Transcriptome reveals the gene expression patterns of sulforaphane metabolism in broccoli florets

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Abstract

Sulforaphane is a new and effective anti-cancer component that is abundant in broccoli. In the past few years, the patterns of variability in glucosinolate content and its regulation in A. thaliana have been described in detail. However, the diversity of glucosinolate and sulforaphane contents in different organs during vegetative and reproductive stages has not been clearly explained. In this paper, we firstly investigated the transcriptome profiles of the developing buds and leaves at bolting stage of broccoli (B52) to further assess the gene expression patterns involved in sulforaphane synthesis. The CYP79F1 gene, as well as nine other genes related to glucoraphanin biosynthesis, MAM1, MAM3, St5b-2, FMO GS-OX1, MY, AOP2, AOP3, ESP and ESM1 were selected by digital gene expression analysis and were validated by quantitative real-time PCR (qRT-PCR). Meanwhile, the compositions of glucosinolates and sulforaphane were detected for correlation analysis with related genes. Finally the RNA sequencing libraries generated 147 957 344 clean reads, and 8 539 unigene assemblies were produced. In digital result, only CYP79F1, in the glucoraphanin pathway, was up-regulated in young buds but absent from the other organs, which was consistent with the highest level of sulforaphane content being in this organ compared to mature buds, buds one day before flowering, flowers and leaves. The sequencing results also presented that auxin and cytokinin might affect glucoraphanin accumulation. The study revealed that up-regulated expression of CYP79F1 plays a fundamental and direct role in sulforaphane production in inflorescences. Two genes of MAM1 and St5b-2 could up-regulated glucoraphanin generation. Synergistic expression of MAM1, MAM3, St5b-2, FMO GS-OX1, MY, ESP and ESM1 was found in sulforaphane metabolism. This study will be beneficial for understanding the diversity of sulforaphane in broccoli organs.
Introduction

In recent years, sulforaphane has attracted much interest due to its anti-cancer activity, and a growing body of epidemiological evidence has shown that increased consumption of sulforaphane or cruciferous vegetables rich in sulforaphane can lower the risk of lung [1], colon [2], pancreatic [3], breast [4], bladder [5] and prostate [6] cancers as well as some geriatric diseases such as Alzheimer’s disease [7] and cardiovascular disease [8, 9]. The chemoprotective function of sulforaphane is due to its ability to induce phase II detoxification enzymes [10, 11], directly resulting in cancer cell apoptosis [12, 13].

Sulforaphane is an isothiocyanate, and it can be synthesized from glucoraphanin through hydrolysis by myrosinase when broccoli is chewed, mechanically damaged, digested by humans, or bitten by insects [14, 15]. Glucoraphanin (4-Methylsulfonylbutyl glucosinolate) is a glucosinolate mostly found in Brassica vegetables, such as broccoli, cabbage (green and red), Chinese kale, Brussels sprouts, kohlrabi, collards, and turnip [16–18]. Among the crucifers tested, broccoli has been reported to be rich in glucoraphanin, and the regulation of glucosinate synthesis has been largely reported in A. thaliana [19–22].

Glucosinolates are mainly synthesized from amino acids Met, Phe and Trp, which accordingly give rise to three groups of glucosinolates: aliphatic glucosinolates, benzenic glucosinolates and indolic glucosinolates [15, 22–24]. Regulation genes of glucosinolate and the pathway have been successfully identified in Arabidopsis [23, 25–27]. Glucoraphanin belongs to aliphatic glucosinolate derived from Met. In the process of chain elongation, it starts with deamination by a BCAT4 giving rise to a 2-oxo-4-methylthiobutanoic acid. The 2-oxo-4-methylthiobutanoic acid then enters a cycle of three successive transformations: condensation with acetyl-CoA by MAM1 and MAM3, isomerization by IPMI-SSU2, 3, and oxidative decarboxylation by IPM-DH, generating 2-Oxo-6-methylthiohexanoic acid [16, 28].

A total of 13 enzymes, representing five different biochemical steps in the formation of the glucosinolate core structure, have been characterized [24, 29]. For the core biosynthetic pathway of aliphatic glucosinolates, CYP79F1 (Met1-6), CYP79F2 (Met 5, 6), CYP83A1, GSTF11, GSTU20, GGP1, SUR1 (C-S lyase), UGT74C1, SOT17 (AtSTb), and SOT18 (AtSTb) play distinct roles in oxidation, conjugation, C-S cleavage, glucosylation and sulfation functions, then 2-oxo-6-methylthiohexanoic acid and dihomomethionine are transferred to 4-methylthiobutyl glucosinolate (glucoerucin). Finally, glucoerucin is oxidized and changed into glucoraphanin by FMO-GSOX1-5 [26, 30].

The following process is secondary modification, and the biological activity of glucosinolates is determined by the structure of the side chain [22, 27]. In aliphatic glucosinolates, 4-methylthiobutyl actually is the precursor of glucoraphanin, is catalyzed to generate 3-pentenyl glucosinolate (gluconapin) by GS-ALK, as well as 4-benzoyloxybutyl glucosinolate by GS-OHB. Glucoraphanin can also be hydrolyzed to sulforaphane catalyzed by myrosinase (MY) or, depending on pH, more sulforaphane is generated in an alkaline environment [28, 31, 32] together with side-chain elongation, secondary modifications are responsible for more than 132 known glucosinolate structures [33, 34], of which there have been 56 putative genes identified in glucosinolate pathway of B. oleracea [http://www.ocri-genomics.org/cgi-bin/bolbase/pathway_detail.cgi?entry=map00966] [35], and 110 in B. rapa [http://brassicadb.org/brad/glucoGene.php] [36, 37].

By 2010, approximately 29 genes have been found in aliphatic glucosinolate pathway [26, 38–40]. Glucosinolate synthesis and its regulation mechanism has been revealed mostly in Arabidopsis. However, glucosinolates are affected by many factors, such as genotypes, organs, development stages, cultivation conditions, soil microbes, and environments [22, 27, 31]. Some research and our previous work have reported that the significant differences of sulforaphane and glucoraphanin happened in different organs of Brassica vegetables [16, 28, 41].
But there are few reports that can explain the diversity of sulforaphane contents in different broccoli organs at various developmental stages [42, 43]. And one of the best methods to elucidate these mechanisms is to study them at the molecular level by transcriptome analysis.

In our study, it was found that the contents of glucoraphanin and sulforaphane were both in a high level with significant differences in developing buds. So the buds at bolting stage were chosen and carried out by transcriptome analysis for exploring the gene expression patterns of sulforaphane. The aims of our research were to (i) identify and validate differential expression of specific genes in developing buds (LN_B1-B4) and leaves (LN_F) individually, and (ii) find genes related to sulforaphane metabolism. Our results would provide new insights into explanation of sulforaphane accumulation in different organs of broccoli.

**Materials and methods**

**Plant material**

Broccoli inbred line B52 was cultured and treated using the method described in our previous research, and this inbred line was bred at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS-IVF) [41]. All plants were planted in greenhouse on August 2, 2015, florets formed on October 15 and bolting on November 22. At the same time, the developmental buds and leaves (LN_F) were collected at bolting stage, and the organs were young buds (LN_B1), mature buds (LN_B2), buds one day before flowering (LN_B3) and flowers (LN_B4) (Fig 1).

**Library construction, sequencing and bioinformatics analysis**

Total RNA was extracted from each sample by using TRIzol reagent (Invitrogen, CA, USA), and its quality was monitored on 1% agarose gels and assessed by a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) with a minimum RNA integrity number (RIN) of 7.0. Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA), and index codes were assigned to each sample. Library quality was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, the prepared libraries were sequenced on an Illumina HiSeq 2500/4000 platform (Illumina, Inc., San Diego, CA, USA), which was conducted by Beijing Allwegene Technology Co., Ltd, China. Before assembly, raw reads of the cDNA libraries were filtered to remove adaptor sequences, low-quality reads containing poly-N and sequences with more than 5% unknown nucleotides. After transcriptome assembly, each unigene was annotated using five databases [44, 45]: NCBI non-redundant protein (Nr), Eukaryotic Ortholog Groups (KOG), Protein family (Pfam), Swiss-Prot, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Blast all software was used to predict and classify the KOG and KEGG pathway-associated unigenes [46, 47], employing BlastX (v.2.2.28C) with an E-value of less than 1e-5. Gene Ontology (GO) annotations were analyzed using GOseq [48].

**Analysis of differentially expressed genes**

All quenching reads for five samples were remapped to the reference sequences using RSEM software, and the abundance of each assembled transcript was evaluated using FPKM [49–50]. For genes with more than one alternative transcript, the longest transcript was selected to calculate the FPKM. The DESeq package (ver.2.1.0) was employed to detect DEGs between sample pairs (LN_B1 versus LN_F, LN_B2 versus LN_F, LN_B3 versus LN_F and LN_B4 versus LN_F [51, 52]. The false discovery rate (FDR) was applied to correct the p-value threshold in
An FDR-adjusted p-value (q-value) ≤ 0.05 and a |log2 Fold Change| > 1 were used as the thresholds for identifying significant differences in gene expression. For convenience, DEGs with higher expression levels in buds compared to leaves were designated up-regulated, whereas those with lower expression were designated down-regulated [50].

**Candidate glucosinolate genes selection and certification of relative expression**

To verify the reliability of the expression analysis, ten candidate glucosinolate genes of *MAM1*, *MAM3*, *CYP79F1*, *St5b-2*, *FMO GS-OX1*, *MY (TGG1)*, *AOP2* and *AOP3*, *ESP* and *ESM1* were selected and quantified by real-time PCR. The primers for these genes were listed in Table 1. Samples of developing buds and leaves were gathered, and qRT-PCR analysis was performed by the method described in our previous study [41]. qRT-PCR was carried out using SYBR Premix Ex TaqII (Tli RNaseH Plus; TAKARA BIO, Inc., Shiga, Japan) on an ABI 7900HT (Applied Biosystems, Carlsbad, CA, USA).

**Investigation of glucosinolate genes associated with sulforaphane**

To gain overall insight into differential gene expression patterns between developing buds and leaves. Ten regulated genes related to the sulforaphane pathway were chosen for confirmation.
by quantitative real-time PCR (qRT-PCR). These genes are MAM1, MAM3, CYP79F1, St5b-2, FMO GS-OX1, AOP2, AOP3, MY, ESP and ESM1.

### Extraction and determination of sulforaphane and glucoraphanin

Five samples were pretreated and dried in a lyophilizer, HPLC and UHPLC–Triple–TOF–MS methods were used for determination of sulforaphane and glucoraphanin separately. The extraction and determination methods of sulforaphane are thoroughly described in our previous study [41, 53]. The methods for analysis of glucoraphanin and the other glucosinolates was carried out by using UHPLC-Triple-TOF-MS. Samples were extracted using 70% methanol and injected after concentration of the standard glucoraphanin. UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm) column was selected with acetonitrile-water (both 0.1% formic acid) as mobile phase. Chromatographic separation was achieved under gradient elution in 10 min. In ESI negative ion mode, TOF-MS scan-IDA-Product ion scan was performed to acquire both MS and MS/MS information from one injection. Based on high resolution TOF-MS, accurate masses of molecular ions and fragment ions were obtained for high accuracy-identification.

### Results

#### Sequencing, assembly and functional annotation

A pooled cDNA library of five samples of developing buds and leaves was analyzed on the Illumina HiSeq 2500/4000 platform (Illumina, Inc., San Diego, CA, USA). The library generated 147.96 million raw reads (Tables 2 and 3), and the assembled raw reads (>95.23%) had Phred-

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**Table 1. The qRT-PCR genes related sulforaphane metabolism and their primers.**

| No | Gene names | Primer sequences |
|----|------------|------------------|
| 1  | MAM1       | Forward primer: GAGTAGACATCATGGAAGTCGGTT  
                  | Reverse primer: AAGTCGCCCTCAATGCTCTATGTGTT |
| 2  | MAM3       | Forward primer: CGAAGTGACGATCAACGGAA  
                  | Reverse primer: GACATTCCAAGCCATCACGAC |
| 3  | CYP79F1    | Forward primer: GTCCAGCCAGAGCAATCAGAA  
                  | Reverse primer: GCCAAGCCCTGTCTTTTCACAAGT |
| 4  | FMO GS-OX1 | Forward primer: GAAAGCAGATCTCATAGCCACA  
                  | Reverse primer: CATAGATTGTTTTGGGGGACTG |
| 5  | AOP2       | Forward primer: AGTAAGAGTGACCAGAGAAAAAGGG |
                  | Reverse primer: GCGACCAGCTTCTGATGAGGAG |
| 6  | AOP3       | Forward primer (homologous domain): AGGTTAAGACCAAGAAAGGGGAA  
                  | Reverse primer (homologous domain): TCGGATATCAGGAGAAGGGA |
| 7  | MY         | Forward primer: GTGTGAGGTGAGGCGGTGAAC  
                  | Reverse primer: GTCTGATAAGGCTAGACGC |
| 8  | St5b-2     | Forward primer: CCCATATACCAACCGGCCTG  
                  | Reverse primer: CCCATGAACTCAGCCACCT |
| 9  | ESP        | Forward primer: GATCGAGTGAGGGGAAAGAG  
                  | Reverse primer: AAGGGTTTCGCTCTGATGCTCTA |
| 10 | ESM1       | Forward primer: AAAGATCCTCAGCAACCTATG  
                  | Reverse primer: TTTGTATTTGCTTCACGATC |
| 11 | actin-12   | Forward primer: GGCTCTATCAGGGGTCTCAGT  
                  | Reverse primer: CCAGATTCATCATACCTCGGCTTC |
like quality scores at the Q20 level (an error probability of 0.01–0.02%). Finally 48,852 unigenes of 150 bp generated based on PE150. The Gene Ontology (GO) database assigned 27,606 unigenes into 30 functional categories. The largest proportion was represented by biological process (GO 0008150, 11.86%) and metabolic process (GO 0008152, 12.39%; S1 Fig). In total, 6656 unigenes were categorized into 4 Clusters of Orthologous Groups of Proteins (COG) classifications (S2 Fig), which was shown and validated by Venn diagram comparisons (Fig 2A) and cluster analysis of differentially expressed genes between leaves and developmental buds (Fig 2B). The 3450 assembled sequences were mapped to the reference canonical pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG). In the top 20 KEGG pathways, the pathway most strongly represented by the mapped unigenes was biological process and metabolism (KO 03010, 263 unigenes) (Fig 3).

Identification and annotation of differentially expressed genes

Approximately 29.88–51.52 million 150 bp paired-end reads were generated through RNA sequencing (S3 Fig). Transcript levels were calculated using fragments per kilobase per million reads (FPKM; Table 3). The GC content from the 10 libraries ranged from 45.09 to 46.23%, and the Q30 values (reads with an average quality scores > 30) were all in the range of 89.81 to 97.90%, indicating that the quality and accuracy of sequencing data were sufficient for further analysis (Table 2). The percentage of sequenced reads from all libraries that remapped to the assembled reference transcripts was nearly ≥ 70% (Table 1). According to the cabbage reference genome, 8539 genes of 45758 unigenes were functionally annotated with an e-value ≤ 1e-5 in at least one database.

Differential expression in young buds (LN_B1), mature buds (LN_B2), buds one day before flowering (LN_B3), flowers (LN_B4) and leaves (LN_F) of broccoli at bolting stage (FPKM > 5.0 in at least one treatment group, fold change ≥ 2.0, P ≤ 0.05) was found for 4775 to 5956 genes. Of these, 2534 to 3101 were up-regulated and 2000 to 2974 were up-regulated in all four groups of developing buds versus leaves (Table 4). The detailed gene numbers at different interval are shown in Table 5, and most genes were within an FPKM Interval 0–1 (49.85%-58.35%), particularly in leaves, followed by the buds one day before flowering, flowers and mature buds (Table 4). As shown in Fig 4A, we found that low expression genes were enriched in leaves, followed by buds one day before flowering, flowers, mature buds and young buds. However, young buds had higher overall gene expression, the second was mature buds, flowers, buds one day before flowering, and leaves showed the least (Fig 2A). Pearson correlations between five organs were calculated to investigate relationship of developing buds and leaves (Fig 4B). There was a gradual decrease in developing buds from young buds to flowers (LN_B1~4), which was consistent with the phenotype. In contrast, leaves displayed a varying relationship, which were most similar to young buds. All the differentially expressed genes were annotated by the databases described above.

Investigation of the glucosinolate genes associated with sulforaphane metabolism

The expression of the glucosinolate genes, including glucosinolate core genes and secondary metabolic genes were confirmed by qRT-PCR. Most of these genes showed similar trends in RNA sequencing and qRT-PCR (Fig 5). In this study, ten genes were investigated and compared with sulforaphane concentrations measured by HPLC. It was found that unlike CYP79F1 and AOP3, the genes MAM1, MAM3, St5b-2, FMO GS-OX1, MY, AOP2, ESP and ESM1 displayed a low expression level compared to the leaf control. There was a significantly higher expression of CYP79F1 in the young buds compared to the other organs at this stage, following by flowers and mature buds and buds one day before flowering, and leaves had at
the lowest expression level (Fig 5). AOP3 also showed a high gene expression similarly to CYP79F1, but the highest-expressing organ was flowers, and second were young buds followed by mature buds and buds one day before flowering, with leaves having the lowest level.

The same changing trends of sulforaphane and glucoraphanin happened to B52 at bolting stage, and there was an obvious decrease in sulforaphane and glucoraphanin concentrations from young buds to leaves (Fig 6A). Also there was a sharp decrease to mature buds from young buds, then another decrease from flowers to leaves. The corresponding sulforaphane contents were 3370.44, 2140.34, 1323.98, 1090.46, 235.82 mg/kg DW, respectively (Fig 6A and 6C). The corresponding contents of glucoraphanin were 43.83, 21.82, 24.65, 11.14 and 2.27 μM/g DW (Fig 6A and 6D). So the generation efficiency of sulforaphane from glucoraphanin was 30.3% to 58.6% in these organs. Except the buds one day before flowering, the other organs showed the similar efficiency, suggesting there should be no difference of myrosinase activity (ESM1) in catalyzing glucoraphanin into sulforaphane. At the same time, another 11 glucosinolates were detected in our study (Fig 6B), and gluconapin, glucotropaeolin, progoitrin and sinigrin were not determination. The result provided a good evidence for previous reports. This result showed the pattern of sulforaphane accumulation in different organs was consistent with our previous reports [41, 53].

**Discussion**

The glucosinolate pathway and sulforaphane metabolism

In the past 30 years, 16 natural glucosinolates in broccoli and 26 glucosinolates in A. thaliana have been elucidated. The total number of documented glucosinolates from plants has been 122 types [54–56].

### Table 2. Comparison of reads and reference sequence.

| Sample | LN_F   | LN_B1  | LN_B2  | LN_B3  | LN_B4  |
|--------|--------|--------|--------|--------|--------|
| Total reads | 70435770 | 79567320 | 51953284 | 45781558 | 48176756 |
| Total mapped | 50627502 (71.88%) | 57043101 (71.69%) | 37400419 (71.99%) | 33219629 (72.56%) | 33514062 (69.56%) |
| Multiple mapped | 1760525 (2.5%) | 1385966 (1.74%) | 1048692 (2.02%) | 894641 (1.95%) | 740476 (1.54%) |
| Uniquely mapped | 48866977 (69.38%) | 55657135 (69.95%) | 36351727 (69.97%) | 32324988 (70.61%) | 32773586 (68.03%) |
| Read-1 | 25396488 (36.06%) | 28907373 (36.33%) | 18828753 (36.24%) | 16742405 (36.57%) | 17539166 (36.41%) |
| Read-2 | 23470489 (33.32%) | 26749762 (33.62%) | 17522974 (33.73%) | 15582583 (35.32%) | 16382702 (34.01%) |
| Reads map to '+' | 24496371 (34.78%) | 27873454 (35.03%) | 18184082 (35%) | 16172019 (35.32%) | 16390884 (34.02%) |
| Reads map to '-' | 24370606 (34.6%) | 27783681 (34.92%) | 18167645 (34.97%) | 16152969 (35.28%) | 16382702 (34.01%) |
| Non-splice reads | 32180436 (45.69%) | 34794098 (43.73%) | 23101224 (44.47%) | 21927513 (47.9%) | 21693113 (45.03%) |
| Splice reads | 16686541 (23.69%) | 20863037 (26.22%) | 13250503 (25.5%) | 10397475 (22.71%) | 11080473 (23%) |

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### Table 3. Sequencing and assembly statistics for the 10 transcriptomes of the B52 inbred line at bolting stage.

| Sample  | Raw Reads | Raw Bases | Clean Reads | Clean Bases | Error Rate | Q20   | Q30   | GC Content |
|---------|-----------|-----------|-------------|-------------|------------|-------|-------|------------|
| LN_F    | 36374237  | 5.45Gb    | 35217885    | 5.28Gb      | 0.01%      | 99.26%| 97.90%| 46.15%     |
| LN_F    | 36374237  | 5.45Gb    | 35217885    | 5.28Gb      | 0.01%      | 97.27%| 94.13%| 46.23%     |
| LN_B1   | 41132570  | 6.16Gb    | 39783660    | 5.97Gb      | 0.01%      | 99.27%| 97.94%| 45.84%     |
| LN_B1   | 41132570  | 6.16Gb    | 39783660    | 5.97Gb      | 0.01%      | 97.31%| 94.23%| 45.90%     |
| LN_B2   | 26796422  | 4.01Gb    | 25976642    | 3.9Gb       | 0.01%      | 99.26%| 97.90%| 45.70%     |
| LN_B2   | 26796422  | 4.01Gb    | 25976642    | 3.9Gb       | 0.01%      | 97.46%| 94.51%| 45.75%     |
| LN_B3   | 23634550  | 3.54Gb    | 22890779    | 3.43Gb      | 0.01%      | 99.22%| 97.81%| 45.09%     |
| LN_B3   | 23634550  | 3.54Gb    | 22890779    | 3.43Gb      | 0.01%      | 97.45%| 94.49%| 45.17%     |
| LN_B4   | 25283977  | 3.79Gb    | 24088378    | 3.61Gb      | 0.01%      | 98.75%| 96.66%| 45.49%     |
| LN_B4   | 25283977  | 3.79Gb    | 24088378    | 3.61Gb      | 0.02%      | 95.23%| 89.81%| 45.75%     |

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The aliphatic pathway, encompassing 29 genes in *Arabidopsis*, was reviewed in 2010 [23, 31, 57]. Homologs for most of these genes can be found in broccoli, but different copies and variations are usually found in *Brassica* plants, such as AOP family genes [58, 59], which are responsible for the conversion of glucoraphanin to gluconapin in *Arabidopsis*. There are 3 AOP copies in broccoli, of which one is functional and two are mutated, whereas three genes in *B. napa* are functional [35]. According to sequence alignments acids, the AOP1 gene has an extra intron in exon 2, produces a smaller predicted protein and may not be functional [58, 60]. The AOP2 gene has few base changes and no function, and there is a large deletion in exon 2 in *AOP3*, but this gene might still retain its function. *AOP3* was not found in *B. napa* [58, 60, 61]. Another gene, *FMO GS-OX1*, is responsible for the conversion of glucoerucin into glucoraphanin, which is important for sulforaphane generation. However, there are few differences between broccoli plants [39]. In *Arabidopsis*, the MAM family contains three tandemly duplicated and functionally diverse members (*MAM1*, *2*, *3*). *MAM1* and *MAM2* catalyze the condensation of the first two elongation cycles for the synthesis of the dominant C3 and C4 side chain aliphatic glucosinolates, respectively [62, 63], whereas *MAM3* is assumed to contribute to the production of all glucosinolate chain lengths [22]. However, in *B. rapa* and *B. oleracea*, *MAM1/MAM2* genes experienced independent tandem duplication to produce C6 and C5 orthologs, respectively [24, 35]. In addition to the *MAM3* homologs in *Brassica*, at least two *MAM3* genes seem to be involved the C-side chain size: *BoGSL-PRO* and *BoGSL-ELONG*, determining glucosinolate of C3 and C4 side chains, respectively [60, 64].

Fig 2. Venn diagram comparisons (A) and cluster analysis of differentially expressed genes between leaves and developmental buds (B). Venn diagram comparison of differentially expressed genes between leaves and developmental buds at bolting stage. Hierarchical cluster analysis of differentially expressed genes among genotypes. The color key represents Lg (RPKM + 1). Red indicates high relative expression and blue indicates low relative expression. LN_F denotes leaves and LN_B (1–4) denotes developmental buds of broccoli at bolting stage.

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In this study, the genes of MAM1, MAM3, CYP79F1, St5b-2, FMO GS-OX1, MY, AOP2, AOP3 (homologous domain), ESP and ESM1 were detected and analyzed by qRT-PCR.

Table 4. The number of differentially expressed genes between different pairs samples.

| Groups/samples   | Total number | Up-regulated | Down-regulated |
|------------------|--------------|--------------|----------------|
| LN_B1 vs LN_F    | 4775         | 2775         | 2000           |
| LN_B2 vs LN_F    | 5454         | 3101         | 2353           |
| LN_B3 vs LN_F    | 5956         | 2982         | 2974           |
| LN_B4 vs LN_F    | 4874         | 2534         | 2340           |

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Combined with the transcriptome data, the study would help us to reveal the gene expression patterns of sulforaphane in the developmental buds at bolting stage.

Glucoraphanin belongs to C4 glucosinolate, which might be produced by MAM1/MAM2 genes. In B. rapa, MAM3 plays an important role in accumulation of C5 glucosinolates, such as glucobrassicanapin [35, 37]. However, our results showed that leaves had a higher level of MAM1 gene expression than the developing buds, which was depending on the cultivar of broccoli (Fig 5), and all the materials in this study had a low level of MAM1 as well as MAM3 expression, with the exception of the flowers, which had a slightly higher expression. This was consistent with the transcriptome results, which showed no significant differences between MAM1 and MAM3. In this study, the sulforaphane and glucoraphanin contents in developing buds were inversely correlated with the developmental stages, which might be caused by low MAM1 gene expression after bolting [31, 65].

A. thaliana with the CYP79F2 gene knocked out showed substantially reduced long-chain aliphatic glucosinolates and increased short-chain aliphatic glucosinolates, and CYP79F1

Table 5. The statistics of gene numbers at different interval level.

| FPKM Interval | LN_F | LN_B1 | LN_B2 | LN_B3 | LN_B4 |
|---------------|------|-------|-------|-------|-------|
| 0–1           | 35975(58.35%) | 30733(49.85%) | 32042(51.97%) | 33284(53.99%) | 33231(53.90%) |
| 1–3           | 4779(7.75%) | 5840(9.47%) | 5558(9.02%) | 5628(9.13%) | 5349(8.68%) |
| 3–15          | 10372(16.82%) | 12451(20.20%) | 12218(19.82%) | 12405(20.12%) | 12054(19.55%) |
| 15–60         | 7365(11.95%) | 9008(14.61%) | 8396(13.62%) | 7332(11.89%) | 7832(12.70%) |
| >60           | 3159(5.12%) | 3618(5.87%) | 3436(5.57%) | 3001(4.87%) | 3184(5.16%) |

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Fig 4. Violin plot of the normalized FPKM values for gene expression in different groups (A). Absolute magnitude (log) of the divergence of absolute magnitude of log (FPKM+1) resulting from leaves (LN_F), young buds (LN_B1), mature buds (LN_B2), buds one day before flowering (LN_B3) and flowers (LN_B4) of broccoli at bolting stage. Pearson correlation between samples of developmental buds (LN_B1−4) and leaves (LN_F) (B).

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Fig 5. RNA sequencing and qRT-PCR results of the expression genes related with sulforaphane metabolism.

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increases both long- and short-chain aliphatic glucosinolates [26, 29]. In our study, CYP79F1 (Bo5g021810-4.2906) was only significantly up-regulated in aliphatic glucosinolate biosynthesis, however this up-regulation was not found in the other developing buds and leaves. Therefore, up-regulation of the CYP79F1 gene might be one of the reasons for causing sulforaphane content being higher in young buds than mature buds, blossom buds, flowers and leaves at bolting stage, and this result was also supported by qRT-PCR. So up-regulation of the CYP79F1 gene might directly affect glucoraphanin accumulation [22, 31]. In growing and developing buds, because there is no up-regulation of the CYP79F1 gene, so the intermediates and precursors of glucoraphanin are gradually consumed. Therefore, the results of our study support de novo synthesis of glucoraphanin in young buds. Recent studies have identified two mechanisms of glucosinolate metabolism in plants: transport and de novo synthesis. The first mechanism is the transport of glucosinolate via the phloem from mature leaves to inflorescences and fruits [66, 67]. Other studies have shown that reproductive organs are likely to generate specific and unique glucosinolates by de novo synthesis in these organs [22, 68]. In fact, divergent glucosinolate composition of seeds and other organs have been widely detected, and there are obviously different amounts in the seeds, higher than the other oranges, which also supports the possibility of de novo synthesis in reproductive organs [69]. Our study provided for evidence in synthesis of glucosinolate in reproductive organs. St5b-2 is numbered K11821 in the KEGG orthology pathway, and it is responsible for tryptophan metabolism, glucosinolate biosynthesis, biosynthesis of secondary metabolites, and 2-Oxocarboxylic acid metabolism. In our study, this gene referred to 4-methylthiobutyl glucosinolate biosynthesis. According to sequence analysis, another gene in this family, ST5a-1, has similar function in tryptophan metabolism, glucosinolate biosynthesis, biosynthesis of secondary metabolites, and 2-Oxocarboxylic acid metabolism. ST5a-1 and St5b-2 have been reported in B. rapa, and their sequences have been analyzed by shotgun sequencing, but still no similar sequence was found in broccoli [35, 37]. In this result, there was a lower level of gene expression in developmental buds compared with in leaves. This might indicate that it supported the accumulation of

Fig 6. Sulforaphane and glucoraphanin concentrations detected in different organs of broccoli at bolting stage (A). Chromatography of sulforaphane (C) and TIC Chromatograph of glucosinolate (B) corresponding to glucoraphanin spectrum (RT) (D).

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4-methylthiobutyl glucosinolate (glucoerucin) for glucoraphanin generated by oxidation by FMO GS-OX1 gene [58].

The FMO GS-OX family (flavin-monooxygenase) contains five genes of FMO GS-OX1–5, two genes of FMO GS-OX2 and FMO GS-OX5 [70–72], and FMOGS-OX1 has been identified as an enzyme in the biosynthesis of aliphatic glucosinolates in Arabidopsis, catalyzing the S-oxygenation of methylthioalkyl to methylsulfinylalkyl glucosinolate. In sulforaphane synthesis, FMOGS-OX1 catalyzes the conversion of 4-methylthiobutyl glucosinolate (glucoerucin) to 4-methylsulfinylbutyl glucosinolate (glucoraphanin), the precursor of sulforaphane [72–73]. Five FMO genes At1g65860 (FMO GS-OX1), At1g62540 (FMO GS-OX2), At1g62560 (FMO GS-OX3), At1g62570 (FMO GS-OX4), and At1g12140 (FMO GS-OX5) have been found within a subclade of the FMO phylogeny [31, 57]. In the study, the gene expression of FMO GS-OX1 was at a low level in developing buds comparing to in leaves, which was similar to the genes of a subclade of the FMO phylogeny [31, 57].

Most of studies have reported the hydrolysis products of glucosinolate are controlled by epithiospecifier protein (ESP), myrosinase (MY), and potentially free iron and pH [21, 74]. Previous conclusions have shown that the system of glucosinolate hydrolysis is complex, and some results suggest that the ESP runs functions via interactions with myrosinase [32]. Myrosinase can catalyze the hydrolysis of the thioglucoside linkage and release a glucose and an unstable aglycone. The aglycone moiety subsequently rearranges to form various products depending on the aglycone structure, myrosinase, pH, ferrous ion, zinc and magnesium concentrations [24, 75–77]. Our results showed a high consistency of the gene expression among MY, FMO GS-OX1, St5b-2 and MAM1. Therefore, the correlations of ten genes in expression level and the contents of sulforaphane and glucoraphanin were analyzed by Pearson correlation test. The result revealed that six genes of MAM1, St5b-2, FMO GS-OX1, AOP2, ESP and ESM1 were highly correlated with correlation coefficients from 0.887 to 0.999 (P<0.01) (Tables 6 and 7). From the contents and consistent changes of gulcoraphanin and sulforaphane, it could be proved that myrosinase and ESP had not influence on sulforaphane generation at bolting stage.

So far, three AOP2 genes have been identified in B. oleracea, two are non-functional due to the presence of premature stop codons, and no AOP3 gene has been found [35]. In contrast, all three AOP2 copies are functional in B. rapa, resulting in conversion of glucoraphanin into gluconapin, which explains why glucoraphanin is abundant in B. oleracea, but not in B. rapa [31, 35]. AOP3 also does not exist in B. rapa, which contains three AOP loci orthologs, each containing two tandem duplicated genes [21, 60]. Studies in Arabidopsis have shown differential AOP leaf expression, whereby a particular accession expresses either AOP2 or AOP3 but not both [70, 78], which has been reported to be due a complete inversion of the AOP2 and AOP3 structural genes in some accessions, causing the AOP3 gene to be expressed from the AOP2 promoter [79]. This conclusion is in conflict with the absence of an AOP3 gene in cabbage [35], but our results support this conclusion in Arabidopsis based on AOP3 gene expression in this study.

According to the gene expression patterns of AOP2 and AOP3, it was found that AOP2 gene, likely MY, FMO GS-OX1, St5b-2 or MAM1, showed a lower level of expression in developing buds than in leaves (Fig 5). However, there was significantly higher AOP3 expression in developmental buds compared to leaves (Fig 5), the highest being in flowers, followed by young buds, mature buds and buds one day before flowering, and leaves were at the lowest level. AOP3 should be present in broccoli plant, however it was detected in the expression of the AOP3 domain, which might provide us new evidence for explaining the diversity of sulforaphane in different broccoli organs. Meanwhile the AOP3 gene plays a role in hydroxylation of glucoraphanin, which might partly explain why there was lower accumulation of
glucoraphanin in flowers compared to the other developmental buds, resulting in low concentration of sulforaphane [41, 60].

Plant hormones in pathways affecting glucoraphanin accumulation

Some studies have reported glucoraphanin and sulforaphane are influenced by genotype, developmental stages and environment effects, and others state that plant hormones, such as IAA and jasmonic acid (JA), also affect glucoraphanin production, resulting in changes in sulforaphane levels in broccoli [22–24, 57]. Several reports indicate that the loss function of CYP79F1 in mutations could end the formation of short-chain methionine-derived glucosinolates, but increase the amounts of IAA and cytokinin [80]. Glucosinolate syntheses also conversely affect the levels of auxin and cytokinin [27, 80]. JA is an elicitor and signaling molecule for glucosinolate biosynthesis, it has been shown to enhance both the production of indolic glucosinolates and their biosynthetic gene transcript levels in Arabidopsis, and the accumulation of glucoraphanin in broccoli could be up-regulated by JA related genes [38, 81].

| Table 6. The correlation analysis of sulforaphane contents and related genes in different organs. |
|------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Pearson Correlation | Sulforaphane | MAM1 | IMS2 | CYP79F1 | FMO GS-OX1 | AOP2 | AOP3 | MY | St5b-2 | ESP | ESM1 |
|-------------------|----------------|----------|-------|---------|-------------|------|------|-----|--------|------|------|
| Sulforaphane       | 1              | -0.61    | -0.373| 0.796   | -0.581      | -0.51| 0.125| -0.468| -0.63   | -0.141| 0.039 |
| MAM1               | -0.61          | 1        | 0.06  | -0.198  | .994**       | .967**| -0.484| .979**| .999**  | 0.868 | 0.757 |
| IMS2               | -0.373         | 0.06     | 1     | -0.022  | 0.093        | -0.002| 0.828| -0.002| 0.055   | -0.124| -0.144 |
| CYP79F1            | 0.796          | -0.198   | -0.022| 1       | -0.123      | -0.045| 0.248| -0.015| -0.224  | 0.282 | 0.464 |
| FMOGS-OX1          | -0.581         | 0.994**  | 0.093 | -0.123  | 1            | .985**| -0.443| .990**| .993**  | .887  | 0.79  |
| AOP2               | -0.51          | .967**   | -0.002| -0.045  | .985**       | 1     | -0.502| .992**| .967**  | .907  | 0.828 |
| AOP3               | 0.125          | -0.484   | 0.828 | 0.248   | -0.443      | -0.502| 1     | -0.498| -0.493  | -0.494| -0.426 |
| MY                 | -0.468         | .979**   | -0.002| -0.015  | .990**       | .992**| -0.498| 1     | .975**  | .939  | 0.862 |
| St5b-2             | -0.63          | .999**   | 0.055 | -0.224  | .993**       | .967**| -0.493| .975**| 1       | 0.855| 0.74  |
| ESP                | -0.141         | 0.868    | -0.124| 0.282   | .887*        | .907* | -0.494| .939* | 0.855   | 1     | .980** |
| ESM1               | 0.039          | 0.757    | -0.144| 0.464   | 0.79         | 0.828 | -0.426| 0.862 | 0.74    | .980  | 1     |

Note: *. Correlation is significant at the 0.05 level (2-tailed) and **. Correlation is significant at the 0.01 level (2-tailed).

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| Table 7. The correlation analysis of glucoraphanin contents and related genes in different organs. |
|------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Pearson Correlation | glucoraphanin | MAM1 | IMS2 | CYP79F1 | FMO GS-OX1 | AOP2 | AOP3 | MY | St5b-2 | ESP | ESM1 |
|-------------------|----------------|----------|-------|---------|-------------|------|------|-----|--------|------|------|
| glucoraphanin     | 1              | -0.634   | -0.444| .768    | -0.587      | -0.473| .061 | -0.470| -0.647 | -0.183| 0.000 |
| MAM1              | -0.634         | 1        | .060  | -0.198  | .994**      | .967**| -0.484| .979**| .999**  | .868 | 0.757 |
| IMS2              | -0.444         | .060     | 1     | -0.022  | .093        | -0.002| .828 | -0.002| 0.055   | -0.124| -0.144 |
| CYP79F1           | .768           | -0.198   | -0.022| 1       | -0.123      | -0.045| .248 | -0.015| -0.224  | .282 | 0.464 |
| FMOGS-OX1         | -0.587         | .994**   | 0.093 | -0.123  | 1            | .985**| -0.443| .990**| .993**  | .887 | 0.79  |
| AOP2              | -.473          | .967**   | -.002 | -.045   | .985**       | 1     | -.502| .992**| .967**  | .907 | .828 |
| AOP3              | .061           | -.484   | .828  | .248    | -.443       | -.502 | 1    | -.498 | -.493   | -.494 | -.426 |
| MY                | -.470          | .979**   | -.002 | -.015   | .990**       | .992**| -.498| 1    | .975**  | .939 | .862 |
| St5b-2            | -.647          | .999**   | .055  | -.224   | .993**       | .967**| -.493| .975**| 1       | .855 | .74  |
| ESP               | -.183          | .868     | -.124 | .282    | .887*        | .907* | -.494| .939* | .855    | 1    | .980** |
| ESM1              | .000           | .757     | -.144 | .464    | .790         | .828 | -.426| .862 | .740    | .980 | 1     |

Note: *. Correlation is significant at the 0.05 level (2-tailed) and **. Correlation is significant at the 0.01 level (2-tailed).

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In this study, plant hormone signal transduction was analyzed by RNA sequencing, and there were 95, 89, 97 and 86 corresponding DEGs with the same 521 background genes based on the developing buds (LN_B1-4) versus leaf expression. According to the differences in plant hormone signal transduction gene expression among four organs in developmental buds, 2 DEGs were found in the auxin signaling pathway, one was up-regulated (Bo5g027930) only in young buds. This finding reminded us the association of young buds with higher sulforaphane content and higher expression of Bo5g027930 only occurring in this organ, which might provide evidence for the importance of CYP79F1. The specific mechanism driving these observations still needs further research. The other auxin signaling gene was down regulated (Bo9g151530), and it occurred in buds one day before flowering and flowers. Thus, it could be inferred that different auxin response might affect the accumulation of glucoraphanin [27, 41].

In the cytokinin signaling pathway, 3 up-regulated genes and 2 down-regulated genes were different in developing buds. A total of 3 up-regulated genes, Bo8g091410, Bo3g107060 and Bo3g035110, only showed higher expression in young buds and were absent in the remaining developing buds. In contrast, 2 genes, Bo5g027070 and Bo8g059410, were down-regulated in buds one day before flowering and flowers, and absent from in young and mature buds. These 5 genes belong to the two-component response regulator ARR-A family, which might be potential genes in affecting glucoraphanin generation [29].

**Conclusions**

In the study, it was found that CYP79F1 plays a fundamental and direct role in sulforaphane production of inflorescences at differential developmental stages, and a low expression level resulted in a decrease of this compound or the precursor glucoraphanin due to competition for the intermediates, such as 2-oxo-6-methylthihexanoic acid or 4-methylthiobutyl (glucoerucin). These genes of MAM1, MAM3, St5b-2, FMO GS-OX1 were in favor of glucoraphanin, MY, ESP and ESM1 played a high efficiency function in sulforaphane generation although with low expression level in this stage. At the same time, the plant hormones auxin and cytokinin might affect glucoraphanin accumulation. The knowledge gained from this study provides a way to study different molecular mechanisms and the diversity of sulforaphane in different organs during broccoli development stages.

**Supporting information**

S1 Fig. The most enriched GO terms. (TIF)

S2 Fig. Patterns of gene expressions in the developmental buds and leaves of B52 by STEM analysis ($P < 0.05$). The green line represents the expression pattern of all the genes. The number of genes belonging to each pattern is labeled above frame. (TIF)

S3 Fig. The distribution of clean reads, containing N, low quality and adapter related reads in the raw reads. (TIF)

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References
1. Jin CY, Moon DO, Lee JD, Heo MS, Choi YH, Lee CM, et al. Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis through downregulation of ERK and Akt in lung adenocarcinoma A549 cells. Carcinogenesis 2007; 28(5): 1058–66. https://doi.org/10.1093/carcin/bgl251 PMID: 17183064
2. Chung YK, Chi-Hung OR, Lu CH, Ouyang WT, Yang SY, Chang CC. Sulforaphane down-regulates SKP2 to stabilize p27 for inducing anti-proliferation in human colon adenocarcinoma cells. J Biosci Bioeng 2015; 119(1): 35–42. https://doi.org/10.1016/j.jbiosc.2014.06.009 PMID: 25070589
3. Kalilifatidis G, Rausch V, Baumann B, Apel A, Beckermann BM, Groth A, et al. Sulforaphane targets pancreatic tumour-initiating cells by NF-kappa B-induced anti-apoptotic signalling. Gut 2009; 58(7): 949–963. https://doi.org/10.1136/gut.2008.149039 PMID: 18829980
4. Azarenko O, Okouneva T, Singletary KW, Jordan MA, and Wilson L. Suppression of microtubule dynamic instability and turnover in MCF7 breast cancer cells by sulforaphane. Carcinogenesis 2008; 29(12): 2360–8. https://doi.org/10.1093/carcin/bgn241 PMID: 18952594
5. Abbaoui B, Riedl KM, Ralston RA, Thomas-Ahner JM, Schwartz SJ, Clinton SK, et al. Inhibition of bladder cancer by broccoli isothiocyanates sulforaphane and erucin: characterization, metabolism, and interconversion. Mol Nutr Food Res 2012; 56(11): 1675–1687. https://doi.org/10.1002/mnfr.201200276 PMID: 23038615
6. Brooks JD, Paton V. Potent induction of carcinogen defence enzymes with sulforaphane, a putative prostate cancer chemopreventive agent. Prostate Cancer Prostatic Dis 1999; 2(S3): S8. https://doi.org/10.1038/sj.pcan.4500334 PMID: 12496788
7. Zhang R, Miao QW, Zhu CX, Zhao Y, Liu L, Yang J, et al. Sulforaphane Ameliorates Neurodegenerative Deficits and Protects the Brain From Amyloid beta Deposits and Peroxidation in Mice With Alzheimer-Like Lesions. Am J Alzheimers Dis Other Demen 2014. 30(2): 183–191. https://doi.org/10.1177/1533317514542645 PMID: 25024455
8. Evans PC. The influence of sulforaphane on vascular health and its relevance to nutritional approaches to prevent cardiovascular disease. EPMA J 2011; 2(1): 9–14. https://doi.org/10.1007/s13167-011-0064-3 PMID: 23199123
9. Sivakumar MR, Sanjeev S. Cerebrovascular manifestations and carotid artery intima medial thickness in Takayasu’s arteritis evaluated by using the Disease Extent Index for TA (DEI.Tak). Clinical and Experimental Rheumatology 2007; 25(2): S120–S120.
10. Alumkal JJ, Slottke R, Schwartzman J, Cherala G, Munar M, Graff JN, et al. A phase II study of sulforaphane-rich broccoli sprout extracts in men with recurrent prostate cancer. Investigational New Drugs 2015; 33(2): 480–489. https://doi.org/10.1007/s10637-014-0189-z PMID: 25431127

11. Angeloni C, Leoncini E, Malaguti M, Angelini S, Hrelia P, Hrelia S. Modulation of phase II enzymes by sulforaphane: implications for its cardioprotective potential. J Agric Food Chem 2009; 57(12): 5615–22. https://doi.org/10.1021/jf900549c PMID: 19456137

12. Asakage M, Tsuno NH, Kitayama J, Tsuchiya T, Yoneyama S, Yamada J, et al. Sulforaphane induces inhibition of human umbilical vein endothelial cells proliferation by apoptosis. Angiogenesis 2006; 9(2): 83–91. https://doi.org/10.1007/s10456-006-9034-0 PMID: 16821112

13. Ferreira de Oliveira JM, Remedios C, Oliveira H, Pinto P, Pinho F, Pinho S, et al. Sulforaphane induces DNA damage and mitotic abnormalities in human osteosarcoma MG-63 cells: correlation with cell cycle arrest and apoptosis. Nutr Cancer 2014; 66(2): 325–34. https://doi.org/10.1080/01635581.2014.864777 PMID: 24405297

14. Angelino D, Dosz EB, Sun J, Hoeflinger JL, Van Tassell ML, Chen P, et al. Myrosinase-dependent and -independent formation and control of isothiocyanate products of glucosinolate hydrolysis. Frontiers in Plant Science 2015; 6: 831. https://doi.org/10.3389/fpls.2015.00831 PMID: 26500669

15. Fahey JW, Holtzclaw WD, Wehage SL, Wade KL, Stephen son KK, Talalay P. Sulforaphane Bioavailability from Glucorapha nin-Rich Broccoli: Control by Active Endogenous Myrosinase. Plos One 2015; 10(11): e0140963. https://doi.org/10.1371/journal.pone.0140963 PMID: 26524341

16. Angelino D, Jeffery E. Glucosinolate hydrolysis and bioavailability of resulting isothiocyanates: Focus on glucoraphan. Journal of Functional Foods 2014; 7: 67–76.

17. Cramer JM, Jeffery EH. A comparison of the bioavailability of sulforaphane from broccoli sprouts and a semi-purified broccoli powder rich in glucoraphan in healthy human males. Faseb Journal 2009; 23.

18. Matusheski NV, Jeffery EH. Comparison of the bioactivity of two glucoraphan hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. J Agric Food Chem 2001; 49(12): 5743–9. PMID: 11743757

19. Agudo A, Ibanez R, Amiano P, Ardanaz E, Barricarte A, Berenguer A, et al. Consumption of cruciferous vegetables and glucosinolates in a Spanish adult population. European Journal of Clinical Nutrition 2008; 62(3): 324–331. https://doi.org/10.1038/sj.ejcn.1602750 PMID: 17426741

20. Ares AM, Bernal J, Nozal MJ, Turner C, Plaza M. Fast determination of intact glucosinolates in broccoli leaf by pressurized liquid extraction and ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. Food Research International 2015; 76(Pt 3): 498–505. https://doi.org/10.1016/j.foodres.2015.06.037 PMID: 28455030

21. Brown PD, Tokuhisa JG, Reichelt M, Gershenzon J. Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. Phytochemistry 2003; 62(3): 471–81. PMID: 12620360

22. Field B, Cardon G, Traka M, Botterman J, Vancanneyt G, Mithen R. Glucosinolate and amino acid biosynthesis in Arabidopsis. Plant Physiology 2004; 135: 828–839. https://doi.org/10.1104/pp.104.039347 PMID: 15155874

23. Grubb CD, Abel S. Glucosinolate metabolism and its control. Trends in Plant Science 2006; 11(2): 89–100. https://doi.org/10.1016/j.tplants.2010.02.005 PMID: 20303821

24. Wittstock U, Halkier BA. Glucosinolate research in the Arabidopsis era. Trends in Plant Science 2002; 7(6): 263–270. PMID: 12049923

25. Guo LP, Yang RQ, Wang ZY, Gu QH, Gu ZX. Glucoraphan, sulforaphane and myrosinase activity in germinating broccoli sprouts as affected by growth temperature and plant organs. Journal of Functional Foods 2014; 9(1): 70–77.

26. Sonderby IE, Geu-Flores F, Halkier BA. Biosynthesis of glucosinolates—gene discovery and beyond. Trends in Plant Science 2010; 15(5): 283–290. https://doi.org/10.1016/j.tplants.2010.02.005 PMID: 20303821
30. Hansen CH, Du LC, Naur P, Olsen CE, Axel sen KB, Hick AJ, et al. CYP83B1 is the oxime-metabolizing enzyme in the glucosinolate pathway in Arabidopsis. Journal of Biological Chemistry 2001; 276(6): 24790–24796.

31. Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, Gershenzon J, et al. Genetic control of natural variation in Arabidopsis glucosinolate accumulation. Plant Physiol 2001; 126(2): 811–25. PMID: 11402209

32. Lambrix V, Reichelt M, Mitchell-Olds T, Kliebenstein DJ, Gershenzon J. The Arabidopsis ethiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences Trichoplusia ni herbivory. Plant Cell 2001; 13(12): 2793–2807. https://doi.org/10.1109/tpc.010261 PMID: 11752388

33. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 2001; 56(1): 5–51. PMID: 11198818

34. Li YM, Sawada Y, Hirai A, Sato M, Kuwahara A, Yan XF, et al. Altered Regulation of MYB Genes Changes the Aliphatic Glucosinolate Accumulation Under Long-Term Sulfur Deficiency in Arabidopsis. Molecular Physiology and Ecophysiology of Sulfur 2015; 195–199.

35. Li ZS, Liu YM, Fang ZY, Yang LM, Zhuang M, Zhang YY, et al. Variation of Sulforaphane Levels in Broccoli (Brassica Oleracea Var. Italica) during Flower Development and the Role of Gene Aop2. Journal of Liquid Chromatography & Related Technologies 2014; 37(9): 1199–1211.

36. Glade MJ, Meguid MM. A Glance at . . . Broccoli, glucoraphanin, and sulforaphane. Nutrition 2015; 31(9): 1175–1178. https://doi.org/10.1016/j.nut.2015.03.003 PMID: 26004191

37. Io R, Bernardi R, Gueyrard D, Rollin P, Palmieri S. Formation of glucoraphanin by chemoselective oxidation of natural glucoraphanin: a chemoenzymatic route to sulforaphane. Bioorg Med Chem Lett 1999; 9 (7): 1047–8. PMID: 10230637

38. Cano-Gomez C, Palero F, Buitrago MD, Garcia-Casado MA, Fernandez-Pinero J, Fernandez-Pacheco P, et al. Analyzing the genetic diversity of teschoviruses in Spanish pig populations using complete VP1 sequences. Infection Genetics and Evolution 2011; 11(8): 2144–2150.

39. Heinl S, Spath K, Egger E, Grabherr R. Sequence analysis and characterization of two cryptic plasmids derived from Lactobacillus buchneri CDO34. Plasmid 2011; 66(3): 159–168. https://doi.org/10.1016/j.plasmid.2011.08.002 PMID: 21907734

40. Clark CG, Beeston A, Bryden L, Wang GH, Barton C, Cuff W, et al. Phylogenetic relationships of Campylobacter jejuni based on porA sequences. Canadian Journal of Microbiology 2007; 53(1): 27–38. https://doi.org/10.1139/w06-099 PMID: 17496947

41. Nolling J, Breton G, Omelchenko MV, Makarova KS, Zeng QD, Gibson R, et al. Genome sequence and comparative analysis of the solvent-producing bacterium Clostridium acetobutylicum. Journal of Bacteriology 2001; 183(16): 4823–4838. https://doi.org/10.1128/JB.183.16.4823-4838.2001 PMID: 11466286

42. Clark RM, Schweikert G, Toomajan C, Osowski S, Zeller G, Shinn P, et al. Common sequence polymorphisms shaping genetic diversity in Arabidopsis thaliana. Science 2007; 317(5836): 338–342. https://doi.org/10.1126/science.1138632 PMID: 17641193

43. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. Bmc Bioinformatics 2011; 12(1): 323.
50. Zhang XL, Liu YM, Fang ZY, Li ZS, Yang LM, Zhuang M, et al. Comparative Transcriptome Analysis between Broccoli (Brassica oleracea var. italica) and Wild Cabbage (Brassica macrocarpa Guss.) in Response to Plasmidiphorabracksicae during Different Infection Stages. Frontiers in Plant Science 2016; 7(R106): 1929.

51. Liu C, Liu ZY, Li CY, Zhang Y, Feng H. Comparative transcriptome analysis of fertile and sterile buds from a genetically male sterile line of Chinese cabbage. In Vitro Cellular & Developmental Biology-Plant 2016; 52(2): 130–139.

52. Xing MM, Lv HH, Ma J, Xu DH, Li HL, Yang LM, et al. Transcriptome Profiling of Resistance to Fusarium oxysporum f. sp conglutinans in Cabbage (Brassica oleracea) Roots. Plos One 2016; 11(2): e0148048. https://doi.org/10.1371/journal.pone.0148048 PMID: 26849436

53. Li ZS, Liu YM, Fang ZY, Yang LM, Zhuang M, et al. Development and Identification of Anti-cancer Component of Sulforaphane in Developmental Stages of Broccoli (Brassica oleracea var. italica). Journal of Food and Nutrition Research 2016; 4(8): 490–497.

54. Agerbirk N, Olsen CE, Chew FS, Orgaard M. Variable glucosinolate profiles of Cardamine pratensis (Brassicaceae) with equal chromosome numbers. J Agric Food Chem 2010; 58(8): 4693–4700. https://doi.org/10.1021/jf904362m PMID: 20334382

55. Gao MQ, Li GY, Yang B, McCombie WR, Quiros CF. Comparative analysis of a Brassica BAC clone containing several major aliphatic glucosinolate genes with its corresponding Arabidopsis sequence. Genome 2004; 47(4): 666–679. https://doi.org/10.1139/g04-021 PMID: 15284871

56. Giamoustas A, Mithen R. The effect of flower colour and glucosinolates on the interaction between oilseed rape and pollen beetles. Entomologia Experimentalis Et Applicata 1996; 80(1): 206–208.

57. Hall C, McCallum D, Prescott A, Mithen R. Biochemical genetics of glucosinolate modification in Arabidopsis and Brassica. Theoretical and Applied Genetics 2001; 102(2): 369–374.

58. Halkier BA, Du LC. The biosynthesis of glucosinolates. Trends in Plant Science 1997; 2(11): 425–431.

59. Hall C, McCallum D, Prescott A, Mithen R. Biochemical genetics of glucosinolate modification in Arabidopsis and Brassica. Theoretical and Applied Genetics 2001; 102(2): 369–374.

60. Li G, Quiros CF. In planta side-chain glucosinolate modification in Arabidopsis by introduction of dioxygenase Brassa homolog BoGSL-ALK. Theoretical and Applied Genetics 2003; 106(6): 1116–1121. https://doi.org/10.1007/s00122-002-1161-4 PMID: 12671761

61. Burrow M, Atwell S, Francisco M, Kerwin RE, Halkier BA, Kleibenstein DJ. The Glucosinolate Biosynthetic Gene AOP2 Mediates Feed-back Regulation of Jasmonic Acid Signaling in Arabidopsis. Molecular Plant 2015; 8(8): 1201–1212. https://doi.org/10.1016/j.molp.2015.03.001 PMID: 25758208

62. Heidel AJ, Clauss MJ, Kryomann J, Savolainen O, Mitchell-Olds T. Natural variation in MAM within and between populations of Arabidopsis lyrata determines glucosinolate phenotype. Genetics 2006; 173(3): 1629–36. https://doi.org/10.1534/genetics.106.056986 PMID: 16702431

63. Piotrowski M, Schemenwitz A, Lopukhina A, Muller A, Janowitz T, Weiler EW, et al. Desulfoglucosinolate sulfotransferases from Arabidopsis thaliana catalyze the final step in the biosynthesis of the glucosinolate core structure. Journal of Biological Chemistry 2004; 279(49): 50717–50725. https://doi.org/10.1074/jbc.M407681200 PMID: 15358770

64. Yabar E, Pedreschi R, Chirinos R, Campos D. Glucosinolate content and myrosinase activity evolution in three maca (Lepidium meyenii Walp.) ecotypes during preharvest, harvest and postharvest drying. Food Chemistry 2011; 127(4): 1576–1583.

65. Textor S, de Kraker JW, Hause B, Gershenson J, Tokuhisa JG. MAM3 catalyzes the formation of all aliphatic glucosinolate chain lengths in Arabidopsis. Plant Physiology 2007; 144(1): 60–71. https://doi.org/10.1104/pp.106.091579 PMID: 17369439

66. Brudell AJP, Griffiths H, Rossiter JT, Baker DA. The phloem mobility of glucosinolates. Journal of Experimental Botany 1999; 50(335): 745–756.

67. Chen S, Andreasson E. Update on glucosinolate metabolism and transport. Plant Physiology and Biochemistry 2001; 39(9): 743–758.

68. Du LC, Halkier BA. Biosynthesis of glucosinolates in the developing silique walls and seeds of Sinapis alba. Phytochemistry 1998; 48(7): 1145–1150.

69. Gorrissen A, Kraut NU, de Visser R, de Vries M, Roelofs H, Vonk RJ. No de novo sulforaphane biosynthesis in broccoli seedlings. Food Chemistry 2011; 121(1): 192–196.

70. Bennett RN, Hick AJ, Dawson GW, Wallsgrove RM. Glucosinolate Biosynthesis—Further Characterization of the Aldoxime Forming Microsomal Monoxygenases in Oilseed Rape Leaves. Plant Physiology 1995; 109(1): 299–305. PMID: 12228596
71. Kong WW, Li J, Yu QY, Cang W, Xu R, Wang Y, et al. Two Novel Flavin-Containing Monooxygenases Involved in Biosynthesis of Aliphatic Glucosinolates. Frontiers in Plant Science 2016; 7(e2068):1292.

72. Li J, Hansen BG, Ober JA, Kliebenstein DJ, Halkier BA. Subclade of Flavin-Monoxygenases Involved in Aliphatic Glucosinolate Biosynthesis. Plant Physiology 2008; 148(3): 1721–1733. https://doi.org/10.1104/pp.108.125757 PMID: 18799661

73. Li J, Kristiansen KA, Hansen BG, Halkier BA. Cellular and subcellular localization of flavin-monoxygenases involved in glucosinolate biosynthesis. Journal of Experimental Botany 2011; 62(3): 1337–1346. https://doi.org/10.1093/jxb/erq369 PMID: 21078824

74. Wittstock U, Kliebenstein DJ, Lambrix V, Reichelt M, Gershenzon J. Glucosinolate hydrolysis and its impact on generalist and specialist insect herbivores. Integrative Phytochemistry: From Ethnobotany to Molecular Ecology 2003; 37: 101–126.

75. Liang H, Yuan QP, Xiao Q. Effects of metal ions on myrosinase activity and the formation of sulforaphane in broccoli seed. Journal of Molecular Catalysis B-Enzymatic 2006; 43(1): 19–22.

76. Fu LL, Wang M, Han BY, Tan DG, Sun XP, Zhang JM. Arabidopsis Myrosinase Genes AtTGG4 and AtTGG5 Are Root-Tip Specific and Contribute to Auxin Biosynthesis and Root-Growth Regulation. International Journal of Molecular Sciences 2016; 17(6): 892.

77. Pang QY, Guo J, Chen SX, Chen YZ, Zhang L, Fei MH, et al. Effect of salt treatment on the glucosinolate-myrosinase system in Thellungiella salsuginea. Plant and Soil 2012; 355(1–2): 363–374.

78. Kliebenstein DJ, Lambrix VM, Reichelt M, Gershenzon J, Mitchell-Olds T. Gene duplication in the diversification of secondary metabolism: Tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in arabidopsis. Plant Cell 2001; 13(3): 681–693. PMID: 11251105

79. Kroymann J, Textor S, Tokuhisa JG, Falk KL, Bartram S, Gershenzon J, et al. A gene controlling variation in arabidopsis glucosinolate composition is part of the methionine chain elongation pathway. Plant Physiology 2001; 127(3): 1077–1088. PMID: 11706188

80. Tantikanjana T, Mikkelsen MD, Hussain M, Halkier BA, Sundaresan V. Functional analysis of the tandem-duplicated P450 genes SPS/BUS/CYP79F1 and CYP79F2 in glucosinolate biosynthesis and plant development by Ds transposition-generated double mutants. Plant Physiology 2004; 135(2): 840–848. https://doi.org/10.1104/pp.104.040113 PMID: 15194821

81. Bak S, Feyereisen R. The involvement of two P450 enzymes, CYP83B1 and CYP83A1, in auxin homeostasis and glucosinolate biosynthesis. Plant Physiology 2001; 127(1): 108–118. PMID: 11553739