Bangladesh J. Bot. **53**(3): 627-633, 2024 (September) Special DOI: https://doi.org/10.3329/bjb.v53i30.76601

FUNCTIONAL CHARACTERIZATION OF CHALCONE REDUCTASE GENE *CHR3* THROUGH RNAi

NAN WU, LONG JIANG, KEXIN YU, XILIN NING, MING LIU AND MINGHAO YUAN*

College of Agronomy, Jilin Agricultural Science and Technology University, Jilin, Jilin 132101, Jilin Province, P. R. China

Keywords: Soybean, Chalcone reductase gene *CHR3*, RNAi expression vector, Isoliquiritigenin, *Phytophthora sojae*, High Performance Liquid Chromatography

Abstract

In this experiment, the soybean genome "Jilin 30" was used as a template to clone the target gene *CHR3* using specific primers. The sense fragment, antisense fragment, and intron of the target gene were ligated into pCAMBIA3300 through double enzyme digestion to form a stem loop structure. Constructed RNAi vector pCAMBIA3301-CHR3 RNAi was transferred into the recipient soybean genome via *Agrobacterium* mediated method, specifically reducing the expression of the target gene *CHR3*. By genetic transformation, positive plants were obtained, and molecular biology methods such as Southern blotting, real-time quantitative PCR, isoliquiritigenin analysis, and identification of resistance to *Phytophthora* root rot were used to detect the expression level of the *CHR3* gene in the positive plants and study its function.The RNAi expression vector pCAMBIA3300-CHR3-RNAi was successfully constructed using the sense and antisense fragments of 428 bp *CHR3* gene and an intron of 110 bp was inserted to form the stem-loop structure of the RNAi vector. PCR was used to detect 6 lines of the T_1 generation and 15 lines of the T_2 generation. The results of Southern analysis confirmed the integration of *CHR3* and marker genes into the soybean genome. The expression *CHR3* gene in the T_1 genotypes decreased by 13.1 to 58.3%; the expression in the root decreased by 0.9 to 47.4%; the expression in the leaf decreased by 20 to 44.2%; and the expression in the stem decreased by 7.6 to 23%. The content of isoliquiritigenin decreased by 26.7% as transgenic shoots had a lower content of isoliquiritigenin and reduced resistance to *Phytophthora sojae*.

Introduction

Chalcone reductase CHR3 exists specifically in the synthetic pathway of isoliquiritigenin, in the synthesis of isoliquiritigenin, and may also have an important effect on the synthesis of soybean genistein (Serra *et al*. 2012, Wang *et al*. 2012). Isoflavin is one of the main components of isoflavones (Welle *et al*. 2011, Zhang *et al*. 2014); is an important physiological active substance with anti-fungal (Subramanian *et al*. 2012; Screevidya *et al*. 2011), antioxidant (Lopez-Lazaro *et al*. 2012) and other characteristics; and can inhibit the growth of microorganisms, such *Phytophthora sojae* (Gomez *et al*. 2011).

RNAi technology has a high degree of sequence specificity and can specifically target genes for silencing, loss of function or reduced expression via mutation; among other fields (Archana *et al*. 2018). The expression of isoflavone synthase gene (*IFS*) in soybean leaves and roots and its effect on isoflavone synthesis rate were studied using RNA interference technology. The results showed that RNA interference technology can analyze the function of *IFS* gene. (Lyle *et al*. 2015, Jiang *et al.* 2013, Senthil *et al.* 2017). used RNAi to inhibit the expression of the *FNSII* gene, which changed the content of flavonoids in soybean root hair root. Graham *et al.* (2015) used RNAi technology to specifically reduce the content of isoliquiritigenin in soybean without affecting other soybean isoflavone constituents and found that the transformed to *Phytophthora sojae* was significantly reduced (Terrence *et al.* 2007, Zhang *et al.* 2015).

*Author for correspondence: <yuanminghao2021@163.com>.

In my paper, the RNAi expression vector of the soybean chalcone reductase *CHR3* gene was constructed successfully and transformed into the soybean genome (Zhang *et al*. 2015). The effect of *CHR3* on the synthesis of daidzein was investigated by analysing the expression level of *CHR3* and the content of isoliquiritigenin in transgenic progeny and by disease resistance identification to explore the function of the soybean chalcone reductase gene *CHR3* and the relationship soybean with *Phytophthora sojae*.

Materials and Methods

Genotypes of soybean cultivated varieties "Jilin 30" was found as materials, and *E. coli* DH5α, *Agrobacterium tumefaciens* strain EHA105, and *Phytophthora sojae* were used as the bacterial strains. Expression vector pCAMBIA3301 were preserved by Agriculture Science and Technology University.

The *CHR3* gene was isolated by Zhang *et al.* (2014) (GenBank ID: KF927169). The specific primers CHR3ZS/CHR3ZAS and CHR3FS/CHR3FAS (Table 1, by the U.S. company Changchun Library Synthesis) were used to amplify the fragment, whose sequence was then aligned via NCBI sequencing.

DNA was extracted from Jinong 18 soybean tender leaf tissue using SoyBase SSR simple sequence repeat primers INS/INAS (Table 1). Simple sequence repeat amplification amplified 110 bp, which transformed via an RNAi expression vector to form a stem loop structure.

PCR was used to amplify positive, sense, antisense and intron fragments, and the basal expression vector pCAMBIA3301 was digested with *Bgl*II and *Bst*EII (restriction endonucleases from TaKaRa). The amplified digested, recovered and purified. Purification of positive, sense, antisense, intron and vector large fragments was performed as follows: a 5 μl mixture of vector + sense + antisense + intron at 50 centigrade for 15 min, the mixture was placed in an ice box, and the fragments were transformed into *E. coli*. The RNAi expression vector pCAMBIA3301-CHR3-RNAi was screened with the herbicide-resistant bar gene. The vector was subjected to PCR detection, double digestion and sequencing. A vector construction diagram shown in Fig. 1.

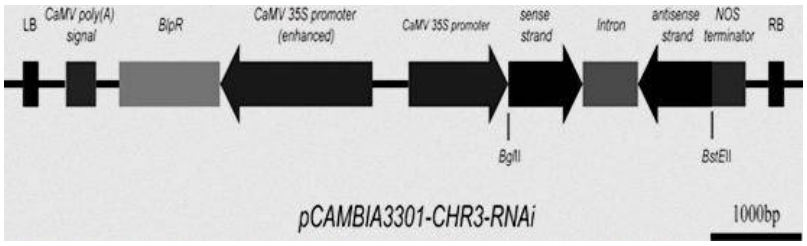

Fig. 1. Diagram of the structure of the RNAi expression vector.

In this study, the plasmid DNA of pCAMBIA3301-CHR3-RNAi was into the JL30 genome. A technology named *Agrobacterium* were infection of soybean and we have obtained positive plants.

The pCAMBIA3301-CHR3-RNAi plasmid vector contained the CAMV35S promoter and the herbicide gene its name is *Bar*. We use software to design primers, and the corresponding primer lengths are shown in Table 1.

Collecting young leaves of genetically modified soybean plants, Genomic DNA was extracted by CTAB and detected by PCR. The PCR amplification of the Bar gene was performed with 25 μl of DNA from an untransformed soybean leaf as negative control. Polymerase chain reaction-positive T_1 and T_2 genomic DNA, and we use the CTAB technology was extracted from the leaves

of transgenic plants, and then we put the DNA liquid into the EP tube with restriction endonuclease *Hin*dIII and we extracted the bar genome as a tube.

The RNA from positive signals was detected by Southern blotting, and cDNA was obtained by reverse transcription and diluted 5 times. The fluorescent quantitative real-time PCR primers QCHR3 and QACHR3 were designed (Table 1). The total RNA of soybean leaf tissue was analysed by Mx 3000P fluorescent quantitative real-time PCR (Jintai Biological Technology) according to the SYBR Premix Ex TaqTM kit (TaKaRa). Using SPSS19.0 software, the difference in the expression of the *CHR3* gene between the PCR and the receptor was analysed.

The technology named High-performance liquid chromatography (HPLC) was used to determine the isoliquiritigenin content. Transformation soybean grain and Jilin 30 untransformed soybean grain as a control were selected, crushed. Then, to 100 mg soybean powder was added 4 mL 80% methanol solution, before the tissue samples were further broken via ultrasound (40°C, 60 min, 70% power). The samples (20 µl) were organic membrane with a mobile phase of 80:20 methanol: water (0-5 min, 30% A; 5-20 min, 45% A; 20-30 min, 45% A; 30-35 min, 30% A; 35-40 min, 30% A). The column temperature was the same as the room temperature. Quantitative analysis of high-performance liquid chromatography was performed (Wu *et al*. 2017, Lidia *et al*. 2016).

Soybean inoculated with Phytophthora sojae was identified, and the incidence of all plants was investigated. Resistance was evaluated and classified as follows: resistant (R), plant mortality is less than or equal to 30%; moderately resistant (MR), plant mortality is 31 ~ 69%; and susceptible (S), plant mortality is more than 70% (Bennett *et al*. 2015, Dorrance *et al*. 2015).

Results and Discussion

The recombinant plasmid BL21-pET28a-CHR3 was used as a template, and its specific primers CHR3ZS/CHR3ZAS were used to amplify a fragment of just 428 bp (Table 1).

Table 1. Primer information.

Name	Sequence (5'→3')
CHR3ZS CHR3ZAS	ACTCTTGACCATGGTAGATCTCGATTACCTTGACCTCTATTTGATCC
CHR3FS CHR3FAS	ATAAGCCTGCACCTGAGAGAAACTTGTGCAATCTT
uINS	TAGTTTATCGCCCCACCTGAGAGAAACTTGTGCAATCTT
INAS	GTCACCTGTAATTCACACGTGCGATTACCTTGACCTCTATTTGATCC
35S	TCTCTCAGGTGCAGGCTTATCTTAAGACAAGTTACAC
35AS	TCAGGTGGGGCGATAAACTAGAACAGGA
BarS	TAGAGGACCTAACAGAAC
BarAS	CCGTGTTCTCTCCAA ATG
QCHR3	TCAAATCTCGGTGACGGGC
QACHR3	ATGAGCCCAGAACGACGC
QFACT	GTGCTAGTCGTGGTCCTAATG
QRACT	CGTACAACCACCTGAGAGAAA
CHR3ZS CHR3ZAS	ATCTTGACTGAGCGTGGTTATTCC
CHR3FS CHR3FAS	GCTGGTCCTGGCTGTCTCC

Similarly, CHR3FS/CHR3FAS were used to amplify the antisense fragment, and the length of the fragment was 428 bp (Table 1).

The genome of the "Jinong 18" was used as a template, the intron fragments were amplified by specific primers INS/INAS , and the length was 110 bp (Table 1).

Extract genomic DNA from PCR positive plants separately and use restriction enzyme *Bam*HI to cleave genomic DNA, As shown in Fig. 2, there was no hybridization signal in the unconverted plants. The detected transgenic Jilin 30 plants showed obvious hybridization signals. The blot shows that the target gene has been integrated into the recipient soybean genome but that the integration site is different.

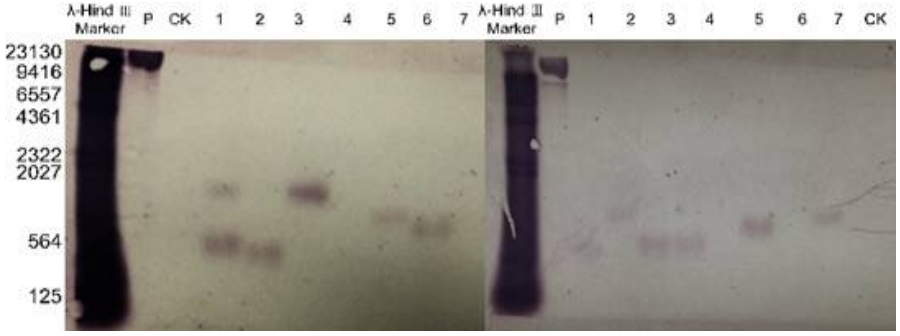

Fig. 2. Southern blotting detection of T_1 and T_2 generation transgenic plants. P: positive control; WT: wild type; Jilin 30 T_1 generation plants(a). Jilin 30 T_2 generation plants(b).

SYBR Green I was used as a dye, and Southern blotting-positive transgenic plants were verified by qRT-PCR. As shown in Fig. 3a, the relative expression of the *CHR3* gene mRNA in the T_1 generation transgenic soybean different from receptor plants, and the expression in the root decreased as follows: 0.342, 0.228, 0.442, 0.318, and 0.204. The expression in the stem decreased by 0.117, 0.020, 0.230, 0.207 and 0.076, respectively, and that in the leaves decreased by 0.276, 0.177, 0.396, 0.218 and 0.104, respectively. As shown in Fig. 3b, the relative expression of the *CHR3* gene in the mRNA of T_2 transgenic soybean plants compared to receptor plants also distinctly differed; the expression in the roots decreased by 0.203, 0.131, 0.282, 0.583, 0.350, and 0.268; that in the stems decreased by 0.115, 0.090, 0.327, 0.474, 0.273, and 0.220; and that in the leaves decreased by 0.019, 0.208, 0.106, 0.488, 0.306, and 0.272.

In JL30-2-1-6 and receptor Jilin 30 soybean plants, the glycyrrhizin content was measured in seeds by HPLC. Using the sample volume of X as the abscissa and the peak area Y as the ordinate, the regression equation of Y=252828 * 106X+0.223424, r=0.999, was used to calculate the content of isoliquiritigenin in soybean seeds.

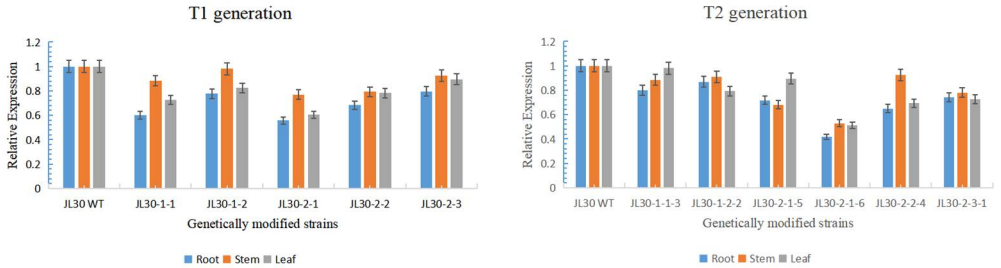

Fig. 3. Relative expression of the *CHR3* gene. a: Relative expression of the *CHR3* gene in T_1 generation positive plants; b: Relative expression of the *CHR3* gene in T_2 generation positive plants.

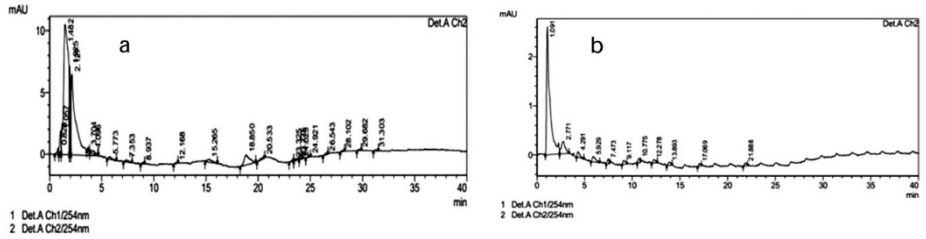

Fig. 4. ISO glycyrrhizin content determination by HPLC. a: HPLC analysis of the ISO glycyrrhizin content in unconverted soybean; b: HPLC analysis of the ISO glycyrrhizin content in soybean.

As shown in Fig. 4a, the content of ISO glycyrrhizin in mature seeds of untransformed plants was 1.332 g/ml. Fig. 4b shows that the content of ISO glycyrrhizin in transformed plants was 0.976 g/ml, decreasing by 26.7%.

In the past two years, the average mortality rate of JL30-1 was 100%, the average mortality rate of JL30-2 was 84%, and the average mortality rate of JL30 was 55% (Figure 5). The resistance of transgenic lines to *Phytophthora sojae* decreased from moderate resistance to susceptible (Table 2).

Table 2. Identification of resistance to *Phytophthora sojae*.

Year	Cultivar and line	Detected No.	Alive No.	Death No.	Death rate (%)	Resistance reaction
T_1 generation (2022)	JL30 WT	10	4	6	60	MR
	JL30-1	5	0	5	100	S
	JL30-2	5	1	4	80	S
T_2 generation (2023)	JL30 WT	30	15	15	50	MR
	JL30-1	15	0	15	100	S
	JL30-2	15	2	13	88	S

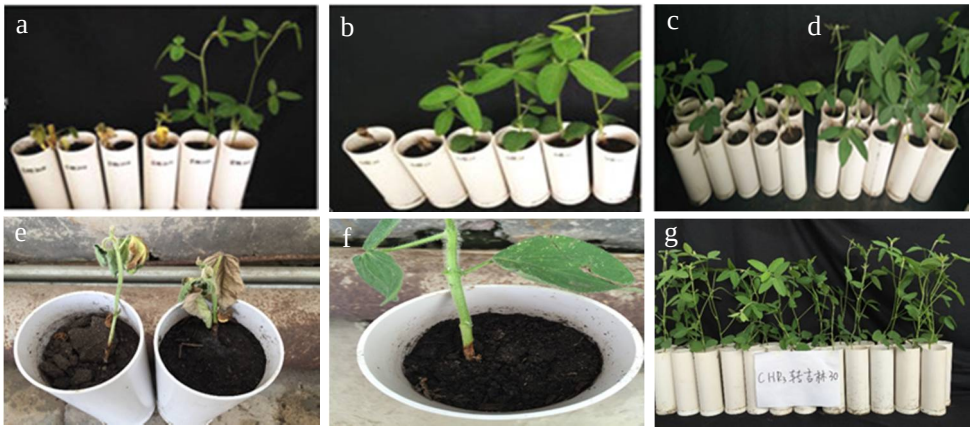

Fig. 5. Identification of resistance to *Phytophthora sojae*. a: The incidence of T_1 generation transgenic plants; b: The incidence of JL30 in the control variety; c: The incidence of T_2 generation transgenic plants; d: The incidence of JL30 in the control variety; e: The degree of decay of the stem base of transgenic plants; f: The degree of decay of the stem base of the control variety JL30; g: Transfer of expression vector plant disease.

The chalcone reductase family is composed of multiple genes need to be further studied (Ballance *et al*. 2005; Sallaud *et al*. 2005). In 2006, Shimada cloned from *Lotus japonicus* the chalcone reductase homologue of the polyketide reductase gene, expressed in petunia flowers, to demonstrate its promotion of isoliquiritigenin formation (Shimada *et al*. 2006). In 2011, six *CHR* genes were found in alfalfa and relatives by Young *et al*. (2011). Liu *et a*l. (2012) cloned the soybean chalcone reductase gene *GmCHR*, and quantitative fluorescence results showed that the gene expression in soybean leaves was the highest, followed by that in the stems. The expression level of the *CHR3* gene in soybean stem was the lowest, followed by that in the roots, as determined by constructing an RNAi expression vector of the chalcone reductase *CHR3* gene and transforming it into soybean via *Agrobacterium*-mediated transformation. Wu Nan cloned the soybean chalcone reductase gene *CHR1* and constructed an RNAi expression vector. The results showed that the content of ISO glycyrrhizin decreased by 38.7% (Nan *et al*. 2017). The results of this study showed that the content of ISO glycyrrhizin decreased by 26.7%. The reason for this difference may be that the efficiency of different genes is different.

To study the theoretical and practical value of genes in a comprehensive way, in 2015, Wu Nan constructed an over-expression vector of the chalcone reductase gene *CHR3* and transformed it into soybean. He obtained positive plants by molecular detection and identification of disease resistance, and the results showed that the relative expression of the *CHR3* gene in transgenic soybean plants significantly increased, from 2 to 20. The expression of the *CHR3* gene was the highest in soybean leaves, followed by the stem. At the same time, as the isoliquiritigenin content increased, the ability of soybean to resist *Phytophthora sojae* also improved. In this study, RNAi technology was used to silence the expression of the *CHR3* gene and reduce the content of ISO glycyrrhizin in soybean without affecting the content of other soybean isoflavones. It was found that the resistance of transformed soybean to *Phytophthora sojae* was significantly reduced.

Acknowledgements

This research was funded by Key R&D projects of Jilin Provincial Department of Science and Technology “Exploration of high-yield and high protein functional genes and cultivation of new soybean varieties based on gene editing technology” (20230202014NC); Jilin Provincial Department of Education “Analysis of expression patterns and response mechanisms of soybean *GmERF* gene under drought stress” (JJKH20230444KJ); College Student Science and Technology Innovation and Entrepreneurship Training Program Project “Molecular mechanism of soybean *GmCHR3* gene resistance to Phytophthora root rot disease” (S202311439010).

References

Archana K and Hada A 2018. RNAi-mediated resistance to yellow mosaic viruses in soybean targeting coat protein gene. Acta Phys Plant **5**(2): 32-40.

Ballance GM and Dixon RA 2005. Medicago sativa cDNAs encoding chalcone reductase. Plant Phy. **107**(3): 1027-1028.

Bennett J, Hondred D and Register JC 2015. Plant Cell Rep. **34**: 1.

Dorrance AE 2015. Phytophthora root and stem rot. In: Hartman GL, Rupe JC, Sikora EF, Domier LL, David JA, and Steffy KL, editors. Compendium of soybean diseases and pests. Amer. Phytopathol. Soc. **102**(3): 73-76.

Gomez-Escribano JP and Bibb MJ 2011. Engineering streptomyces coelicolor for heterologous expression of secondary metabolite gene clusters. Micro. Biot. **4**(2): 207-215.

Graham TL and Madge Y 2007. Senthil Subramanian, RNAi silencing of genes for elicitation or biosynthesis of 5-deoxyisoflavonoids suppresses race-specific resistance and hypersensitive cell death in infected tissues. Plant Phy. **144**(3): 728-740.

Jiang YN 2013. Study on the content of clone and RNA interference of soybean isoflavonesynthase gene regulation of flavonoids. Shanghai Jiao Tong Univ. **29**(07): 1-3.

Lidia M 2016. chromatography coupled to diode array and tandem mass spectrometry detection. Sci. Dir. **3**(24): 145-159.

Liu J 2012. Cloning of soybean chalcone reductase gene *GmCHR* and Research on Agrobacterium mediated genetic transformation of soybean. Nanjing Agricul. Univ. **37**(30): 1-6.

Lopez M 2012. Flavonoids as anticancer agents: structure-activity relationship study. Curr. Med. Chem.**2**(24): 691-714.

Lyle R and Senthil S 2015. Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using soybean Type I and Type II chalcone isomerases. Plant Physiol. **137**(3): 1375-1388.

Sallaud C, EL-Turk J and Breda C 2005. Differential expression of cDNA coding for chalcone reductase, a key enzyme of the 5-deoxyflavonoid pathway, under various stress conditions in Medicago sativa. Plant Sci. **109**(6): 179-190.

Screevidya VS, Srinivasa Rao C, Sullia SB, Ladha JK and Reddy PM 2011. Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia. Experimental Bot. **57**(9): 1957-1969.

Senthil S, Terrence L, Madge Y and Oliver Y 2017. RNAi silencing of genes for elicitation or biosynthesis of 5-deoxyisoflavonoids suppresses race-specific resistance and hypersensitive cell death in phytophthora sojae infected tissues. Plant Physiol. **144**: 728-740.

Serra A, Macià A and Maria R 2012. Metabolic pathways of the colonic metabolism of flavonoids (flavonols, flavones and flavanones) and phenolic acids. Food Chem. **130**(2): 383-393.

Shimada N, Aoki T and Sato S 2006. A cluster of genes encodes the two types of chalcone isomerase involved in the biosynthesis of general flavonoids and legume-specific 5-deoxy(iso)flavonoids in Lotus japonicus. Plant Phy. **131**(3): 941-951.

Subramanian S, Graham MY, Yu O, Graham TL 2005. RNA interference of soybean isoflavone synthase genes lead to silencing in tissues distal to the transformation site and to enhanced susceptibility to phytophthora sojae. Plant Physiol. **137**(4): 1345-1353.

Wang Y, Wu L and Sun M 2012. Analysis of gene expression underlying soybean isoflavone synthesisrelative enzymes at different growth stages. Soybean Sci. **31**(6): 887-893.

Welle R, Schroder G, Schiltz E, Grisebach H and Schroder J 2011. Induced plant responses to pathogen attack analysis and heterologous expression of the key enzyme in the biosynthesis of phytoalexins in soybean. Europ. J. Bio. **196**(2): 423-430.

Wu N 2014. Functional study of the CHR1 gene of chalcone reductase in soybean. Heredity. **36**(07): 707-712.

Wu N, Wang PW, Lin N, Lu S, Feng YQ, Rong J, Zhang Z and Qu J 2017. Construction of a chalcone reductase expression vector and transformation of soybean plants. Mol Med Rep, 16(5): 6178-6183.

Young ND, Debellé F, Oldroyd GED and Geurts R 2011. The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature **480**(14): 520-524.

Zhang Z. 2014. Cloning and functional identification of soybean chalcone reductase gene (gmchr4). Chin J Oil Crop Sci. **36** (6): 720-727.

Zhang C, Wang P and Zhang Z 2015. Cloning and catalytic activity analysis of chalcone reductase 3 gene (CHR3) in soybean. J. Northwest Agricul. Forestry Univ. **43**(9):813-820.

(Manuscript received on 29 January, 2024; revised on 10 August, 2024)