3*) gene, partial cds	998	0.0	99%
KT252909.1	*Silybum marianum* chalcone synthase 3 (*CHS3*) mRNA, partial cds	484	9e-133	98%
AF511471.1	*Chrysanthemum chanetii* chalcone synthase (*CHS1*) gene, partial cds	326	6e-85	79%
AF511461.1	*Chrysanthemum indicum* chalcone synthase (*CHS24*) gene, partial cds	320	3e-83	78%
LC223140.1	*Dahlia pinnata DvCHS3-2* gene for chalcone synthase-like, complete cds, cultivar: Yuino	283	4e-72	76%
DQ366576.1	*Sargentodoxa cuneata CHS1* gene, partial cds	137	3e-28	75%
DQ901397.2	*Pyrus communis* chalcone synthase mRNA, complete cds	110	6e-20	79%
XM009378072.2	Predicted: *Pyrus* x *bretschneideri* polyketide synthase 5 (LOC103965056), mRNA	99.0	1e-16	75%
JN870843.1	*Pyrus pyrifolia* chalcone synthase (*CHS2*) mRNA, complete cds	99.0	1e-16	75%
JX403948.1	*Pyrus communis* chalcone synthase (*CHS*) mRNA, complete cds	99.0	1e-16	78%

(MG751178.1) gene sequence and the sequence of *CHS2* (JN182806.1) (Fig. 4B). The number of SNPs between the *CHS3* gene partial sequence (MG751181.1) and *CHS3* (JN182807.1) were four (Fig. 4C). The obtained results were supported by the findings of the previous studies, where, **El-Garhy et al, (2016)** isolated and sequenced *CHS2* and *CHS3* genes from *S. marianum* cDNA moreover they demonstrated that these genes are elicitor responsive and involved in silibin A and B synthesis pathway. In addition, **Sanjari et al. (2015)** identified *SmCHS1*, *SmCHS2* and *SmCHS3* genes, which are probably involved in the silymarin biosynthetic pathway in *S. marianum*, using PCR and rapid amplification of cDNA end (RACE) techniques, and determined their transcript levels.

Multiple sequence alignment (MSA) of DNA sequences and proteins

MSA was performed using the best search hits for each of *CHS1*, *CHS2*, and *CHS3* DNA sequences and putative proteins. This analysis (http://www.ncbi.nlm.nih.gov/) was performed to identify nucleotides and proteins in other organisms homologous to the *CHS1, 2* and *3* DNA sequence and putative proteins in *S. marianum*. The best 10 hits for the three DNA sequences (Tables 2, 3, and 4, respectively) (Fig. 5A, B and C, respectively) and proteins (Fig. 6A, B, and C, respectively) with the lowest e-values and high identity percent. Results of the most closely amino acid sequences to the three chalcone synthase from *S. marianum* are, *CHS1* (JN182805.1), *CHS2* (JN182806.1) and *CHS3* (JN182807.1) of *S. marianum* (e-value 0.0) for all. These results indicated that the characterized DNA sequences and putative protein of the current study in *S. marianum* are members of silymarin biosynthesis pathway.

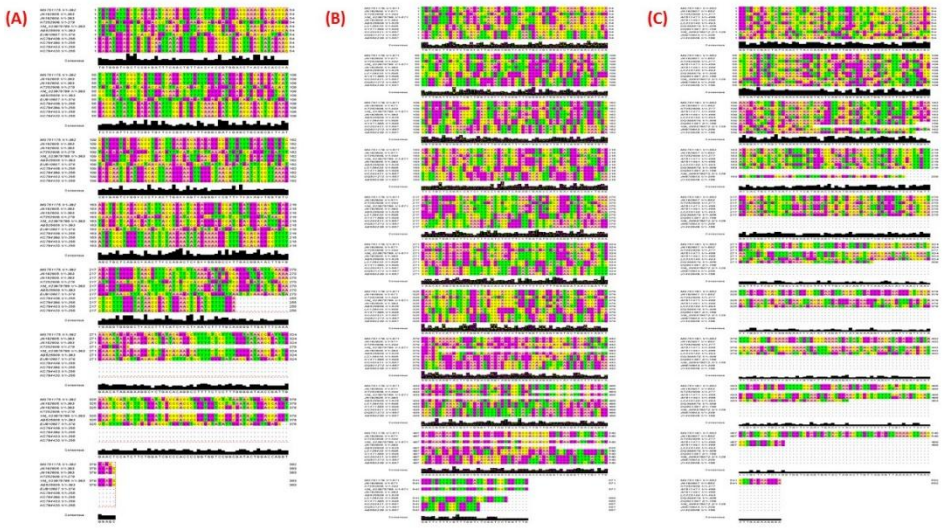

Fig.5. Multiple sequence alignment of the nearest ten different hits of chalcone synthesase (*CHSs*) DNA sequences in the NCBI with the obtained *CHSs* genes sequences of *Silybum marianum* A: *CHS1* (MG751175.1), B: *CHS2* (MG751178.1) and C: *CHS3* (MG751181.1).

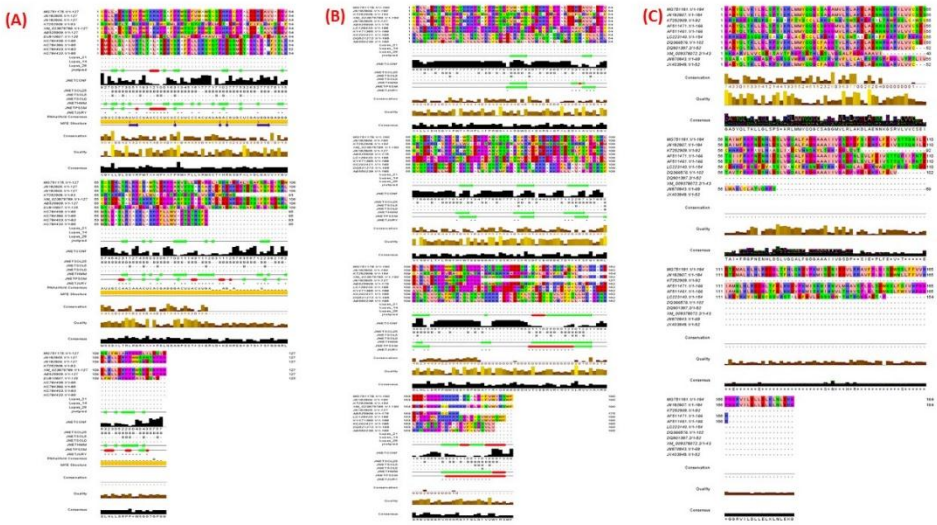

Fig.6. Multiple sequence alignment of the nearest ten different hits of chalcone synthase (*CHSs*) protein sequences in the NCBI with the obtained CHSs deduced amino acids sequence of *Silybum marianum* A: CHS1 (MG751175.1), B: CHS2 (MG751178.1) and C: CHS3 (MG751181.1).

Differential expression of CHS1, 2 and 3 genes in response to abiotic elicitors

To investigate the effect of 200 and 600GY doses of gamma irradiation on the expression of *CHS1, 2, 3* genes in *S. marianum*, the abundance of the transcripts was determined using qRT-PCR (Fig.7). In general, the expression of *CHS1, 2* and *3* genes was up-regulated in response to both treatments.

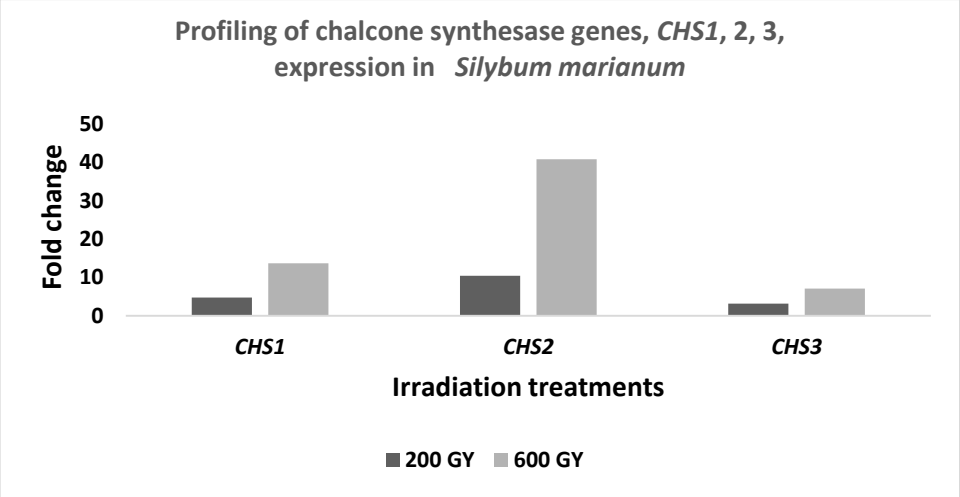

Fig. 7. Expression profiling of chalcone synthase genes *CHS1, CHS2, CHS3* of *Silybum marianum*, showing different responses under different irradiation doses

However, the obtained results showed that *CHS2* was more elicited (10.4, 40.8-fold increase) with 200 and 600GY irradiation treatments, respectively than the *CHS1* and *3* genes. Moreover, the transcripts of *CHS2* had the highest abundance (40.8-fold increase) under the effect of 600 GY. Studying molecular pathways involved in using gamma irradiation and different elicitors revealed that it is an effective way to generate stress tolerance in plants, activate multiple signaling pathways of intracellular defense mechanisms, and regulate related genes expression hence accumulate important metabolites such as silymarin production in *S. marianum* plants (**Jimenez-Garcia *et al.*, 2013; El Sherif *et al.*, 2013; Katar *et al.*, 2013; El-Garhy *et al.*, 2016**). It has been stated that after treating living cells with irradiation, numerous mechanisms are stimulated via differential expression of genes, which can be measured with qRT-PCR **(Roy *et al.*, 2009)**. Therefore, using gamma irradiation could be a promising approach to enhance genetic variation for the selection of a stable mutated genotype with a high content of silymarin. However, there is no major report on how irradiation may alter the expression of genes involved in the silymarin biosynthetic pathway. Our results agreed with **El-Garhy *et al.*, (2016)** who found that exposure of *S. marianum* seeds to six doses of gamma irradiation (100, 200, 400, 600, 800 and 1000GY) increased the expression of the *CHS1, CHS2* and *CHS3* genes in the leaves which positively correlated with an increase of silybin A and B content in the fruits. Using qRT-PCR, **Sanjari et al. (2015)** showed that *SmCHS, SmCHS2* and *SmCHS3* genes from *S. marianum* are involved in the silymarin biosynthetic pathway. Our results also agree with **Sanchez-Sampedro *et al.* (2005),** who observed that Methyl Jasmonate (MeJA) enhanced *CHS* activity and increased silymarin levels in cell cultures of *S. marianum*. Also, **Torres and Corchete (2016)** found a relationship between the expression of silymarin (Sm) pathway genes (*SmCHS*) and the production of these metabolites in *S. marianum*. Their results indicated that the observed increase in silymarin accumulation may contribute to the differential expression of the studied genes in response to different elicitors. HPLC analysis of transgenic roots showed a 9-fold increase in the accumulation of total silymarin. The accumulation of secondary metabolites plays an important role in plant protective reactions during biotic and abiotic stresses **(Rahnama *et al.*, 2013; Dehghan *et al.*, 2014).**

Conclusion

Using gamma irradiation could be a promising approach to enhance genetic variation for the selection of a stable mutated genotype with a high content of silymarin. However, there is no major report on how irradiation may alter the expression of genes involved in the silymarin biosynthetic pathway. The accumulation of the secondary metabolites plays an important role in plant protective reactions during biotic and abiotic stresses. Results of qRT-PCR revealed up-regulation of the *CHS1, 2* and *3* genes expression in response to both treatments. However, the obtained results showed that *CHS2* was more elicited with 200 and 600GY irradiation treatments than the *CHS1* and 3 genes. Moreover, the transcripts of *CHS2* had the highest quantity under the effect of 600 GY. In general, our results confirmed that exposure of *S. marianum* seeds to different doses of gamma irradiation increased the expression of the *CHS1, CHS2* and *CHS3* genes in the leaves which positively could be correlated with an increase of secondary metabolites content in the fruits.

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