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Seperation and identification of glucosinolates in Chinese kale by LC/ESI-MS and comparison of their contents in ten cultivars of Chinese kale (*Brassica alboglabra* L.H. Bailey)

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ABSTRACT

A little information is available on the kinds and contents of glucosinolate (GS) in Chinese kale (*Brassica alboglabra* L.H. Bailey), a kind of healthy food widely grown in China. The objectives were to identify the glucosinolates in Chinese kale and determine the variety of glucosinolates in ten cultivars widely grown in China. Eleven glucosinolates were identified in bolting stem of Chinese kale among ten cultivars by LC/ESI-MS. The GSs identified include seven aliphatic GSs and four indolyl GSs. Four very important glucosinolates for cancer chemoprotection, i.e. glucoiberin, glucoraphanin, sinigrin and glucobrassicin, were all detected in Chinese kale. Total glucosinolate content in bolting stem varied significantly ($P < 0.05$) and ranged from 5.21 to 11.92 $\mu\text{mol g}^{-1}$ DW among ten cultivars of Chinese kale. The aliphatic GSs were predominant, representing 81.63% of the total glucosinolate content on average, while indolyl glucosinolates represented 18.37%. Gluconapin was the major glucosinolate in ten cultivars except cv.GZ2. The presence of high concentration of sinigrin, glucoraphanin, glucoiberin, and glucobrassicin in bolting stems warrant further research into their potential use to enhance the level of these important phytochemicals in Chinese kale.

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KEYWORDS

Brassica alboglabra
L.H. Bailey;
Bolting stem;
Glucosinolates (GSs);
LC/ESI-MS;
Cultivars;
Aliphatic GSs;
Indolyl GSs.

INTRODUCTION

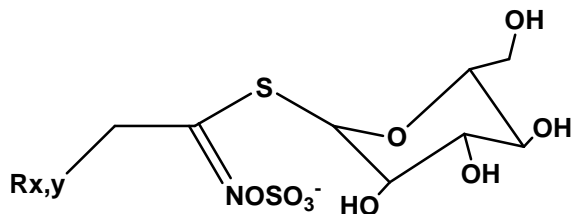
Brassica vegetables are a family of important vegetables species consumed in high quantities throughout the world, being very important for human daily dietary nutrition. Epidemiological data indicated that the risk of

a number of cancers can be significantly reduced by an intake of cruciferous vegetables such as broccoli, Brussels sprouts and cauliflower as little as 10 g day⁻¹[1-4]. More and more researches showed that the cancer-chemoprotective activity of those vegetables is mainly recognized to be due to their high content of

FULL PAPER

glucosinolates (GSs)^[1,5-8]

GSs are a group of plant secondary metabolites mainly detected in six families of angiosperm species and



Note: The side chain $R_{x,y}$ is derived from amino acids

Figure 1 : General structure of glucosinolates

more than 120 GSs has been reported so far^[9]. The variations of glucosinolates structures main occur only in the side-chain $R_{x,y}$ derived from amino acids (Figure 1)^[10]. They usually can be classified into three groups according to the precursor from which they originate, i.e. aliphatic, aromatic and indolyl groups^[11]. When the glucosinolate-containing plant tissue is damaged by food preparation or chewing, GSs are brought into contact with the endogenous enzyme “myrosinase”, results in the formation of a complex variety of biologically hydrolysis active compounds including isothiocyanates, thiocyanates and nitriles, and so on^[12]. Isothiocyanates aroused broad interest, for not only they are chemo-preventive agents against carcinogenesis of the lung and other tissues in laboratory animals, but also they exert protective effects such as inhibition of carcinogen activating enzymes, enhancement of carcinogen detoxifying enzymes and induction of apoptosis^[13]. Chemopreventive properties of GSs and their metabolites have been the motivation for recent efforts to better understand factors that affect their production within the plant.

The concentration and chemical form of GSs in plants can be strong influenced by both genetic and environmental factors^[8,14]. Genetic factors have a direct influence on kinds and content of GSs in vegetables, while environmental conditions may modify the expression of the GSs. However, the genetic background of the product is the major determining factor. The kinds and content of GSs in vegetables depends both quantitatively and qualitatively on their genetic information. In *Brassica.L*, GS patterns in broccoli and cauliflower, green pyramidal cauliflower, violet broccoli, violet and green cauliflower showed higher contents of indolyl GSs than green broccoli, whereas the alkyl GS glucoraphanin was mainly found in green broccoli type compared with other

broccoli and cauliflower types^[15]. However, in radish, no cultivar differences in the GS patterns occurred. The alkenyl GS glucoraphasatin was always the main GS present although in different concentrations^[8].

Chinese kale belongs to *Brassica.L* is only produced in China and planted in China. In the past decades, Chinese kale has spread quickly and shows increasing economic potential because of its good taste and rich content in carotene, VC, microelements human needs, and falconoid in bolting stems that are key components of the human diet of Chinese kale. However, there is a little information available on the kinds and their contents in bolting stem of Chinese kale, especially the contents among different cultivars^[16-18]. With the increased interest in diet and human health, it is necessary to have information of profiles and levels of GSs in Chinese kale. These compounds are of interest to improve the quality of the product with the aim to develop new cultivars with an appropriate GS profile from which high quality products with an added value can be produced. The most promising varieties for future breeding purposes would be those with the high total GS content and, especially, with the high content of GSs related to beneficial effects.

The objectives of this study were: (1) to separate and identify the GSs in bolting stem of Chinese kale and (2) to evaluate the content in ten cultivars of Chinese kale popular planted in China.

EXPERIMENTAL

Plant growth

The seeds of cv.GZ1, GZ2, GZ3 and GZ4 were bought from Guangzhou vegetable research center, while other cv. ST1, ST2, ST3, ST4, ST5 and ST6 were provided by Linong vegetable seeds research center. Each cultivar seeds of Chinese kale (*Brassica alboglabra L. H. Bailey*) were sown in a container full of peat and vermiculite (3:1) and allowed to germinate in the greenhouse with computer controlled growing conditions on Huajiachi Campus of Zhejiang University in Hangzhou, China. The growth conditions were as follow: relative humidity of 65%, day/night temperature of 23/18°C and photosynthetically active radiation of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h per day. After four weeks, the seedlings with three true leaves were transferred into the field. Before transplant, 120 kg N ha⁻¹, 60 kg P₂O₅ ha⁻¹ and 100 kg k₂O ha⁻¹ fertilizers were applied to the soil. There were four random split plots

with 2.25 m² for each cultivar. The distance for each plant was 25 cm. After 35 days, four plants in each pot were selected for GS analysis. Bolting stems of Chinese kale were cut and frozen immediately in liquid nitrogen. After being lyophilized and ground into powder, the samples were stored in a desiccator at -20°C prior to analysis.

Extract preparation of GSs

Samples for GS analysis were prepared according to the procedure described by Xu's description with some modifications^[19]. Duplicate of freeze-dried powder samples (0.1 g) were extracted with 1.5 mL methanol–water (70:30, v/v). The extracts were heated at 70 °C in a heating bath for 10 min. Then the extracts were centrifuged at 5000 g for 10 min at 4°C. The supernatant was refrigerated while the pellet was extracted a second time with 3 mL of methanol–water (70:30, v/v), heated at 70°C and centrifuged using the previous conditions. The two supernatants were combined and refrigerated. The desulphation reaction was performed with mini-columns prepared with DEAE Sephadex A-25 (100 mg) (170170-01, Amersham Biosciences, Sweden) and 0.5 mol L⁻¹ pyridine acetate to have a 0.5 mL bed volume. Columns were washed with 0.5 mol L⁻¹ pyridine acetate and with ultra-pure water. Subsequently, 2.0 mL of the GS extract was added to the column. The unbound material was removed washing with 0.02 mol L⁻¹ pyridine acetate (pH 4) and ultra-pure water, then 100 µL sulphatase (S9626, Sigma-Aldrich Co., MO, USA) was loaded in the column and desulphation was performed overnight (16 h) at room temperature. The desulpho-GSs were eluted with 2.0 mL ultra-pure water and stored -20 °C before analysis. 2-Propenyl GS (sinigrin) (E.C. 3.1.6.1, type H-1, Helix pomatia, Sigma-Aldrich Co., MO, USA) was used as an external standard.

HPLC analysis

20 µL of the desulphation extract were analyzed by HPLC (Beckman Coulter, Inc. System Gold HPLC, Beckman, America) at a flow rate of 1 mL min⁻¹ using a Hypersil ODS2 column (250 mm × 4.6 mm, 5 µm; Elite, China) and the temperature of the column was set at 35°C. The eluate was detected at 227 nm by a UV detector (Beckman). The mobile phase was a mixture of ultra-pure water (A) and acetonitrile (B). Desulpho-GSs elution was achieved using the solvent program consisted of constant a linear gradient from 0 to 20% in 18 min, then kept constant at 20% B for 15 min^[20].

ESI-MS analysis

The mass spectrometry (MS) data were obtained by electrospray under atmospheric pressure using a HPLC-MSD system (Agilent 1100 LC/MSD, Agilent Company, USA). The conditions used for the electrospray source were described as follows: ionspray voltage, 4.8 KV (positive mode); orifice voltage, 40 V; capillary voltage, 4 KV; nebulizer gas, air; curtain gas, nitrogen; drying gas flow, 13 L min⁻¹, desolvation gas temperature, 350 °C^[18].

Statistical analysis

For data analysis, one-way analysis of variance (ANOVA) was performed with a significance level of $P < 0.05$ by software SPSS for windows, version 11.0 (SPSS INC., Chicago, IL).

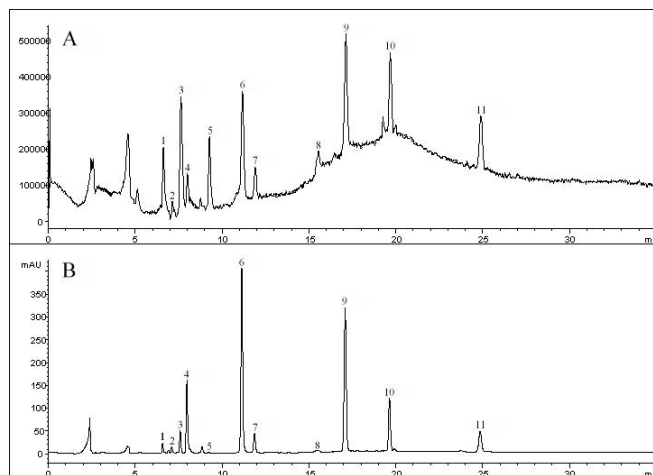
RESULTS AND DISCUSSION

HPLC profile of desulphoGSs separated and total ion current (TIC) chromatogram of bolting stem in vegetable Chinese kale are shown in Figure 2. The desulpho-GSs share a common glucosyl structure, which produces the loss of a neutral glucosyl fragment (G, m/z 162.1) under collision activation conditions, so their protonated molecular ions [M-G+H]⁺ fragmentation is a typical indicator in the identification of desulpho-GSs in ESI spectrum^[21]. Moreover, protonated molecular ions [M+H]⁺, [M+Na]⁺ and [M+K]⁺ were also available in ESI spectrum for the identification of desulpho-GSs^[22]. The molecular ion region of electrospray ionization mass spectrometry of aliphatic desulpho-glucoraphanin and indolyl desulpho-glucobrassicin in bolting stem of Chinese kale was shown in Figure 3 as detailed examples.

Identified GSs

Eleven GSs were identified, and their retention time, side-chain structure, trivial names, desulpho MW, and their response factors are given in TABLE 1. All the GSs were identified by analyzing the chemical structure of the aglucone chain *R* (Figure 1), and they will be described according to their trivial names used internationally for a long time. The 14 GSs response factors were determined by the International Organization for Standardization (ISO) scientifically in 1992 to calculate the individual GS content by HPLC corresponding

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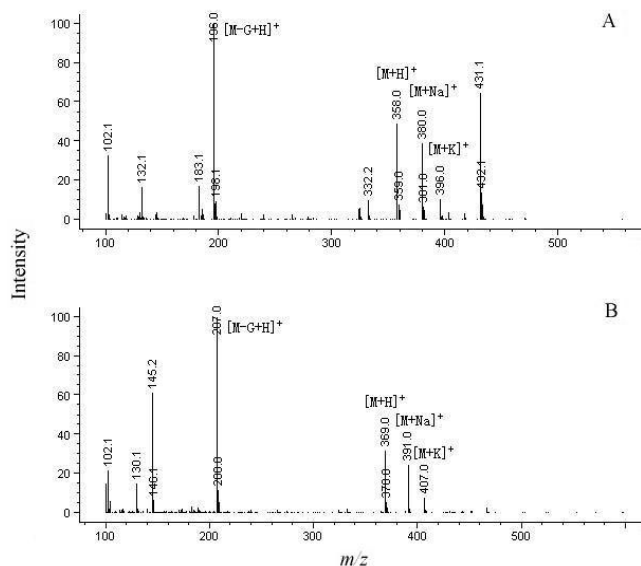


Note: A, Total ion current of protonated molecules; B, absorbance at 227 nm. Peak numbers refer to GSs listed in TABLE 1.

Figure 2 : Typical HPLC elution profile of desulpho-GSs in bolting stem in Chinese kale

to sinigrin as a standard^[23]. And the response factors of the GSs unreported by ISO were denoted by 1.00. By using these response factors, the GSs content was calculated from the peak area of a compound with an unknown proportion relative to that of sinigrin.

In our study, 11 peaks were detected by LC/ESI-MS in the collection of Chinese kale varieties studied consisted of consisted of 7 aliphatic GSs (1: glucoiberin, 2: progoitrin, 3: glucoraphanin, 4: sinigrin, 5: glucoalyssin, 6: gluconapin and 8: glucoerucin) and 4 indolyl GSs (7: 4-hydroxyglucobrassicin, 9:



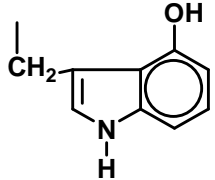
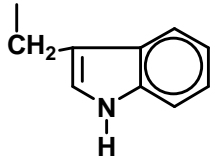
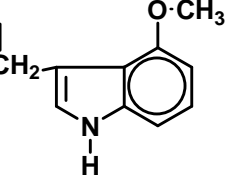
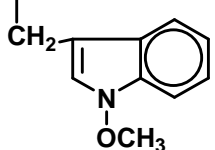
Note: (A) Spectrum of desulpho-glucobrassicin ($[M+H]^+$, m/z 368); (B) Spectrum of desulpho-glucobrassicin ($[M+H]^+$, m/z 369). Spectrum were recorded between m/z 100 and 600.

Figure 3 : Molecular ion region of electrospray ionization mass spectrometry of desulpho-glucoraphanin and desulpho-glucobrassicin isolated from bolting stem of Chinese kale

glucobrassicin, 10: 4-methoxyglucobrassicin and 11: neoglucobrassicin). There were a few reports about the GSs in Chinese kale. He *et al.*^[16] detected 7 intact GS in bolting stem of Chinese kale. Recently, Chen *et al.*^[17] reported another three new aliphatic desulpho-GSs in Chinese kale. In this study, a new kind of aliphatic GSs (glucoerucin) was firstly detected in bolting

TABLE 1 : Identified desulpho-GSs in bolting stem of Chinese kale

NO.*	Retention time (min)	Side-chain structure	Trivial name	Desulpho-MW	Response factor [†]
1	6.58	$\text{CH}_2\text{CH}_2\text{CH}_2-\text{S}-\text{CH}_3$ O	Glucoiberin	343	1.07
2	7.10	$\text{CH}_2\text{CH}(\text{OH})\text{CH}=\text{CH}_2$	Progoitrin	309	1.09
3	7.59	$\text{CH}_2(\text{CH}_2)_3-\text{S}-\text{CH}_3$ O	Glucoraphanin	357	1.07
4	7.98	$\text{CH}_2\text{CH}=\text{CH}_2$	Sinigrin	279	1
5	9.24	$\text{CH}_2(\text{CH}_2)_4-\text{S}-\text{CH}_3$ O	Glucoalyssin	371	1.07
6	11.13	$\text{CH}_2\text{CH}_2\text{CH}=\text{CH}_2$	Gluconapin	293	1.11

NO.*	Retention tme (min)	Side-chain structure	Trivial name	Desulpho-MW	Response factor [†]
7	11.86		4-Hydroxyglucobrassicin	384	0.28
8	15.54	$\text{CH}_2(\text{CH}_2)_2\text{CH}_2\text{-S-CH}_3$	Glucocerucin	341	1 [‡]
9	17.08		Glucobrassicin	368	0.29
10	19.64		4-Methoxyglucobrassicin	398	0.25
11	24.86		Neoglucobrassicin	398	0.2

*Note: Elution order of desulphoGSs from HPLC. † The International Organization for Standardization (ISO 9167-1, 1992). ‡ Not yet decided by the ISO.

stem of Chinese kale besides all the GSs compounds reported before in Chinese kale.

GSs content

11 GSs were detected in all the ten cultivars. Chinese kale showed significant differences for total GS content and for all individual GSs ($P < 0.05$). The total GS content ranged from $5.21 \mu\text{mol g}^{-1}$ DW (cv.GZ1) to $11.92 \mu\text{mol g}^{-1}$ DW (cv.ST5) (TABLE 2), with a mean value of $8.38 \mu\text{mol g}^{-1}$ DW. The levels are lower than those found in He's study^[16], but very similar to Chen's and La's results^[17,18].

Aliphatic GSs were predominant, representing 81.63% of the total GS content on average, while indolyl GSs represent 18.37% (Figure 4). The value of indolyl GSs was much smaller because the wavelength of the maximum absorption of indolyl desulpho-GSs is in the short range (219-221 nm) compared to that of other desulpho-GSs (225-230 nm)^[24]. Gluconapin was the major GS in each of the ten cultivars except cv.GZ2 (TABLE 2). In cv.GZ2, glucoraphanin account for 29.92% of total GS content, a little more than gluconapin that accounted for 29.34% of total GSs content. The

presence of glucoraphanin in bolting stem of Chinese kale should be studied more extensively, because this aliphatic GS is the precursor of sulforaphane which is considered to be one of the most potent inducers of phase ϕ proteins^[5]. The third and fourth GS in cv.GZ2 is progoitrin and glucocerucin, accounted for 8.92% and 6.55% respectively. Progoitrin, described as potentially goitrogenic for livestock, did not appear to have a health risk associated with consumption of these vegetables for human^[25]. Glucocerucin possess direct antioxidative activity because of its ability to decompose hydroperoxides and hydrogen peroxide^[26].

In cv.GZ1, ST2, ST3, ST5, GZ3 and GZ4, sinigrin was the second GS in abundance, which represented 17.65% of total GS content on average, followed by glucoraphanin in cv.GZ1, GZ3, GZ4 and ST3 while followed by glucoiberin in cv.ST5 and 4-Methoxyglucobrassicin in cv.ST2. Isothiocyanates derived from the hydrolysis of sinigrin and glucoiberin has been found to have anti-carcinogenic and anti-mutagenic effects^[27,28]. Moreover, it is known that isothiocyanates derived from sinigrin can cause a reduction in the cholesterol levels in mice^[29]. Another beneficial effects at-

FULL PAPER

tributed to sinigrin is its role as suppressor of the growth of nematodes, fungi, and other soil microorganisms^[8], although this GS also contribute, as well as glucoiberin and gluconasturtiin, to the presence of some specialist pests. In cv.ST1 and ST4, glucoraphanin was the second GS, followed by sinigrin.

Glucobrassicin is the parent compound of indole-3-carbinol which has been proven along with the sulforaphane as the most potent anticancer compounds found in cruciferous vegetables^[30]. In cv. ST6, glucobrassicin was the third GS in abundance following glucoraphanin, which represented 9.60% of total GS content. However, the highest glucobrassicin content ($1.03 \mu\text{mol g}^{-1} \text{DW}$) in the ten cultivars of Chinese kale is the variety ST2.

Other GSs detected in bolting stem of Chinese kale such as glucoalyssin, 4-hydroxyglucobrassicin was in a small amount (TABLE 2).

Because of the beneficial effects on human health attributed to sinigrin, glucoraphanin, glucoiberin and glucobrassicin, the most promising varieties for future breeding purposes would be those with the highest contents in the GSs above mentioned besides the total GS content. In ten cultivars, the highest content of total GSs

is cv. ST5 ($11.92 \mu\text{mol g}^{-1} \text{DW}$) while the highest content of the four mentioned important GSs is cv. GZ2 ($4.71 \mu\text{mol g}^{-1} \text{DW}$). Moreover, plant stages and plant parts should be considered when planning harvest or when making breeding selections for GS concentrations, because GS concentration changes with plant age and developmental stage^[31,32].

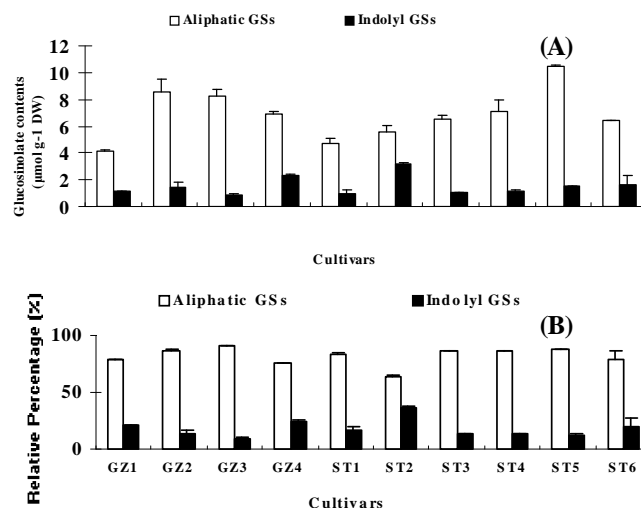


Figure 4 : The content (A) and relative percentages (B) of total aliphatic and total indolyl GS content in bolting stem of Chinese kale among 10 cultivars

TABLE 2 : Total and individual GSs content ($\mu\text{mol g}^{-1} \text{DW}$) in bolting stem of 10 Chinese kale varieties

Cultivars	GIB	PRO	GRA	SIN	GAL	GNP	4HGB	GRU	GBS	4MGB	NGBS	Total GSs
ST1	0.46±0.04c	0.14±0.03c	0.91±0.14de	0.64±0.07bc	0.11±0.04c	2.24±0.19ef	0.037±0.01bc	0.16±0.05cd	0.44±0.11cd	0.30±0.09c	0.22±0.07de	5.66±0.68e
ST2	0.33±0.01cd	0.31±0.06c	0.76±0.07e	1.09±0.06ab	0.08±0.00c	2.88±0.32de	0.02±0.01c	0.16±0.01cd	1.03±0.18a	1.08±0.19a	1.03±0.05a	8.76±0.48bcd
ST3	0.68±0.04b	0.81±0.02ab	0.87±0.05e	1.53±0.10a	0.11±0.08c	2.45±0.41e	0.05±0.00bc	0.11±0.00d	0.51±0.01c	0.34±0.00c	0.14±0.04e	7.59±0.26d
ST4	0.64±0.15b	0.49±0.04bc	1.2±0.25cd	0.73±0.14bc	0.24±0.07b	3.35±0.25bcd	0.03±0.01bc	0.44±0.01b	0.47±0.06cd	0.37±0.01bc	0.26±0.00d	8.24±0.91cd
ST5	1.13±0.11a	1.02±0.55a	1.75±0.05b	0.61±0.04c	0.43±0.05a	5.03±0.54a	0.03±0.01bc	0.45±0.00b	0.72±0.07b	0.50±0.16bc	0.23±0.01de	11.92±0.24a
ST6	0.20±0.07d	0.29±0.04c	0.90±0.03de	0.52±0.16c	0.16±0.04bc	4.05±0.18b	0.12±0.03a	0.29±0.06c	0.77±0.06b	0.62±0.08b	0.15±0.02e	8.07±0.59cd
GZ1	0.19±0.02d	0.10±0.03c	0.93±0.23de	0.97±0.05bc	0.11±0.02c	1.58±0.09f	0.09±0.00a	0.22±0.00cd	0.45±0.03cd	0.36±0.05bc	0.22±0.02de	5.21±0.18e
GZ2	0.20±0.05d	0.89±0.15ab	2.97±0.17a	0.56±0.04c	0.35±0.08a	2.92±0.25cde	0.05±0.02bc	0.66±0.17a	0.32±0.08d	0.62±0.26b	0.44±0.04c	9.97±1.32b
GZ3	0.46±0.02c	0.96±0.11a	1.28±0.05c	1.45±0.06a	0.07±0.00c	3.93±0.47b	0.06±0.00b	0.11±0.03d	0.35±0.03cd	0.29±0.00c	0.19±0.03de	9.15±0.39bc
GZ4	0.29±0.02d	0.20±0.03c	0.88±0.09e	1.46±0.20a	0.05±0.02c	3.58±0.09bc	0.10±0.01a	0.47±0.07b	0.80±0.04b	0.61±0.02b	0.77±0.09b	9.21±0.34bc

Within each column values followed by the same are not significantly different at $P < 0.05$. Values are mean \pm standard deviation ($n = 3$). GIB, glucoiberin; PRO, progoitrin; GRA, glucoraphanin; SIN, sinigrin; GAL, glucoalyssin; GNP, gluconapin; 4HGB, 4-hydroxyglucobrassicin; GRU, glucoerucin; GBS, glucobrassicin; 4MGB, 4-methoxyglucobrassicin; NGBS, neoglubrassicin.

CONCLUSIONS

Among ten cultivars of Chinese kale, 11 kinds of GSs were identified in bolting stem including 7 aliphatic GSs and 4 indolyl GSs. Among ten cultivars, there were significant differences for total GS content and for all individual GSs in bolting stem among them ($P < 0.05$). The total GSs content in bolting stem among ten culti-

vars ranged from 5.21 to $11.92 \mu\text{mol g}^{-1} \text{DW}$, with a mean value of $8.38 \mu\text{mol g}^{-1} \text{DW}$. The aliphatic GSs represented 81.63% of the total GS content on average, while indolyl GSs represent 18.37%. Gluconapin was the major GS in each of the ten cultivars except cv.GZ2. In cv.GZ2, glucoraphanin account for 29.92% of total GS content, a little more than gluconapin that accounted for 29.34% of total GSs content. Regarding both the high content of sinigrin, glucoraphanin,

glucoiberin, and glucobrassicin and total GS content, two varieties ST5 and GZ2 could be good sources of beneficial GSs for further breeding.

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