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Development and Identification of Anti-cancer Component of Sulforaphane in Developmental Stages of Broccoli (*Brassica oleracea* var. *italica* L.)

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Abstract Broccoli is a rich source of sulforaphane, an anti-cancer component. In the study, four broccoli lines were chosen to characterize the variation of sulforaphane among different organs and developmental stages by RP-HPLC. The result showed different changing rule of sulforaphane contents in the whole develop period. The contents in leaves were in the lowest level of the whole period, peaking 28 to 33 days after planting. The content of sulforaphane in florets was correspondingly higher in leaves part during mature stage. An interesting phenomenon happened in developmental buds, and the contents of sulforahane in buds decreased gradually from the top buds to mature buds, to buds one day before flowering and to flowers at bolting stage. Another new finding was that they increased gradually in seed pod and decreased in pod areas at developmental pod stage. Sulforaphane in seedlings decreased gradually in first ten days and then began to rise. Ripe seed contained the highest sulforaphane (3051.03 to 4624.63 mg·kg^{-1} FW). The study revealed the variation of sulforaphane content in developmental stages of broccoli, and the result provided scientific basis for anti-cancer research and human nutrition.

Keywords: *Sulforaphane, Broccoli, Glucoraphanin, Development cycle, HPLC*

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1. Introduction

Recent evidence from epidemiological and experimental studies has shown that cruciferous vegetables, such as broccoli, cabbage, kale and Brussels sprouts, are rich in glucosinolate, especially the phytochemical, glucoraphanin [1,2,3,4]. Glucoraphanin (4-methylsulfinylbutyl glucosinolate) is methionine-derived glucosinolate. When cruciferous vegetables are chewed or chopped, glucoraphanin can be hydrolyzed by myrosinase enzyme (thio-glucoside glucohydrolase) to form sulforaphane (4-methylsulfinyl-3-butenyl isothiocyanate), and its formation depends on pH, hydrolysis time and temperature [5,6]. There are different breakdown products in different condition, such as isothiocyanates (ITCs), thiocyanates, nitriles, epithionitriles and oxazolidines [7,8]. At the same time, some studies have proved that the main hydrolyzed product at neutral condition is sulforaphane [9,10]. Evidence indicates that the consumption of broccoli (*Brassica oleracea* var. *italica* L.), such as florets, seedling and their extract sulforaphane, can reduce the risk of several kinds of cancers, such as stomach [11], lung [12], breast [13] and colorectal [14], as well as myocardial infarction and other cardiovascular diseases [15].

A great deal of research into sulforaphane and anti-carcinogens in broccoli has been reported [16,17]. According to animal experiments, broccoli and its extracted sulforaphane are effective functional foods or anti-carcinogens through inducing Phase II enzymes coupled with inhibiting a Phase I enzyme (cytochrome P450) [18]. Some studies have also found that sulforaphane, acting as an indirect antioxidant, can induce cell cycle arrest and apoptosis of many cancer cell types, such as colon, prostate, lymphocyte, stomach and mammary [19].

Current studies on glucoraphanin or sulforaphane have mostly focused on *Brassicaceae* such as broccoli, cabbage, Brussels sprouts, collards, kale, turnip greens and radishes. Glucosinolates and isothiocyanates were first found in mustard seeds at the beginning of the 17th century. At present, over 120 types of glucosinolate have been found in *Brassicaceae* plants [2,20]. Kushad examined a set of 24 cultivars and 26 inbred lines of broccoli and found a glucoraphanin content from 1.5 to 21.7 μmol/g DW for cultivars and from 0.8 to 13.8 μmol/g DW for inbred lines [21]. Some studies reported that the sulforaphane content in broccoli (14.6 mg/kg FW) was generally higher than in cabbage (3.0 mg/kg FW); and that the average sulforaphane content in broccoli florets (12.9 mg/kg FW) was higher than in stems (5.1 mg·kg^{-1} FW) and in leaves (1.5 mg·kg^{-1} FW) [9]. Most studies have agreed that broccoli seeds contain higher levels of sulforaphane or glucoraphanin than other plants with the highest content reported as 1.4 mg/kg DW [22]. Some studies have found a higher level of sulforaphane or glucoraphanin content in

broccoli seedlings (1 to 2 days after germination) and that these levels decreased during growth from 2 to 14 days [16,23]. Gorissen have demonstrated that sulforaphane was not biosynthesised *de novo* during the first week of seedling development using a unique stable isotope technique (C^{12}/C^{13}- cross experiments) and also proved that sulforaphane originates exclusively from the initial accumulation in the seed [24]. The study also explained why broccoli seed usually contains the highest content of sulforaphane or glucoraphanin.

Most reports on sulforaphane or glucoraphanin usually focus on one or several periods during broccoli growth or comparing some plant organs during one period. The variation of sulforaphane content in different organs or tissues during the broccoli growth and development cycle is unclear. The first objective of the present study was comprehensively to determine the sulforaphane content in different organs and tissues of broccoli during its growth and development cycle. The second objective was as simply to determine if sulforaphane content is cultivar dependent. The third objective was to reveal the preliminary changing rule of sulforaphane contents in its growth and development cycle of broccoli, finally according to the result to explain any new phenomena and make further conclusions.

2. Materials and Methods

2.1. Plants and Reagents

Broccoli (*Brassica oleracea* L. var. *italica*) seeds were cultivated by the Institute of Vegetables and Flowers, Chinese Academy Agricultural and Sciences (IVF, CAAS). Four broccoli lines of cultivars were chosen for the study; two inbred lines (B691 and B692) and two F_1 hybrids (B693 and B694).

The sulforaphane standard was purchased from LKT Laboratories Inc (St. Paul, MN, USA). The HPLC-grade methanol was supplied by Sigma-Aldrich Company (St. Louis, MO, USA) and ultra-pure water using a Milli-Q quality water system (Millipore, Bedford, MA, USA). Analytical grade phosphates and ethyl acetate were purchased from Beijing Chemical Works (Beijing, China).

A LC-20A HPLC system (Shimadzu, Kyoto, Japan) equipped with an SPD-20 UV detector and a reverse-phase C_{18} column (250×4.6mm, 5μm, Shiseido, Tokyo, Japan) and a Rotavapor (RII, Büchi Labortechnik AG, Flawil, Switzerland).

2.2. Plant Growth and Development

Sprout period: The young sprout plants were collected on August 8th, 2013, and then every five days until being planted in the field.

Growing period: The broccoli leaves were sampled on September 10th, then again every 5 days for a total of twelve times.

Mature period: Mature florets and leaves were gathered from more than five plants from the 3 batches from November 2nd to 10th, 2013.

Bolting period: When all the plants had reached the bolting period, different development buds and flowers were collected from the same branch, to include young buds, mature buds, flowers one day before flowering and flowers.

Silique period: After twenty days of pollination, enough pod areas and silique-form seeds had been separated and collected. Then every five days, the different development pod areas and silique-form seeds were collected consecutively.

Seed period: Ripe seeds of the inbred and hybrid broccoli lines were recovered at the appropriate time, about sixty to seventy days after pollination.

Seedling period: Broccoli seeds were sown on water-soaked filter paper, and the soil substrate contained vermiculite, peat and clay (1:3:4). An alternating photoperiod of day and night (16/9) at 25 ± 1°C and a relative humidity of 80 % were used for seedling with the light intensity of 2000 lux. The first sample seedling was collected one day after germination and on each of the following fourteen days.

2.3. Sample Preparation

All the fresh materials were gathered and lyophilized using a freeze drier. The dried samples and seeds were crushed into powder and held at a low temperature (4°C) in sealed bags.

2.4. Extraction of Sulforaphane

The method was referenced Liang's report [9], at the same time, we had optimized the extraction method by orthogonal test including factors of materials to buffer (PBS - Phosphate Buffered Saline), pH value, reaction time at 25°C temperature (unpublished).

2.5. HPLC Analysis

The Shimadzu LC-20A HPLC system was equipped with a SPD-20 UV detector and a reverse-phase C_{18} column (250×4.6 mm, 5 μm, Shiseido). The gradient mobile phase consisted of 5 % tetrahydrofuran for pump A and 100 % methanol for pump B. The solvent for pump B was initially set at 40 %, and then changed linearly to 60 % by the tenth minute, then subsequently returned to full methanol (100 %) after a further 10 minutes, kept at 100 % for 15 minutes at a flow rate of 0.80 $mL \cdot min^{-1}$, finally returning to the initial condition. The absorbance value was 254 nm and the column oven temperature was at 32°C [9,10].

Ten milligram (mg) of a sulforaphane standard was dissolved in 10 mL methanol to generate a dilution series: 5, 25, 50, 100 and 200 $\mu g \cdot mL^{-1}$. The precision of the system was measured by standard peak areas (n = 6, 100 $\mu g \cdot mL^{-1}$) and the recovery was defined by adding standard samples (100 $\mu g \cdot mL^{-1}$) at known concentration (5.51, 12.55, 20.43, 45.51, 60.27, 80.69 $\mu g \cdot mL^{-1}$) of the samples (n = 6).

2.6. Data Treatment

The experimental data and all the derived figures were treated and created using Sigma Plot 10.0 (version 10.0). After one-way ANOVA, the Tukey post-hoc test was performed to test the significance of the differences between mean values using a probability level of $p < 0.05$. The test data were shown as mean values ± SD (n = 3).

3. Results and Discussions

3.1. HPLC System Accuracy

The calibration results showed that there was a good linear relationship in the range of the 5 to 300 μg/mL standard solutions: the regression equation was:

$$Y = 3.422 \times 10^{-4} \times X + 0.940 \left(R^2 = 0.9999 \right)$$

Where Y stands for sulforaphane concentration (mg·L^{-1}), X stands for peak area (UV/min). The peak areas and relative standard deviation (RSD) of 0.01% (peak area: 380000, 380056, 380100, 380110, 380072 and 380090) indicated that the precision was acceptable. Comparing the known samples and the standard concentrations, the average recovery from six tests was 96.3% (recovery: 98.1, 95.1, 95.3, 96.4, 95.2 and 97.7%) (RSD = 1.3%, n = 6). The limit of quantification was 0.045%.

The results from RP-HPLC showed that the peaks from four lines were consistent with the standard, and that the retention time was 6.10 min with no impurity interference (Figure 1).

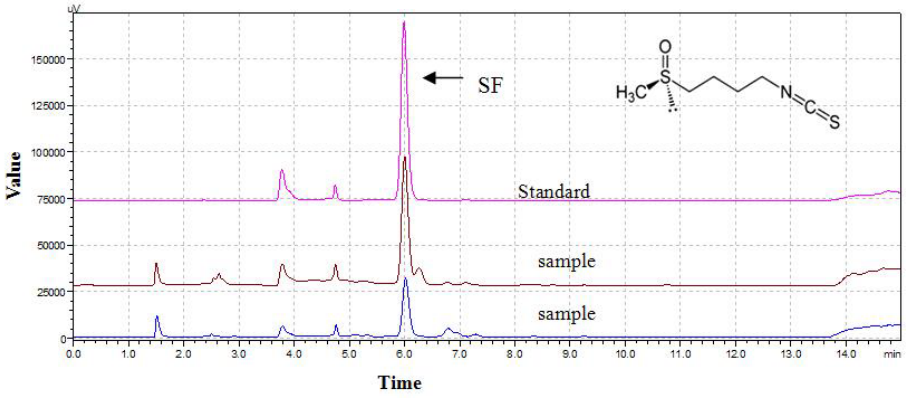

Figure 1. HPLC chromatograms of sulforaphane (SF) in standard and extract samples

3.2. Diversity of Sulforaphane during the Broccoli Growth and Development Cycle

3.2.1. Sprout Period

The data showed that the sulforaphane content of broccoli leaves from the seedling period ranged from 11.42 to 92.23 mg·kg^{-1} FW before being planted in the field. The leaves from B691, B692, B693 and B694 had significantly different levels from the 1st day to the 21st day (Figure 2). The two highest levels occurred during this period on days 6 and 21. The highest content was found on the 6th day: for B691, B692, B693 and B694, these were 92.23, 59.97, 78.15 and 76.29 mg·kg^{-1} FW respectively. The highest content was B691, and the lowest was B692. The second highest content occurred on the 21st day, but with lower contents from the four lines than those collected on the 6th day. Sulforaphane contents were lower between the 6th and 21st days. The phenomenon might be affected by expression genes of glucoraphanin regulation genes or decreasing activity of myrosinase. The glucoraphanin generation was regulated by many family genes, such as *BCAT*, *MAM*, *IPMI*, *CYP*, *GST*, *FMO*, and *AOP* and so on. In the sprout stage, vegetative growth and differentiation of organs and tissues were dominant, so these family genes were not only responsible for the generation of glucoraphanin, but also for their family members of indole and aromatic glucosinolate. Meanwhile in the stage, monooxygenase activities were found in young expanding leaves with the highest activity, but the activities decreased rapidly as the leaves reached full expansion and matured. So the changes of sulforaphane in sprout were detected during these days, which should be a good expansion in the special stage.

The sulforaphane content in the seeds was higher than in the leaves, because during seed germination, most sulforaphane is broken down, and not re-synthesized. Therefore the sulforaphane content was higher in seedlings before field planting than in the leaves after field planting [24].

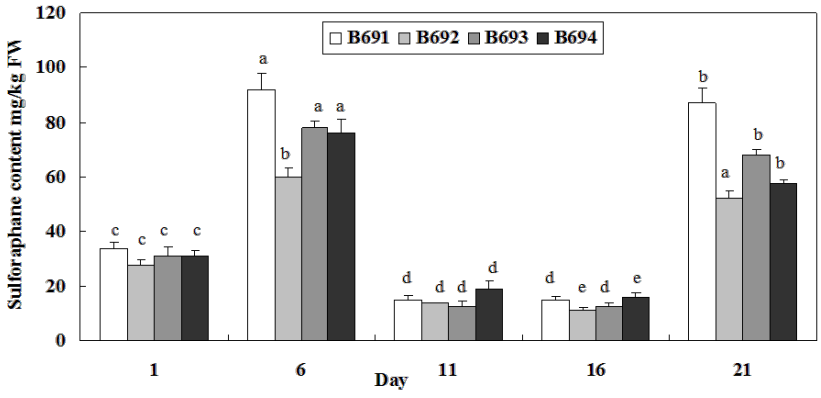

Figure 2. Variation in sulforaphane content of broccoli leaves from young plants with time (1 to 21 days before planting) and lines. Values shown by bars with different letters are significantly different in different days among the same lines ($p < 0.05$)

3.2.2. Leaves in Adult Plant

The sulforaphane content of leaves in the four broccoli lines ranged from 0 to 306.23 mg·kg^{-1} FW, with significant differences among 56 days (p<0.05) (Table 1). There were large changes during the period, the early and late sections with lower contents and the middle section with a higher content. From Table 1, it can be observed that B691 had a low sulforaphane content (4.51 mg·kg^{-1} FW) in the 1st day, while no sulforaphane was detected in the other three lines. By the 6th day, sulforaphane was detected in all four lines, the highest level in B693 (62.39 mg·kg^{-1} FW) and the lowest in B692 (11.57 mg·kg^{-1} FW), with B691 and B694 at 44.40 and 32.00 mg·kg^{-1} FW, respectively. By the 21st day, the contents in all lines had greatly risen. B691 had increased to 306.23 mg·kg^{-1} FW, almost ten times higher than in the 16th day sample with the other three lines all relatively higher. By the 26th day, the content in B691 had fallen to 137.70 mg·kg^{-1} FW, with the other three lines, especially B694 showing rising levels. The content fell to very low levels by the 36th and 41st day. After rising on the 46th day and a downward trend on the 51st day, by the 56th day, the content began to rise again. From the consistent change of all plants, the result might be affected by lower temperature in these days, the temperature of these days (15 to 20°C) were lower than earlier days (18 to 27°C), and then the temperature changed from 18 to 28°C on 56th day. Broccoli sprouts grown at 25°C contained higher content of sulforaphane than at 20 and 30°C [26]. In addition, the contents of glucosinolate were affected by developmental stages, the older leaves usually had lower glucosinolate concentrations than younger leaves in broccoli [27].

Table 1. Variation in sulforaphane contents in leaves of adult plants use superscript for statistical letters

Lines [a]	Sampling of days [b]											
	1	6	11	16	21	26	31	36	41	46	51	56
B691	4.51 ± 0.07 [f]	44.40 ± 5.16 [d]	45.62 ± 8.71 [d]	30.28 ± 1.41 [e]	306.23 ± 26.02 [a]	137.70 ± 8.92 [b]	113.32 ± 4.46 [c]	2.49 ± 0.02 [g]	ND	15.23 ± 1.24 [e]	ND	103.09 ± 12.27 [c]
B692	ND [d]	11.57 ± 1.08 [g]	18.21 ± 3.47 [g]	32.00 ± 1.12 [f]	82.10 ± 3.66 [c]	85.22 ± 4.94 [b]	102.49 ± 4.02 [a]	2.89 ± 0.18 [h]	2.61 ± 0.07 [h]	47.59 ± 1.51 [e]	ND	90.63 ± 2.16 [b]
B693	ND	62.39 ± 5.02 [d]	26.41 ± 2.83 [f]	41.46 ± 2.00 [e]	90.92 ± 3.28 [c]	187.58 ± 26.91 [a]	106.60 ± 3.71 [b]	0.52 ± 0.01 [h]	1.47 ± 0.60 [h]	24.48 ± 1.04 [f]	2.96 ± 0.21 [g]	59.01 ± 2.19 [d]
B694	ND	32.00 ± 3.49 [f]	27.49 ± 1.27 [g]	38.76 ± 5.89 [f]	214.26 ± 17.84 [b]	249.41 ± 10.26 [a]	111.15 ± 2.57 [d]	ND	ND	142.52 ± 5.43 [c]	2.13 ± 0.87 [h]	48.93 ± 1.06 [e]

Note: [a] stand for plant lies. [b] stand for sampling of days. Values were shown by mean ± SD with different minuscule letters of significantly differences in the level of 5% among the same lines in adult plants with time (1 to 56 days after planting) and lines. [d] stand for no detection.

Table 2. Variation of sulforaphane contents during the pod period of broccoli

Lines [a]	Sampling of days [b]									
	1	6	11	16	21	26	31	36	41	46
B691J	81.65 ± 4.35 [d]	58.10 ± 3.27 [f]	82.57 ± 3.02 [d]	67.90 ± 2.65 [e]	118.65 ± 3.63 [b]	89.22 ± 5.48 [c]	210.76 ± 7.02 [a]	79.12 ± 5.09 [d]	64.26 ± 2.61 [e]	55.41 ± 3.16 [g]
B691Z	215.62 ± 8.14 [j]	462.64 ± 13.04 [i]	946.56 ± 32.92 [h]	1696.63 ± 66.17 [g]	2895.02 ± 122.91 [f]	3068.99 ± 125.69 [e]	3582.36 ± 129.71 [d]	3884.15 ± 171.48 [c]	4124.22 ± 140.84 [b]	4564.25 ± 188.01 [a]
B692J	404.52 ± 14.59 [a]	385.93 ± 11.05 [c]	398.74 ± 11.55 [b]	362.54 ± 14.14 [d]	401.46 ± 12.66 [b]	358.16 ± 15.97 [d]	381.48 ± 10.88 [c]	348.14 ± 15.58 [e]	284.14 ± 14.08 [f]	224.01 ± 9.74 [g]
B692Z	541.33 ± 25.19 [f]	538.23 ± 24.99 [f]	440.44 ± 12.18 [g]	283.45 ± 11.05 [h]	1562.45 ± 66.94 [e]	1869.77 ± 75.92 [d]	2252.46 ± 84.85 [c]	2864.55 ± 121.72 [b]	3015.89 ± 127.62 [a]	3014.25 ±107.56 [a]
B693J	2025.24 ± 93.03 [a]	203.56 ± 8.94 [b]	122.53 ± 4.08 [f]	123.89 ± 4.83 [f]	139.19 ± 5.13 [e]	174.72 ± 7.81 [c]	166.53 ± 5.79 [d]	176.11 ± 5.87 [d]	60.15 ± 3.35 [g]	51.87 ± 1.02 [h]
B693Z	1627.91 ± 60.74 [f]	262.79 ± 10.05 [g]	219.39 ± 7.56 [h]	191.46 ± 7.47 [i]	3066.36 ± 129.59 [e]	4091.35 ± 169.56 [d]	4127.59 ± 160.98 [c]	4341.69 ± 159.33 [b]	4504.35 ± 155.67 [a]	4549.68 ± 157.44 [a]
B694J	625.76 ± 21.66 [a]	522.60 ± 18.38 [b]	172.61 ± 4.73 [f]	214.14 ± 8.35 [e]	286.50 ± 12.17 [d]	193.67 ± 6.55 [e]	323.69 ± 12.62 [c]	122.19 ± 3.77 [g]	99.41 ± 4.88 [h]	68.21 ± 3.66 [i]
B694Z	287.81 ± 12.80 [g]	354.56 ± 10.83 [f]	282.61 ± 9.02 [g]	361.17 ± 14.09 [f]	3064.48 ± 129.51 [e]	3279.14 ± 147.89 [d]	3588.71 ± 129.96 [c]	3712.77 ± 124.80 [b]	3689.65 ± 153.90 [b]	3861.37 ± 130.59 [a]

Note: [a] stand for plant lies, and 'J' stand for pod area, 'Z' stand for seed. [b] stand for sampling of days. Values were shown by mean ± SD with different minuscule letters of significantly differences in the level of 5% among the same lines in developmental pod stage.

In the early days after field planting, the content of all the lines were at low levels. Samples from the 21st to the 26th day showed higher level, possibly because of the synthesis of glucoraphanin at this stage, genotypes and growth stages could affect sulforaphane contents in broccoli [28]. The samples in 21st all showed a higher content, there were individually 306.23, 82.10, 90.92 and 214.26 mg·kg^{-1} FW. The content of the leaves was higher than at other stages of the whole development cycle. We might therefore infer that the highest level in leaves appeared one month after planting in the field. This conclusion could also indicate a vigorous synthesis of glucosinolates in the leaves [29].

3.2.3. Florets in the Mature Period

The data showed that the contents in florets (B691, B692, B693 and B694) were respectively: 123.35^{a}, 89.34^{d}, 113.07^{b} and 106.32^{c} mg·kg^{-1} FW (p<0.05), and in the leaves: 63.89^{a}, 31.95^{b}, 24.82^{c} and 22.13^{c} mg·kg^{-1} FW. We could see florets were higher leaves, which consistent with other reports [9,30].

3.2.4. Buds and Leaves during the Bolting Period

The data showed significant differences among four lines with different development bus (p<0.05) (Figure 3). There was a notable change: it decreased sequentially

from top buds, to mature buds, to flower buds one day before flowering and to the open flowers. The highest content in top buds was 1127.61 mg·kg^{-1} FW (B694) and the lowest 377.76 mg·kg^{-1} FW (B692). At the same time, open flowers in all lines showed a higher content than the florets in bulk. Their contents were 267.08, 219.64, 328.97 and 437.18 mg·kg^{-1} FW for B691, B692, B693 and B694, respectively. The corresponding contents in leaves were 48.17, 60.93, 58.57 and 40.83 mg·kg^{-1} FW. The results indicated high content in buds during the bolting period, so this could be a topic for more detailed research on the mechanism of its development as an anti-cancer component.

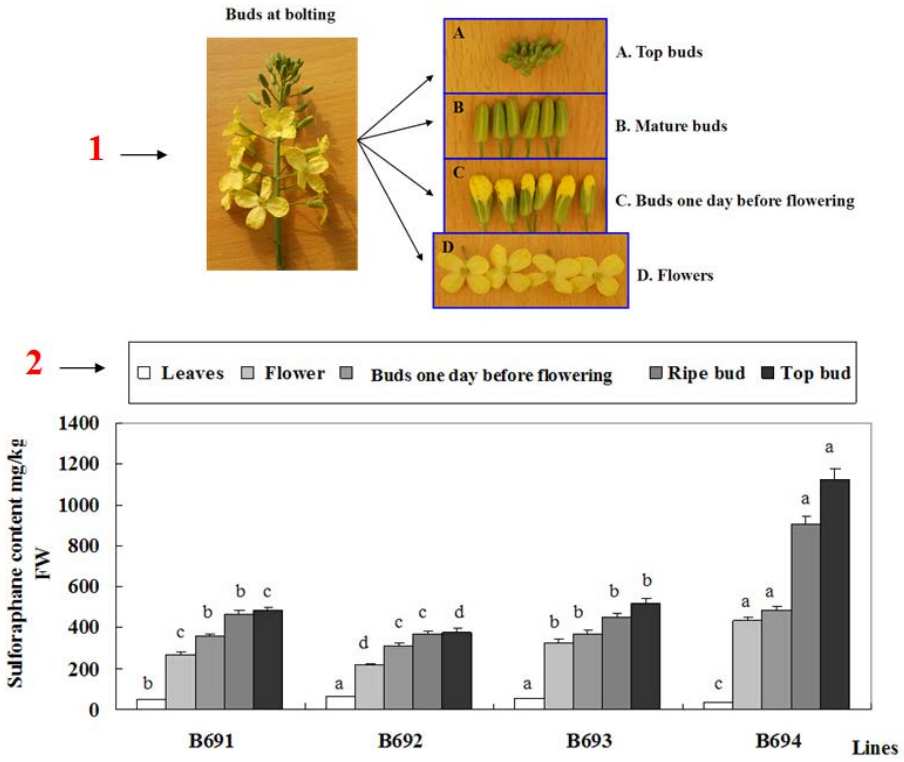

Figure 3. Development of buds at the bolting stage (1) and sulforaphane content of buds, flowers and leaves at the bolting stage for different lines (2). Values shown by bars with different letters are significantly different during the lines ($p < 0.05$)

During the bolting period, there was a new pattern in the variation of sulforaphane content: this was a negative correlation between sulforaphane content and the developmental maturity of the buds. This result occurred in all materials, with the highest levels in top buds, followed by adult buds, buds one day before flowering to the lowest levels in flowers. At the same time, exception leaves, two hybrid lines, especially B694, showed obvious heterosis in developmental buds (Figure 3-(2)), and the performance happened in the sprout stage, which proved again there was the dominant allele gene loci resulting in difference of B694 higher than B693 (Figure 3-(2)), which suggested there were expression differences of regulation genes in different organs.

The present study is the first to report this phenomenon, and there was no other related research. So the study might provide a new way to study the relationship between glucosinolate (glucoraphanin) and flower development. Meanwhile the new found implied some regulation mechanism of the flower development interact with glucosinolate in bolting stage. During the period, reproductive growth and differentiation was fast, and there were also large changes in some chemical constituents such as vitamins, glucosinolates, reactive oxygen species, and function oxygenases, following with multiple cross reaction [31,32]. From the variation of sulforpahane in development buds, we could speculate and get the conclusion, tender bud contain the highest content of sulforaphane, and the ingredient was translated into other components as the buds changing to flowers.

3.2.5. Pods in Development Stage

The pod stage is crucial for seed maturity. The immature seeds and pod area were artificially and carefully separated from pods in the development stage (Figure 4). Samples were collected and analyzed until the 46th day after artificial pollination, when the pods were almost mature. The initial values of content in B691, B692, B693 and B694 were 215.62, 541.33, 1627.91 and 287.81 mg·kg^{-1} FW respectively in young seed. The corresponding contents in pod areas were 81.65, 404.52, 2025.24 and 625.76 mg·kg^{-1}FW (Table 2). Thus the initial contents in young seeds were nearly all lower than those in pod areas, but contents on the 46th day showed the reverse. On the 46th day, the data showed that contents in seeds of B691, B692, B693 and B694 were respectively 4564.25, 3014.25, 4549.68 and 3861.37 mg·kg^{-1} FW. The corresponding values in pod areas were 55.41, 224.01, 51.87 and 68.21 mg·kg^{-1} FW. These final results showed seeds were even higher than pod areas and initial young seeds. This great difference between mature seeds and pod areas indicated that seeds did originate from the external pod areas, but could have been generated by endogenous gene. It might also be concluded that contents declined from the initial young to mature pod areas and, at the same time, they increased from the initial young seeds to ripe

seeds. From the opposite trend, we could proved that there was an accumulation process in developmental seeds, so these data also improved understanding why ripe seed contained the highest sulforaphane. So from the rapid accumulation process of sulforaphane in ripe seed, we could prove that glucoraphanin, the precursor of the sulforaphane, play an important role in seed storage and vigor. And the conclusion was also indirectly proved by seedling. The conclusion indicated glucoraphanin was rapidly accumulated after pollination, especially thirty days before ripe (Table 2). Furthermore the final content in four plants was also even higher than florets in bulk.

In this part, we firstly detected the differences of sulforaphane in seed pod and pod area, meanwhile the opposite change trend in seed pod and pod area were provided in this stage. The data not only broadening sulforaphane changes in broccoli organs, but also strengthened the difference of organs, and provide us a target to comprehensively study the regulation mechanism of sulforaphane.

3.2.6. Seeds at the Ripe Stage

In the stage, the contents of B691, B692, B693 and B694 were respectively 4584.52^{b}, 3051.03^{d}, 4624.23^{a} and 3654.63^{c} mg·kg^{-1} FW ($p<0.05$), while the leaves were respectively 38.46^{c}, 46.31^{a}, 42.01^{b} and 35.95^{c} mg·kg^{-1} FW. So the ripe seeds were further higher than the leaves, at the same time, it was also the highest organ in the whole development cycle, which was also consistent with other reports [24,33].

Most studies have reported sulforaphane in ripe seed was higher than the other organs, such as florets, leaves and stems [9,24,30,34]. The present study agreed the conclusion. The different lines exhibited different contents, which were mainly depended on genotype and organs [10,28,35]. Therefore investigating broccoli seeds rich in sulforaphane may be of great scientific value and practical utilization for cancer research and vegetable breeding.

3.2.7. Seedling Stage

The ripe seeds were sown in 15-cm diameter dishes. The first sampling was collected one day after sowing and then seedlings were collected every day until day 15. The 1st day samples had a high content: 4515.34, 2752.35, 3818.57 and 2852.94 mg·kg^{-1} FW respectively from B691, B692, B693 and B694 ($p<0.05$) (Figure 4). Overall, all the plants gradually decreased over the ten days after sowing. The B691 and B694 lines showed the lowest level on the 11th day (320.82 and 311.23 mg·kg^{-1} FW), while B692 and B693 on the 13th day (298.58 and 227.19 mg·kg^{-1} FW). All the materials began to rise after these low points, which indicated that no generation during the first ten days, and the conclusion has been provided by some experiments [24].

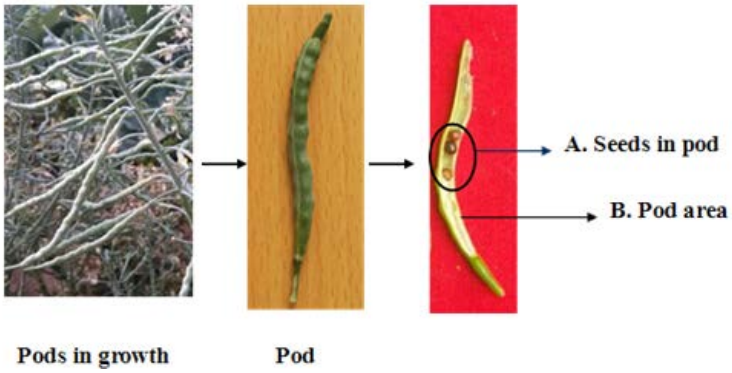

Figure 4. Seeds in pod (A) and pod area (B) of developmental seed pod

In the stage, there showed a declining trend during the first fifteen days after sowing. During the first 5 days, the content of four plants was over 1000 mg·kg^{-1} FW and then declined rapidly (Figure 5). This indicated there was no synthesis in the first 10 days. From the result, we could find the content mainly depended on genotype, and sulforaphane content declined gradually as seedling growth in first ten days and then began to rise after tenth day (10th day to 15th day) to some extent. So the human should consider to early seedling as health meal.

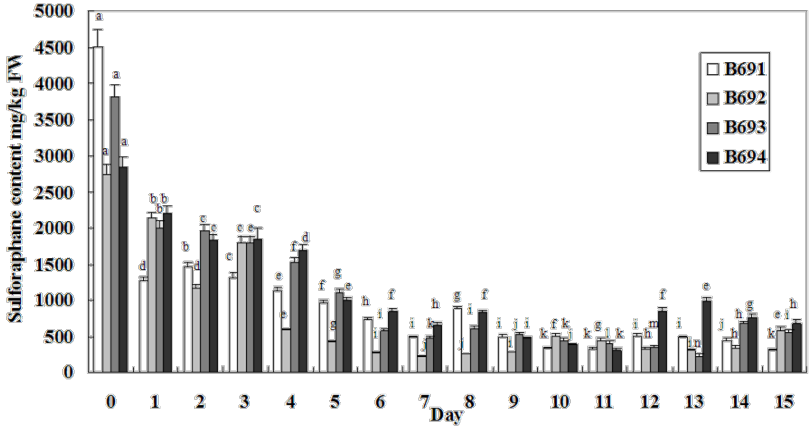

Figure 5. Variation in sulforaphane content in broccoli seedlings with time and lines. Values shown by bars with different letters are significantly different in different days among the same lines ($p < 0.05$)

4. Conclusion

This study has described the variation of sulforaphane content in different organs during the whole cycle of broccoli development. The result showed that there were different change rule in the whole growth and development period, and different organs of broccoli presented diversity changing of sulforaphane content in different growth and development stages. According to the result, several conclusions were proved and found. Ripe seed and the early seedling (1 to 5 days) contained higher content of sulforaphane, especially ripe seed. There might be no correlation between seed pod and pod area in developmental pod stage, and they showed opposite change in sulforaphane content. The contents of sulforaphane in developmental buds not only showed a regular change, but also were found with higher contents of sulforaphane. Leaves in adult plant might be considered to be feed for poultry and livestock and early seedling should be considered as healthy meal. At the same time, the result also proved that sulforaphane content mainly depended on genotype, and there was tissue-specific restriction in broccoli. Therefore more research studies are required to elucidate the mechanism of sulforaphane in different organs of broccoli.

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References

[1] Cramer, J.M., Jeffery, E.H., A comparison of the bioavailability of sulforaphane from broccoli sprouts and a semi-purified broccoli powder rich in glucoraphanin in healthy human males. *Faseb Journal*, 2009, 23.

[2] Fahey, J.W., Zalcmann, A.T., Talalay, P., The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry*, 2001, 56:5-51.

[3] Angelino, D., Jeffery, E., Glucosinolate hydrolysis and bioavailability of resulting isothiocyanates: Focus on glucoraphanin. *J Funct Foods*, 2014, 7:67-76.

[4] Agerbirk, N., Olsen, C.E., Chew, F.S., Orgaard, M., Variable glucosinolate profiles of Cardamine pratensis (Brassicaceae) with equal chromosome numbers. *Journal of agricultural and food chemistry*, 2010, 58:4693-700.

[5] Bertl, L., Bartsch, H., Gerhauser, C., Antiangiogenic properties of sulforaphane, an isothiocyanatc derived from broccoli. *Cancer Epidem Biomar*, 2003, 12:1347s-s.

[6] Campas-Baypoli, O.N., Sanchez-Machado, D.I., Bueno-Solano, C., Ramirez-Wong, B., Lopez-Cervantes, J., HPLC method validation for measurement of sulforaphane level in broccoli by-products. *Biomedical chromatography: BMC*, 2010, 24:387-92.

[7] Bones, A.M., Rossiter, J.T., The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry*, 2006, 67: 1053-67.

[8] Tang, L., Paonessa, J.D., Zhang, Y.S., Arnbrosone, C.B., McCann SE., Total isothiocyanate yield from raw cruciferous vegetables commonly consumed in the United States. *J Funct Foods*, 2013, 5: 1996-2001.

[9] Liang, H., Yuan, Q.P., Dong, H.R., Liu, Y.M., Determination of sulforaphane in broccoli and cabbage by high-performance liquid chromatography. *J Food Compos Anal*, 2006, 19:473-6.

[10] Li, Z.S., Liu, Y.M, Fang, Z.Y., Yang, L.M., Zhuang, M., Zhang, Y.Y., Variation of Sulforaphane Levels in Broccoli (Brassica Oleracea Var. Italica) during Flower Development and the Role of Gene Aop2. *J Liq Chromatogr R T*, 2014, 37: 1199-211.

[11] Fahey, J.W., Haristoy, X., Dolan, P.M., Kensler, T.W., Scholtus, I., Stephenson, K.K., Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. *Proceedings of the National Academy of Sciences of the United States of America*, 2002, 99: 7610-5.

[12] Conaway, C.C., Wang, C.X., Pittman, B., Yang, Y.M., Schwartz JE, Tian D., Phenethyl isothiocyanate and sulforaphane and their N-acetylcysteine conjugates inhibit malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice. *Cancer research*, 2005, 65:8548-57.

[13] Pawlik, A., Wiczk, A., Kaczynska, A., Antosiewicz, J., Herman-Antosiewicz, A., Sulforaphane inhibits growth of phenotypically different breast cancer cells. *European journal of nutrition*, 2013, 52:1949-58.

[14] Kaminski, B.M., Loitsch, S.M., Ochs, M.J., Reuter, K.C., Steinhilber, D., Stein, J., Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells via induction of the TGF-beta/Smad signaling pathway. *Molecular nutrition & food research*, 2010, 54:1486-96.

[15] Evans, P.C., The influence of sulforaphane on vascular health and its relevance to nutritional approaches to prevent cardiovascular disease. *The EPMA journal*, 2011, 2:9-14.

[16] Nakagawa, K., Umeda, T., Higuchi, O., Tsuzuki, T., Suzuki, T., Miyazawa, T., Evaporative light-scattering analysis of sulforaphane in broccoli samples: Quality of broccoli products regarding sulforaphane contents. *Journal of agricultural and food chemistry*, 2006, 54:2479-83.

[17] Saha, S., Hollands, W., Teucher, B., Needs, P.W., Narbad, A., Ortori, C.A., Isothiocyanate concentrations and interconversion of sulforaphane to erucin in human subjects after consumption of commercial frozen broccoli compared to fresh broccoli. *Molecular nutrition & food research*, 2012, 56:1906-16.

[18] Matusheski, N.V., Jeffery, E.H., Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. *Journal of agricultural and food chemistry*, 2001, 49:5743-9.

[19] Chiao, J.W., Chung, F.L., Kancherla, R., Ahmed, T., Mittelman, A., Conaway, C.C., Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. *International journal of oncology*, 2002, 20:631-6.

[20] Josefsson, E., Effects of variation of heat treatment of conditions on the nutritional value of low-glucosinolate rapeseed meal. *Journal of the science of food and agriculture*, 1975, 26:157-64.

[21] Kushad, M.M., Brown, A.F., Kurilich, A.C., Juvik, J.A., Klein, B.P., Wallig, M.A., Variation of glucosinolates in vegetable crops of Brassica oleracea. *Journal of agricultural and food chemistry*, 1999, 47:1541-8.

[22] Liang, H., Yuan, Q.P., Xiao, Q., Effects of metal ions on myrosinase activity and the formation of sulforaphane in broccoli seed. *J Mol Catal B-Enzym*, 2006, 43:19-22.

[23] Zang, Y.X., Kim, H.U., Kim, J.A., Lim, M.H., Jin, M., Lee, S.C., Genome-wide identification of glucosinolate synthesis genes in Brassica rapa. *The FEBS journal*, 2009, 276:3559-74.

[24] Gorissen, A., Kraut, N.U., Visser, R., Vries, M., Roelofsen, H., Vonk, R.J., No de novo sulforaphane biosynthesis in broccoli seedlings. *Food chemistry*, 2011, 127:192-6.

[25] Bennett R., Ludwigmuller, J., Kiddle, G., Hilgenberg, W., Wallsgrove, R., Developmental Regulation Gf Aldoxime Formation in Seedlings and Mature Plants of Chinese-Cabbage (Brassica-Campestris Ssp Pekinensis) and Oilseed Rape (Brassica-Napus) - Glucosinolate and Iaa Biosynthetic-Enzymes. *Planta*, 1995, 196:239-44.

[26] Guo, L.P., Yang, R.Q., Wang, Z.Y., Guo, Q.H., Gu, Z.X.. Glucoraphanin, sulforaphane and myrosinase activity in germinating broccoli sprouts as affected by growth temperature and plant organs. *J Funct Foods*, 2014, 9:70-76.

[27] Brown, P.D., Tokuhisa, J.G., Reichelt, M., Gershenzon, J., Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. Phytochemistry, 2003, 62:471-81.

[28] Farnham, M.W., Wilson, P.E., Stephenson, K.K., Fahey, J.W., Genetic and environmental effects on glucosinolate content and chemoprotective potency of broccoli. *Plant Breeding*, 2004, 123:60-5.

[29] Bennett, R.N., Hick, A.J., Dawson, G.W., Wallsgrove, R.M., Glucosinolate Biosynthesis-Further Characterization of the Aldoxime Forming Microsomal Monooxygenases in Oilseed Rape Leaves. *Plant physiology*, 1995, 109:299-305.

[30] Li, C.F., Liang, H., Yuan, Q.P., Hou, X.D., Optimization of sulforaphane separation from broccoli seeds by macroporous resins. *Separ Sci Technol*, 2008, 43:609-23.

[31] Li, J.C., Maezawa, S., Nakano, K., Correlations between antioxidative enzyme activities and antioxidative substrates and senescence in broccoli (Brassica oleracea L.) flower buds at different storage temperatures. *J Jpn Soc Hortic Sci*, 2004, 73: 399-403.

[32] Starzynska, A., Leja, M., Mareczek, A., Physiological changes in the antioxidant system of broccoli flower buds senescing during short-term storage, related to temperature and packaging. *Plant Science*, 2003, 165:1387-95.

[33] Liang, H., Li, C., Yuan, Q., Vriesekoop, F., Separation and purification of sulforaphane from broccoli seeds by solid phase extraction and preparative high-performance liquid chromatography. *Journal of agricultural and food chemistry*, 2007, 55:8047-53.

[34] Lopez-Cervantes, J., Tirado-Noriega, L.G., Sanchez-Machado, D.I., Campas-Baypoli ON, Cantu-Soto EU, Nunez-Gastelum JA. Biochemical composition of broccoli seeds and sprouts at different stages of seedling development. *Int J Food Sci Tech*, 2013, 48: 2267-75.

[35] Ares, A.M., Bernal, J., Martin, M.T., Bernal, J.L., Nozal, M.J., Optimized Formation, Extraction, and Determination of Sulforaphane in Broccoli by Liquid Chromatography with Diode Array Detection. *Food Anal Method*, 2014, 7:730-40.