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ORIGINAL ARTICLE

Improving sulforaphane content in transgenic broccoli plants by overexpressing *MAM1*, FMO_{GS-OX2}, and *Myrosinase*

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Abstract

Sulforaphane (SF) is a potential secondary metabolite with anticancer activity and its metabolic pathway has been revealed in the model plant *Arabidopsis thaliana*. Generally, the SF extraction cycle from broccoli is long and the yield is low, which means that it no longer meets cancer treatment requirements. Therefore, the SF content needs to be urgently improved.
In this study, *MAM1*, FMO_{GS-OX2}, and *Myrosinase*, which are required for SF biosynthesis, were introduced into broccoli by *Agrobacterium tumefaciens*-mediated transformation. To obtain plants with a higher SF content, broccoli was transformed using *Myrosinase-*FMO_{GS-OX2}*-MAM1* (*M-F-A*) triple genes in tandem and by adding each of the genes on their own. The results showed that the SF contents in the *MAM1*, FMO_{GS-OX2}, or *Myrosinase* transgenic plants improved by 1.7–3.4, 1.6–2.7, and 3.7-fold compared to the wild type (WT), respectively, However, the SF contents in transgenic plants with all three genes improved by 1.86–5.5fold. Furthermore, we examined the anticancer effect of the SF extracts from transgenic plants via cell viability detection of HCT116 colon cancer cells. The results showed that SF extracts from transgenic plants had more obvious anticancer activity than SF extracts from WT, and the SF extracts from the multiple genes plants had stronger anticancer activity than the single gene plants. In summary, the multiple genes transformation increased the SF content in broccoli more than single gene introductions.

Key message

The aim of this research was to improve sulforaphane content by introducing FMO_{GS-OX2}, *MAM1*, and *Myrosinase* genes, and *Myrosinase-*FMO_{GS-OX2}*-MAM1* (*M-F-A*) serial triple genes into broccoli.

Keywords Anticancer activity · Broccoli · Genetic transformation · Overexpression · Sulforaphane

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Introduction

Secondary metabolites are routinely and effectively used to prevent and treat cancer. Glucosinolates are amino acid-derived secondary metabolites that play important roles in plant defense and resistance to pathogen invasion (Sonderby et al. 2010). Glucosinolates are converted into isothiocyanates (ITCs) (Juge et al. 2007). Sulforaphane (SF) is a metabolite of methionine-derived 4-methylsulfinylbutyl glucosinolate [also called as glucoraphanin (GRA)] (Mikkelsen et al. 2010). It can influence cancer cells through inhibition of phase 1 enzymes and nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated induction of phase 2 detoxification enzymes and is an ITC that is well-known for its anticancer activity (Cheng et al. 2016). SF is found in edible cruciferous vegetables, such as *Brassica oleracea* (broccoli, cabbage, cauliflower), *Brassica napus,* and *Arabidopsis thaliana* and the SF content is high relatively in broccoli, but it is still too low to satisfy market requirements. Genetic engineering can effectively increase the SF content and recent progress in broccoli transformation technology has made it possible to produce broccoli cultivars with high SF contents. There have been reports that the SF contents in the Brassicaceae were improved by the genetic transformation of a single gene (Huang et al. 2016; Wu et al. 2013). The SF content was raised by varying degrees, but the effect was still limited. This may be due to the complex metabolism of SF in plants and the overexpression of a single gene does not seem to have a great impact on the final content. Tandem co-expression of multiple genes may be a strategy that could be used to obtain broccoli plants with high SF contents(Ye 2000; Datta et al. 2002; Farhi et al. 2011), but there have been few reports about this subject.

The glucosinolate metabolism pathway has been reported in detail and is divided into three parts: side chain elongation of precursor amino-acids, formation of the core glucosinolate structure, and side-chain decoration. Most of the regulatory genes involved in glucosinolate synthesis in *Arabidopsis* have been cloned and characterized (Halkier and Gershenzon 2006). In this study, the methylthioalkyl malate synthase1 (*MAM1*), *Myrosinase*, and flavin-monooxygenase enzyme-2 (FMO_{GS-OX2}) genes were selected as research subjects in the complicated SF metabolic regulatory network, as shown in Fig. 1. One to six elongation cycles for methionine side chains are catalyzed by the *MAM* family, which determine the diversity of the glucosinolates structures. *MAM1*, a member of the *MAM* family, has been reported to promote the condensation reactions of the first two methionine elongation cycles and also participates in the synthesis of GRA (Benderoth et al. 2006; Kroymann et al. 2001). The FMO_{GS-OX} gene family

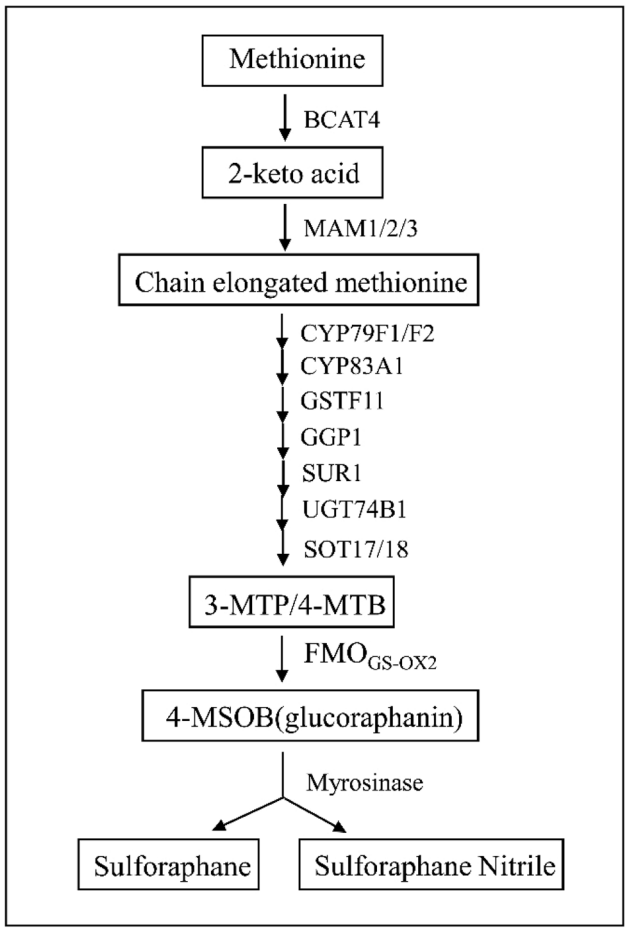

Fig. 1 Biosynthetic pathyway of sulforaphane. The biosynthetic pathyway of sulforaphane can be divided into three categories: side chain elongation of precursor amino-acids, formation of the core glucosinolate structure, and side-chain decoration. The hydrolysis of glucoraphanin by myrosinase to form sulforaphane, sulforaphane nirtle. *BCAT4* branched-chain amino acid aminotransferase 4, *MAM1/2/3* methylthioalkyl malate synthase1/2/3, *CYP79F1/F2* cytochrome P450 79F1/F2, *CYP83A1* cytochrome P450 83A1, *GSTF11* glutathione S-transferase F11, *GGP1* gamma-glutamyl peptidase 1, *SUR1* C–S lyase, *UGT74B1* UDP-glucosyl transferase 74B1, *SOT17/18* sulfotransferase 17/18, FMO_{GS-OX2} flavin-monooxygenase enzyme-2

takes part in the modification of the glucosinolate side chains and mainly catalyzes the S-oxygenation of methylthioalkyl to methylsulfinylalkyl GSLs (Li et al. 2008). In addition, studies have shown that the anticancer activity of cruciferous vegetables was related to S-oxygenation of aliphatic glucosinolates. Consequently, the FMO_{GS-OX} family has great biotechnological potential. FMO_{GS-OX} is a member of FMO family and mainly promotes S-oxygenation of a precursor to GRA. Myrosinase is the last rate-limiting enzyme during SF biosynthesis and directly converts GRA into SF (Chiang et al. 1998).

In this study, the FMO_{GS-OX2}, *MAM1*, and *Myrosinase* genes, and *Myrosinase*-FMO_{GS-OX2}-*MAM1*(*M-F-A*) serial triple genes were introduced into broccoli by the *Agrobacterium*-mediated genetic transformation method to obtain transgenic broccoli plants with high SF contents. The overexpression of these genes, which are involved in SF synthesis, resulted in the accumulation of SF.

Materials and methods

Plant materials and growth conditions

The broccoli seeds were purchased from the Shanxi Academy of Agricultural Sciences. They were immersed in 70% ethanol for 30 s and 0.1% $HgCl_2$ for 5 min. The seeds were washed five times with sterile water for 5 min each time and dried using filter paper. Then the seeds were inoculated onto 1/2 MS solid medium and cultured at 23 °C, 60% relative humidity, and under a 16/8 h (light/dark) photoperiod with 160 μEmm-2 s-1 light illumination.

Primer design and construction of the overexpressing vector

Primer design

The FMO_{GS-OX2}, *MAM1* and *Myrosianse* sequences are not available for broccoli in the NCBI database. Therefore, the nucleotide sequences were aligned using other Brassica species. The sequences for Brassica oleracea var. oleracea, Chinese cabbage and alboglabra were selected for the three unknown genes, respectively and were used as templates when we designed degenerate primers to amplify FMO_{GS-OX2}, *MAM1*and *Myrosianse* genes, which was based on their homology with broccoli.

Construction of the single gene overexpressing vectors

The basic plasmids, XF-350 and XF-246, consisted of a kanamycin resistant gene (*kan*) and a hygromycin phosphotransferase gene (*hpt*), which acted as plant selectable markers, and were provided by Cao Xiaofeng from the Institute of Genetic and Developmental Biology, Chinese Academy of Sciences. Full-length sequences of the *MAM1*, FMO_{GS-OX2}, and *Myrosinase* genes were obtained from previously published coding sequences (GenBank accession numbers: LOC10632463, LOC106318728 and DQ767973.1) (Guo et al. 2016; Xie et al. 2008). Broccoli cDNA was used as the template for PCR amplification and the primers were pp1762–1763, pp1764–1765, and pp1870–1871 (Table S1). The sequences were amplified and connected to *Bam*HI and *Eco*RI in XF-350 by homologous recombination according to the manual (5 × ligation-FreeCloning, abm). The plasmids were transformed into DH5α competent cells. Positive recombinant plasmids were identified by restriction enzyme digestion and sequenced at the Beijing Genomics Institute.

Construction of triple genes overexpressing vector

Recombinant plasmids XF350-*Myrosinase*, XF350-FMO_{GS-OX2}, and XF350- *MAM1* were as templates, which had been successfully constructed. The promoter and terminator, but not the gene fragments, were also amplified so that they could be correctly expressed. The PCR schemes for amplification were as follows: (*Nco*I + HA tag + *Myrosinase* + Nos3′UTR), FMO_{GS-OX2} (CaMV 35S + HA tag + FMO_{GS-OX2} + Nos3′UTR), and *MAM1* (CaMV 35S + HA tag + *MAM1* + *Pst*I). The primers are shown in Table S1. The *BoM-F-A* triple genes were ligated to the *Pst*I and *Nco*I sites of the binary vector XF-246 by multiple fragments homologous recombination according to the manufacturer's instructions (Hieff Clone Plus Multi One Step Cloning Kit, Yeasen, shanghai, China). The recombinant plasmid was verified by enzyme digestion and sequenced at the Beijing Genomics Institute. Successfully constructed recombinant plasmids XF350-*Myrosinase*, XF350-FMO_{GS-OX2}, XF350-*MAM1*, and XF246-*M-F-A* were transformed into EHA105. Positive clones that had been correctly identified were stored at − 80 °C for genetic transformation.

Establishment of the high-efficiency regeneration system in broccoli

We selected four broccoli cultivars: Duofu 70 broccoli, Lvjian No. 3 broccoli, broccoli, and Guanjun 80 days, and compared the effect of 6-BA and NAA at different concentrations and combinations on regeneration efficiency in the different broccoli cultivars. Sterile seeds from the broccoli cultivars were cultured in 1/2 MS medium and 6-day old cotyledons were chosen as explants. These were explanted in differentiation medium and left to grow for 20 days in media containing a combination of 6-BA (2 mg L^{-1}, 3 mg L^{-1}, or 4 mg L^{-1}) and 0.1 mg L^{-1} NAA, 2 mg L^{-1} 6-BA and 0.2 mg L^{-1} NAA. The average number and the regeneration frequency of the regenerated shoots were counted in the media, which contained different hormones (Table1). The formulas used were "the total number of adventitious buds on the explants/the total number of explants containing adventitious buds" and "the total number of explants containing adventitious buds/the total number of explants on the medium," respectively. When the regenerated buds grew to 3 cm in length, the buds were cut from the cotyledon petiole and explanted into 1/2 MS medium containing IBA (0.1 mg L^{-1}, 0.2 mg L^{-1}, 0.3 mg L^{-1}, 0.4 mg L^{-1}, or 0.5 mg L^{-1}). The rooting plantlets were transplanted into soil

Table 1 Effects of different hormone rates and genotypes on broccoli regeneration efficiency

Genotyping	Hormone (mg L^{-1})		Number of explants	Average number of buds	Regeneration frequency (%)
	6-BA	NAA			
Duofu 70 broccoli	2	0.1	100	1.3	37.2
	2	0.2	100	1.7	42.6
	3	0.1	100	2.8	92.3
	4	0.1	100	3.2	93.1
Lvjian No. 3 broccoli	2	0.1	100	1.1	10.5
	2	0.2	100	1.3	23.1
	3	0.1	100	1.7	33.8
	4	0.1	100	2.1	62.3
Broccoli	2	0.1	100	1.2	8.2
	2	0.2	100	1.0	12.9
	3	0.1	100	1.3	14.3
	4	0.1	100	1.6	24.8
Guanjun 80 days	2	0.1	100	2.2	70.5
	2	0.2	100	2.5	75.9
	3	0.1	100	2.5	50.6
	4	0.1	100	1.9	25.2

when the regenerated buds had been induced to form strong roots. The rooting rates for the regeneration seedlings were counted (the number of rooted plantlets /the total number of regenerated buds).

Establishment of genetically transformed broccoli

The most common method used to transform broccoli is the *Agrobacterium*-mediated method (Hiei et al. 1994). In this study, we evaluated the effects of preculture time, microbial concentration, infection time, and kanamycin concentration in order to establish a highly efficient transformation system. An individual impact factor was considered to be a single variable and the different impact factors associated with broccoli transformation efficiency were investigated. The following number of preculture days were investigated: 1 day, 2 days, and 3 days; the OD_{600} for the microbial concentration treatments were 0.2, 0.3, and 0.4; the time gradient for infection time was 5 min, 7 min, 10 min, and 15 min; and the concentration gradient for the kanamycin concentrations in the shoot-inducing medium was 0, 2.5 mg L^{-1}, 5 mg L^{-1}, 7.5 mg L^{-1}, 10 mg L^{-1}, and 15 mg L^{-1}. The 6-day old broccoli cotyledons were infected by controlling a single variable. Shoot inductivity and *Agrobacterium* growth were recorded after 30 days and the measurements were repeated three times in each experiment. There were 50 explants in each treatment.

PCR, RT-PCR, and Q-PCR

The transgenic broccoli genomic DNA was extracted by CTAB (Wood 1983). DNA as template that can amplify fragments using a vector-specific upstream primer and a gene-specific downstream primer (primers shown in Table S1).

The RNA was extracted using RNAiso Plus (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The RNA was reversed into cDNA by All-In-One RT MasterMix (Abm, Nanjing, China) and the cDNA used as a template for the RT-PCR and Q-PCR to determine gene expression levels. The method was the same as the one used by Shen et al (2013). (primers shown in Table S1).

Determination of the SF contents in transgenic broccoli

The determination method was the same as the one used in a previous study (Zhang et al. 2019). Totally 10 g of fresh broccoli stem and leaf mixture are washed and dryed when transgenic broccolis grew to four months. The SF content was determined by 1200 HPLC (C18 column, Innoval ODS-2 4.6 mm × 250 mm, 5 µm, Agilent, made in China). The determination parameters were set up as follow: detection wavelength: 201 nm, column temperature: 35 °C and flow rate: 1 mL min^{-1}, sample volume: 20 µL.

Cell culture and MTT analysis

HCT116 colon cancer cells were cultured in F12 medium containing 10% FBS/1% penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. Cells in the exponential growth phase were plated at a cell density of 5000 cells per well in 96-well tissue culture plates. After attachment overnight, the cells were treated at the concentrations, which was equivalent to

diluting the SF extracts from transgenic broccoli by a factor of about 20. After the end of the 48 h treatment, 10 µL of MTT (5 mg L^{-1}) was added to each well and the cells were allowed to incubate for 4 h at 37 °C. DMSO (100 µL) was added to each well, and their absorbance at 570 nm was read by a microplate. The cells incubated with the control medium were considered 100% viable (model 680, Bio-Rad).

Trypan blue staining

HCT116 cells were plated at a density of 5000 cells per well for 24 h in new 24-well culture dishes. The cells were treated with SF standard. After the end of the 48 h treatment, the cells were washed with phosphate buffered saline (PBS) and digested by trypsin enzyme. Then single cell suspension was mixed with 1% trypan blue solution at a ratio of 9:1 and photographed under a microscope.

Statistical analysis

The data are presented as means ± SEM and the data represents at least three independent experiments. Statistical comparisons were made using one-way ANOVA, and the significance level was set at $p < 0.05$ (*) and $p < 0.01$ (**).

Results

Construction of the overexpression vectors

To improve the SF content in broccoli, we constructed a single gene overexpressing vector that contained the *MAM1*, $FMO_{GS\text{-}OX2}$, or the *Myrosinase* gene, which are involved in SF synthesis. The *MAM1*, $FMO_{GS\text{-}OX2}$, and *Myrosinase* genes were amplified and inserted into XF-350. After the digestion of recombinant XF-350 plasmids by *Bam*HI and *Eco*RI restriction enzymes, the fragments with approximately 1518 bp, 1386 bp, and 1647 bp were released and they were consistent with the expected sizes (Fig. 2c–e). Sequences obtained from the sequencing results were identical to the sequences from NCBI database. The different effect of the genetics between single gene overexpression and triple genes co-expression were investigated by exploring the complex SF metabolic pathway. The *M-F-A* triple genes set were inserted into XF-246 by multiple fragments homologous recombination. The recombinant plasmid was digested by *Nco*I and four fragments that were 2337 bp, 1.000 bp, 982 bp, and 600 bp in size were released. In addition, a 2817 bp fragment was also obtained by enzyme digestion with *Pst*I (Fig. 2f). All the fragments were in line with expectations. Sequence analysis of *M-F-A* indicated that the three fragments had been ligated successfully into XF-246, and the construct was called XF-246-*Myrosinase*-$FMO_{GS\text{–}OX2}$-*MAM1*.

Broccoli regeneration

The broccoli regeneration efficiency was closely related to genotype and hormone concentration. The 6-day old cotyledons from Duofu 70 broccoli, Lvjian No.3 broccoli, broccoli, and Guanjun 80 days were selected by adjusting the 6-BA and NAA hormone combination to establish a high-efficiency regeneration system. The results indicated that the shoot differentiation rates for Duofu 70 broccoli and Guanjun 80 days were relatively high at 93.1% and 75.9%, respectively and the average number of regeneration shoots was 3.2 and 2.5, respectively. The broccoli regeneration efficiency was relatively low, with the lowest efficiency being only 8.2%. Furthermore, the lowest average number of regenerated shoots was one (Table 1). Root culturing and transplantation are crucial when attempting to establish a plant regeneration system. Adventitious shoots from Duofu 70 broccoli and Guanjun 80 days were placed in different concentrations of rooting medium. The results indicated that the Duofu 70 broccoli and Guanjun 80 days rooting rates were 89.7% and 90.8%, respectively, on the medium containing 0.5 mg L^{-1} IBA (Table 2). Consequently, Duofu 70 broccoli was used for the genetic transformation experiment after taking into account the average number and regeneration frequency of the regenerated shoots, and the rooting rate for the regenerated shoots (Fig. 3).

Molecular characterization and culture of resistant broccoli plants

A single gene and triple genes in tandem gene set were introduced into broccoli by *Agrobacterium*-mediated genetic transformation to obtain transgenic broccoli with a high SF content. The cotyledons were infected by co-cultivation with *Agrobacteria* for 2 days and were cultured in selecting media containing antibiotics. Resistant plants were transferred to soil after 2 months. A total of 12, 11, 6, and 3 resistant plants that overexpressed *BoMAM1*, $BoFMO_{GS\text{–}OX2}$, *BoMyrosianse* genes, or the *BoM-F-A* triple genes set were obtained, respectively. The genomic DNA from the WT and resistant plants were extracted to identify whether the target genes had been integrated into the broccoli genome. The extracts were used as templates to amplify target genes by a vector-specific upstream primer and a gene-specific downstream primer. The results indicated that the band for the target gene appeared in *BoMAM1* resistant broccoli, except for lines 1, 3, and 5, and the WT (Fig. 4a). In addition, PCR products from the target genes were also observed in resistant plants that overexpressed $BoFMO_{GS\text{–}OX2}$, *BoMyrosianse*, and the *BoM-F-A* triple genes set, whereas the band was not

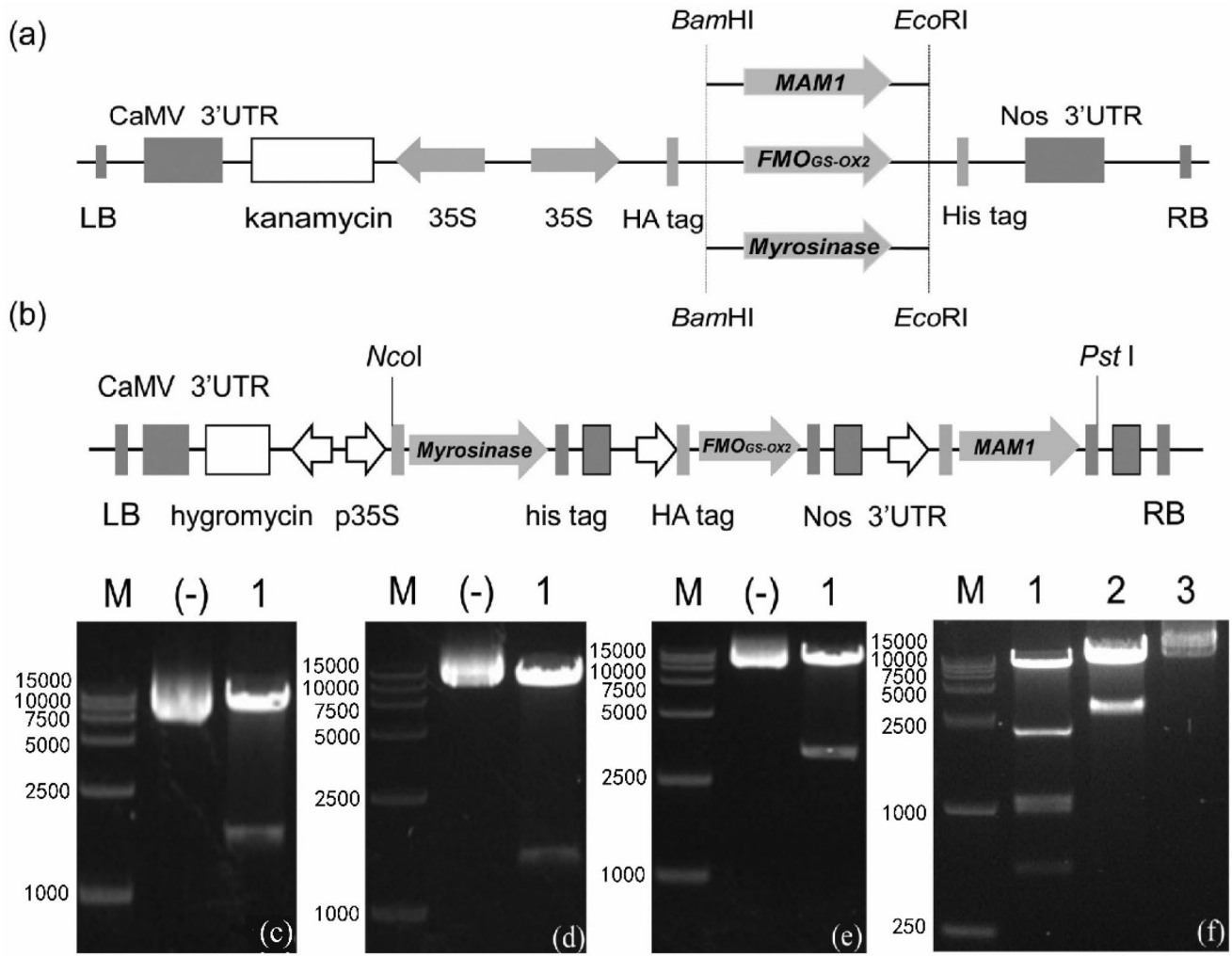

Fig. 2 Construction of the overexpression vector for *MAM1*, FMO_{GS-OX2}, and *Myrosinase* **a** XF-350 vector backbone T-DNA region of the expression vector for *MAM1*, FMO_{GS-OX2}, and *Myrosinase*; *RB* right border, *LB* left border, *p35S* CaMV35S promoter, kanamycin, a screen selection marker; Tnos, nopaline synthase terminator; **b** T-DNA region of the overexpression vector backbone for XF-246-*M-F-A*. HPT, hygromycin phosphotransferase; **c–e** Enzyme digestion identification of recombinant plasmids for *MAM1*,FMO_{GS-OX2}, and *Myrosinase*; M: DNA Marker15000; (-) empty vector; 1: plasmid digested by *Bam*H I and *Eco*R I. **f** Enzyme digested identification of the recombination plasmid for XF-246-*M-F-A*; M: DNA Marker15000; 1: plasmid digested by *Nco*I; 2: plasmid digested by *Pst*I; 3: empty vector

detected in the WT (Fig. 4b–d). This initially proved that the target genes had been integrated into the broccoli genome. There were no obvious phenotype differences between the overexpression plants and the WT (Fig. 4e).

Expression analysis in resistant broccoli

The RNA was extracted from the WT and resistant plants to further determine whether the expression levels of the objective genes in resistant plants had improved. The expression levels of the target genes were confirmed by semi-quantitative RT-PCR analysis and Q-PCR. The results showed that gene expression notably improved in the transgenic broccoli that overexpressed the *BoMAM1*, $BoFMO_{GS-OX2}$, *BoMyrosinase*, and *BoM-F-A* genes compared to the WT. In addition, the rise in the expression levels of the target genes in the different transgenic broccoli varied. The relative gene expression levels increased by approximately 2- to 13-fold for *BoMAM1*, 3- to 21-fold for $BoFMO_{GS-OX2}$, 2- to 41-fold for *BoMyrosianse*, and 1.6- to 20-fold for *BoM-F-A* compared to the WT (Fig. 5).

Determination of SF content in transgenic plants

High performance liquid chromatography (HPLC) was used to determine the SF content in the WT and transgenic plants. The results showed that gene expression increased in transgenic broccoli and that this led to a rise in SF content.

Table 2 Effects of different hormone concentrations on root generation

Genotyping	Plant growth regulation (mg L^{-1}) IBA	Rooting rate of regeneration seedling (%)
Duofu 70 broccoli	0.1	56.3
	0.2	62.1
	0.3	63.7
	0.4	72
	0.5	89.7
Guanjun 80 days	0.1	43.5
	0.2	78.2
	0.3	86.7
	0.4	88.2
	0.5	90.8

The SF content increased by 1.7- to 3.4-fold for *BoMAM1*, whereas there was no significant change in the line 7 *BoMAM1* overexpression plant, which was in accordance with the change in the corresponding gene expression level (Figs. 5e, 6a). The SF content increased by 1.6- to 2.7-fold for $BoFMO_{GS\text{–}OX2}$, 3.7-fold for *BoMyrosinase*, and 5.5-fold for *BoM-F-A* (Fig. 6b–d). Therefore, the results showed that the multi-gene co-transformed plants produced more SF than the single gene transformed plants.

Effect of SF extraction on the HCT116 colon cancer cell survival rate in transgenic broccoli

We examined the effect of SF extraction on the survival rate of HCT116 colon cancer cells in WT and transgenic broccoli

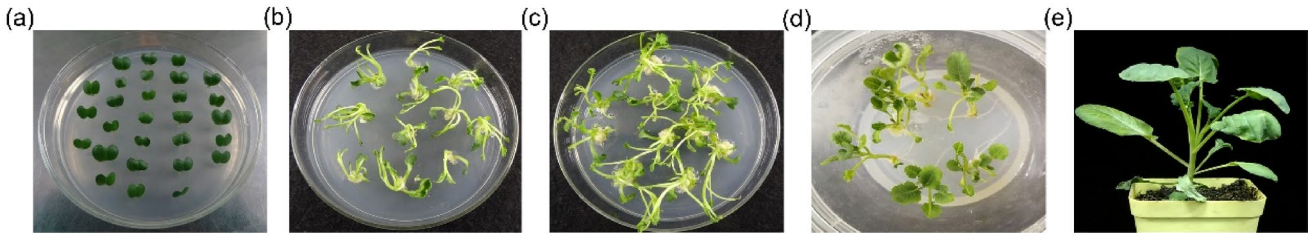

Fig. 3 Growth of regenerated broccoli seedlings **a** Cotyledons grown for 6 days as explants, **b** formation of the regenerated shoots over 10 days, **c** further growth of regenerated shoots over 20 days, **d** regenerated shoots were placed on the rooting medium, and **e** transplanted broccoli seedlings

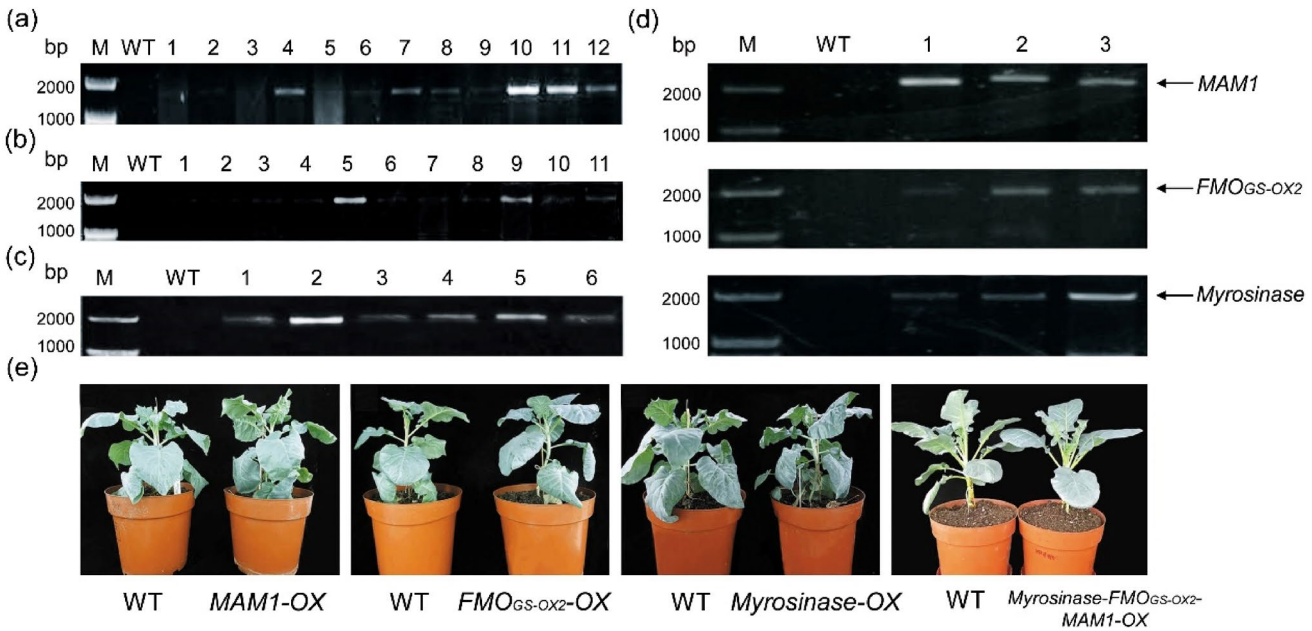

Fig. 4 DNA identification in resistant broccoli plants **a–c** DNA identification in broccoli plants that overexpressed the *BoMAM1*,$BoFMO_{GS\text{–}OX2}$, *BoMyrosinase*, and *BoM-F-A* genes. *M* marker, *WT* wild type (negative control), **a** 1–12—*BoMAM1* resistant plants, **b** 1–11—$BoFMO_{GS\text{–}OX2}$ resistant plants, **c** 1–6—*BoMyrosinase* resistant plants, **d** 1–3—*BoM-F-A* resistant plants; and e. phenotypes of the wild type and resistant broccoli plants

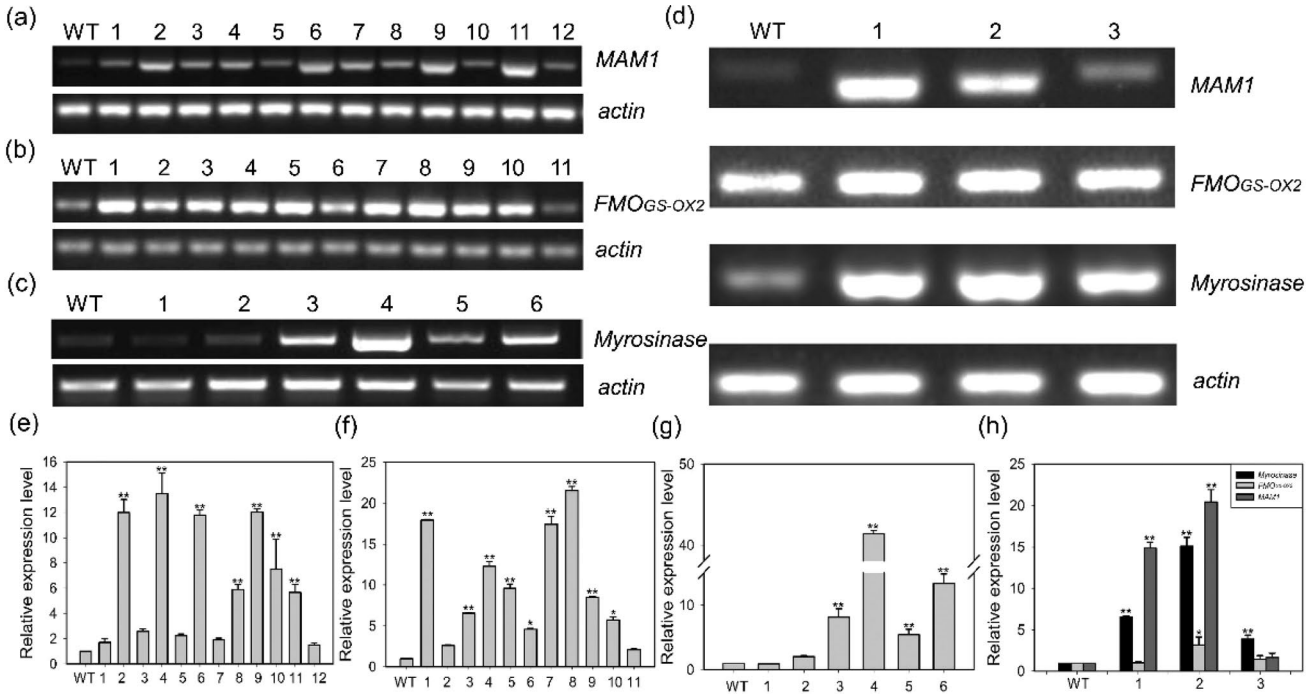

Fig. 5 Identification of gene expression levels in resistant broccoli **a**–**d** RT-PCR analysis of resistant broccoli that overexpressed the *BoMAM1, BoFMO*$_{GS-OX2}$, *BoMyrosinase,* and *BoM-F-A* genes. Actin (ACT1 broccoli); *WT* wild type, **a** 1–12—*BoMAM1* resistant plants, **b** 1–11—*BoFMO*$_{GS-OX2}$ resistant plants, **c** 1–6—*BoMyrosinase* resistant plants, and **d** 1–3—*BoM-F-A* resistant plants. **e**–**h** Q-PCR analysis of resistant broccoli that overexpressed the *BoMAM1, BoFMO*$_{GS-OX2}$, *BoMyrosinase*, and *BoM-F-A* genes. Data were mean ± SEM of RNA extracted from three independent samples. Significance levels were set at $p < 0.05$ (*) and $p < 0.01$ (**)

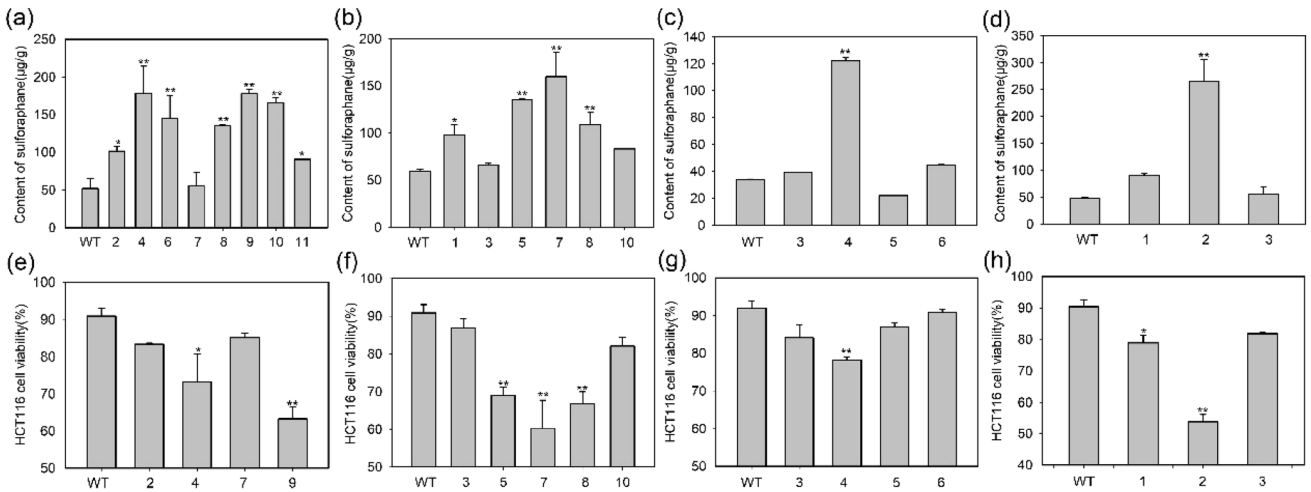

Fig. 6 Determination of the SF contents in transgenic broccoli and the effect of SF extracts on HCT116 colon cancer cell survival rates Fig. 6a–d show that the SF contents in transgenic broccoli that overexpressed the *BoMAM1*, *BoFMO*$_{GS-OX2}$, *BoMyrosinase*, and *BoM-F-A* genes. **a** *WT* wild type, Lines 2, 4, 6, 7, 8, 9, 10, and 11 are the *BoMAM1* transgenic plant lines, **b** *WT* wild type, Lines 1, 3, 5, 7, 8, and 10 are the *BoFMO*$_{GS-OX2}$ transgenic plant lines, **c** *WT* wild type, lines 3, 4, 5, and 6 are the *BoMyrosinase* transgenic plant lines; **d** *WT* wild type, lines 1, 2, and 3 are the *BoM-F-A* transgenic plant lines. Figures 6e–h show the effects of the SF extracts on HCT116 colon cancer cell survival rate. **e** *WT* wild type, lines 2, 4, 7, and 9 the *BoMAM1* transgenic plant lines, **f** *WT* wild type, lines 3, 5, 7, 8, and 10 are the *BoFMO*$_{GS-OX2}$ transgenic plant lines, **g** *WT* wild type, lines 3, 4, 5, and 6 are the *BoMyrosinase* transgenic plant lines, **h** *WT* wild type, lines 1, 2, and 3 are the *BoM-F-A* transgenic plant lines. SF content were determined from stems and leaves mixtures of 4-month-old transgenic broccoli. Data were means ± SEM and three technological repeats, significance levels were set at $p < 0.05$ (*) and $p < 0.01$ (**)

because SF shows anticancer activity. The results indicated that the cancer cell survival rates were significantly reduced by the SF extracted from transgenic plants compared to the WT plants. The HCT116 survival rate reductions in transgenic broccolis that overexpressed *BoMAM1*, *BoFMO*$_{GS-OX2}$, and the *BoMyrosinase* genes were 25.2%, 28.6%, and 14.8%, respectively (Fig. 6e–g). Interestingly, the significant SF content improvement in line two of the *BoMAM1* transgenic broccolis had no obvious effect on the HCT116 survival rate (Fig. 6e). This may be because SF can be unstable. In addition, the largest reduction in the HCT116 survival rate was 40.4% in transgenic plants that overexpressed the *BoM-F-A* triple gene series (Fig. 6 h). SF standard exhibited inhibitory effects on cell number and survival rate of HCT116 cells in a dose-dependent manner (Figs. S1-S2). Consequently, the results showed that *BoMAM1*, *BoFMO*$_{GS-OX2}$, and *BoMyrosinase* were involved in the regulation of SF synthesis.

Discussion

Glucosinolate metabolites have anticancer activity and are involved in plant defense (Sonderby et al. 2010). SF is a glucosinolate metabolite, and it has been reported to be a very effective anticancer drug. The SF content could be improved by genetic engineering, which is an efficient way of improving SF levels in broccoli. Currently, SF extraction is expensive and broccoli yields are low. In this study, we selected *BoMAM1, BoFMO*$_{GS-OX2}$, and *BoMyrosinase*, which regulate SF synthesis. They were introduced into broccoli by *Agrobacterium*-mediated transformation as either single genes or as part of a triple multiple gene series. The results showed that transformation with multiple genes increased SF content in broccoli more than single gene introductions.

Previous studies have shown that the co-expression of multiple regulatory genes increases the content of provitamin A (p-carotene) in rice endosperm more than single gene introductions (Ye 2000). In subsequent studies, co-expression multiple regulation gene transformations have been applied to tobacco, rice, soybean, maize, and potato, etc. to improve crop quality (Datta et al. 2002; Farhi et al. 2011; Jobling et al. 2002; Wu et al. 2002; Zhu et al. 2008). Furthermore, modifying regulation genes that are involved in SF synthesis has become an effective way of improving the SF content in plants. It was also reported that modifying single genes was not an efficient way to improve SF content (Huang et al. 2016; Wu et al. 2013). This study showed that it was much more effective to introduce multiple genes into broccoli. Consequently, *BoMyrosinase*, *BoFMO*$_{GS-OX2}$, and *BoMAM1* were introduced into broccoli as single genes and as a triple genes co-expression series in this study. The results showed that transformations using multiple genes in tandem improved SF content more than single gene transformations (Fig. 6a–d). This might be similar to a complex water pipeline, in which the water yield is affected by multiple easily blocked nodes. Only dredging one of them may have little effect on the final water yield.

The SF contents were raised by different amounts depending on which *BoMAM1*, *BoFMO*$_{GS-OX2}$, and *BoMyrosinase* transgenic broccoli lines were tested. In addition, the SF content improvement was different in transgenic broccoli that overexpressed *BoMAM1*, *BoFMO*$_{GS-OX2}$, or the *BoMyrosinase* genes (Fig. 6a–c). This may be due to the different roles played by the genes during SF synthesis. *MAM1* mainly promotes the condensation reactions in the first two methionine elongation cycles (Kroymann et al. 2001) and *FMO*$_{GS-OX2}$ may promote S-oxidation of methylthioalkyl to methylsulfinylalkyl GSLs which are linked to cancer-preventive properties of cruciferous vegetables (Li et al. 2008). And *BoMyrosinase* is the last rate-limited enzyme in the SF synthesis pathway, the SF contents in *BoMyrosinase* transgenic broccolis were lower than in those plants containing the *BoMAM1* and *Bo FMO*$_{GS-OX2}$ genes (Fig. 6a–c). This may be because enzyme activities during SF extraction were affected by the instability of Myrosinase. Furthermore, the expression levels and SF contents varied among the different transgenic plant lines. This is probably due to the position effects when the target genes were integrated into the broccoli genome.

SF biosynthesis in cruciferae species is very complex and may be regulated by dozens, or even hundreds, which mainly divided in two categories: one is identified functional genes. Zang et al. (2008) reported that total aliphatic GSL content could be increased by overexpressing MAM1 in Chinese cabbage. Gene silencing of MAM gene results in a induction in C3 and reduction in C4, C5 side chain aliphatic GSLs in B. napus (Liu et al. 2011). There also have been indicated that C4 side chain aliphatic glucosinolates (2OH3B and 3-Butenyl GSL) content had significantly improved by overexpression of BnMAM1 compared with in wild type (Zang et al. 2015). SF, the isothiocyanate derivative of GRA found in cruciferae species. In our study, SF contents were improved in broccoli with ectopic overexpression of MAM1. This is consistent with previous studies and further elucidates the importance of MAM1 in SF biosynthesis. The other is transcription factors, which could regulate functional genes. The amount of aliphatic GSLs is regulated by R2R3-MYB transcription factors (MYB28, MYB29, MYB76) (Hirai et al. 2007). Sonderby et al. (2007) reported that double mutant plant of *myb28myb29* in *Arabidopsis* eliminates all aliphatic glucosinolates. These studies provide a new ideal and make a theoretical foundation for improving SF content of broccoli by genetic engineering.

In conclusion, the SF contents in *BoM-F-A* transgenic broccoli improved more than when only *BoMAM1*, *BoFMO*$_{GS-OX2}$, and *BoMyrosinase* genes were introduced. These

results should improve the breeding of new broccoli cultivars with higher SF contents, which may lead to the mass production of SF.

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Author contributions HYC, YXP and ZPJ designed the experiments and wrote the manuscript. HYC and RNL performed the experiments. HYC, ZQL and JHZ analyzed the data. SHF and GDY revised the manuscript. All authors read and approved the final manuscript.

Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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