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Full Length Article

Characterization and Expression Analysis of Myrosinase for Sulforaphane Synthesis in Broccoli

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Abstract

Broccoli (*Brassica oleracea* L. var. *italica* planck) is one of the most important import vegetables in developed countries. It has high nutrition value and rich in sulforaphane that is a bioactive component with the highest content for anti-cancer in vegetables. Sulforaphane is synthesized through the hydrolysis of glucoraphanin by myrosinase (MY). In the present work, we analyzed the sulforaphane content in transgenic broccoli under the conditions of overexpression and RNAi knockdown of *MY* gene. Totally 64, 49 and 47 positive transformated plants with over-expression and RNAi knockdown of MY gene as well as empty vector (pCAMBIA1301) were obtained. The relative expression of *MY* gene in transgenic broccoli was analyzed by real-time PCR, and revealed an obvious increase by 16.5% in over-expressed plants and a significant decrease by 23.8% in RNAi knockdown plants. Similarly, the contents of sulforaphane were increased by 92.77% in *MY* gene overexpressed broccoli head and decreased by 35.82% in RNAi knockdown plants. The results indicated that *MY* gene should participate in the synthesis of sulforaphane.

Keywords: Broccoli; Functional analysis; Myrosinase; Sulforaphane

Introduction

Myrosinase (MY) is present in most dicotyledonous plants. In plants containing glucosinolates, especially cruciferous plants, myrosinase, as a kind of hydrolase, can hydrolyze the glucosinolates into isothiocyanates that are benefit for human health (Ingrid, 2010). Sulforaphane in broccoli is generated from the hydrolysis of 4-methylethylidene sulfonyl-butyl glucosinolate (glucoraphanin) by MY.

Myrosinase (EC 3.2.3.147) is widely present in most plants and is an important defense system (Atle *et al*., 1996). In cruciferous plants, MY is more important due to its enzymatic hydrolysis function for glucosinolates and the generation of isothiocyanate beneficial to human health (Keck *et al*., 2004). The cloned gene of MY has been acquired from a variety of cruciferous vegetables (Xue *et al*., 1992; Masakazu *et al*., 2000). The molecular weight of MY is in the range of 62–77 kDa. Various compositions of MY from different sources have been determined (Atle *et al*., 1989). Three gene codes of MY have been identified, which include MA as the code of soluble enzymes, and MB and MC as the codes of insoluble enzymes (Frauke *et al*., 2002).

The activity of MY can be affected by many factors so that the MY from plants with different genotypes reveals various activities (Andrew *et al*., 1984). In addition, ascorbic acid can be used as the activator of MY to improve the enzymatic reaction rate by changing protein conformation (Tsuruo and Hata, 1968). Moreover, Zn^{2+} also can promote the activity of MY; in contrast, Cu^{2+}, Mg^{2+}, Fe^{2+} and other metal ions may reduce its activity (Liang *et al*., 2006).

In broccoli, glucoraphanin can be hydrolyzed by MY to generate sulforaphane（Ouda *et al*., 2008). The content of glucoraphanin is high in broccoli and kale (He *et al*., 2002). In our laboratory, the full-length gene of MY from broccoli and kale has been successfully cloned and first reported in 2006 (Xie *et al*., 2008).

In this study, the gene of MY from broccoli was transferred to broccoli. The characterization and expression analysis of myrosinase for sulforaphane synthesis in broccoli were conducted on the basis of the common gene for MY from broccoli.

Materials and Methods

Experimental Material

The gene of MY and its plasmids as well as DH5α cell line was reserved in our laboratory. The BOP49 inbred broccoli was selected in this study. Kanamycin (Kan), rifampicin (Rif), ampicillin (Amp) and hygromycin (Hyg) were purchased from Fuzhou Dingguo Biotech Co., Ltd. The

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rTaq enzyme, ExTaq enzyme, PrimerStar high-fidelity enzyme and restriction enzyme were purchased from Takara Company.

Construction of Expression Plasmids

The double digestion of vector pSPROK was conducted by *Hin*d III and *Eco*R I. The 35S-Tnos expression cassette with multiple cloning sites was purified. Meanwhile, pCAMBIA1301 was also digested by *Hin*d III and *Eco*R I. The larger fragment was purified. Both purified fragments were ligated to obtain the recombinant plasmid, which was named as 1301-35S and validated by restricted enzyme digestion.

According to the base sequence of MY, the specific primers were designed at both ends of open reading frame, which were MY-F (5'-CGCGGATCCCAACACAACACATACATCAAC-3') and MY-R (5'-CCGGAGCTCGAGTAAGTAGGAAGAGTGAGAG-3'). The length of the cloned fragment was approximately 1750 bp and included *Bam*H I and *Sac* I restriction sites, as shown in the underlined parts. By using specific primers from the recombinant vector pET-myr, the gene of MY was amplified, purified and sequenced. Then, the gene of MY was ligated into pCAMBIA1301 vector to obtain the recombinant plasmid of 1301-MY (Fig. 1).

According to the gene sequence of MY, two pairs of primers were designed in opposite directions to accomplish the forward and reverse amplification of RNAi and insert the small fragment with a length of approximately 375 bp. The pJM007 vector was digested and the larger fragment was purified. The reversely amplified fragment and the larger fragment from pJM007 were ligated to construct the recombinant plasmid of 007-MP(-). The forward amplified fragment was ligated into the larger fragment from pJM007 to construct the plasmid of 007-MY RNAi. The expression cassette of MY RNAi was achieved by using a single enzyme Pst I digestion. The vector pCAMBIA1301 and MY RNAi expression cassette were ligated to obtain the recombinant plasmid of 1301-MY RNAi (Fig. 2).

Cultivation of Sterile Broccoli

The healthy broccoli seeds were selected and disinfected by 70% ethanol for 90 s and 10% NaClO for 10 min. The disinfected seeds were washed by sterile water for 5 times and dried by filter paper. The treated seeds were inoculated on MS solid medium and cultivated at 25°C in an incubator with light illumination (3000 lux) for 7 days.

Genetic Transformation of MY from Broccoli

The carrier 1301-MY, 1301-MY RNAi and pCAMBIA1301 plasmids were transformed into LBA4404 agrobacteria and the positive bacilli were stored at -80°C. The agarobacteria were inoculated in kan-resistant YEB plate and cultured at 28°C in a light-free environment for 48 h. The single colony was then cultured in YEB + Kan + Rif media at 28°C with shaking speed of 220 rpm until $OD_{(600)}$ of 0.5 for explants.

According to the modified method from Huang Ke (Huang *et al*., 2011), non-sterile seedlings with growth period of 7 days were selected and cut into 2–3 cm of hypocotyl stem segments. The agrobacteria was pre-arranged media for 3 days. Agrobacterium broth carrying 1301-MY, 1301-MY RNAi and pCAMBIA 1301 plasmids was dip for 8 min. The explants were evenly arranged in the media to develop for 2 days. Then, explants were transferred to differentiation medium to induce the growth of kan-resistant buds. When the kan-resistant bud grew to 3 cm in length, the bud was cut from homogeneous callus and cultivated in the root-generating medium. The plants were transplanted to outside for the growth when the plants had 3 leaves.

Pre-culture medium (MS + 0.02 mg/L NAA + 4 mg/L 6-BA + 2% sucrose + 0.8% agar, (pH = 5.8); co-culture medium (MS + 0.02 mg/L NAA + 4 mg/L 6-BA + 2% sucrose + 0.8% agar + 5 mg/L Hyg (pH = 5.8); Differentiation medium (MS + 0.02 mg/L NAA + 4 mg/L 6-BA + 2% sucrose + 0.8% agar + 5 mg/L Hyg + 500 mg/L Amp (pH 5.8); Root-generating medium (MS + 0.2 mg/L NAA + 2% sucrose + 0.8% agar + 5 mg/L Hyg (pH 5.8).

PCR analysis of Transgenic Plants

Broccoli genomic DNA extraction was referred to previous methods (Sambrook and Russell, 2001). A pair of primers was synthesized according to the GUS gene sequence of T-DNA region in pCAMBIA1301 vector for PCR amplification.

Identification of Gene Expression by Quantitative PCR

TRIzol RNA extraction kit method such as TAKARA kit (DRR037S) was used to reversely transcribe the RNA into cDNA. The cDNA samples at 30 ng/μL were used to prepare PCR reaction solution by using TAKARA fluorescence quantitative reaction kit (DRR081A).

Determination of GUS Staining in the Tissue of Transgenic Seedlings

Short roots of transgenic seedlings soaked in GUS staining solution were incubated at 37°C overnight. On the next day, the roots were taken out and soaked in 70% ethanol for one day. The tissue staining was examined by a microscope. GUS staining formula was referred to Jefferson's method (Richard, 1987).

Southern Blot Analysis of Transgenic Plants

According to the slightly modified method for Southern blot

analysis, the *Hin*d III digestion of genomic DNA was conducted by using non-transgenic plants as the control. The capillary method was used for DNA transfer. The design of primers was same as GUS probe.

Determination of Sulforaphane in Transgenic Plants

The determination method was referred to previous method with slight modification (Nathan *et al*., 2004): Totally 10 g of fresh flowers were subjected to grind in liquid nitrogen. Then, 1 g of power sample was accurately weighed, transferred to the flask with 65°C deionized water and incubated for 5 min. Pre-heated sample was immediately cooled on ice and at room temperature for 4 h. The products were extracted by 40 mL of dichloromethane with shaking at the interval time of 5 min. After 15 min, the shaking was repeated for 2 times. The anhydrous sodium sulfate was added to dichloromethane fraction for further purification of sulforaphane in funnel. After filtration, the filtered solution was subjected to vacuum drying in rotary evaporator at 35°C. The dried products were eluted by 3 mL of acetonitrile (HPLC grade). The eluted sulforaphane was cryopreserved at -20°C. Before determination, the eluted solution was filtered by 0.45 μm filter to autosampler vial. The content of sulforaphane was determined by using Agilent 1100 Series high-performance liquid chromatography. The determination parameters were set up as: mobile phase: 20% acetonitrile and 80% water; wavelength: 205 nm; column temperature: 30°C; injection volume: 10 μL; column: C18 reversed-phase column; flow rate: 1 mL/min; time: 12 min.

Results

Construction and Validation of Recombinant Expression Plasmids

Construction of 1301-MY plasmid for overexpression: After the digestion of recombinant 1301-35S plasmid by *Hin*d III and *Eco*R I restriction enzymes, a small fragment with approximately 1200 bp was obtained, which was consistent with the expected size. The fragment of MY with the size of approximately 1740 bp was obtained by PCR amplification through specific primers such as MY-F and MY-R, which was consistent with the original template.

By using specific primers, MY-F and MY-R, for PCR amplification, a plaque-resistant fragment was amplified to be 1740 bp, which was consistent with the expected bands (Fig. 3). The bacteria-resistant colonies were screened. The positive colonies were selected for DNA extraction and validated by the digestion of *Bam*H I and *Sac* I. After double digestion, a small fragment of approximately 1740 bp was obtained, which was consistent with the expected results, indicating that the fragment had been successfully integrated into the recombinant MY 1301-35S plasmid (Fig. 4).

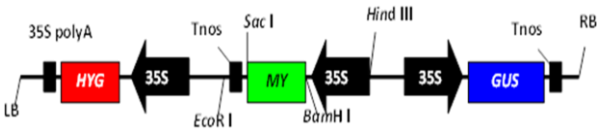

Fig. 1: Construction of *MY* overexpression vector1301-MY

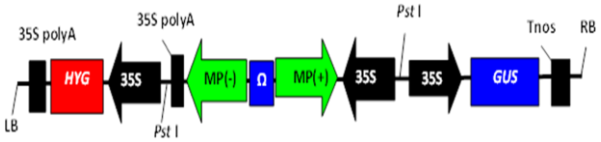

Fig. 2: Construction of *MY* RNAi expression vector 1301-MY RNAi

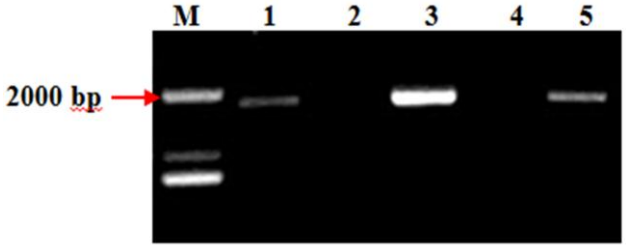

Fig. 3: PCR product of DH5 α colony with 1301-MY plasmid, M: DNA Marker2000; 1 and 3: positive colony; 2: negative colony; 4: negative control; 5: positive control.

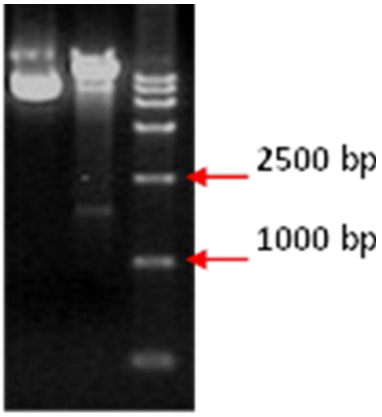

Fig. 4: Enzyme digestion identification of recombinant plasmid 1301-MY, M: DNA Marker15000; 1: plasmid 1301-MY; 2: the plasmid 1301-MY digested by *Bam*H I and *Sac* I

Construction of 1301-MY RNAi expression plasmid: The positive small fragments MP(-) and MP(+) with approximately 375 bp of anti-MY were amplified by reverse primer with insertion of *Bam*H I and *Not* I restriction sites and forward primer with insertion of *Xba* I restriction site, which was consistent with the expected fragment size. Meanwhile, the sequencing results showed that the base sequence is fully consistent with the original template.

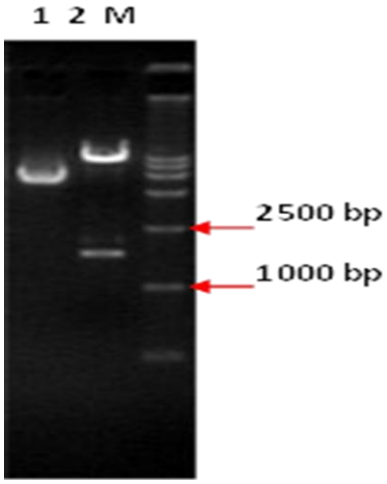

Fig. 5: Enzyme digestion identification of recombinant plasmid 1301-MY RNAi, M: DNA Marker15000; 1: plasmid 1301-MY RNAi; 2: the plasmid 1301-MY RNAi digested by *Pst* I

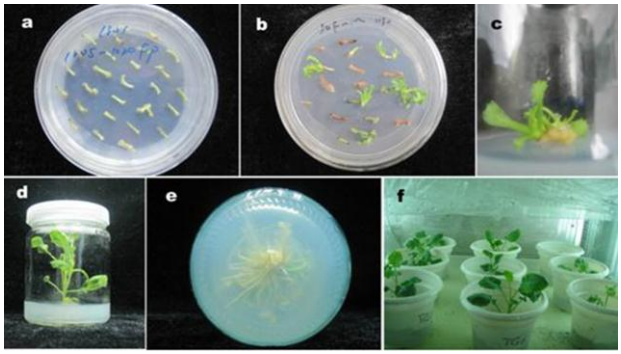

Fig. 6: Broccoli regeneration protocol of *MY* gene function analysis, a: Calli formation; b: Formation of the anti-HYG regenerated bud; c: Further growth of a regenerated bud; d and e: Root formation in the regenerant; f: The transplantation of a regenerated broccoli seedling

The colonies of amp-resistant recombinant plasmid containing pJM007 and MP(-) fragments were grown on a plate. The positive colonies amplified by MP(-)-F and MP(-)-R primers were selected and validated by restriction enzymes including *Bam*H I and *Not* I. A fragment of approximately 375 bp was achieved, suggesting that MP(-) had been successfully inserted to pJM007 vector.

Both 007-MY RNAi plasmids were digested by *Xba* I and a small fragement, MP(+), of approximately 375 bp was obtained, which was consistent with the expected band. After sequencing analysis, the insertion of both recombinant plasmids was forward insertion. Their base sequences were same as the original template, indicating that MP(+) forward fragment had been successfully integrated into 007-MP(-). The 007-MY RNAi was digested by *Pst* I and a small fragment of approximately 1700 bp was obtained, suggesting that MY RNAi expression cassette was successfully constructed.

The 1301-MY RNAi plasmid was digested by *Pst* I and a samll fragment of approximately 1800 bp was obtained (Fig. 5). The sequencing analysis revealed the accurate restriction sites, suggesting that 1301-MY RNAi expression plasmid was successfully constructed.

Cultivation of Transgenic Broccoli Seedlings

According to the established genetic transformation system, Hyg-resistant buds were screened and then cultured to grow up as plants. Totally 71 transgenic seedlings with 1301-MY plasmid were achieved with a transformation rate of 21.13%; 57 transgenic seedlings with 1301-MY RNAi plasmid was obtained with a transformation rate of 20.36% and 53 transgenic seedlings with the control plasmid of pCAMBIA1301 were obtained with a transformation rate of 21.81% (Table 1 and Fig. 6).

PCR Verification of Transgenic Broccoli

Genomic DNA of transgenic broccoli was extracted using CTAB method to obtain broccoli genomic DNA with high purity. The specific primers for GUS gene such as GUS-F and GUS-R were used for PCR amplification. The positive plants were screened, which were same as the positive control with a band of approximately 375 bp (Fig. 7). The positive transformation rate of each plasmid was higher than 85%.

Southern blot analysis of transgenic plants: Five PCR-positive transgenic plants with excellent growth status for each plasmid were selected for Southern blot analysis. The transformation rates for transgenic plants with overexpression plasmid and RNAi expression plasmid were 80%; in contrast, the transformation rate of transgenic plants with empty vector was 100%. Southern blot analysis showed that exogenous fragments have been integrated into the genome of broccoli at the format of single copy (Fig. 8).

GUS staining of transgenic broccoli: The X-Gluc staining of the tissues from transgenic broccoli was examined by a Nikon microscope (10X). The results indicated that blue root tips were observed in transgenic plants, but no GUS staining for non-transgenic plants (Fig. 9). Therefore, GUS gene was successfully transferred to broccoli and the genes of MY and its RNA expression cassette was integrated into the genome of broccoli. The number of positive GUS-stained transgenic plants with 1301-MY, 1301-MY RNAi and pCAMBIA1301 plasmids were 58, 44 and 40, with positive staining rates of 81.69, 77.19 and 75.47%, respectively.

Quantitative fluorescence detection of transgenic broccoli: The total DNA of transgenic plants with 1301-MY, 1301-MY RNAi and pCAMBIA1301 plasmids was used as the templates for the synthesis of cDNA through reverse transcriptase. The fluorescent quantitative PCR

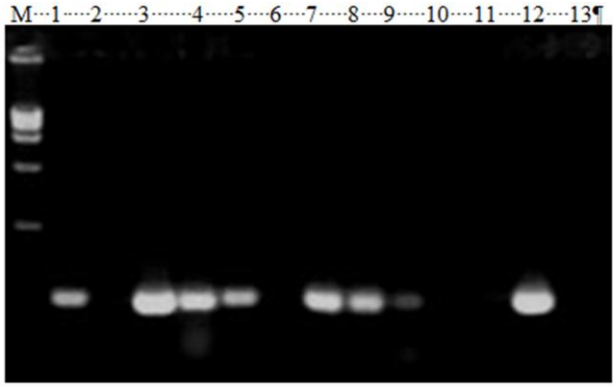

Fig. 7: PCR analysis of transgenic broccoli, M: DNA Marker15000; 1-4: broccoli overexpression *MY* gene by vector 1301-MY. 1, 3 and 4 is positive plant, 2 is negative plant; 5-8: broccoli RNAi *MY* gene by vector 1301-MY RNAi. 5, 7 and 8 is positive plant, 6 is negative plant; 9-11: broccoli transformed by empty vector pCAMBIA1301; 12: positive control; 13: negative control

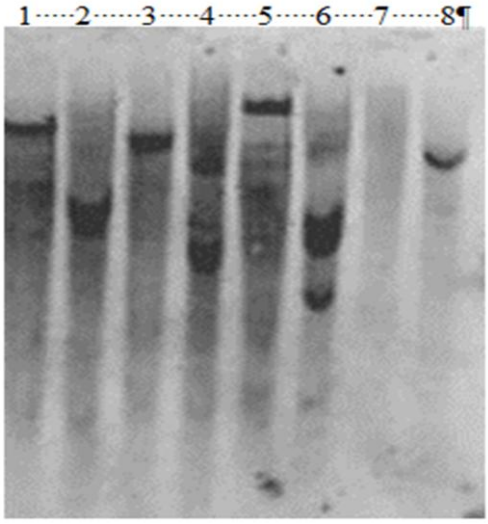

Fig. 8: Southern blot of transgenic broccoli 1 and 2 are transgenic broccoli transformed by 1301-MY, 3 and 4 are transgenic broccoli transformed by 1301-MY RNAi; 5 and 6 are transgenic broccoli transformed by pCAMBIA1301; 7 is non-transgenic broccoli (negative control); 8 is positive control

amplification of MY (F: 5'-GATGGGCGAACTCAATGCTAC-3'; R: 5'-CACTCCCCTACTCACCTTTCCTT-3') and 18S (F: 5'-CGAGACCTCAGCCTGCTAACTAG-3'; R: 5'-TCAAACTTCCTTGGCCTAAACG-3') genes were conducted at the condition with cDNA concentration of 30 ng/μL. The sequences of designed primers were listed as follows:

The amplification fusion curve was a single peak with good reproducibility, indicating that the designed primers were specific without the production of non-specific amplification and primer dimer.

According to the Ct value of 18S housekeeping gene, the Ct values of transgenic plants with 1301-MY, 1301-MY RNAi and pCAMBIA1301 plasmids were corrected. The Ct value of non-transgenic plants was set up to be zero as the control. The gene expression level of MY in transgenic plants with 1301-MY plasmid revealed an enhancement of

Table 1: the percentage of each vector's transformation

Vector name	Number of calli	Number of resistance bud	Number of seedling	Transformation efficiency (%)
1301-MY	336	84	71	21.13
1301-MY RNAi	280	68	57	20.36
pCAMBIA1301	243	58	53	21.81

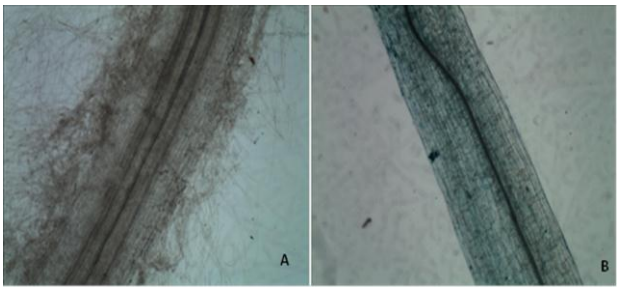

Fig. 9: GUS expression in root of transgenic broccoli A: root of normal broccoli under microscope (10X); B: root of transgenic broccoli under microscope (10X)

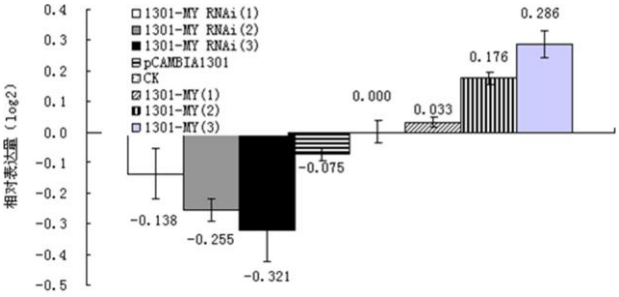

Fig. 10: Relative expression of *MY* gene in transgenic broccoli

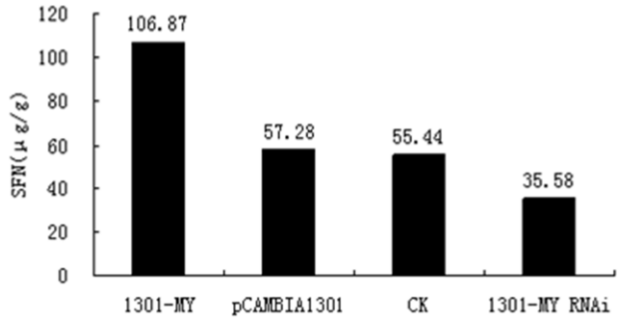

Fig. 11: Content of SFN in transgenic broccoli

0.033, 0.176 and 0.286 fold, which revealed a significant difference between the transplants containing 1301-MY and CK. Compared with the control, the gene expression of MY in transgenic plants with 1301-MY RNAi plasmid exhibited a reduction of 0.138, 0.255 and 0.321 fold. On the other hand, the gene expression of non-transgenic plants did not exhibit an obvious change (Fig. 10).

Determination of Sulforaphane in Transgenic Plants

The transgenic plants with positive PCR, Southern blot, GUS staining and quantitative PCR reaction were selected for the extraction of sulforaphane (SFN). The content of SFN in curd tissue of transgenic broccoli was determined by high performance liquid chromatography. The SFN contents in transgenic plants with MY overexpression (1301-MY), RNAi expression plasmid (1301-MY RNAi) and empty vector (pCAMBIA1301) as well as non-transgenic plants (CK) were 106.87, 57.28, 55.44 and 35.58 μg/g, respectively. Compared with the empty vector transformed plants and CK plants, the contents of SFN revealed the increase by 86.57% and 92.77%, respectively. Meanwhile, the content of SFN in RNAi plants revealed the reduction by 37.88% and 35.82%, respectively. No significant difference in SFN content was observed in transgenic plants with empty vector and non-transgenic plants (Fig. 11).

Discussion

Southern blot results showed that the target gene fragment was successfully integrated into the genome of broccoli in the form of a single copy (Fig. 8). RT-PCR showed that the gene expression of MY in transgenic plants with MY plasmid revealed an average enhancement by 16.5% when compared with that of non-transgenic plants; In contrast, the gene expression of MY in RNAi-expressing transgenic plants revealed an average reduction of 23.8% (Fig. 10). So, the amplified fragment has been successfully inserted in transgenic plants to complete its expression function.

SFN content analysis showed that the transgenic plants with overexpression of MY gene exhibited a significant improvement (approximately 92.77% increase) of SFN than the transgenic plants with empty vector or non-transgenic plants. However, RNA interference of MY in transgenic plants could result in a significant reduction (35.82% reduction) of SFN content when compared with the transgenic plants with empty vector or non-transgenic plants (Fig. 11). It is well known that SFN is generated from the hydrolysis of glucosinolates by MY. Therefore, the MY gene in kale and broccoli is highly correlated with the accumulation of SFN and participate in the synthesis pathway of SFN. During our experiments, the MY gene expression in the transgenic plants with empty vector also revealed a decrease by 7.5% when compared with that of non-transgenic plants. Its mechanisms remain to be further investigated.

In the process of construction of plant expression plasmids, recombinant plasmids were transformed into *E. coli* on LB plates containing antibiotics and cultured at 37°C. It is prone to result in false positive colonies during the actual experimental process, especially for the smaller carrier. The PCR identification of colonies can be rapidly identified without bacterial amplification prior to the transformation of *E. coli* (Sambrook and Russell, 2001). In the present study, during the process of plasmid construction, the PCR identification of the colonies has been conducted. The screened positive colonies can be further verified by restriction enzyme and sequencing analyses, thus obtaining the results in the early stage without the requirements of common cell culture procedures.

During the experimental design of genetic transformation in this study, the transgenic plants with pCAMBIA1301 empty vector were used as the reference, which can exclude the influence of T-DNA sequences inserted into the genome of plants on gene expression so that it can provide more reliable results.

Due to the existence of MY gene in the genome of broccoli, two kinds of expression plasmids with overexpression and RNAi gene of MY were designed in this study. It is difficult and cumbersome for the direct detection of transgenic plants by using specific primers of MY gene, because the sequence in same T-DNA region can be integrated into the genome of plants to a certain extent. Therefore, in this study, the detection of GUS gene in the same region of T-DNA into plants was used to verify the successful transformation. If the GUS gene and corresponding expression activity were observed in the tissues of transgenic plants, the transformation of the fragment MY or its RNAi expression cassette should be completed. Meanwhile, the gene expression of MY also can be verified by fluorescent quantitative PCR from mRNA level. However, prior to the transformation, the concentration of cDNA generated from reverse transcription should be adjusted to the same level as the original template. The results showed that the gene of MY in transgenic seedlings with overexpressed MY plasmid have been significantly improved; in contrast, the gene of MY in transgenic seedlings with RNAi MY plasmid has been significantly reduced (Fig. 10). Therefore, the overexpression and RNAi technology has been applied in this study.

The T-DNA region of pCAMBIA1301 has GUS gene of CaMV35S promoter. During the construction of 1301-MY and 1301-MY RNAi expression plasmids, MY and GUS genes are in the same T-DNA region. Thus, the application of GUS staining in transgenic plants is relatively straightforward. During the gene expression of GUS, a blue substance can be generated by the hydrolysis of X-Gluc at the condition of active enzyme, which can be observed by naked eyes. In the present study, the GUS staining of roots in transgenic seedlings was applied due to its lighter background color, which is easier to identify when compared with the leaves and stems. In addition, in the

present study, the difference between PCR identification results and GUS staining was observed during the detection of positive transformation in transgenic plants, which may be due to the CaMV35S promoter. The gene silence may occur during the gene expression induced by 35S promoter so that GUS staining cannot be observed in some positive transgenic seedlings (Wang *et al*., 2008).

In this study, a combinatorial method using overexpression and RNAi technology was used to explore the functions of MY. The overexpression of MY can result in the increased content of SFN (92.77%) in broccoli, while RNA interference can result in the reduction of gene expression of MY and reduced content of SFN (35.82%). MY is a key enzyme for the synthesis of SFN in kale and broccoli. The MY genes in both plants have high homology so that the gene of MY in kale should also play an important role in the synthesis of SFN through hydrolyzing glucoraphanin in broccoli.

Sulforaphane is a product from the enzymatic hydrolysis of glucoraphanin by MY. However, in plants, MY and glucoraphanin are present in different locations. When plants are damaged or at the environment of high temperature, the MY can hydrolyze glucoraphanin to produce sulforaphane. During the preservation process of vegetables, the activity of MY remains a continuous decrease so that the amount of SFN is dependent on the preservation time and conditions. The investigation of gene functions and structure analysis of MY will reveal great significance for the production of SFN. Currently, the preparation for the extraction and production of SFN is being extensively investigated to improve the utilization efficiency of resources without the requirement of fresh samples.

During the enzymatic hydrolysis process of MY, radish nitrile instead of SFN can be generated at the condition of inappropriate hydrolysis. Radish nitrile has much less health effect than SFN. Currently, the hydrolysis conditions of MY are still debated. The optimal pH for enzymatic hydrolysis of glucoraphanin by MY has been explored as pH 7 (Steven *et al*., 2005). Similarly, the impact of metal ions on the enzymatic digestion capability of MY has also been explored and Zn^{2+} is favorable to the production of SFN (Liang *et al*., 2006). However, the report has also demonstrated that the optimal enzymatic hydrolysis conditions for MY are pH 5 in Tris-HCl buffer at 15°C. In addition, one previous report has stated the enzymatic hydrolysis conditions of MY at 60°C for 5–10 min facilitate to the formation of SFN (Nathan *et al*., 2004). Therefore, it is highly necessary to systematically explore the optimal hydrolysis conditions of MY.

In conclusion, the sulforaphane content in transgenic broccoli under the conditions of overexpression and RNAi knockdown of *MY* gene. The relative expression of *MY* gene revealed an obvious increase by 16.5% in over-expressed plants and a significant decrease by 23.8% in RNAi knockdown plants. Similarly, the contents of sulforaphane were increased by 92.77% in *MY* gene overexpressed broccoli head and decreased by 35.82% in RNAi knockdown plants. The results indicated that *MY* gene should participate in the synthesis of sulforaphane.

Acknowledgments

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