Food Chemistry 168 (2015) 321–326

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Variation of glucosinolates and quinone reductase activity among different varieties of Chinese kale and improvement of glucoraphanin by metabolic engineering

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ARTICLE INFO

Article history:
Received 11 February 2014
Received in revised form 15 June 2014
Accepted 14 July 2014
Available online 23 July 2014

Keywords:
Chinese kale
Glucosinolates
Glucoraphanin
Quinone reductase activity
AOP2
Metabolic engineering

ABSTRACT

The variation of glucosinolates and quinone reductase (QR) activity in fourteen varieties of Chinese kale (*Brassica oleracea* var. *alboglabra* Bailey) was investigated in the present study. Results showed that gluconapin (GNA), instead of glucoraphanin (GRA), was the most predominant glucosinolate in all varieties, and QR activity was remarkably positively correlated with the glucoraphanin level. AOP2, a tandem 2-oxoglutarate-dependent dioxygenase, catalyzes the conversion of glucoraphanin to gluconapin in glucosinolate biosynthesis. Here, antisense *AOP2* was transformed into Gailan-04, the variety with the highest gluconapin content and ratio of GNA/GRA. The glucoraphanin content and corresponding QR activity were notably increased in transgenic plants, while no significant difference at the level of other main nutritional compounds (total phenolics, vitamin C, carotenoids and chlorophyll) was observed between the transgenic lines and the wide-type plants. Taken together, metabolic engineering is a good practice for improvement of glucoraphanin in Chinese kale.

1. Introduction

Glucosinolates are a group of sulphur- and nitrogen-containing secondary metabolites that are mainly found in the order of Brassicales and related groups of dicotyledonous angiosperms (Hansen, Møller, Sørensen, & de Trejo, 1995; Yan & Chen, 2007). Glucosinolates and the hydrolytic myrosinase (β-thioglucosidase) are stored separately under normal situations, but they come into contact with each other when tissues are damaged, and then the glucosinolates are hydrolysed into several degradation products, such as isothiocyanates and nitriles (Andréasson, Jørgensen, Höglund, Rask, & Meijer, 2001). Among these products, sulforaphane, the isothiocyanate derivative of glucoraphanin, is the focus of numerous studies because it has been proven as the most potent monofunctional inducer of quinone reductase (QR), one of the main phase II enzymes which detoxify toxic intermediates in carcinogenic metabolism, in murine hepatoma cells (Zhang, Talalay, Cho, & Posner, 1992). Recent studies have further confirmed the preventive and therapeutic effects of sulforaphane on stomach cancer and colon cancer (Halkier & Gershenzon, 2006; Lozanovski, Houben, Schemmer, & Büchler, 2013).

The direct assay of the QR activity in murine hepatoma cells has been developed to assess anticarcinogenic activity of cruciferous vegetables (Prochaska, Santamaria, & Talalay, 1992). A colorimetric bioassay for QR activity in Hepa lclc7 murine hepatoma cells was also used as a versatile tool to rapidly monitor methylsulfinylalkyl glucosinolate content in plant extracts (Gross, Dalebout, Grubb, & Abel, 2000). In our previous studies, the QR activity bioassay system was established in broccoli and Chinese kale (Xu, Guo, Yuan, Yuan, & Wang, 2006; Zhang, Xiang, Wang, & Wang, 2006), and the effects of different preharvest and postharvest treatments on glucoraphanin content and induction of QR activity in broccoli florets were investigated (Xu et al., 2006, 2010).

Chinese kale (*Brassica oleracea* var. *alboglabra* Bailey) is an original Chinese cruciferous vegetable which is distributed widely in southern China and southeast Asia. It is generally grown for its bolting stem as a common edible part. Besides good flavour, the bolting stem also exhibits a high nutritional value because of its high levels of main antioxidants and anticarcinogenic compounds, including total phenolics, vitamin C, carotenoids and glucosinolates (Sun, Yan, Liu, Wei, & Wang, 2012; Sun, Yan, Zhang, & Wang, 2012). Our previous studies also showed that the most

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http://dx.doi.org/10.1016/j.foodchem.2014.07.073
0308-8146/

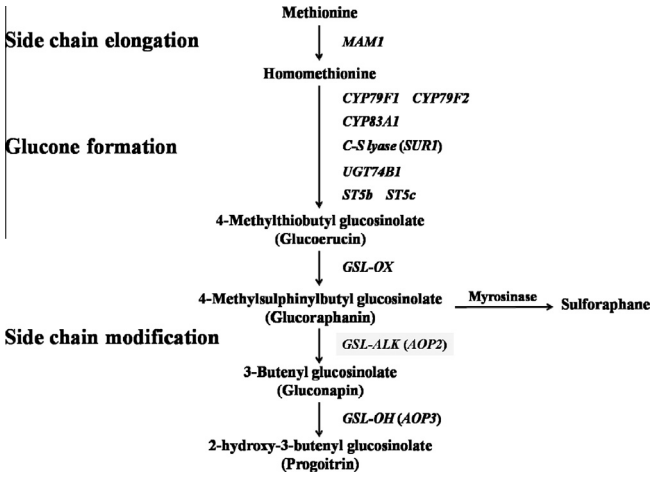

Fig. 1. The metabolic pathway and the related genes involved in biosynthesis of glucoraphanin in *Brassica oleracea* vegetables.

abundant glucosinolate in Chinese kale is gluconapin, the alkenyl product of glucoraphanin (Fig. 1). As the degradation product of gluconapin is associated with the flavour of pungency and bitterness (Padilla, Cartea, Velasco, de Haro, & Ordás, 2007), the improvement of glucoraphanin in Chinese kale is essential for both better flavour and cancer-prevention.

In recent years, great progress has been achieved in elucidation of glucosinolate biosynthesis; 4C aliphatic glucosinolates are the major aliphatic glucosinolates in *Brassica oleracea* vegetables, and the biosynthetic pathways include side chain elongation, glucone formation and side chain modification (Fig. 1) (Grubb & Abel, 2006; Halkier & Du, 1997; Halkier & Gershenzon, 2006). The *AOP2* gene, identified from *Arabidopsis* and other *Brassica* plants (Kliebenstein, Lambrix, Reichelt, Gershenzon, & Mitchell-Olds, 2001; Li & Quiros, 2003), encodes 2-oxoglutarate-dependent dioxygenase, which is responsible for the conversion of methylsulfinylalkyl glucosinolate (glucoraphanin) to its alkenyl form (gluconapin) (Hansen et al., 2008; Neal, Fredericks, Griffiths, & Neale, 2010). In our former study, an antisense *AOP2* gene fragment was transformed into several Chinese kale varieties, including Gailan-04 via *Agrobacterium tumefaciens* in order to disrupt the AOP2 function (Xu, Sun, Pan, & Wang, 2008). Here, we aim to further evaluate the glucosinolate profiles and QR activity in fourteen typical Chinese kale varieties, and investigate the effects of antisense *AOP2* gene transformation on the levels of glucoraphanin, QR activity and other nutritional compounds in Chinese kale.

2. Material and methods

2.1. Plant material

Chinese kale seeds of fourteen local varieties were sown in trays containing peat and vermiculite (3:1) in a greenhouse of Zhejiang University (Hangzhou, China). The seedlings were grown in the greenhouse at a day temperature of 25 °C and a night temperature of 20 °C. After three weeks, the seedlings with 3–5 true leaves were transplanted into an agricultural field with 40 × 30 cm spacing arranged in a completely randomized design. Water, fertilizer, and pesticides were applied as necessary.

Bolting stems (free of any insects and mechanical damage) were harvested as a replicate from 5 bolting plants with inflorescence as tall as the apical leaves. For each sampling, three independent replicates were taken for analysis. Samples were harvested in early morning, placed on ice, and immediately transported to the laboratory. The bolting stems were weighed freshly, and then lyophilized in an ultralow −80 °C freezer to determine the ratio of fresh weight (FW) to dry weight (DW). The lyophilized samples were ground into a fine powder, using a coffee mill, and stored at −20 °C for further analyses of glucosinolate content and quinone reductase activity.

2.2. Transformation of antisense AOP2 gene in Chinese kale

Transgenic Chinese kale plants carrying the antisense *AOP2* gene fragment were produced via *Agrobacterium*-mediated plant transformation (Xu et al., 2008). Briefly, the DNA fragment of *AOP2* gene in Chinese kale (Supplemental Fig. 1A) was cloned by using specific primers (Upper: 5′ ATAGGATCCTGAAGTATGTAG-CACCAC 3′, lower: 5′ GAGTCTAGATAACACCTCCAAACCTTC 3′) from important conservative functional domains of *AOP2* from collard (AY044425) and *Arabidopsis thaliana* ecotype Cape Verde Islands (Atcvi, AF417858). The *AOP2* gene fragment cloned contains an important domain of the Fe (II)-dependent oxygenase superfamily (Supplemental Fig. 1B). The *35Spro: antisense-AOP2* was prepared by inserting the PCR-amplified coding sequence of *AOP2*, with confirmed correct size and sequence, in the antisense orientation, into the BamH I site and Xba I site of the binary vector pCAMBIA2301 under the control of cauliflower mosaic virus 35S promoter (Supplemental Fig. 1C).

The 'Gailan-04' hypocotyl explants, pre-cultured for 3 days in MS medium with 1 mg/l of BA, 1 mg/l of 2, 4-D, 0.8% phytagar, were infected with the overnight-cultured *Agrobacterium* strain EHA105 by immersion for 15–30 s. Explants were co-cultivated with *Agrobacterium* strain EHA105 for 3 days, as in pre-culture, and then transferred to the plates with differential medium. Kanamycin-resistant shoots, regenerated on differential medium, were transferred to rooting medium in plastic bottles. After several months, kanamycin-resistant plantlets with well-developed roots were acclimatized and transplanted into the greenhouse for further analysis and recovery of seeds. PCR assay confirmed that the antisense *AOP2* was integrated into the Chinese kale genomes in transgenic plants (Xu et al., 2008).

Three genetically stable T_2 transgenic plant lines (L1, L4 and L5), and the 'Gailan-04' non-transformed wild-type plants, were grown and sampled as described in the former section. The lyophilized samples were used for analyses of glucosinolate content and quinone reductase activity, while some fresh samples were used to analyse the contents of total phenolics, vitamin C, carotenoid, and chlorophyll in transgenic lines and wild-type plants.

2.3. Glucosinolate assay

Glucosinolates were extracted and analysed as previously described (Sun, Yan, Liu, et al., 2012). Briefly, freeze-dried samples (100 mg) were boiled in 4 ml of water for 10 min. The supernatant was collected after centrifugation (5 min, 7000×*g*), and the residues were washed once with water (4 ml), centrifuged and then combined with the previous extract. The aqueous extract was applied to a DEAE-Sephadex A-25 (40 mg) column (pyridine acetate form) (GE Healthcare, Piscataway, NJ). The column was washed three times with 1 ml of pyridine acetate (20 mM) and twice with 1 ml of water. The glucosinolates were converted into their desulpho analogues by overnight treatment with 100 μl of 0.1% (1.4 units) aryl sulphatase (Sigma), and the desulphoglucosinolates were eluted with 2 × 0.5 ml of water. HPLC analysis of desulphoglucosinolates was carried out using a Waters HPLC instrument equipped with a Model 2996 PDA absorbance detector (Waters, USA). Samples (20 μl) were separated at 30 °C on a Waters Spherisorb C18 column (250 × 4.6 mm i.d.; 5 μm particle size) using acetonitrile and water at a flow rate of 1.0 ml min^{-1}. The

procedure employed isocratic elution with 1.5% acetonitrile for the first 5 min; a linear gradient to 20% acetonitrile over the next 15 min, followed by isocratic elution with 20% acetonitrile for the final 10 min. Absorbance was detected at 226 nm. Ortho-nitrophenyl-β-D-galactopyranoside (Sigma) was used as an internal standard for HPLC analysis.

2.4. Quinone reductase activity assay

Quinone reductase activity was analysed as previously described (Xu et al., 2006; Zhang et al., 2006). A sample of 20 mg was homogenised in a 1.5 ml plastic tube containing 200 μl of extraction buffer (5 mM K_2HPO_4–KH_2PO_4, 1 mM EDTA, pH 7.6). The pestle was rinsed with 300 μl of extraction buffer and the homogenate was centrifuged twice at 16,000×*g* for 10 min to recover the supernatant, which was then filtered with sterile non-pyrogenic filters (0.22 μm) and stored at −80 °C until used.

Hepa 1c1c7 murine hepatoma cells were cultured in a minimal essential medium supplemented with 10% foetal calf serum in a humidified incubator in 5% CO_2 at 37 °C. To monitor the QR inducer potency of plant extracts, 10,000 Hepa 1c1c7 murine hepatoma cells were seeded into each well of 96-well microtitre plates, and grown for 24 h, and then incubated for 24 h by exposure to 200 μl of fresh medium containing 0.5% serial-diluted plant extract to be assayed.

QR activity was measured in cell lysates after induction of murine hepatoma cells for 24 h. The cell culture medium was decanted and 50 μl of lysing solution (0.4% Nonidet P-40) were added to each well. Cells were lysed for 10 min at 37 °C, followed by shaking (100 rpm) for 10 min at room temperature. For measurement of QR activity, 200 μl of assay solution, which contains 25 mM Tris buffer (pH 7.4), 1 mM glucose-6-phosphate, 50 μM menadione, 30 μM NADP, 5 μM FAD, 0.07% (w/v) bovine serum albumin, 0.03% (w/v) MTT, 0.01% (v/v) Tween-20, and 1 unit/ml of yeast glucose-6-phosphate dehydrogenase, were added to each well, using an octapipet. The reaction mixtures were incubated for 10 min at room temperature and then reactions were terminated by the addition of 50 μl of 0.1 M HCl. Absorbances were measured by scanning the microtitre plates at 595 nm. QR activity was analysed for both inducer potency and induction ratio. The QR inducer potency was expressed as unit g^{-1} dry weight. One unit of QR inducer activity is defined as the amount of plant material required to double the specific QR activity in a microtitre well containing 200 μl of cell culture medium. QR induction ratio was expressed as the ratio of QR activity measured in treated (plant extract) and untreated murine hepatoma cells.

2.5. Total phenolics assay

Total phenolics were extracted with 50% ethanol and incubated at room temperature for 24 h in the dark. The suspension was centrifuged at 10,000 rpm for 10 min at room temperature and the supernatant was collected. Phenolic compounds were determined, using Folin–Ciocalteu reagent, by reading the absorbance at 760 nm with the spectrophotometer as previously described (Wei, Miao, & Wang, 2011; Yuan, Wang, Guo, & Wang, 2010). Gallic acid was used as a standard and the results were expressed as mg gallic acid equivalent (GAE)/100 g fresh weight.

2.6. Vitamin C assay

Vitamin C content was analysed as previously described with minor modifications (Sun, Yan, Liu, et al., 2012). Frozen bolting stems (5 g) were ground to a fine powder in liquid nitrogen and were extracted twice with 20 ml of 1.0% (w/v) oxalic acid and centrifuged at 5000 rpm for 5 min. Each sample was filtered through a 0.45 μm cellulose acetate filter. HPLC analysis of vitamin C was carried out using a Waters instrument with a Model 2996 PDA detector (Waters Inc., Milford, USA). Samples (20 μl) were separated at room temperature on a Waters Spherisorb C18 column (250 × 4.6 mm id; 5 μm particle size), using a solvent of 0.1% oxalic acid at a flow rate of 1.0 ml min^{-1}. The amount of vitamin C was calculated from absorbance values at 243 nm, using authentic ascorbic acid as a standard. Results were expressed as mg 100 g^{-1} FW.

2.7. Carotenoid and chlorophyll assay

The contents of carotenoid and chlorophyll were determined according to the HPLC method of Lefsrud, Kopsell, Wenzel, and Sheehan (2007). Briefly, 0.1 g of sample was hydrated with 0.8 ml of deionized water in a 40 °C water bath for 20 min. After hydration, 0.8 ml of the internal standard, ethyl-β-apo-8′-carotenoate and 2.5 ml of tetrahydrofuran (THF) were added and the mixture was homogenised before being placed in a clinical centrifuge for 3 min at 500×*g*. The supernatant was transformed into a test tube, capped and held on ice. The sediment was re-suspended in 2.0 ml of THF and homogenised for the second time. The sample was then centrifuged for 3 min at 500×*g* and the supernatant was collected and combined with the first extracted supernatant. The combined supernatants were reduced in volume under a stream of nitrogen gas, and brought to a final volume of 5 ml with methanol. A 2 ml aliquot was filtered prior to HPLC analysis. Samples (20 μl) were separated at 16 °C on a RP C18 column (250 × 4.6 mm i.d.; 5 μm particle size), using gradient elution at a flow rate of 0.7 ml min^{-1}. Eluted carotenoids and chlorophyll compounds were separately detected at 452 nm (carotenoids and internal standard), 652 nm (chlorophyll *b*), and 665 nm (chlorophyll *a*). Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from photodiode array detection, using external standards.

2.8. Statistical analysis

Statistical analysis of data was performed using the SPSS package program version 11.5 (SPSS Inc. Chicago, IL, USA). Data were analysed by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test at a significance level of 0.05. Different letters within the same group indicate significant difference at the 5% level.

3. Results

3.1. Glucosinolate contents in different varieties of Chinese kale

The total glucosinolate content in bolting stems of 14 Chinese kale varieties ranged from 2.95 to 11.6 μmol g^{-1} DW, with an average value of 7.10 μmol g^{-1} DW (Table 1 and Supplemental Fig. 2). Significant difference in total glucosinolate content was observed among different varieties. The aliphatic glucosinolates were predominant, with gluconapin, the alkenyl product of glucoraphanin, being the most abundant one, representing over 60% of the total glucosinolate content in all varieties tested. The highest contents of glucoraphanin and total glucosinolate were found in Gailan-01, while the lowest were detected in Gailan-25. The highest content of gluconapin, as well as the highest ratio of the GNA/GRA, was observed in Gailan-04 (Table 1), which meant that the alkenyl modification was more intensive in this variety than that in other varieties. Moreover, the results also indicated that flower colour and maturity exerted little influence on glucosinolate content.

Table 1
The composition and contents (μmol g^{-1} DW) of total and main aliphatic glucosinolates in bolting stems of Chinese kale among different varieties.

Variety	Flower colour	Maturity	Total glucosinolates	Gluconapin (GNA)	Glucoraphanin (GRA)	Ratio GNA/GRA
Gailan-01	White	Early	11.6 a	6.84 a	2.89 a	2.37
Gailan-02	White	Early	5.98 de	3.71 d	0.56 ef	6.63
Gailan-03	White	Early	7.79cd	5.99 b	0.62 ef	9.66
Gailan-04	White	Early	8.32 c	7.12 a	0.69 e	10.3
Gailan-05	White	Early	6.31 de	3.15 e	1.05 c	3.00
Gailan-06	White	Early	6.08 de	3.32 e	1.04 c	3.19
Gailan-07	White	Early	5.68 de	3.92 cd	0.56 f	7.00
Gailan-08	White	Early	5.15 e	2.78 f	0.82 d	3.39
Gailan-09	White	Early	4.53 e	2.09 g	0.62 ef	3.37
Gailan-10	White	Late	9.72 b	5.16 b	1.61 b	3.20
Gailan-15	White	Late	6.94 d	4.05 c	1.54 b	2.63
Gailan-23	White	Late	9.80 b	5.95 b	1.56 b	3.81
Gailan-24	Yellow	Late	8.56 c	5.37 b	1.11 c	4.84
Gailan-25	Yellow	Late	2.95 f	1.46 h	0.33 g	4.42

Each value represents a mean ($n = 3$). Values in the same column followed by the same letter are not significantly different at $p < 0.05$.

Table 2
QR inducer potency and QR induction ratio in murine Hepa lclc7 cells by extracts of bolting stems of Chinese kale among different varieties.

Variety	Dry weight ratio	QR inducer potency (units/g)	QR induction ratio (treated/control)
Gailan-01	7.8	21358 a	7.36 a
Gailan-02	7.3	5405 ef	2.25 ef
Gailan-03	7.5	6285 ef	2.46 ef
Gailan-04	7.0	6716 e	3.08 e
Gailan-05	7.6	10731 c	3.93 c
Gailan-06	7.8	10369 c	3.89 c
Gailan-07	7.4	5405 f	2.24 f
Gailan-08	7.4	8288 d	3.18 d
Gailan-09	7.2	6015 ef	2.29 ef
Gailan-10	7.9	19448 ab	6.81 ab
Gailan-15	7.5	16220 b	5.92 b
Gailan-23	7.3	15654 b	5.76 b
Gailan-24	7.6	11344 c	4.88 c
Gailan-25	7.4	3322 g	1.45 g

Each value represents a mean ($n = 3$). Values in the same column followed by the same letter are not significantly different at $p < 0.05$.

3.2. QR activity in different varieties of Chinese kale

As shown in Table 2, the QR inducer potency and induction ratio of Chinese kale varied markedly among different varieties. The highest QR inducer potency was found in Gailan-01 and Gailan-10, while Gailan-25 exhibited the lowest value. The trend of QR induction ratio was similar to that of QR inducer potency (Table 2).

The relation between QR activity and total/individual glucosinolate contents are shown in Supplemental Fig. 3 and Table 3. The content of glucosinolate appeared to be positively related to QR inducer potency, whereas the ratio of GNA/GRA was negatively related to it (Supplemental Fig. 3). Detailed statistical analysis indicated that QR inducer potency correlated with the glucoraphanin content very well, with a positive correlation coefficient of 0.9431, followed by total glucosinolate content (0.7977), while the correlation coefficient between the QR inducer potency and the gluconapin content was the lowest (0.4946). Similar results were also observed in correlation between QR induction ratio and glucosinolate contents.

3.3. Glucosinolate contents and QR activity in antisense AOP2 transgenic plants

The glucosinolate contents and QR activity in bolting stems of three anti-*AOP2* transgenic Gailan-04 lines (L1, L4 and L5) were analysed. The glucoraphanin content in all three transgenic lines was markedly higher than that in wild-type. Specifically, the glucoraphanin content increased by 0.33-fold, 1.28-fold, and 3.09-fold in lines L1, L4 and L5, respectively, when compared with the wild-type plants. As for the level of gluconapin in transgenic plants, there was either a moderate rise (line L4) or no obvious change (lines L1 and L5) when compared with the wild-type plants. Therefore, the ratio of GNA/GRA was notably declined in all three transgenic lines, especially in L5 (Table 4). The effects of anti-*AOP2* transformation on other main glucosinolates, including three indolic glucosinolates (glucobrassicin, 4-methoxy glucobrassicin and neoglucobrassicin) and two aliphatic glucosinolates (progoitrin and glucoerucin), were also investigated, and no significant difference in the contents of these glucosinolates was observed between transgenic lines and wild-type plants (Table 4), indicating that anti-*AOP2* transformation had no notable influence on glucosinolates other than glucoraphanin.

Significant increases in both QR inducer potency and QR induction ratio were found in transgenic lines (L4 and L5) when compared with the wild-type plants (Table 4).

3.4. The contents of total phenolics, vitamin C, carotenoids and chlorophyll in antisense AOP2 transgenic plants

The effects of anti-*AOP2* transformation on the contents of normal nutritional compounds (total phenolics, vitamin C, carotenoids and chlorophyll) in bolting stems of transgenic plants were also investigated in the current study. As shown in Table 5, no

Table 3
Correlation coefficients between glucosinolate content and QR activity in bolting stems of Chinese kale.

	Total glucosinolates	Gluconapin	Glucoraphanin	QR inducer potency	QR induction ratio
Total glucosinolates	1.0000				
Gluconapin	0.8985	1.0000			
Glucoraphanin	0.8023	0.5288	1.0000		
QR inducer potency	0.7977	0.4946	0.9431	1.0000	
QR induction ratio	0.8241	0.5424	0.9284	0.9925	1.0000

Table 4
The composition and content of glucosinolate, QR inducer potency and QR induction ratio in different transgenic lines of Chinese kale.

	Gluconapin (GNA)[1]	Glucoraphanin (GRA)[1]	Ratio GNA/GRA	Progoitrin[1]	Glucoerucin[1]	Glucobrassicin[1]	4-Methoxy glucobrassicin[1]	Neoglucobrassicin[1]	QR inducer potency[2]	QR induction ratio[3]
L1	6.28 b	0.99 c	6.34	0.53 a	0.40 a	0.81 a	0.31 a	0.41 a	10158 c	3.52 b
L4	10.96 a	1.69 b	6.49	0.49 a	0.45 a	0.71 a	0.30 a	0.35 a	19598 b	6.93 a
L5	9.08 b	3.03 a	3.00	0.47 a	0.44 a	0.76 a	0.25 a	0.34 a	24538 a	7.89 a
WT	7.88 b	0.74 c	10.65	0.38 a	0.37 a	0.85 a	0.27 a	0.35 a	8316 c	3.28 b

L1, L4, and L5 are three antisense *AOP2* transgenic plant lines; WT is wild-type plants. Each value represents a mean ($n = 3$). Values in the same column followed by the same letter are not significantly different at $p < 0.05$.
[1] Glucosinolate in μmol g^{-1} DW.
[2] QR inducer potency in units/g.
[3] QR induction ratio in treated/control.

Table 5
The contents of total phenolics, vitamin C, carotenoids and chlorophyll in bolting stems of Chinese kale among different transgenic lines and wild-type.

	Total phenolics (mg GAE 100 g^{-1} FW)	Vitamin C (mg 100 g^{-1} FW)	Lutein (mg 100 g^{-1} FW)	β-carotene (mg 100 g^{-1} FW)	Carotenoids (mg 100 g^{-1} FW)	Chlorophyll *a* (mg 100 g^{-1} FW)	Chlorophyll *b* (mg 100 g^{-1} FW)	Chlorophyll (mg 100 g^{-1} FW)
L1	42.4 a	67.9 a	1.63 a	0.79 a	2.42 a	10.5 a	3.11 b	13.6 a
L4	39.9 a	69.7 a	1.84 a	0.70 a	2.53 a	11.4 a	4.55 ab	15.9 a
L5	48.6 a	73.9 a	1.69 a	0.77 a	2.46 a	10.1 a	5.53 a	15.7 a
WT	46.7 a	67.5 a	1.54 a	0.72 a	2.26 a	10.9 a	3.31 ab	14.2 a

L1, L4, and L5 are three antisense *AOP2* transgenic plant lines; WT is wild-type plants. Each value represents a mean ($n = 3$). Values in the same column followed by the same letter are not significantly different at $p < 0.05$.

statistically significant difference in the contents of these compounds was observed between transgenic lines and wild-type plants.

4. Discussion

In the present study, glucosinolate contents and QR activity in fourteen varieties of Chinese kale were investigated. Striking differences in glucosinolate content were found among different varieties. Similar results were also reported in several other *Brassica* species, such as broccoli, cauliflower, kale, cabbage, and turnip (He, Chen, & Schnitzler, 2002; Kushad, Cloyd, & Babadoost, 2004; Padilla et al., 2007; Sun, Liu, Zhao, Yan, & Wang, 2011; Verkerk et al., 2009). QR activity also exhibited notable difference among different varieties. Extremely high positive correlation existed between QR activity and glucoraphanin content in the current survey (Supplemental Fig. 3 and Table 3), which was also reported in *Arabidopsis* and broccoli (Gross et al., 2000; Xu et al., 2006). Therefore, the QR assay we have developed could be a feasible approach to detect anticancer activity and glucoraphanin content in *Brassica* vegetables.

The variation of glucosinolate content among different varieties of Chinese kale in the current survey is in accordance with previous reports on broccoli and other *Brassica* vegetables and ornamental plants (Verkerk et al., 2009). The genetic variation provides the potential to produce new varieties of Chinese kale with optimal glucosinolate composition and content. The breeding goals in Chinese kale are to achieve milder flavour and to improve health benefits, such as anticarcinogenic potency. But, in view of the fact that gluconapin, a bitterness-related glucosinolate, was the primary glucosinolate, with a level ranging from 46.1% to 85.6% of total glucosinolates among different varieties of Chinese kale in our study, it is difficult to achieve breeding goals through conventional breeding.

The biosynthetic pathway of aliphatic glucosinolates has been basically elucidated. Three important genetic loci (*GSL-OX*, *GSL-ALK*, and *GSL-OH*), identified in *Brassica* species and *Arabidopsis*, contribute to the clarification of the side chain modification pathway of aliphatic glucosinolates (Fig. 1). Flavin monooxygenase (FMO), at the *GSL-OX* locus, is responsible for the conversion of methylthiol glucosinolates to methylsulphinylalkyl glucosinolates. (Hansen, Kliebenstein, & Halkier, 2007). The *AOP2* gene, at the *GSL-ALK* locus, is involved in the alkenylation of methylsulphinylalkyl glucosinolates by removing the methylsulphinyl group and inserting a double bond (Hansen et al., 2008; Li & Quiros, 2003; Neal et al., 2010). The *AOP3* gene, at the *GSL-OH* locus, regulates the hydroxylation of alkenyl glucosinolates (except propenyl glucosinolates) (Hansen et al., 2008; Kliebenstein et al., 2001). Here, in our current survey, gluconapin, an alkenyl product of glucoraphanin, was the major aliphatic glucosinolate in the bolting stems of Chinese kale, while its precursor, glucoraphanin, was detected at low level (Table 1). The results implied that Chinese kale owns a functional *GSL-ALK* allele, which is similar to those reported in *Brassica rapa* and *B. oleracea* (Padilla et al., 2007; Verkerk et al., 2009). By contrast, glucoraphanin is abundant in broccoli as this botanical variety lacks functional alleles at the *GSL-ALK* locus (Neal et al., 2010; Verkerk et al., 2009). Understanding of glucosinolate biosynthesis provides a potential approach to alter levels of specific glucosinolates in plants by metabolic engineering. In the *CYP83A1*-overexpressed transgenic Chinese cabbage plants, the levels of all aliphatic glucosinolates were elevated (Zang, Kim, Park, Kim, & Hong, 2008). It was also reported that the absence of functional *AOP2* and *AOP3* led to the accumulation of methylsulfinylalkyl glucosinolates in *A. thaliana* (Kliebenstein et al., 2001). Our study is the first report about the effect of anti-*AOP2* transformation on glucosinolate profiles and QR activity in Chinese kale. Both glucoraphanin content and QR activity were significantly increased via anti-*AOP2* transformation in Chinese kale. Therefore, improvement of *Brassica* vegetables via metabolic engineering of glucosinolate is feasible.

Although the content of glucoraphanin was increased, the content of its alkenyl product, gluconapin, did not decline, but notably increased in line L4 (Table 4). It is possible that feedback regulation was activated when the *AOP2* gene lost normal function in transgenic plants. Other genes might play roles similar to the *AOP2* gene in transgenic plants, although the alkenyl function is weaker than *AOP2* under normal conditions. Similar results were also observed in the biosynthesis of flavonoids, where anthocyanidin synthase

(ANS), flavonol synthase (FLS) and flavanone 3β-hydroxylase (FHT) had overlapping substrate and product selectivities (Turnbull et al., 2004). Previous studies reported that *AOP2* and *AOP3*, which belong to the 2-oxoacid-dependent dioxygenases genes family, evolved into different functions in the side chain modification of glucosinolates in *Arabidopsis*. However, whether *AOP3* could partly replace the function of *AOP2*, and the difference in evolution of *AOP3* and *AOP2* between *Arabidopsis* and Chinese kale are still unclear. Further investigations are needed to answer these questions and to clarify the mechanism involved.

In conclusion, the total glucosinolate content ranged from 2.95 to 11.6 μmol g^{-1} DW, and gluconapin was the primary glucosinolate in the bolting stems of 14 Chinese kale varieties. QR inducer potency was significantly positively correlated with glucoraphanin content, with a correlation coefficient as high as 0.9431. The glucoraphanin content and QR inducer potency in the anti-*AOP2* transgenic line L5 were notably 4.09-fold and 2.95-fold of those in the wild-type, respectively. However, no significant differences in the contents of other glucosinolates, total phenolics, vitamin C, carotenoids or chlorophyll were found between transgenic plants and wild-type. Therefore, anti-*AOP2* transformation could be an efficient method to enhance the glucoraphanin level and anticancer capacity in Chinese kale.

Acknowledgements

We are grateful to the 985-Institute of Agrobiology and Environmental Sciences of Zhejiang University for providing access to analytical instruments. This work was supported by National High-tech R&D Program of China (863 program 2008AA10Z111), National Natural Science Foundation of China (30320974), and Fok Ying Tong Education Foundation (104034).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.07.073.

References

Andréasson, E., Jørgensen, L. B., Höglund, A.-S., Rask, L., & Meijer, J. (2001). Different myrosinase and idioblast distribution in Arabidopsis and *Brassica napus*. *Plant Physiology, 127*(4), 1750–1763.

Gross, H. B., Dalebout, T., Grubb, C. D., & Abel, S. (2000). Functional detection of chemopreventive glucosinolates in *Arabidopsis thaliana*. *Plant Science, 159*(2), 265–272.

Grubb, C. D., & Abel, S. (2006). Glucosinolate metabolism and its control. *Trends in Plant Science, 11*(2), 89–100.

Halkier, B. A., & Du, L. (1997). The biosynthesis of glucosinolates. *Trends in Plant Science, 2*(11), 425–431.

Halkier, B. A., & Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *The Annual Review of Plant Biology, 57*, 303–333.

Hansen, B. G., Kerwin, R. E., Ober, J. A., Lambrix, V. M., Mitchell-Olds, T., Gershenzon, J., et al. (2008). A novel 2-oxoacid-dependent dioxygenase involved in the formation of the goitrogenic 2-hydroxybut-3-enyl glucosinolate and generalist insect resistance in Arabidopsis. *Plant Physiology, 148*(4), 2096–2108.

Hansen, B. G., Kliebenstein, D. J., & Halkier, B. A. (2007). Identification of a flavin-monooxygenase as the S-oxygenating enzyme in aliphatic glucosinolate biosynthesis in Arabidopsis. *The Plant Journal, 50*(5), 902–910.

Hansen, M., Møller, P., Sørensen, H., & de Trejo, M. C. (1995). Glucosinolates in broccoli stored under controlled atmosphere. *Journal of the American Society for Horticultural Science, 120*(6), 1069–1074.

He, H. J., Chen, H., & Schnitzler, W. H. (2002). Glucosinolate composition and contents in *Brassica* vegetables. *Scientia Agricultura Sinica, 35*(2), 192–197.

Kliebenstein, D. J., Lambrix, V. M., Reichelt, M., Gershenzon, J., & Mitchell-Olds, T. (2001). Gene duplication in the diversification of secondary metabolism: Tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in Arabidopsis. *The Plant Cell, 13*(3), 681–693.

Kushad, M. M., Cloyd, R., & Babadoost, M. (2004). Distribution of glucosinolates in ornamental cabbage and kale cultivars. *Scientia Horticulturae, 101*(3), 215–221.

Lefsrud, M., Kopsell, D., Wenzel, A., & Sheehan, J. (2007). Changes in kale (*Brassica oleracea* L. var. *acephala*) carotenoid and chlorophyll pigment concentrations during leaf ontogeny. *Scientia Horticulturae, 112*, 136–141.

Li, G., & Quiros, C. (2003). In planta side-chain glucosinolate modification in Arabidopsis by introduction of dioxygenase *Brassica* homolog BoGSL-ALK. *Theoretical and Applied Genetics, 106*(6), 1116–1121.

Lozanovski, V., Houben, P., Schemmer, P., & Büchler, M. W. (2013). Sulforaphane and related mustard oils in focus of cancer prevention and therapy. *Wiener Medizinische Wochenschrift, 163*(3–4), 80–88.

Neal, C., Fredericks, D., Griffiths, C., & Neale, A. (2010). The characterisation of AOP2: A gene associated with the biosynthesis of aliphatic alkenyl glucosinolates in *Arabidopsis thaliana*. *BMC Plant Biology, 10*(1), 170.

Padilla, G., Cartea, M. E., Velasco, P., de Haro, A., & Ordás, A. (2007). Variation of glucosinolates in vegetable crops of *Brassica rapa*. *Phytochemistry, 68*(4), 536–545.

Prochaska, H. J., Santamaria, A. B., & Talalay, P. (1992). Rapid detection of inducers of enzymes that protect against carcinogens. *Proceedings of the National Academy of Sciences, 89*(6), 2394–2398.

Sun, B., Liu, N., Zhao, Y., Yan, H., & Wang, Q. (2011). Variation of glucosinolates in three edible parts of Chinese kale (*Brassica alboglabra* Bailey) varieties. *Food Chemistry, 124*(3), 941–947.

Sun, B., Yan, H., Liu, N., Wei, J., & Wang, Q. (2012). Effect of 1-MCP treatment on postharvest quality characters, antioxidants and glucosinolates of Chinese kale. *Food Chemistry, 131*(2), 519–526.

Sun, B., Yan, H., Zhang, F., & Wang, Q. (2012). Effects of plant hormones on main health-promoting compounds and antioxidant capacity of Chinese kale. *Food Research International, 48*(2), 359–366.

Turnbull, J. J., Nakajima, J.-I., Welford, R. W., Yamazaki, M., Saito, K., & Schofield, C. J. (2004). Mechanistic studies on three 2-oxoglutarate-dependent oxygenases of flavonoid biosynthesis. *Journal of Biological Chemistry, 279*(2), 1206–1216.

Verkerk, R., Schreiner, M., Krumbein, A., Ciska, E., Holst, B., Rowland, I., et al. (2009). Glucosinolates in *Brassica* vegetables: The influence of the food supply chain on intake, bioavailability and human health. *Molecular Nutrition & Food Research, 53*(S2), S219.

Wei, J., Miao, H. Y., & Wang, Q. M. (2011). Effect of glucose on glucosinolates, antioxidants and metabolic enzymes in *Brassica* sprouts. *Scientia Horticulturae, 129*, 535–540.

Xu, C. J., Sun, B., Pan, G., & Wang, Q. M. (2008). Improvement of Chinese kale by metabolic engineering of glucosinolate. In: *The 1st international conference on plant secondary metabolism*, 89–90.

Xu, C. J., Guo, R. F., Yan, H. Z., Yuan, J., Sun, B., Yuan, G. F., et al. (2010). Effect of nitrogen fertilization on ascorbic acid, glucoraphanin content and quinone reductase activity in broccoli floret and stem. *Journal of Food, Agriculture & Environment, 8*(1), 179–184.

Xu, C. J., Guo, D. P., Yuan, J., Yuan, G. F., & Wang, Q. M. (2006). Changes in glucoraphanin content and quinone reductase activity in broccoli (*Brassica oleracea* var. *italica*) florets during cooling and controlled atmosphere storage. *Postharvest Biology and Technology, 42*(2), 176–184.

Yan, X., & Chen, S. (2007). Regulation of plant glucosinolate metabolism. *Planta, 226*(6), 1343–1352.

Yuan, G. F., Wang, X. P., Guo, R. F., & Wang, Q. M. (2010). Effect of salt stress on phenolic compounds, glucosinolates, myrosinase and antioxidant activity in radish sprouts. *Food Chemistry, 121*, 1014–1019.

Zang, Y. X., Kim, J. H., Park, Y. D., Kim, D. H., & Hong, S. B. (2008). Metabolic engineering of aliphatic glucosinolates in Chinese cabbage plants expressing Arabidopsis MAM1, CYP79F1, and CYP83A1. *BMB Reports, 41*(6), 472–478.

Zhang, Y., Talalay, P., Cho, C. G., & Posner, G. H. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. *Proceedings of the National Academy of Sciences, 89*(6), 2399–2403.

Zhang, H. F., Xiang, X., Wang, P., & Wang, Q. M. (2006). Rapid detection of anticancer activity of Chinese Kale by quinone reductase bioassay. *Chinese Journal of Cell Biology, 2*, 028.