Indian J. Genet., 75(3): 357-362 (2015)
DOI: 10.5958/0975-6906.2015.00056.5

Homocysteine S-methyl transferase regulates sulforaphane concentration in *Brassica oleracea* var. *italica*

Canhong Gao†, Juncheng Lin[1,†], Liangxia Zhao, Fule Xu[2] and Shuiming Zhang*

College of Agriculture, Anhui Agricultural University, Hefei 230036, China; [1]College of the Environment & Ecology, Xiamen University, Xiamen 361102, China; [2]Chaoda Modern Agriculture Group, Fuzhou 350003, China

(Received: November 2014; Revised: July 2015; Accepted: July 2015)

Abstract

Homocysteine S-methyl transferase (HMT, EC 2.1.1.10) is a ubiquitous enzyme in angiosperms that plays an important role in sulfur nutrition status. We used over-expression and RNA interference (RNAi) to study the regulatory function of *BoHMT1* on sulforaphane concentration in broccoli. Agrobacterium-mediated transforrmation was used to produce 63 putatively transgenic broccoli plants; of these, 51 were positive for the gene construct by PCR selection, and 44 were confirmed by GUS staining. Over-expression of *BoHMT1* caused a significant increase in the relative expression level of this gene, and five independent transgenic plants had expression levels >4-fold higher than in the control and they also showed an increase in sulforaphane concentration. Similarly, expression of an RNAi construct decreased the relative expression of *BoHMT1*, and four independent transgenic lines had expression levels >4-fold lower than in the control, and also showed a reduction in sulforaphane concentration. It is concluded that the *BoHMT1* gene regulates sulforaphane concentration in broccoli and that there is a positive relationship between the expression level of the gene and the sulforaphane content.

Key words: *Brassica oleracea* var. italica, homocysteine S-methyltransferase, RNA interference, sulforaphane

Introduction

Brassicas are important cash crops that show high intra-specific variation in morphological and chemical traits (Hanson et al. 2009). Glucosinolates (GSs), a group of structurally diverse, nitrogen- and sulfur-containing secondary metabolites, are widespread within the Brassicaceae (Kabouw et al. 2010). *Brassica oleracea* var. *italica* (broccoli) contains relatively high levels of glucoraphanin (a type of glucosinolate), which is synthesized from methionine (Met) (Kushad et al. 1999; Brown et al. 2002; Rangkadilok et al. 2002). The degradation product of glucoraphanin is sulforaphane (SF), which has cancer chemoprotective attributes and other disease prevention properties (Yeh and Yen 2005; Liu and Talalay 2013; Lenzi et al. 2014). Therefore, increasing the SF content in broccoli is of interest to plant breeders. Homocysteine S-methyl transferase (HMT, EC 2.1.1.10) is a protein that uses S-methyl methionine (SMM) as a methyl donor for the methylation of homocysteine to produce Met (Kocsis et al. 2003). HMT is ubiquitous in angiosperms, and its expression responds to changes in plant sulfur nutrition status (Bourgis et al. 1999; Ranocha et al. 2000). Ranocha et al. (2001) cloned two homologous *HMT* genes from *Arabidopsis*, designated *AtHMT-1* and *AtHMT-2*. The deduced polypeptides encoded by these two genes are similar in size (36 kDa), share a zinc-binding motif, lack obvious targeting sequences, and are 55% identical in protein sequence. However, *AtHMT-1* is strongly inhibited by Met, whereas *AtHMT-2* is not. *BoHMT1*, a homologue of *HMT*, was cloned from broccoli, and shares a high level of primary sequence homology with *AtHMT-1* (Lyi et al. 2007). These authors demonstrated that *BoHMT1* promoted sulfur assimilation in *E. coli*, but was not dramatically affected by Met. They also showed that *BoHMT1* is a single copy gene in the broccoli genome and is regulated by changes in plant sulfur status. Higher levels of expression of *BoHMT1* are found in roots, young leaves, flowers, and seeds than in older leaves, suggesting that this gene is expressed during

*Corresponding author's e-mail: cdbroccoli@126.com
†These authors contributed equally to this work
Published by the Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
Online management by indianjournals.com

development, and is involved in sulfur assimilation or biotransformation.

As described above, the precursor of SF is synthesized from Met and contains a sulfur atom. Consequently, our hypothesis is that *BoHMT1* can regulate the synthesis of glucoraphanin and is associated with the accumulation of SF. Here, we used over-expression and RNA interference (RNAi) technology to evaluate the relationship between *BoHMT1* mRNA levels and SF concentration. This study will provide us with new insights into the improvement of SF content in broccoli, and extend our knowledge of *HMT* function in *Brassica oleracea*.

Materials and methods

Plant material and culture

The inbred broccoli cultivar 'BOP49' was used in this study. For transgenic experiments, disinfected seeds were sown on MS solid medium (Huang et al. 2011) and cultured at 25°C in an incubator with light illumination (3000 lux) for 7 days.

RNA extraction and gene cloning

RNA was extracted using TRIzol® reagent (Invitrogen; 15596-026), and first-strand cDNA was synthesized using a PrimeScript® RT reagent kit according to the manufacturer's instructions (Takara; DRR037A). Specific primers (HMT-XbaI-F and HMT-KpnI-R; Table 1) were designed for amplifying the open reading frame (ORF) of *BoHMT1* (GenBank accession DQ679980.1). For PCR amplification, each 10 μl reaction contained 50 ng of cDNA, 1.5 mM dNTPs, 1.5 μM of each primer, 1 μl of 10×PCR buffer, and 1U of PrimerSTAR® HS DNA polymerase (Takara, DR010S). The PCR conditions were 5 min at 94°C, followed by 30 cycles of 94°C for 30s, 56°C for 30s, and 72°C for 1 min, with a final extension of 72°C for 5 min.

Construction of plant gene expression vectors

The purified *BoHMT1* gene fragment was inserted into plasmid 1301-35S as previously described (Wu et al. 2013). The over-expression vector (1301-HMT) was confirmed by digestion with restriction enzymes *Xbal* and *Kpn*I (embedded between CaMV 35S promoter and Nos-terminal element). For the RNAi vector, two pairs of primers (RHF-1/RHR-1 and RHF-2/RHR-2; Table 1) were designed to amplify two DNA fragments of ~360 bp, which were designated HR(-) and HR(+). The two fragments were used to construct a dsRNA targeted to silence *BoHMT1*. The pJM007 (Schattat et al. 2004) vector was digested with *Bam*HI and *Not*I and the linearised vector was gel-purified. HR(-) was ligated with the linearised pJM007 to construct the recombinant plasmid called 007-HR(-). HR(+) was ligated into 007-HR(-) that had been linearized by *Xba*I digestion, resulting in construction of the plasmid named 007-HMT-RNAi. The HMT-RNAi expression cassette was obtained from 007-HMT-RNAi by *Pst*I digestion. The vector pCAMBIA1301 and the HMT-RNAi expression cassette were ligated together to produce the recombinant plant transformation vector 1301-HMT-RNAi.

Generation of transgenic plants

The hypocotyledonary axis of 7-day-old sterile seedlings was used to generate callus tissue, which was then co-cultured with *Agrobacterium tumefaciens* strain LBA4404 that carried the plant expression vector plasmids. The transformation method followed was previously described by Huang et al. (2011) and Lin (2011).

Validation of putative transgenic plants

Genomic DNA from wild type and putative first generation transgenic broccoli was extracted as described by Sambrook and Russell (Sambrook and Russell 2001). Because *BoHMT*1 is an endogenous gene in broccoli, we used the GUS gene as a target to screen for potential transgenic plants (primer sequences are given in Table 1). GUS enzyme staining was performed as a further validation procedure according to Jefferson (1987). Briefly, short roots of transgenic seedlings were soaked in GUS staining solution and incubated at 37°C overnight. Then the stained roots were washed in 70% ethanol for 1 day and examined under microscope.

Quantifying gene expression

RNA samples from transgenic plants were treated with DNaseI (Takara; D2210) and reverse transcribed into cDNA. Real-time PCR analysis was conducted using the ABI7500 Real-time PCR system with SYBR® Premix Ex Taq II (Takara; RR041A). The 18S ribosomal RNA gene was used as the endogenous control. The relative expression of specific genes was quantified using the fold-change=$2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and were normalized by logarithm. Each sample had 3 biological replicates. The primers used are shown in Table 1.

Table 1. A list of Oligonucleotide primers used in the study

Primers	DNA Sequence (5'3')	Enzyme sites
HMT-XbaI-F	attaTCTAGAATGGGTTTGG AGAAGAAGTC	*Xba* I
HMT-KpnI-R	attaGGTACCCTGAGGAAC TCATCTTCG	*Kpn* I
RHF-1	gccGGATCCCTACAAAAGA GCGTGAAACT	*Bam*H I
RHR-1	atatGCGGCCGCAGCATTCT CACCATCTACAG	*Not* I
RHF-2	gcgTCTAGACTACAAAAG AGCGTGAAACT	*Xba* I
RHR-2	gcgTCTAGAAGCATTCTCA CCATCTACAG	*Xba* I
GUS-F	ACGTCCTGTAGAAACCCCA ACC	-
GUS-R	TCCCGGCAATAACATAC GGCGT	-
18S-qtf	CGAGACCTCAGCCTGCT AACTAG	-
18S-qtr	TCAAACTTCCTTGGCCT AAACG	-
HMT-qtf	CATTTGCGCTGTTGGCATTAA	-
HMT-qtr	TGGAGAATTTGCGGATCAGAT	-

Note: Restriction enzyme recognition sites are underlined

Determination of sulforaphane concentration

Determination of sulforaphane was performed as previously described with slight modifications (Nathan et al. 2004). Briefly, 10 g of fresh leaves of transgenic plants were ground to a powder in a chilled mortar and pestle with liquid nitrogen. Then, 1 g of powdered sample was mixed with 10 ml deionized water and incubated at 65°C for 5 min. The pre-heated sample was immediately cooled on ice and incubated at room temperature for 4 h. The products were extracted three times with 40 mL of dichloromethane. After filtration, the solution was subjected to vacuum drying in a rotary evaporator at 35°C. The dried products were dissolved in 3 mL of acetonitrile and stored at –20°C. The concentration of sulforaphane was determined on an Agilent 1100 Series high-performance liquid chromatography (HPLC) system. The instrument parameters were as follows: mobile phase, 20% acetonitrile and 80% water; wavelength, 205 nm; column temperature, 30°C; injection volume, 5μL; column, C18 reversed-phase column flow rate, 1 ml/min; time, 12 min. The sulforaphane standard was purchased from Sigma (CAS: 4478-93-7). The relative concentration of sulforaphane was calculated by sample/control, which showed the fold-change.

Results

Plant expression vector construction

A 981bp ORF of *BoHMT1* were cloned by PCR (Fig. 1). Three clones were sent for sequencing. The results showed that the 3 sequences were the same and shared 99% nucleotide sequence identity with the original sequence obtained from GenBank (DQ 679980.1). The predicted protein sequence of our clone was identical to that predicted from DQ679980.1. The construction of the over-expression and RNAi vectors are shown in Fig. 2.

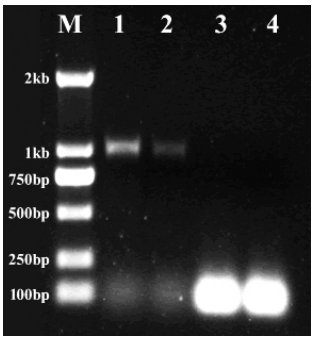

Fig. 1. PCR amplification of BoHMT ORF. M: DL2000 size marker. Lanes 1-2: products of PCR amplification. Lanes 3-4: negative controls. The low molecular products may be primer dimers

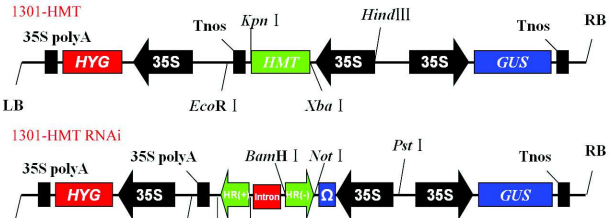

Fig. 2. Schematic of plant transformation vectors 1301-HMT and 1301-HMT-RNAi

Confirmation of transgenic lines

The *GUS* gene was co-expressed in the opposite polarity in both the over-expression and RNAi plant transformation vectors. Therefore, amplification of the *GUS* gene was used to select for transgenic plants. The percentage of regenerated shoots from the 1301-HMT, 1301-HMT-RNAi, and pCAMBIA1301 vectors that tested positive were 80%, 89.3%, and 60%, respectively (Table 2). GUS staining of the roots was positive in 72%, 71.4%, and 60% of the regenerated plants, respectively (Table 2).

Table 2. Statistics of broccoli transformation

Vector	No. of calli	No. of regenerated plants	Positive PCR plants	Positive GUS staining
1301-HMT	100	25	20	18
1301-HMT RNAi	100	28	25	20
pCAMBIA1301	30	10	6	6
Total	230	63	51	44

Changes in gene expression

The *BoHMT1* mRNA levels in the over-expressing (OVE) plants varied. Compared to the control, transcription levels in five of the over-expressing plants showed significant increases of >4-fold (Fig. 3). Importantly, the relative transcription of *BoHMT1* in OVE-6 was 18.76-fold higher than in the control, suggesting that the introduced copy of *BoHMT1* was efficiently expressed in this line. Moreover, a visible reduction in the relative transcription level of *BoHMT1* was also observed among the RNAi plants (Fig. 3). Four of these plants showed a significant reduction in transcription of >4-fold. Our results also showed that the HMT-RNAi construct was efficiently expressed in transgenic plants RNAi-11, RNAi-14 and RNAi-16.

Sulforaphane concentration

We further tested the SF concentration in the leaves of transgenic seedlings. The SF concentration was increased in the OVE plants (Fig. 4). SF concentrations in the OVE plants were significantly increased except for plants OVE-7 and OVE-8. The plant with the highest level of *BoHMT1* expression, OVE-6, accumulated much more SF than did the other transgenic plants.

Also, SF concentration was reduced in the RNAi plants. The reduction was significant in all lines except for RNAi-9 and RNAi-20. Predictably, the SF concentration in RNAi-14, the plant with the lowest level of *BoHMT1* expression, was reduced more than in the others (Fig. 4).

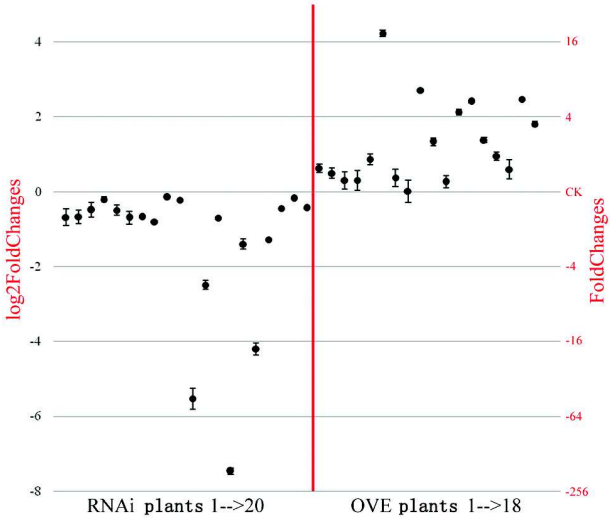

Fig. 3. Relative expression levels of the *BoHMT1* gene in transgenic broccoli plants (RNAi = plants expressing gene silencing construct; OVE = plants transformed with construct for over-expression of *BoHMT1*)

Discussion

The results presented here strongly suggest that over-expression and RNAi-induced gene silencing of *BoHMT1* in broccoli can increase or reduce the level of gene transcription, and also alter the SF content in

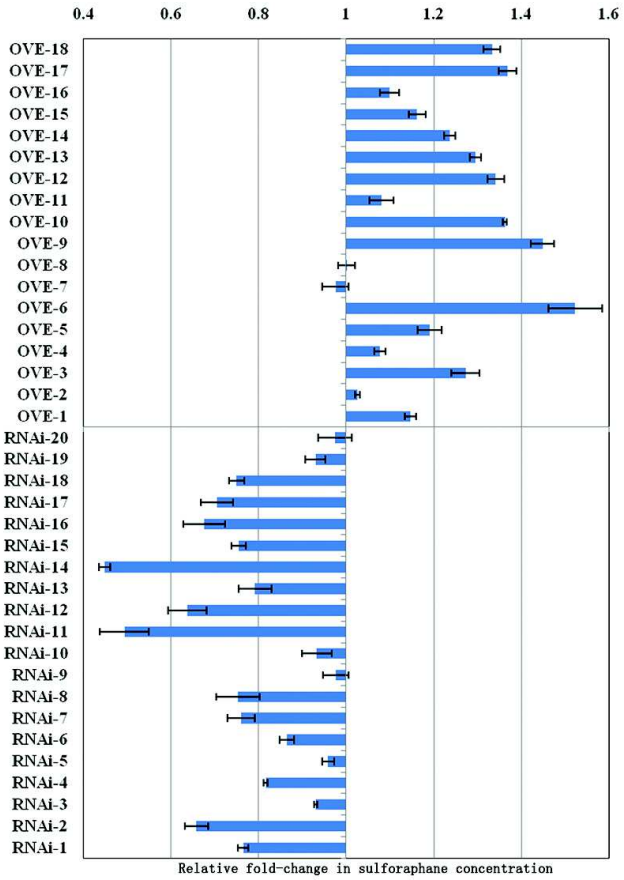

Fig. 4. Relative fold-changes in sulforaphane concentration in the transgenic broccoli plants (CK = the level of sulforaphane in the control plants)

broccoli leaves. These results are in agreement with some prior gene expression studies in *Arabidopsis* (Gigolashvili et al. 2007) and broccoli (Lin 2011). Previously published studies have also used the same two technologies to change the active ingredient contents in plants (Chen et al. 2003; Liu et al. 2011).

The present study did not find a 100% correlation between transgenic plants that screened positive by PCR and those that were positive by GUS staining. There are many possible explanations for this, one of which could be methylation of the *CaMV35S* promoter in the genome (Wang et al. 2008).

Unlike *AtHMT-1*, the expression of *BoHMT1* was not dramatically affected by Met, and was only slightly increased (Lyi et al. 2007). We hypothesize that this could be due to differences in the glucosinolate compounds between broccoli and Arabidopsis (Brown et al. 2002, 2003). Understanding the function of *HMT* in plant cells, the differences between the expression of *AtHMT-1* and *BoHMT1* could suggest that when Met accumulates to high levels in tissue, *BoHMT1* may participate in the SMM cycle (Kocsis et al. 2003), which would provide a high substrate concentration for subsequent synthesis of glucosinolates and SF.

BoSMT was previously shown to have its highest methyltransferase activity with DL-selenocysteine as a methyl acceptor (Lyi et al. 2005). The BoSMT protein shares 51.7% amino acid identity with *BoHMT1*, and also shares close sequence homology to AtHMT-2 (Lyi et al. 2007). Previous studies have shown that organoselenium compounds have a chemoprotective effect against some cancers (Abdulah et al. 2009). However, competitive inhibition between sulfur and selenium assimilation and utilization reduced the SF content by 85% when broccoli was fertilized with selenium (Finley et al. 2005; Robbins et al. 2005; White et al. 2004, 2007). We demonstrated previously that *BoSMT* can relieve the toxicity of selenium to SF accumulation in broccoli (Lin 2011). Combined with the results present here, this suggests that *BoHMT1* and *BoSMT* have a clear functional separation with respect to sulfur and selenium utilization in broccoli.

Initially it was reported that there is a positive relationship between transcription of *BoHMT1* and SF content in broccoli. Although the present study could not detect a linear relationship between these two parameters but the present results should provide new insight into the bioengineering of improving SF content in broccoli.

Acknowledgments

This research was supported by grants from the National Natural Science Foundation of China (30900971), the Natural Science Foundation of Fujian Province (2013J01091), and the Stability and Introduction of Talents Foundation of Anhui Agricultural University (YJ 2013-13).

References

Abdulah R., Faried A., Kobayashi K., Yamazaki C., Suradji E. W., Ito K., Suzuki K., Murakami M., Kuwano H. and Koyama H. 2009. Selenium enrichment of broccoli sprout extract increases chemosensitivity and apoptosis of LNCaP prostate cancer cells. BMC Cancer, **30**: 9:414.

Bourgis F., Roje S., Nuccio M. L., Fisher D. B., Tarczynski M. C., Li C., Herschbach C., Rennenberg H., Pimenta M. J., Shen T. L., Gage D. A. and Hanson A. D. 1999. S-methylmethionine plays a major role in phloem sulfur transport and is synthesized by a novel type of methyltransferase. Plant Cell, **11**: 1485-1498.

Brown A. F., Yousef G. G., Jeffery E. H., Klein B. P., Wallig M. A. and Kushad M. M. 2002. Glucosinolate profiles in broccoli£ºVariation in levels and implication in breeding for cancer chemoprotection. J. Am. Soc. Hort. Sci., **12**: 807-813.

Brown P. D., Tokuhisa J. G., Reichelt M. and Gershenzon J. 2003. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry, **62**: 471-81.

Chen Z., Young T. E., Ling J., Chang S. C. and Gallie D. R. 2003. Increasing vitamin C content of plants through enhanced ascorbate recycling. Proc. Natl. Acad. Sci. U.S.A., **100**: 3525-3530.

Finley J. W., Sigrid-keck A., Robbins R. J. and Hintze K. J. 2005. Selenium enrichment of broccoli: interactions between selenium and secondary plant compounds. J. Nutr., **135**: 1236-1238.

Gigolashvili T., Yatusevich R., Berger B., Müller C. and Flügge U. I. 2007. The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant. J., **51**: 247-261.

Hanson P., Yang R., Chang L., Ledesma L. and Ledesma D. 2009. Contents of carotenoids, ascorbic acid, minerals and total glucosinolates in leafy brassica pakchoi (*Brassica rapa* L. *chinensis*) as affected by season and variety. J. Sci. Food Agric., **89**: 906-914.

Huang K., Wu Q. Y., Lin J. C. and Zheng J. G. 2011. Optimization of a plant regeneration protocol for broccoli. Afr. J. Biotechnol., **10**: 4081-4085.

Jefferson, R. A. 1987. Assaying chimeric genes in plants: the gus gene fusion system. Plant Mol. Biol. Rep., **5**: 387-405.

Kabouw P., Biere A., van der Putten W. H. and van Dam N. M. 2010. Intra-specific Differences in Root and Shoot Glucosinolate Profiles among White Cabbage (Brassica oleracea var. capitata) Cultivars. J. Agric. Food Chem., **58**: 411-417.

Kocsis M. G., Ranocha P., Gage D. A., Simon E. S., Rhodes D., Peel G. J., Mellema S., Saito K., Awazuhara M., Li C., Meeley R. B., Tarczynski M. C., Wagner C. and Hanson A. D. 2003. Insertional inactivation of the methionine s-methyltransferase gene eliminates the s-methylmethionine cycle and increases the methylation ratio. Plant Physiol., **131**: 1808-1815.

Kushad M. M., Brown A. F., Kurilich A. C., Juvik J. A., Klein B. P., Wallig M. A. and Jeffery E. H. 1999. Variation of glucosinolates in vegetable crops of Brassica oleracea. J. Agric. Food. Chem., **47**: 1541-1548.

Lenzi M., Fimognari C. and Hrelia P. 2014. Sulforaphane as a promising molecule for fighting cancer. Cancer Treat Res., **159**: 207-23.

Lin J. C. 2011. Function analysis of sulforaphane accumulation relative gene my and smt in broccoli. Fuzhou. Master Dissertation of Fujian Agriculture and Forest University. (In Chinese).

Liu H. and Talalay P. 2013. Relevance of anti-inflammatory and antioxidant activities of exemestane and synergism with sulforaphane for disease prevention. Proc. Natl. Acad. Sci. U. S. A., **110**: 19065-19070.

Liu Z., Hammerlindl J., Keller W., Mcvetty P. B. E., Daayf F., Quiros C. F. and LI G. 2011. *MAM* gene silencing leads to the induction of C3 and reduction of C4 and C5 side-chain aliphatic glucosinolates in *Brassica napus*. Mol. Breeding, **27**: 467-478.

Livak K. J. and Schmittgen T. D. 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta C}$T Method. Methods, **25**: 402-408.

Lyi S. M., Heller L. I., Rutzke M., Welch R. M., Kochian L. V. and Li L. 2005. Molecular and biochemical characterization of the selenocysteine Se-methyltrasferase gene and Se-methylselenocysteine synthesis in broccoli. Plant Physiol., **138**: 409-420.

Lyi S. M., Zhou X., Kochian L. V. and Li L. 2007. Biochemical and molecular characterization of the homocysteine S-methyltransferase from broccoli (*Brassica oleracea* var. *italica*). Phytochemistry, **68**: 1112-1119.

Nathan V. M., John A. J. and Elizabeth H. J. 2004. Heating decreases epithiospecifier protein activity and increases sulforaphane formation in broccoli. Phytochemistry, **65**: 1273-1281.

Rangkadilok N., Tomkins B., Nicolas M. E., Premier R. R., Bennett R. N., Eagling D. R. and Taylor P. W. J. 2002. The effect of postharvest and packaging treatments on glucoaphanin concentration in broccoli (*Brassica oleracea* var. *italica*). J. Agric. Food Chem., **50**: 7386-7391.

Ranocha P., Bourgis F., Ziemak M. J., Rhodes D., Gage D. A. and Hanson A. D. 2000. Characterization and functional expression of cdnas encoding methionine-sensitive and -insensitive homocysteine S-methyltransferases from Arabidopsis. J. Bio. Chem., **275**: 15962-15968.

Ranocha P., Mcneil S. D., Ziemak M. J., Li C. J., Tarczynski M. C. and Hanson A. D. 2001. The S-methylmethionine cycle in angiosperms: ubiquity, antiquity and activity. Plant J., **25**: 575-584.

Robbins R. J., Keck A. S., Banuelos G. and Finley J. W. 2005. Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate and sulforaphane content of broccoli. J. Med. Food., **8**: 204-214.

Sambrook J. and Russell D. W. 2001. Molecular cloning: a laboratory manual, 3rd edition. Cold Spring Harbor Laboratory Press, New York, USA.

Schattat M. H., Klösgen R. B. and Marques J. P. 2004. A novel vector for efficient gene silencing in plants. Plant Mol. Bio. Rep., **22**: 145-153.

Wang H. H., Wu S. J., Li F. F., Chen T. Z., Jiang Y. J., Ju M., Zhao J., Lü Y. H., Zhang T. Z. and Guo W. Z. 2008. Transgene silencing caused by 35S promoter methylation in upland cotton (*Gossypium hirsutum*). Cotton Sci., 20: 274-280. (In English with Chinese abstract).

White P. J., Bowen H. C., Marshall B. and Broadley M. R. 2007. Extraordinarily high leaf selenium to sulfur ratios define 'Se-accumulator' plants. Ann. Bot., **100**: 111-118.

White P. J., Bowen H. C., Parmaguru P., Fritz M., Spracklen W. P., Spiby R. E., Meacham M. C., Mead A., Harriman M., Trueman L. J., Smith B. M., Thomas B. and Broadley M. R. 2004. Interactions between selenium and sulphur nutrition in *Arabidopsis thaliana*. J. Exp. Bot., **55**: 1927-1937.

Wu Q. Y., Lin J. C., Huang K. and Liu M. Y. 2013. Characterization and expression analysis of myrosinase for sulforaphane synthesis in broccoli. Int. J. Agric. Biol., **15**: 83-89.

Yeh C. T. and Yen G. C. 2005. Effect of sulforaphane on metallothionein expression and induction of apoptosis in human hepatoma *HepG2* cells. Carcinogenesis, **26**: 2138-2148.