Genome-Wide Analysis of Coding and Long Non-Coding RNAs Involved in Cuticular Wax Biosynthesis in Cabbage (Brassica oleracea L. var. capitata)

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Abstract: Cuticular wax is a mixture of very long chain fatty acids (VLCFAs) and their derivatives, which determines vital roles for plant growth. In cabbage, the cuticular wax content of leaf blades is an important trait influencing morphological features of the head. Understanding the molecular basis of cuticular wax biosynthesis can help breeders develop high quality cabbage varieties. Here, we characterize a cabbage non-wax glossy (nwgl) plant, which exhibits glossy green phenotype. By cryo-scanning electron microscope analysis showed abnormal wax crystals on the leaf surfaces of nwgl plants. Cuticular wax composition analyzed by GC-MS displayed severely decreased in total wax loads, and individual wax components in nwgl leaves. We delimited the NWGL locus into a 99-kb interval between the at004 marker and the end of chromosome C08 through fine mapping. By high-throughput RNA sequencing, we identified 1247 differentially expressed genes (DEGs) and 148 differentially expressed IncRNAs in nwgl leaves relative to the wild-type. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that the DEGs and cis-regulated target genes for differentially expressed IncRNAs were significantly enriched in wax and lipid biosynthetic or metabolic processes. Our results provide the novel foundation to explore the complex molecular basis of cuticular wax biosynthesis.

Keywords: cabbage; nwgl phenotype; fine mapping; cuticular wax biosynthesis; RNA-seq; differential expressed genes; IncRNAs

1. Introduction

The aerial surface of land plants is covered with a hydrophobic cuticle layer, which is composed of two major lipid components, the cutin polyester and the non-polymerized cuticular waxes. Cuticular waxes are predominantly composed of very long chain fatty acids (VLCFAs) and their derivatives, including aldehydes, alcohols, alkanes, ketones, and esters [1,2]. The biosynthesis of cuticular wax begins with de novo synthesized C16 and C18 fatty acids, which are then elongated into VLCFAs by a fatty acid elongase (FAE) complex, consisting of β-ketoacyl-CoA synthase, β-ketoacyl-CoA reductase, β-hydroxyacyl-CoA dehydratase, and enoyl-CoA reductase. The formed VLCFAs are further converted into various derivatives via two independent pathways, the alkane-forming and alcohol-forming pathways [3,4]. Cuticular wax protects plants against numerous biotic and abiotic stresses, such as pathogen infection and non-stomatal water loss [5,6]. It also plays important roles in
normal plant developmental processes, including the pollen–pistil interaction [7]. Therefore, identifying plants with cuticular wax deficiency and elucidating the regulatory mechanisms controlling cuticular wax biosynthesis will be beneficial for improving agricultural crops.

To date, many genes associated with cuticular wax biosynthesis pathway have been isolated and characterized in different plant species. For example, CER1 encodes an alkane-forming enzyme and may physically interact with CER3 and cytochrome b5 isoforms (CYTB5s) to catalyze the synthesis of very long chain alkanes [8,9]. CER4 is an alcohol-forming fatty acyl-coenzyme A reductase (FAR) in the synthesis of primary alcohols [10]. In addition, several APETALA2/ETHYLENE-RESPONSIVE FACTOR (AP2/EFR) type transcription factors have also been reported to be involved in cuticular wax biosynthesis through regulating the expression of wax biosynthesis genes [11–17]. For instance, WRINKLED4 (WRI4) activates the expression of LACS1, KCR1, PAS2, ECR, and WSD1 by direct binding to their promoters [16].

In recent years, several RNA transcripts with no protein coding potential have been reported that have a key role in regulation of cuticular wax biosynthesis. These noncoding RNAs include some small RNA species, such as small interfering RNA (siRNA) and microRNA (miRNA), mediated silencing transcription of the wax biosynthetic genes [18–22]. Cuticular wax biosynthesis also involves long noncoding RNAs (lncRNAs). LncRNAs are a class of non-coding RNA transcripts with a length of more than 200 nt. In wheat, INHIBITOROFWAX1 (Iw1) and its homolog Iw2 serve as miRNA precursors that produce an miRNA, miRW1, which represses the expression of the putative carboxylesterase genes that are necessary for β-diketone deposition in wheat [22].

For cabbage, several cuticular wax deficiency mutants have been reported. The phenotype of 10Q-961 and g21-3 mutants is controlled by a same recessive locus, which was finely mapped to chromosome C08 through classical linkage analysis [23,24]. A dominant 10Q-974gl mutant gene BoGL1 was also fine mapped to chromosome C08 [25]. Moreover, both the BoWAX1 and Cgl2 genes were mapped to chromosome C01 [26,27]. In addition, the Cgl2 gene in cabbage had been cloned and could rescue the phenotype of the Arabidopsis cer4 mutant [27]. However, the cuticular wax biosynthesis genes have not been fully explored in cabbage.

In this study, we report the phenotypic and characterization of the non-wax glossy (nwgl) plant characterized by a glossy appearance of the cabbage. The nwgl plants exhibited a marked reduction in amounts of total wax and changes in wax components. We performed fine mapping and delimited the NWGL gene responsible for the nwgl phenotype to the end of chromosome C08. By conducting RNA-seq, we identified differential expressed genes (DEGs) and lncRNAs in leaf tissues of nwgl and wild-type plants. Moreover, we predicted both cis- and trans- regulatory target genes for the differential expressed lncRNAs. Functional analysis of the DEGs and cis-regulated targets genes for differential expressed lncRNAs revealed significantly enriched categories and pathways related with wax and lipid biosynthesis and/or metabolism, providing novel implication underlying cuticular wax biosynthesis in cabbage.

2. Results

2.1. Characterization of the Plant Exhibiting nwgl Phenotype

The plant exhibiting nwgl phenotype was identified from the inbred line G287. The prominent phenotype of nwgl plants was their altered wax coatings, resulting in light green leaves, stems, and flower buds (Figure 1a–f). The plant was designated nwgl based on its non-wax and glossy appearance. Cryo-scanning electron microscopy analysis revealed that the cuboid wax crystals were distributed densely on the leaf surface of the wild-type, whereas only fewer granular wax crystals were deposited on the leaf surface of nwgl plants (Figure 1g,h). Therefore, these results indicate that the nwgl mutation might cause abnormalities in specific components of the cuticular wax of cabbage plants.
2.2. Cuticular Wax Composition of nwgl Plants

To investigate why the plants exhibiting nwgl phenotype had a significant depletion in wax crystals, we examined the content and the constitution of cuticular waxes from leaf blades of nwgl and wild-type plants by gas chromatography-mass spectrometry (GC-MS). Compared with wild-type plants, the total amount of all wax loads on nwgl leaf blades was reduced by 90.7% (Table 1). The contents of VLCFA, alkanes, and primary alcohols decreased by 45.8%, 98.3%, and 42.5% in nwgl leaf blades, respectively (Table 1). The secondary alcohol and ketone were undetectable in nwgl leaf blades (Table 1). However, the content of aldehyde exhibited slightly increased in nwgl leaf blades (Table 1). Further analysis revealed significantly reduced contents of C26 VLCFA, C27 and C29 alkanes, and C28 primary alcohol. Meanwhile, neither C28 secondary alcohol nor C29 ketone was detected in nwgl leaf blades (Figure 2). Taken together, these findings suggest that the reduced wax crystals in nwgl leaf blades result from decreased levels of VLCFAs and their individual derivatives.

Figure 2. Cuticular wax composition in leaves from wild-type and nwgl plants. Error bars indicate SD (n = 3). * p < 0.05; ** p < 0.01.
Table 1. Cuticular wax composition of leaf blades from wild-type and nwgl plants.

| Components | Wild-type (µg/g) | nwgl (µg/g) |
|------------|-----------------|-------------|
| Fatty acid | 0.59 ± 0.04     | 0.32 ± 0.14 * |
| Aldehyde  | 12.93 ± 4.33    | 27.33 ± 13.15 |
| Alkanes   | 334.36 ± 110.66 | 5.52 ± 2.97 * |
| 1-Alcohols| 35.19 ± 2.74    | 20.23 ± 12.52 |
| 2-Alcohol | 14.20 ± 2.49    | -           |
| Ketone    | 174.06 ± 43.09  | -           |
| Total     | 571.33 ± 152.22 | 53.40 ± 28.31 ** |

Data are mean ± SD (n = 3). * p < 0.05; ** p < 0.01; Student's t-test.

2.3. Fine Mapping of NWGL

Genetic analysis showed that the nwgl phenotype is a dominant mutation controlled by a single gene, with one quarter of the F₂ progeny displaying a normal waxy appearance (208:61, χ² = 0.67, p > 0.05; 270:91, χ² = 0.04, p > 0.05). To isolate the NWGL gene, we generated two F₂ mapping populations by crossing plants exhibiting nwgl phenotype with two wild-type inbred lines G306 and G274, respectively. The NWGL locus was mapped preliminarily between simple sequence repeat (SSR) marker at 040 and the end of chromosome C08 (Figure 3). Using newly developed InDel and SSR markers, the NWGL gene was fine-mapped into a 99-kb DNA segment between marker at004 and the end of chromosome C08 (Figure 3). There were 16 putative open reading frames (ORFs) within the 99-kb genomic region according to the cabbage gene annotation database (www.ocri-genomics.org/bolbase, accessed on: 9 June 2019) (Figure 3). Except for Bol018503 and Bol018504, the functions of these genes are either unknown or do not involve in cuticular wax biosynthesis (Table S1). Bol018503 encodes a CER1-L1 homolog, which belongs to fatty acid hydroxylase superfamily in Arabidopsis, and Bol018504 encodes an aldehyde decarbonylase (CER1), which is known to promote wax very long chain alkane biosynthesis in various plant species [8,9,28,29]. To define which of these carries the nwgl mutation, we sequenced the two candidate genes, including their 5′ and 3′ flanking sequences, but no DNA sequence alteration was found. However, examination of gene expression of all the 16 ORFs within the fine mapping region only showed a down-regulation of Bol018504 in nwgl leaves but not in the wild-type, indicating that the decreased expression of Bol018504 might cause the nwgl phenotypes (Figure 4b, Table S2). This speculation was in accordance with a recent study, in which the same expression abundance decrease of Bol018504 was identified in an investigation of a dominant glossy green cabbage mutant 10Q-974gl [25].

Figure 3. Fine mapping of NWGL. The NWGL locus was mapped onto chromosome C08. Molecular markers and ORFs in the mapping region are shown.
Figure 4. Analysis of DEGs in the RNA-seq libraries. (a) Volcano plot showing significant DEGs. Red and green dots represent up and down-regulated DEGs, respectively (FDR < 0.05). Black dots are genes that were not differently expressed. (b) Gene expression of DEGs involved in wax biosynthesis. The data are shown in base 2 logarithmic form (log2 FPKM). Three biological replicates of wild-type (WT) and nwgl leaves are exhibited. (c) Simplified pathways for wax biosynthesis. LACS, long chain acyl-CoA synthetase; KCS, β-ketoacyl-CoA synthase; KCR, β-ketoacyl-CoA reductase; HCD/PAS2, β-hydroxyacyl-CoA dehydratase; ECR, enoyl-CoA reductase; CER, ECERIFERUM; CYTB5, cytochrome b5 isoform; MAH, mid-chain alkane hydroxylase; WSD, wax synthase/diacylglycerol acyltransferase; WRI4, WRINKLED4. Down-regulated and up-regulated genes in nwgl vs. wild-type plants are marked in green and red color, respectively.
These results suggest that the Bol018504 might be regulated by the NWGL locus or other mechanisms, such as an epigenetic manner. We further investigated the DNA methylation level of Bol018504 by employing three methylation sensitive restriction enzymes. For McrBC-PCR analysis, we found that the middle region of Bol018504 corresponding to the fragment “C” (+905 to +1615) was unmethylated in the wild-type but slightly methylated in nwgl plants (Figure S1a,b). The HpaII, MspI-PCR analysis was used to detect the methylation level of CCGG sites within fragments “A” (−1113 to −408), “B” (+189 to +1069), and “C” (+905 to +1615). The results indicated that the C of the CCGG sites within fragments “B” (+189 to +1069) and “C” (+905 to +1615) might be hypermethylated in nwgl plants compared with the wild-type (Figure S1a,c).

2.4. RNA-seq Analysis of Plants Exhibiting nwgl Phenotype

To further explore the global impacts of the nwgl mutation which could reduce cuticular wax contents in plants exhibiting nwgl phenotype, we conducted a transcriptome comparison of nwgl and wild-type leaves by high-throughput RNA sequencing (RNA-seq). In total, 1247 protein coding genes were differentially expressed in nwgl leaves relative to wild-type, including 913 up-regulated genes and 334 down-regulated genes (Figure 4a, Table S2).

We then performed Gene Ontology (GO) analysis to assess the function of the DEGs. Notably, the down-regulated genes most significantly enriched were in the wax biosynthetic process (GO: 0010025), while the up-regulated genes most significantly enriched were in response to chitin (GO: 0010200) (Tables S3 and S4). In addition, a significant fraction of up-regulated genes were involved in lipid-related GO categories, including lipid localization (GO: 0010876), positive regulation of fatty acid metabolic/biosynthetic process (GO: 0045923 and GO: 0045723), glycerolipid metabolic process (GO: 0046486), neutral lipid metabolic process (GO: 0006638) and sphingolipid catabolic process (GO: 0030149) (Table S4), suggesting that NWGL might be involved in wax biosynthesis through regulating the expression of lipid-related genes.

To further understand the metabolic pathways associated with NWGL, the DEGs were mapped to the KEGG database to analyze the enriched pathways. There were nine pathways highly enriched for down-regulated genes, and the most significantly enriched encoded proteins were involved in ‘tyrosine metabolism’ (KEGG ID: ko00350) (Table S5). The ‘cutin, suberine and wax biosynthesis’ (KEGG ID: ko00073), ‘linoleic acid metabolism’ (KEGG ID: ko00591), and ‘sphingolipid metabolism’ (KEGG ID: ko00600) were significantly enriched pathways in up-regulated genes (Table S6), supporting the notion that NWGL functions in wax and lipid metabolism.

When focusing on the wax biosynthetic process, nine genes showed an altered expression in nwgl leaves relative to the wild-type. Only two genes associated with wax biosynthesis were down-regulated in nwgl leaves (genes encoding CER1 (Bol018504) and CER4 (Bol013612)), while seven genes were up-regulated: genes encoding another two CER1 proteins (Bol025251 and Bol025577), two Long-chain-fatty-acyl-CoA reductases (Bol035700 and Bol036039), one 3-ketoacyl-CoA synthase (Bol024192) and two AP2-like factors (Bol038193 and Bol038557) (Figure 4b,c).

2.5. Identification and Characterization of lncRNAs in Cabbage

To screen out potential lncRNAs involved in regulating wax biosynthesis, we thoroughly analyzed the cabbage noncoding transcriptome and identified 4459 putative lncRNAs (Figure 5a, Table S7). The 4459 lncRNA sequences were compared with mRNAs obtained in this study. These lncRNAs were shorter than mRNAs, 76.3% of which being <1000 bp in length (Figure 5b). Most lncRNAs (88.0%) had two or three exons in their transcripts and were fewer than protein-coding transcripts (Figure 5c). Interestingly enough, the two features of lncRNAs in cabbage, shorter lengths and fewer exons, have also been detected in other Brassica species, including Chinese cabbage, rapeseed, kale, and non-heading Chinese cabbage [30–32]. Moreover, lncRNAs also had shorter ORFs compared with mRNAs, with approximately 92.2% at <100 amino acids (Figure 5d).
Figure 4. Analysis of DEGs in the RNA-seq libraries. (a) Volcano plot showing significant DEGs. Red and green dots represent up and down-regulated DEGs, respectively (FDR <0.05). Black dots are genes that were not differently expressed. (b) Gene expression of DEGs involved in wax biosynthesis. The data are shown in base 2 logarithmic form (log 2 FPKM). Three biological replicates of wild-type (WT) and nwgl leaves are exhibited. (c) Simplified pathways for wax biosynthesis. LACS, long chain acyl-CoA synthetase; KCS, β-ketoacyl-CoA synthase; KCR, β-ketoacyl-CoA reductase; HCD/PAS2, β-hydroxyacyl-CoA dehydratase; ECR, enoyl-CoA reductase; CER, ECERIFERUM; CYTB5, cytochrome b5 isoform; MAH, mid-chain alkane hydroxylase; WSD, wax synthase/diacylglycerol acyltransferase; WRI4, WRINKLED4. Down-regulated and up-regulated genes in nwgl vs. wild-type plants are marked in green and red color, respectively.

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Figure 5. Characteristics of lncRNAs identified in cabbage. (a) The 4459 lncRNAs in cabbage were identified by an intersection of results obtained via three softwares CNCI (Coding-Non-Coding Index), CPC (Coding Potential Calculator), CPAT (Coding Potential Assessment Tool) and a protein database Pfam analysis. (b) Length distribution (base pair, bp) of lncRNAs and mRNAs. (c) Exon number in lncRNAs and mRNAs. (d) ORF length in amino acids (aa) of lncRNAs and mRNAs.

2.6. Identification of Differentially Expressed lncRNAs between nwgl and Wild-Type Plants

A total of 148 lncRNAs were found to be significantly differentially expressed (Figure 6a, Table S8). In detail, 96 lncRNAs in nwgl leaves were up-regulated and 52 were down-regulated compared with the wild-type (Figure 6a, Table S8). LncRNAs have been shown to regulate protein coding gene expression via cis- and trans-action [33]. In total, 929 annotated target genes for 148 differentially expressed lncRNAs were predicted, of which 906 were cis-regulatory and 23 were trans-regulatory (Tables S9 and S10). Gene Ontology (GO) terms were identified for the 906 cis-regulated target genes (Figure 6b). The top enriched GO terms in the biological process, cellular component, and molecular function categories were organic cyclic compound biosynthetic process (GO: 1901362), nucleus (GO: 0005634), and protein binding (GO: 0005515), respectively (Table S11). In addition, the GO term glycerolipid metabolic process (GO: 0046486) was highly enriched (Table S11). Moreover, the 906 cis-regulated target genes were significantly enriched in KEGG pathways, consisting of the pentose phosphate pathway (KEGG ID: ko00030), endocytosis (KEGG ID: ko04144), ribosome (KEGG ID: ko03010), glycolysis/gluconeogenesis (KEGG ID: ko00010), degradation of aromatic compounds (KEGG ID: ko01220), and oxidative phosphorylation (KEGG ID: ko00190) (Table S12).

To further analyze the function of differentially expressed lncRNAs, we investigated the co-expression correlation between differentially expressed lncRNAs and their predicted target genes.
A total of 206 interaction relationships (111 positive and 95 negative correlations) were discovered between 97 lncRNAs and 163 target genes (Table S13). Notably, some of the correlated target genes were involved in lipid-related processes, including fatty acid metabolic process, fatty acid beta-oxidation, fatty acid degradation, and fatty acid homeostasis (Figure 6c).

Figure 6. Differentially expressed lncRNAs in *nwgl* vs. wild-type plants. (a) Hierarchical clustering analysis of the differentially expressed lncRNAs. The data are expressed in base 10 logarithmic form (log10 FPKM+0.000001). (b) GO classification of 906 *cis*-regulated target genes of differentially expressed lncRNAs. (c) The co-expression differentially expressed lncRNAs and target genes involved in lipid-related processes. The data are exhibited in base 2 logarithmic form (log2 FPKM). Three biological replicates of wild-type (WT) and *nwgl* plants are shown.

3. Discussion

The content of cuticular waxes is an important agronomic trait for cabbage breeding. However, there are only a few reports regarding wax biosynthesis in cabbage [23–27]. The phenotype and characterization of the *nwgl* plants reported here showed that NWGL is a factor for cuticular wax biosynthesis in cabbage. The *nwgl* leaf blades exhibited decreased contents of VLCFA, alkanes and primary alcohols, and the abortion of secondary alcohol and ketone (Table 1), indicating that NWGL acted as a major regulator for VLCFA elongation and the two distinct pathways, the alkane-forming and alcohol-forming pathways. The involvement of NWGL in the synthesis of various wax compounds was reconfirmed by the RNA-seq analysis. Firstly, GO and KEGG pathway analysis showed that DEGs between plants exhibiting *nwgl* phenotype and the wild-type were enriched in categories related to wax and lipid synthesis/metabolism (Tables S3–S6). Secondly, nine DEGs which encode proteins involved in VLCFA production, alkane-forming and alcohol-forming pathways of wax biosynthesis are regulated by NWGL (Figure 4c). Thirdly, five of the target genes that were found to be strongly co-expressed...
with the differentially expressed IncRNAs were annotated to be involved in lipid-related processes (Figure 6c). Collectively, our data suggest that NWGL is involved in multiple wax biosynthetic processes, from upstream fatty acid synthesis to subsequent wax production.

Our results demonstrated that the glossy phenotype of the nwgl plants is determined by a single dominant locus within approximately 99-kb between a004 and the end of chromosome C08 (Figure 3). Therefore, these results indicated that the NWGL gene may be an allele of the previously studied BoGL1 gene [25]. Unfortunately, we failed to identify DNA lesion of Bol018504 in nwgl plants, except its reduced expression abundance and altered DNA methylation level, which was located in the fine mapping region and encodes an alkane-forming enzyme (BoCER1). Previous studies have reported that the Arabidopsis thaliana CER1 protein is an essential element of wax alkane synthesis [8,9]. The Arabidopsis cer1 mutant exhibited a dramatic decrease in alkanes and nearly depleted of secondary alcohols and ketones, accompanied by a slight increase in aldehyde content as compared to wild-type plants [8,34–36]. Interestingly, our GC-MS analyses found significant reductions in total wax in nwgl leaf blades, especially for C27 and C29 alkanes, and the undetected C28 secondary alcohol and C29 ketone, indicating that Bol018504 played a critical role in NWGL-regulated cuticular wax biosynthesis. However, we cannot rule out the possibility that other unknown genes within the fine mapping region may be responsible for NWGL, which may prove another interesting area for further investigation. For instance, the VLCFA component, which cannot be catalyzed by CER1, also decreased significantly in nwgl leaf blades.

The interaction of NWGL with other genes was investigated via transcriptome analysis. Based on our RNA-seq data, the expression of nine genes, whose homologs in Arabidopsis thaliana are key factors involved in wax biosynthesis, was down-regulated or up-regulated in nwgl leaves [8,10,16,37,38]. Except for Bol018504, the expression of Bol013612 was down-regulated in nwgl leaves. Bol013612, which encodes fatty acyl-CoA reductase, is homologous to Arabidopsis thaliana CER4 [10]. The wax components of the cabbage Bol013612 loss-of-function mutant were lacking in primary alcohols and wax esters [27]. Moreover, the nwgl leaf blades showed significant reduction in C28 primary alcohol, suggesting that the effect of NWGL on wax biosynthesis might require Bol013612 at the mRNA level. However, the expression levels of five genes, which encode one 3-ketoacyl-CoA synthase (Bol024192), another two CER1 proteins (Bol025251 and Bol025577), and two fatty-acyl-CoA reductases (Bol035700 and Bol036039), were up-regulated in nwgl leaves. These results indicated that the altering wax amount in the nwgl mutant resulted in feedback, causing up-regulation of some genes in fatty acid elongation, alkane-forming, and alcohol-forming pathways of wax biosynthesis. In addition, transcriptional control is considered to be the major mechanism for determining the total wax deposition in Arabidopsis thaliana [2]. The remaining two up-regulated genes—Bol038193 and Bol038557—in nwgl leaves, which encode AP2-like factors, are 81.2% and 88.0% homologous to Arabidopsis thaliana WR4 transcription factor, respectively. WR4 is a transcriptional activator and functions in the up-regulation of cuticular wax biosynthesis in Arabidopsis stems [16]. Therefore, these findings suggest that the up-regulated genes in nwgl leaves might be controlled by NWGL through WR4 transcription factor.

Although the number of known plant IncRNAs is increasing dramatically, the identification of IncRNAs as regulators in cuticular wax biosynthesis in plants is still lacking [22,30–32,39–46]. In this study, we identified 148 differentially expressed IncRNAs between plants exhibiting nwgl phenotype and wild-type (Figure 6a, Table S8), revealing specific responses of the noncoding transcriptome to cuticular wax loads reduction. The fact that these IncRNAs changed expression pattern in nwgl leaves suggested that at least some of them have regulatory roles in wax biosynthesis. IncRNAs can regulate gene expression by cis- and trans-acting [33]. We predicted the cis-regulated and trans-regulated target genes of the differentially expressed IncRNAs and performed a co-expression analysis between them to explore the putative regulatory functions of the IncRNAs. Five of the target genes, which were found to be strongly co-expressed with the differentially expressed IncRNAs, were annotated to be involved in lipid-related processes. Unfortunately, we did not find any target genes of the differentially expressed
lncRNAs directly involved in cuticular wax biosynthesis. It would be difficult, but worthwhile, to investigate the exact roles of these differentially expressed lncRNAs in cuticular wax biosynthesis.

In summary, we fine-mapped the NWGL locus underlying the non-wax glossy phenotype in cabbage. Through genome-wide transcriptome studies, we revealed that many protein-coding genes involved in wax biosynthesis were regulated by the NWGL gene. Perhaps the most striking finding by this work was genome-wide identification of a set of lncRNAs differentially expressed in plants exhibiting nwgl phenotype, and may reveal a more sophisticated molecular regulation mechanism of cuticular wax biosynthesis in cabbage.

4. Materials and Methods

4.1. Plant Materials

The cabbage (Brassica oleracea L. var. capitata) plant exhibiting (non-wax glossy) nwgl phenotype was derived from inbred line G287. Two F₂ mapping populations were generated by crossing the plant exhibiting nwgl phenotype with inbred line G306 or G274, respectively. Cabbage plants were cultivated in the experimental field at the Shanghai Academy of Agricultural Sciences (Shanghai, China) during the natural growing season.

4.2. Cryo-scanning Electron Microscopy (Cryo-SEM)

Fresh leaf samples from nwgl and wild-type plants were fixed in specimen holders of a Hitachi S4800 cryo-transfer system (Hitachi, Tokyo, Japan) with glue, immediately frozen with liquid nitrogen. The samples were transferred to a preparation chamber under vacuum for coating. Photographs of the sample surface were taken using its carrying camera.

4.3. Cuticular Wax Analysis

For the wax composition studies, cabbage rosette leaf segments were collected at the harvesting stage and immersed in liquid nitrogen for storage. For wax extraction, 200–400 mg of freeze-dried samples were dipped into 15 mL n-hexane for 30 s at 60 °C and dried under a stream of nitrogen. By adding 100 µL n-hexane, 100 µL N,O-bis (trimethylsilyl) fluorocacetamide (BSTFA), and 2 µL n-tetracosane (20 mg/mL) as an internal standard, samples were shocked for 1 min and subsequently incubated for 30 min at 90 °C. These derivatized samples were then analyzed with an Agilent 7890B-5977B GC-MS (Agilent, Santa Clara, CA, USA).

4.4. Fine Mapping of NWGL

Genomic DNA was isolated from young seedlings with normal phenotype. The NWGL gene was first mapped to the end of chromosome C08 using 46 F₂ normal individuals selected from G306×nwgl or G274×nwgl, respectively. A total of 706 recessive normal individuals selected from the F₂ population G274×nwgl were used for fine mapping. To identify candidate genes, the corresponding DNA fragments were amplified from both nwgl and wild-type plants and sequenced. The primer sequences are listed in Table S14.

4.5. RNA Isolation, Library Construction and Illumina Sequencing

Total RNA was extracted from collected leaves of nwgl and WT plants at harvesting stage using RNAPrep Pure Plant kit (Polysaccarides&Polyphenolics-rich) (Tiangen, Beijing, China) according to the manufacturer’s instructions. RNA yield and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Six cDNA libraries (three biological replicates per genotype) were constructed using NEBNextUltra™ Directional RNA library Prep kit (NEB, Ipswich, MA, USA) for Illumina (Illumina, San Diego, CA, USA). RNA sequencing was performed by IlluminaHiSeq platform by Biomarker Technologies Co. Ltd. (Biomarker, Beijing, China).
4.6. Read Mapping and Transcriptome Assembly

Raw data were filtered using inhouse perl scripts available with Biomarker Technologies Co. Ltd. (Biomarker, Beijing, China). In total, 163.35 Gb clean data were generated from six libraries (on average, more than 26.48 Gb clean data for each sample). Clean reads were aligned to the cabbage genome (www.ocri-genomics.org/bolbase, accessed on: 13 July 2018) using HISAT 2 [47]. The mapped reads were assembled using the StringTie with gff compare program annotation [48]. The raw data were deposited in the GEO database (https://www.ncbi.nlm.nih.gov/geo/, accessed on: 28 April 2019) and the GEO accession number is GSE130405.

4.7. Identification of IncRNAs

The unknown transcripts were used for putative IncRNA identification. The IncRNAs candidates were identified followed rigorous criteria: (1) The transcripts length must be ≥200 bp; (2) The transcripts must be with two or more exons; (3) The transcripts must be with FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) value ≥0.1. Then, the predicted IncRNAs were further screened using four computational approaches, including CPC, CNCI, Pfam and CPAT to ensure that the IncRNAs did not have protein-coding ability [49–52].

4.8. Analysis of Differential Expression of Genes and IncRNAs

Genes and IncRNAs expression level was estimated by calculating FPKM values using StringTie (v1.3.1) [48]. Differentially expressed genes and IncRNAs were identified using the DESeq R package (v1.10.1) with |log2(fold change)|≥1 and a false discovery rate (FDR) <0.05. The FPKM values of genes and IncRNAs have been deposited in the GEO database (https://www.ncbi.nlm.nih.gov/geo/, accessed on: 28 April 2019) and the GEO accession number is GSE130405.

4.9. Target Gene Prediction of IncRNAs

The neighboring genes within 100 Kb upstream and downstream of IncRNAs on the genome were considered as potential cis-regulated target genes. The trans-regulated target genes were searched using LncTar software based on mRNA sequence complementary and RNA duplex energy prediction [53].

4.10. Functional Enrichment Analysis

For all the differentially expressed genes (DEGs) and cis-regulated target genes of differentially expressed IncRNAs, Gene Ontology (GO) was analyzed using topGO R packages and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was carried out using the KOBAS software [54,55]. Significant enriched Go terms and KEGG pathways were identified with p value < 0.05.

4.11. Co-expression Analysis

We calculated the Spearman correlation coefficients between the expression levels of differentially expressed IncRNAs and their target genes to analyze their co-expression.

4.12. McrBC-, HpaII, MspI-PCR

McrBC is an endonuclease which cleaves DNA containing methylcytosine and does not act upon unmethylated DNA. However, McrBC cannot recognize HpaII/MspI endonuclease sites (CCGG) in which the internal cytosine is methylated. For McrBC, HpaII, MspI-PCR analysis of Bol018504, 1000 ng of genomic DNA was digested using 20 units of the three endonucleases, respectively. The volume of enzyme reaction mixes was 50 µL. The reaction time was 0, 0.5, 3, and 8 h for McrBC digestion, but 0, and 3 h for HpaII and MspI digestion. The digested DNAs were used for PCR amplification, and the products were isolated by agarose gel electrophoresis.

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