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

Hongmei Qian [1], Bo Sun [1], Huiying Miao, Congxi Cai, Chaojiong Xu, Qiaomei Wang *

Department of Horticulture, Zhejiang University, Hangzhou 310058, China
Key Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Ministry of Agriculture, Hangzhou 310058, China

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

* Corresponding author. Address: Department of Horticulture, Zhejiang University, 866 Yuhangtang Road, Hangzhou, Zhejiang 310058, China. Tel.: +86 571 8590 9333; fax: +86 571 8898 2278.
E-mail address: qmwang@zju.edu.cn (Q. Wang).
[1] Both authors contributed equally.

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

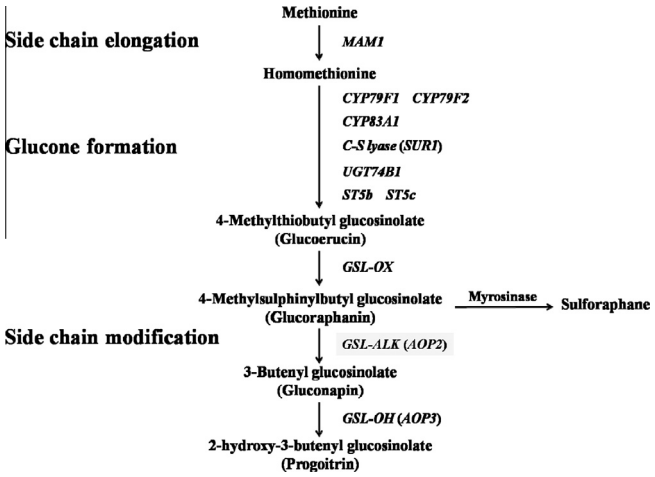

Fig. 1. The metabolic pathway and the related genes involved in biosynthesis of glucoraphanin in *Brassica oleracea* vegetables.

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