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The methylation pattern of DNA and complex correlations with gene expressions during *TuMV* infection in Chinese cabbage

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Abstract

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is one of the most important economic crops. However, its yield and quality can be severely threatened by *Turnip mosaic virus* (*TuMV*). Emerging evidence indicates that epigenetic mechanisms, especially DNA methylation, play an important role in regulating gene expression. Therefore, identification of resistance genes modified by DNA methylation during the virus infection would provide a critical clue for improving disease resistance breeding programs. Here, we present detailed analysis for the correlation of DNA methylation and gene expression involved in several anti-pathogen pathways. We also found that different methylation patterns exist in different methylation sites (CG, CHG, and CHH, where H represents A, G, or T) and genomic regions. Furthermore, we identified disease-resistant genes related to the nucleotide binding site-leucine-rich repeats family, auxin, salicylic acid signaling transduction, cell wall biosynthesis, and protein degradation among the different methylated genes (DMGs) suggesting that these genes may be modified by DNA methylation and work together to activate an immune response. The identified DMGs are a valuable resource for discovering resistance genes. Our study not only provides valuable data for future biotechnology research and epigenetic studies, but also helps to explore how the epigenetic mechanisms modify antiviral pathways.

Additional key words: auxin, *Brassica rapa*, disease resistance, epigenetic mechanisms, NBS-LRR, salicylic acid.

Introduction

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is an of important economic crop. *Turnip mosaic virus* (*TuMV*) belonging to the *potyviruses*, does a great harm to *Brassica* species and causes epidemic disease in Asia, North America, and Europe (Tomlinson 1987). It causes serious systemic vein clearing, necrosis, stunting, and even plant death, resulting in a decline of yield and quality. Plant disease symptoms in hosts are usually combined actions of molecular, cellular, and physiological changes. Virus infection has a complex and various impacts on host gene expressions. Hence, comprehensive information on virus-host interactions and better understanding the plant immune response is required to establish a theoretical basis for breeding to resistance.

Epigenetics is defined as heritable changes, modifying DNA and associated proteins without changing the DNA sequence itself (Jones *et al*. 2002). Epigenetic mechanisms include various contexts like DNA methylation, histone

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Abbreviations: ARF - auxin response factor; CC - coiled-coil; CpG - cytosine-phosphate-avidin; DMG - differentially methylated gene, DMR - different methylation region; *GO - gene ontology*; H - adenine, guanine, or thymidine; IAA - indole-3-acetic acid; *KEGG - Kyoto encyclopedia of genes and genomes*; LRR - leucine-rich repeats; MeDIP - methylated DNA immunoprecipitation sequencing; NBS - nucleotide binding site; NPR - non expresser of PR gene; PR - pathogenesis-related; RNAi - RNA interference; SA - salicylic acid;; siRNA - small interfering RNA; TIR - Tolland interleukin-1 receptor; *TuMV - Turnip mosaic virus;* UPS - ubiquitin proteasome system.
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modifications, and microRNAs. These epigenetic pathways play an important role in maintenance of normal gene expressions (Chan *et al*. 2005, Law and Jacobsen 2010, Gan *et al*. 2013). Although it is conserved in eukaryotes, DNA methylation in plants is slightly different from animals regarding to the methylation patterns and methylation machinery (Chan *et al*. 2005). It usually occurs in a cytosine of a cytosine-phosphate-avidin (CpG) dinucleotide, then a methyl library is added to position 5 of a cytosine ring under the action of DNA methyltransferases (Jones and Baylin 2002). Most of the CpG sites are depleted in the genome and exist in the form of an island, called the CpG island. A mass of studies revealed that methylation of CpG islands in promoter regions often leads to gene silencing (Bird 2002, Jones and Baylin 2002).

The aim of this study was to screen the methylation profile and to detect mRNA transcriptional activity of different methylated genes (DMGs) of Chinese cabbage. Therefore, two DNA libraries were constructed: one before and one after *TuMV* infection. The present study was aimed to explore the epigenetic mechanisms of plant disease resistance and identify differentially methylated genes potentially involved in disease resistance. Our study can serve as a valuable resource for functional validation and contribute to the development of molecular breeding.

Materials and methods

Plants and *TuMV* inoculation: After germination in a hole tray, Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) plants were grown in a mixture of soil and *Vermiculite* (3:1) in a climate chamber at a temperature 22 °C, a relative humidity of 50 %, a 16-h photoperiod, and an irradiance of 300 μmol m^{-2} s^{-1} for seven weeks. Plants were either inoculated with *TuMV* or treated with water (controls). The inoculation method used was the one described by Ayaka *et al* (2016). At 21 d post inoculation, the leaves of *TuMV*-infected (*TuMV*) and water-treated (control) plants were collected and frozen in liquid nitrogen. Each sample from three individuals was mixed in equal amounts for analysis.

Preparation of DNA and library construction: The DNA from leaves of *TuMV* and control plants was isolated using a universal genomic DNA extraction kit (*Takara*, Dalian, China) and sheared to 100 - 500 bp using a *Bioruptor* sonicator (*Diagenode*, NJ, USA). End reparation, base addition and adaptor ligation were performed using a *Methyl-Seq 1* kit (*Takara*). Adaptor-ligated sheared DNA was immunoprecipitated by a 5-methylcytidine antibody with a magnetic methylated DNA immuno-precipitation kit (*Diagenode*) to construct methylated DNA immunoprecipitation sequencing (*MeDIP*) libraries. After PCR amplification and size selection (usually 200 - 300 bp), the qualified libraries were subjected to high-throughput sequencing by the *Illummina HiSeq 2000* platform.

Read alignments and methylated DNA immunoprecipitation sequencing analysis: Sequencing data were filtered and the adapter sequences, contamination,and low-quality reads were removed from raw reads. Clean data were stored in a fastQ format and mapped to *B. rapa* genome, which was downloaded from the *Brassica* database (http://brassicad b.org brad/) by *SOAP2.21*. The methylated DNA immunoprecipitation sequencing (*MeDIP-Seq*) data were mapped to the reference genome, only unique alignments with no more than two mismatches were considered for further analysis. The uniquely mapped data were retained for read distribution analysis including the distribution in different genome components and methylated sites (CG, CHG, and CHH, where H represents A, G, or T). Clean reads can be mapped to the genome by the following three ways: uniquely mapped, multiply mapped, and unmapped. All the following analyses were based on uniquely mapped reads. The *MeDIP-Seq* reads were aligned using mapping and assembly with qualities, and only genome-wide methylation peak scanning was conducted by *MACS 1.4.0* (http://liulab.dfci.harvard.Edu/MACS/). Peaks of samples from *TuMV* and control plants were merged as candidate different methylation regions (DMRs) using *MACS1.4.0* ($P < 0.01$) and at least a 2-fold change in sequence counts. For each candidate DMR, the number of reads of each sample was calculated and then assessed with chi-square statistics and false discovery rate (FDR) statistics to get true DMRs ($P < 0.05$, FDR < 0.01 and at least a 2-fold change in sequence counts).

Functional classification of DMGs by gene ontology and *KEGG*: To investigate the biological significance of the changes in methylation provoked by *TuMV* infection in *Brassica rapa*, the enrichment of *gene ontology* (*GO*) categories and *Kyoto encyclopedia of genes and genom*es (*KEGG*) from the DMGs was assessed. The *GO* database is a relational database comprising the gene ontologies and the annotations of genes and gene products to terms in the *GO*. Differential genes were functionally clustered *via* GO analysis to explore their biological function (http://www.ge neontology.org). Enrichment of *GO* categories among the DMGs was assessed using the *BINGO* software. The *KEGG* is a database resource for understanding high-level functions and utilities of the biological system. A pathway mapped Q value ≤ 0.05 was defined as the pathway of significant enrichment. The biochemical pathways involved in the DMGs were predicted using *KEGG*.

Synthesis of cDNA and gene expression validation: For validation and identification of anti-pathogen related genes from DMGs in Chinese cabbage, real-time quantitative PCR analysis of mRNA expression was performed in *TuMv* and control plants. Total RNA was extracted from a mixture of the leaf tissue of three individual plants as one replication using a *Trizol* reagent (*Takara*) and then subjected to cDNA synthesis using a *PrimeScript*™ first-strand cDNA synthesis kit (*Takara*) according to the manufacturer's protocol. Quantitative PCR was performed using a standard *SYBR H Premix Ex TaqT*™ *II* (*Takara*) on an *ABI PrismR 7900HT* (*Applied Biosystems*, Foster City, USA) according to the manufacturer's instructions. The amplification procedure was performed as following:

pre-denaturation at 94 °C for 10 s, next 94 °C for 30 s, 40 cycles at 60 °C for 30 s, and finally a melting curve (61 cycles at 65 °C for 10 s). Three biological and three technical replicates were performed for each gene. The sequences of Chinese cabbage were downloaded from the *Brassica rapa* database, and gene-specific primers were designed according to the gene sequences using *Becon Designer vision 7.9* (Table 1 Suppl.). The *actin* gene (*Bra028615*) was used as an internal control (Dheda *et al*. 2004). The relative expressions were calculated using the $2^{-\Delta\Delta CT}$ method (Livak *et al*. 2008). Differences at $P < 0.05$ were considered significant. *Bubble Chart* was performed using the *Omic Share* tools, a free online platform for data analysis (www.omicshare.com/tools).

Table 1. Sequencing and assembly statistics for the methylated DNA immunoprecipitation sequencing data before and after *Turnip mosaic virus* (*TuMV*) infection.

Categories	Control	*TuMV*
Total reads	97959184	97959184
Length of reads	49 bp	49 bp
Mapped reads	73645014	77367558
Mapping rate [%]	75.18	78.98
Effective chain depth	12.67	13.31
Unique mapped reads	27419578	33851819
Unique mapping rate	27.99	34.56

Results

To evaluate the global methylation pattern in Chinese cabbage, the samples from *TuMV* and control plants were collected, and two *MeDIP-Seq* libraries (*TuMV* and control) were constructed. In this study, 97 959 184 raw reads from *MeDIP-Seq* were screened from the two libraries, approximately 75.18 % (control) and 78.98% (*TuMV*) of clean reads were mapped to raw reads, and approximately 27.99 % (control) and 34.56 % (*TuMV*) of the reads were uniquely mapped to clean reads (Table 1). The distribution of MeDIP-seq reads in different CG density regions varied. The densities of 15 to 25 CpGs showed a highest percentage of reads in the*TuMV* library. There existed a fluctuation in the control library, which was concentrated in a higher density of CpGs.

The methylation sites in the genome mainly include three types (CG, CHG, and CHH, where H represents A, G, or T). Here, the proportion of three methylation sites with a varied sequencing depth has been measured. Sequencing depth was defined as the ratio of the total number of bases obtained by sequencing to the genome size, which is one of the indicators for evaluating the extent of sequencing. The proportion of three methylation sites in the genome has increased in *TuMV* samples (Fig. 1). A recent work indicated that CHH (H means A, G, or T) methylation plays an important role in various plant developmental processes, which was reported in *Arabidopsis* endosperm, and maize and cotton fibers (Zhang *et al.* 2006, Hsieh *et al*. 2009). Thus, we inferred that a relatively higher proportion of newly identified CHH methylations may influence gene expressions.

The distribution of *MeDIP-Seq* reads in different genomic regions presented distinct methylation patterns.

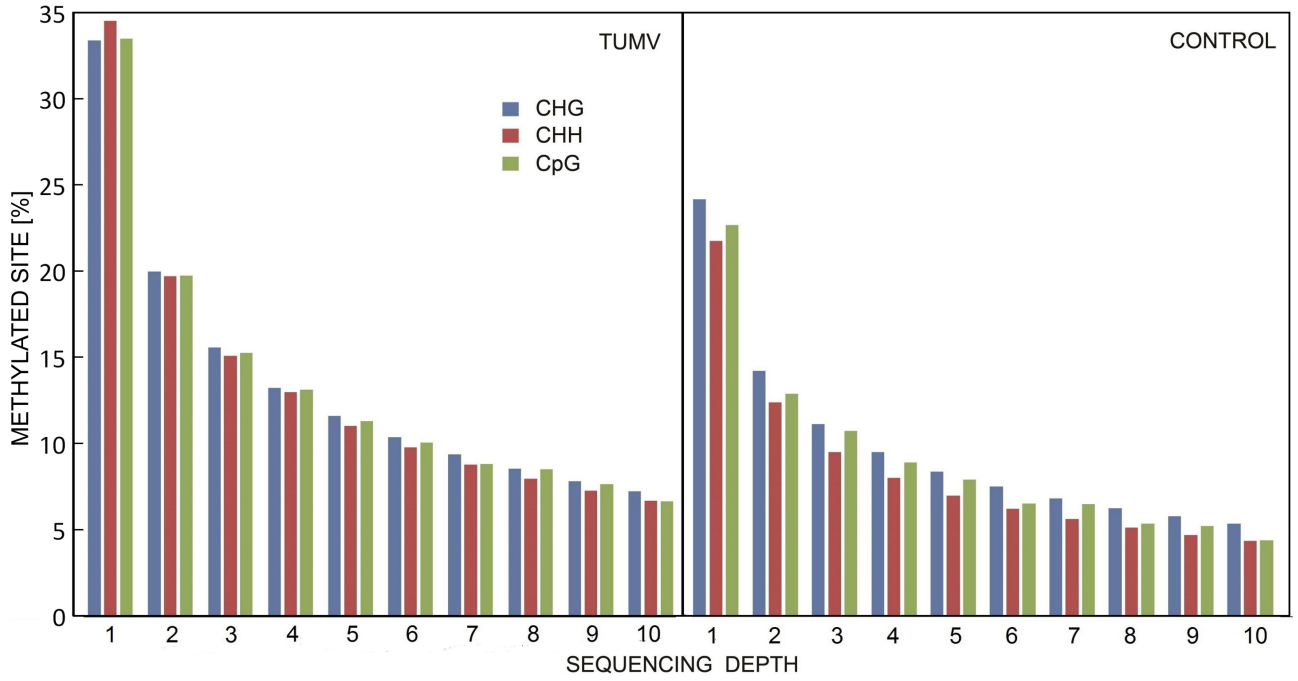

Fig. 1. The percentage of identified CHG, CHH, CG (H means A, G, or T) sites in a genome with various sequencing depths in *Turnip mosaic virus* and control libraries. The *x*-axis indicates the sequencing depth, the *y*-axis indicates the percentage of methylation sites.

We calculated the proportion of *MeDIP-Seq* reads in CpG islands, coding sequences, gene bodies, introns, promoters downstream 2kb and upstream 2kb, untranslated regions 5'UTR and 3'UTR, and each class of repetitive elements. The analysis of read distributions in different genome components showed that the most reads were enriched in introns as their total length was larger than of other elements (Table 1 Suppl.). The CpG islands always presented a low methylation level. However, we found a decreased methylation in CpG islands after *TuMV* infection, whereas methylation in promoters increased, which had a major impact on gene expressions. Besides, we observed different methylations in different repeat elements. More specifically, we analyzed methylation status around gene bodies and CpG islands. The DNA methylation decreased dramatically before the upstream 2kb (promoter) and increased sharply towards the gene body regions and plateaued subsequently until the downstream 2kb. The DNA methylation in CpG islands exhibited opposite trends (Fig. 1 Suppl.). Methylation of DNA in a gene body region was reported to impede transcription elongation in *Arabidopsis thaliana* (Zilberman *et al*. 2007). We assumed that a lower methylation in gene bodies might affect gene transcriptions. On the other hand, the discovered higher methylation of promoters in the *TuMV* library might alter the expressions of some "anti-pathogen" genes (Jia *et al*. 2000, Belkhadir *et al*. 2004, Li *et al*. 2016). To get detailed information on the methylation profile in the two libraries, we scanned highly methylated regions and identified 17 955 (control) and 22 891 (*TuMV*) peaks, respectively. The number and percentage of peaks of various lengths were calculated. The results show that there existed a major distribution of 200 bp peaks. Furthermore, we observed that peak distribution varied in different densities of CpGs. Generally, the density of CpGs in most peaks were focused on the range of 20 to 25. The methylation rate in different components showed that: *1*) coding sequences gained the highest methylation (91.35 % in *TuMV* and 92.52 % in control); and *2*) promoter methylation increased significantly after *TuMV* infection (Table 2 Suppl.).

Table 2. Distribution of differential methylated genes on different gene elements across *Turnip mosaic virus* and control libraries. CDS - coding sequence, UTR - untranslated region.

Categories	Down-methylated	Up-methylated
Upstream 2kb	21	5272
Five-UTR	0	0
CDS	26	2014
Intron	157	5941
Three-UTR	0	0
Downstream 2kb	20	3862

Peaks of the two libraries were merged as candidate DMRs (http://www.gene ontology.org/). We analyzed the DMRs between the *TuMV* and control libraries. The results show that a total of 5 293 (promoters) and 8 139 (gene bodies) DMGs were identified, of which 21 (promoters) and 187 (gene bodies) genes were down-methylated, 5 272 (promoters) and 7 955 (gene bodies) genes were up-methylated in the *TuMV* library compared to the control library (Table 2).

We utilized the *MeDIP-seq* to survey the DNA methylation patterns and calculate global methylation before and after *TuMV* infection. To further investigate the possible mechanism of *TuMV* toleration, the *GO* enrichment of DMGs (http://www.genenontology.org/) was conducted (Fig. 2*A*,*B*). The total DMGs were classified into three clusters: *1*) biological processes, *2*) molecular functions, and *3*) cellular components. Most up-methylated genes belonged to cellular components. In the cellular components, membrane organelles and cell part terms were over-represented, which was possibly associated with virus reproduction and assembly. Although most down-methylated genes were concentrated on

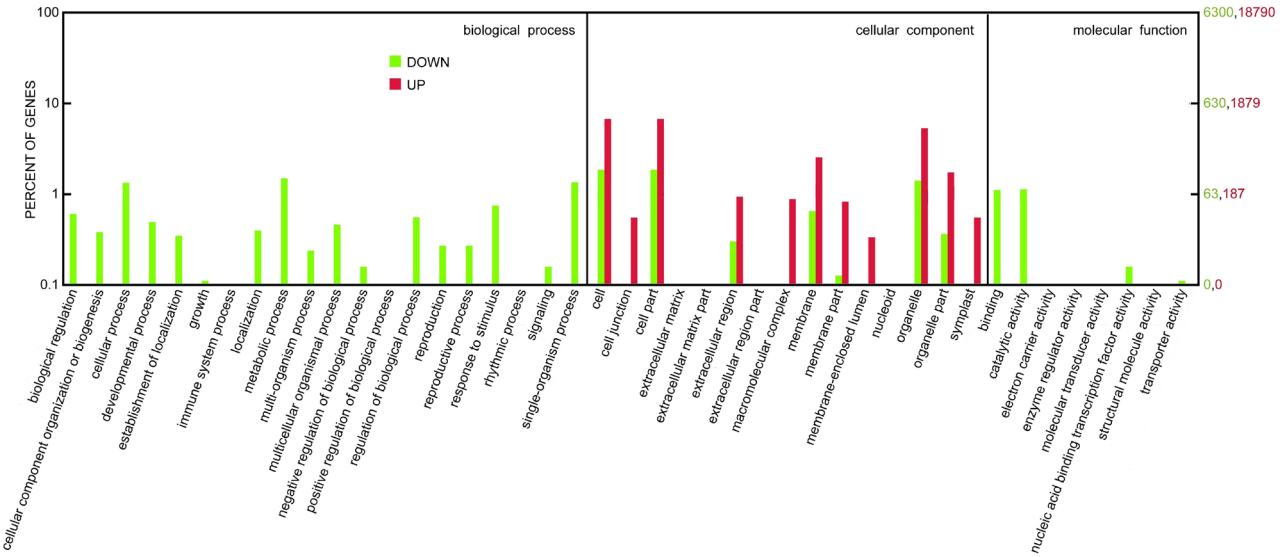

Fig. 2. *Gene ontology* (*GO*) classification for differentially methylated genes. The *x*-axis indicates *GO* items divided into biological processes, cellular components, and molecular functions; the *y*-axis indicates the number of differential methylated genes.

Table 3. The number of differential methylated genes (DMGs) in top 20 enriched *Kyoto encyclopedia of genes and genomes* pathways across *Turnip mosaic virus* and control libraries. DEGs - differentially expressed genes, ID - identity, *P* - probability.

Pathway	DEGs with pathway annotation (2795)	All genes with pathway annotation (21620)	*P*-value	Pathway ID
Metabolic pathways	635	4679	0.08003572	ko01100
Biosynthesis of secondary metabolites	351	2405	0.007000176	ko01110
Plant-pathogen interaction	223	1651	0.2432854	ko04626
Plant hormone signal transduction	218	1518	0.04729572	ko04075
Protein processing in endoplasmic reticulum	85	598	0.6480496	ko04141
Starch and sucrose metabolism	75	593	0.9561471	ko00500
Ribosome	66	613	0.9561471	ko00310
Phenylpropanoid biosynthesis	66	461	0.2022477	ko00940
RNA transport	62	501	0.6654612	ko03013
Protein export	62	281	0.5338896	ko03060
Ubiquitin mediated proteolysis	55	427	0.5338896	ko04120
Purine metabolism	49	376	0.4993233	ko00230
Pyrimidine metabolism	47	364	0.5275207	ko00240
Stilbenoid, diarylheptanoid, and gingerol biosynthesis	44	314	0.3058102	ko00945
Amino sugar and nucleotide sugar metabolism	43	263	0.06138821	ko00520
Flavonoid biosynthesis	41	312	0.1478863	ko00941
Glycolysis/gluconeogenesis	39	228	0.04046097	ko00010

biological processes, the over-represented *GO* terms were primarily divided into four clusters: *1*) cellular processes, *2*) metabolic processes, *3*) responses to stimulus, and *4*) single-organism process terms. These *GO* terms were involved in the metabolism and synthesis pathways. The abundance of DMGs on these *GO* terms is probably due to the fact that virus infection disrupted plant growth, metabolism, and other life activities. To better understand the biological functions of the DMG responses to *TuMV* infection, we performed *KEGG* pathway enrichment analysis. The total DMGs were mapped to pathway annotations involved in more than one hundred metabolic pathways. The top 20 *KEGG* pathways with the highest representation of DMGs are displayed in Fig. 3. Among them, the metabolic pathway, biosynthesis of secondary metabolites pathway, the plant pathogen interaction pathway, as well as the plant hormone transduction pathway were over-represented. Our result indicates that genes related to pathogen responses were significantly enriched, including plant pathogen interaction pathway and plant hormone transduction pathway. We discovered a mass of resistance genes in the two pathways including plant pathogen interaction, auxin, salicylic acid (SA) signaling, cell wall biosynthesis, and protein degradation pathway (Table 3, Fig. 3). To further confirm that the expressions of these resistance genes were modified by DNA methylation, real-time quantitative PCR was performed. The detailed formation of candidate resistance genes including chromosome position, gene definition, and relative expression are listed in Table 3 Suppl. The expression changes of resistance genes before and after *TuMV* infection are depicted in Fig. 4.

Discussion

In the plant-pathogen interaction pathway of DMGs, most plant resistance genes encoding nucleotide-binding leucine-rich repeat (NBS-LRR) proteins are abundant. The NBS-LRR proteins take part in , which is an important part of plant resistance to pathogens (DeYoung and Innes 2006, Ting *et al*. 2008). The NBS domain activates a kinase and other proteins in the pathogen response, and the LRR domain is known as a primary determinant of pathogen recognition specificity or its downstream events (Belkhadir *et al*. 2004). The NBS-LRR proteins usually bind with a toll/interleukin-1receptor (TIR) domain or a coiled coil domain (CC), called TIR-NBS-LRR or CC-NBS-LRR. It influences distant downstream signaling or directly recognizes pathogen effectors, like avirulence proteins (Jia *et al*. 2000). The NBS-LRR proteins are also important in plant innate immune systems. In the plant-pathogen interaction pathway, we found 12 *NBS-LRR* genes. Among them, seven were up-regulated, containing three *CC-NBS-LRR* genes and four *TIR-NBS-LRR* genes. Five were down-regulated, containing a *NBS* gene, a *TIR-NBS-LRR* gene, and three *CC-NBS-LRR* genes. Besides, we found a lot of *LRR* family genes and randomly selected eight *LRR* genes for validation. Among them, four were up-regulated and three were down-regulated. We found that two of four LRR receptor-like serine/threonine related genes were up-regulated. The enhanced expression of resistance genes might trigger activation of effector-triggered immunity and subsequent defense responses.

Plant defense and growth are largely controlled by multiple plant hormones. In this study, we found a plenty of DMGs associated with auxin and SA signaling.

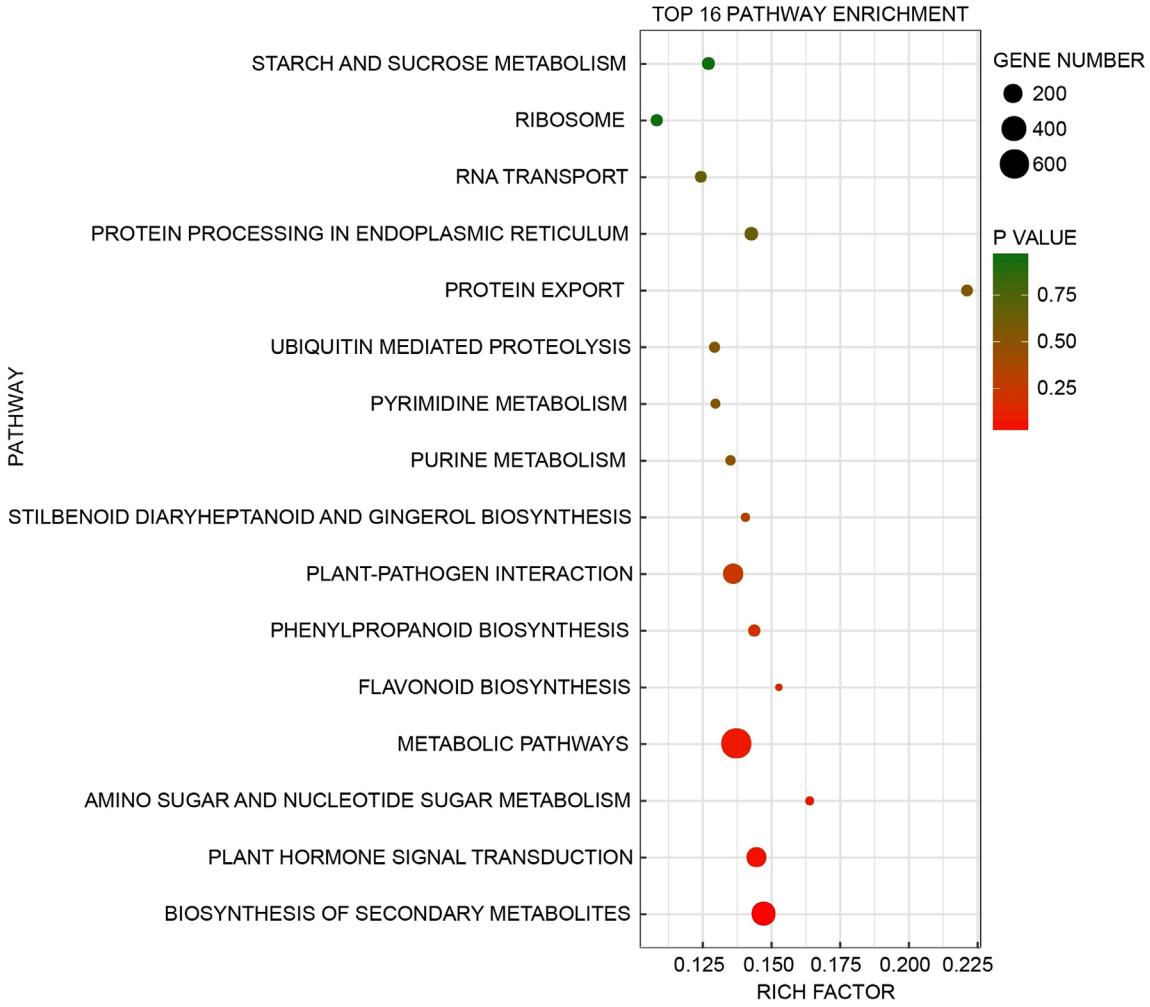

Fig. 3. Top 16 *Kyoto encyclopedia of genes and genomes* pathways with the highest representation of common differential methylated genes from pairwise comparisons between *Turnip mosaic virus* and control libraries.

It is widely accepted that auxin plays an important role in balancing plant defense responses and development in both monocotyledonous and dicotyledonous plants (Li *et al*. 2016). Auxin functions as a negative regulator of plant immune response, and actively modifies plant defense. Recent studies have shown that negatively regulating mRNAs for the F-box receptor could block the auxin pathway, which further facilitates antibacterial resistance in *Arabidopsis* (Navarro *et al*. 2006). In this study, we detected six auxin-responsive family (*BrARFs*) genes from DMGs. Among them, one gene was up-regulated and four genes were down-regulated. Especially, the *ARF* (*Bra016606*) gene was significantly down-regulated. Among auxins, mainly indole-3-acetic acid (IAA) regulates many aspects of growth and metabolic processes such as root, flower, and leaf formation, plant vasculature development, and seed germination (Kieffer *et al*. 2010). Simultaneously, we discovered two of three auxin/IAA family genes and two *Gretchen Hagen 3* family genes, which were down-regulated and an *indole-3-acetic acid* (*Bra035185*) gene, which was significantly down-regulated. These might be explained by the hypothesis that the plant repressed the auxin pathway to activate plant immunity in the manner of DNA methylation.

Pathogenesis-related (*PR*) genes encode a kind of inducible defense-related proteins in many plant species, playing an important role in immune surveillance mechanisms and helping plants to fight against invasion by microorganisms (Van Loon *et al*. 2006). It is widely recognized that *PR* expression is highly responsive to SA and bacterial pathogens (Pajerowska-Mukhtar *et al*. 2013). The *NPR1*, a key regulator of the SA mediated pathway, is located upstream of *PR* and downstream of SA and can act as a transcriptional co-activator in plant innate immunity in concert with TGACG motif binding / basic domain-leucine zipper transcription factors to activate *PR1* transcriptional activity (Kesarwani *et al*. 2007). In this study, we found five PR proteins, an NPR1 (*Bra013950*), and two TGA/bZIP transcription factors (*Bra0372350* and *Bra039631*), which were up-regulated. Besides, NPR3 (*Bra025093*) as well as NPR4 (*Bra013416*), considered as a NPR1 homolog and the receptor of SA, were also up-regulated. Two systemic acquired resistance regulator proteins NIMIN-1 (*Bra000910* and *Bra032601*) were up-

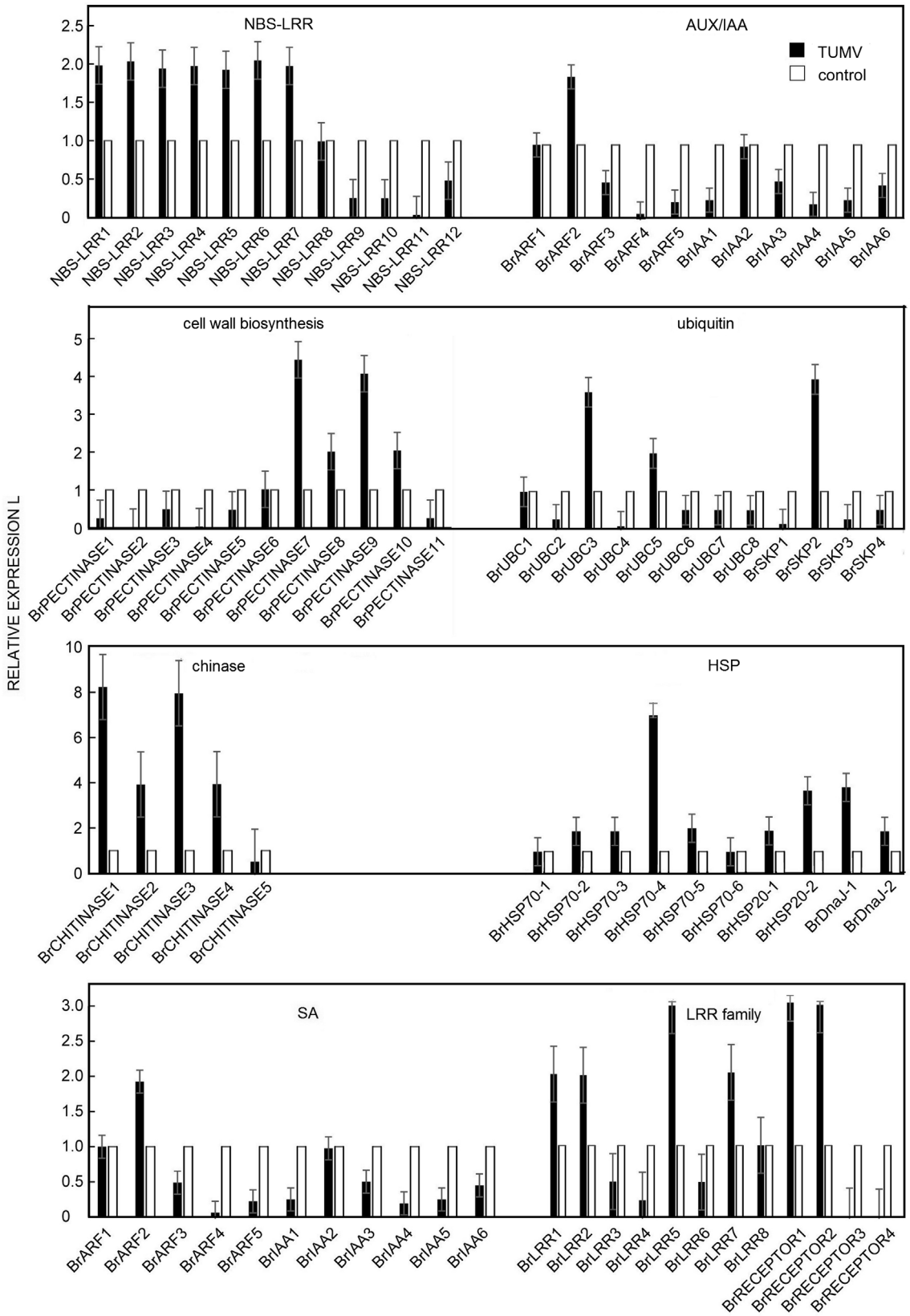

Fig. 4. Real-time quantitative PCR validation of resistance genes related to nucleotide binding site-leucine-rich repeats (NBS-LRR), salicylic acid (SA), auxins (AUX/IAA), heat shock proteins (HSPs), and cell wall biosynthesis pathways from differential methylated genes. *Actin* (*Bra028615*) was used as an internal control (Hsieh *et al*. 2009). The transcriptions were normalized to those of the control library. *Error bars* represent standard deviations from three independent biological samples.

regulated. Taken together, we show that the expression of *NPRs*, *PRs*, and *TGA/bZIP* transcription factors may be enhanced *via* repressing DNA methylation to activate SA signaling and to gain systemic acquired resistance. It is widely accepted that the auxin pathway can interact with the SA pathway. Wang *et al*. (2007) has found that the auxin pathway can down-regulate the defense response by repressing the SA signal pathway and that inhibition of auxin signaling is part of the SA-mediated disease resistance in *Arabidopsis.* Our results are consistent with these previous findings (Wang *et al*. 2007). We assume that DNA methylation potentially modified the key genes to restrict the auxin pathway and to promote SA signal pathway, finally triggering defense response.

Except the plant pathogen interaction pathway and the plant hormone signal pathway, DMGs involved in starch and sucrose metabolism and phenylpropanoid biosynthesis pathways were significantly enriched. Within starch and sucrose metabolism, five of six genes encoding pectinesterase, an enzyme which can hydrolyze cell wall and participate in D-galacturonate biosynthesis, are down-regulated (Sterling *et al.* 2001). Peroxidase is involved in synthesis of guaiacyl and syringyl, which are key components of cell wall. In terms of phenylpropanoid biosynthesis pathway, four of five genes associated with peroxidase were up-regulated. Chitinase, a kind of PR-like proteins, can catalyze hydrolysis of chitin, which is a main component of cell wall. Hydrolysis of chitin could trigger defense responses against fungal pathogens (Xian *et al*. 2012) Furthermore, we found that four of five genes encoding chitinase were up-regulated. Taken together, these results imply that the cell wall biosynthesis pathway was enhanced and its modification could repress *TuMV* invasion.

Viral multiplication and movement depend on the properties of host cell, so energy metabolism and enzymes in host cells can influence the defense response (Wang *et al*. 2016b). The ubiquitin proteasome system (UPS) controls the abundance of key regulatory proteins and enzymes taking part in proteolysis and regulates many processes in plants including development, hormone signaling, and plant-pathogen interactions *via* regulation of immune receptors and immune signaling components (Smalle *et al*. 2004, Trujillo *et al*. 2010, Li *et al*. 2014). Protein ubiquitination is a common form of post-translational modification. The process is achieved by a three-enzyme cascade: ubiquitin-activating enzyme, ubiquitin-conjugating enzymes, and ubiquitin-protein ligases (Wang *et al*. 2016a). Recent studies have also demonstrated that plant *NBS-LRR* genes are controlled by multiple mechanisms, and UPS mediated proteolysis plays a key role in this process (Bieri *et al.* 2004, Holt *et al*. 2005, Cheng *et al.* 2011, Gou *et al.* 2012, Huang *et al.* 2014, 2016, Xu *et al.* 2015, Park *et al.* 2016). Especially, ubiquitin-protein ligases , responsible for special recognition of a target protein, has been reported to affect immunity *via* interacting with NBS-LRR (Wang *et al*. 2016a). In the ubiquitin mediated proteolysis pathway, we detected 12 genes encoding ubiquitin enzymes. Among them, six of seven *ubiquitin-conjugating enzymes*_genes and three of four *ubiquitin-protein ligases* (*skp1*) genes were down-regulated, whereas an *ubiquitin-activating enzyme* gene was up-regulated. In addition, we detected some genes encoding heat shock proteins within protein processing in the endoplasmic reticulum pathway. Heatb shock protein 70 functions as a molecular chaperone, which is indispensable for preventing aggregation and assisting re-folding of non-native proteins under stress conditions (Wang *et al*. 2016b). Heat shock proteins are considered to regulate the function of NBS-LRR (Belkhadir *et al*. 2004). Here, four of six genes encoding heat shock protein 70 were up-regulated. Besides, two chaperone *Dnaj* (*heat shock protein 40*)genes as well as two *eat shock protein 20* genes were also up-regulated. We conjecture that these key genes were modified to prevent a metabolism disorder and enhance immune response.

Basically, antiviral silencing based on RNA interference (RNAi) is regarded as a major mechanism for plant antiviral defense. The antiviral mechanism was as follows: During RNA virus infection, the viral RNA is replicated, and its intermediates induce the double-stranded RNA. Then, the long double stranded RNA is cut into different long small interfering RNA (siRNA) duplexes by different Dicer-like enzymes (Tang *et al*. 2003). The short and long siRNAs are amplified with plant RNA-dependent RNA polymerases (Dalmay *et al*. 2000, Tang *et al*, 2003). Then, a library of the short siRNA duplexes is incorporated into the RNA-induced silencing complex, and the siRNA-directed RNA-induced silencing complex degrades viral RNA. Extensive study suggested that both methylated DNA and RNAi are molecules naturally protecting plants from uncontrolled replication of viruses (Pikaard and Scheid 2014). Moreover, Mattiske and Mann (2012) found that RNAi performs an important function in establishing and maintaining epigenetic states in various mammalian cell types, especially on the establishment of DNA methylation in germ cells and embryonic stem cells of mice. Despite there have been no relevant discoveries so far, we speculate that RNAi influences the establishment and maintenance of DNA methylation also in plant cells. Both RNAi and DNA methylation are thought as a kind of a gene silencing mechanism, which could directly or indirectly regulate gene expressions. Given that RNAi was identified as a major mechanism for plant antiviral defense, we inferr that RNAi was involved in the process of plants against virus infection as the manner of DNA methylation partially induced by RNAi, which is pending for further study.

Conclusions

We firstly investigated epigenetic modification potentially affecting the anti-pathogen pathways and provided evidence that genes related to the NBS-LRR family auxin, SA signaling, cell walls biosynthesis, as well as protein degradation - were modified through DNA methylation to enhance the plant resistance to pathogens. We believe our results would provide a more accurate picture of the events triggered by *TuMV* infection.

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