Mapping of the Waxy Gene in *Brassica napus* L. via Bulked Segregant Analysis (BSA) and Whole-Genome Resequencing

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Abstract: Plant cuticular wax is the covering of the outer layer of the plant. It forms a protective barrier on the epidermis of plants and plays a vital role like a safeguard from abiotic and biotic stresses. In the present study, *Brassica napus* L. materials with and without wax powder were observed. Genetic analysis showed that the separation ratio of waxy plants to waxless plants was 15:1 in the F2 population, which indicated that the wax powder formation was controlled by two pairs of genes. In order to identify the candidate genes associated with the wax powder trait of *B. napus* L., bulked segregant analysis (BSA) was performed. The homozygous waxy plants, the homozygous waxless plants, and plants from three parents were selected for establishing five DNA pools for genome-wide resequencing. The results of the resequencing showed that the site associated with wax powder trait was located in the region of 590,663–1,657,546 bp on chromosome A08. And 48 single nucleotide polymorphisms (SNPs) were found between the DNA sequences of waxy plants and waxless plants in this region. These SNPs were distributed across 16 gene loci. qRT-PCR analysis was conducted for the 16 candidate genes and three genes (*BnaA08g01070D*, *BnaA08g02130D*, and *BnaA08g00890D*) showed significantly differential expression between waxy and waxless parents. *BnaA08g01070D* and *BnaA08g02130D* were significantly down-regulated in the waxless parent, while *BnaA08g00890D* was significantly up-regulated in the waxless parent. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses revealed that the *BnaA08g02130D* gene was enriched in lipid biosynthetic or metabolic processes. All the results in our study would provide valuable clues for exploring the genes involved in wax powder development.

Keywords: *Brassica napus* L; bulked segregant analysis; whole-genome resequencing; waxy; waxless

1. Introduction

A plant cuticle constitutes the interface between the leaf and the external environment, and is an important barrier for plants to cope with biotic and abiotic stresses. The cuticle, comprising wax, is highly hydrophobic and impermeable to water and other solutes. The cuticle is composed of an insoluble membrane impregnated by and covered with soluble waxes such as cutin [1], cutan [2], and other epicuticular waxes. The functions of cuticular wax include sealing any aerial organ's surface to protect the plant against uncontrolled water loss or water stress [3–5], preventing UV damage [6–9], resisting extreme temperatures [10], maintaining a clean and water-repellent surface [11], and protecting plants from the attack of pests and diseases [12,13]. Moreover, the cuticular wax content in *Arabidopsis thaliana* and *Brassica* plants was also closely related to pollen fertility [14,15]. The cuticle also plays a very important role in growth and development.

Plant cuticular wax is composed of very-long-chain fatty acids (VLCFAs) with 20 to 34 carbon atoms and their derivatives (including alkanes, alcohols, aldehydes, ketones, and
esters), of which primary alcohols and esters are mainly produced from VLCFAs via the acyl reduction pathway, while alkanes, secondary alcohols, and ketones are mainly formed from VLCFAs through the decarbonylation pathway [16]. The cuticular wax content of a plant is closely related to its developmental status and the surrounding environment. The composition and total amount of epidermal wax differ significantly between different tissues and organs of a plant in the same or different species [17]. The regulation of the formation of cuticular wax involves the coordination of multiple genes [18]. Several genes related to the synthesis, transport, and regulation of wax have been identified and cloned in the model plant *A. thaliana*. The *Arabidopsis lacs1* and *lacs2* mutants showed a significantly reduced cutin content and increased wax content, and the *lacs1* and *lacs2* double mutant displayed an organ fusion phenotype [19]. The KCS gene family plays an important role in the catalytic synthesis of VLCFAs. KCS6 encodes a VLCFA-condensing enzyme. The wax content in the stem of the *kcs6* mutant plant was reduced to only 6% to 7% of that of the wild type plant [16,20]. In *Arabidopsis*, the CER2 gene encoding a soluble protein was the first cloned wax synthesis-associated regulatory gene [21,22]. CER2 was located in the nuclei and expressed at the highest level in the early stage of plant development, with tissue-specificity [23]. CER6 mutation caused defective VLCFAs on the surface of pollens and stems, wax content reduction, and sterile pollen. The over-expression of CER6 increased the wax content in the epidermis of plants [20]. Moreover, the ethylene response factor (ERF), *WAX INDUCER 1* (*WIN1*) gene was cloned and its over-expression in *A. thaliana* significantly increased the wax content in leaves and stems [24]. The over-expression of the ERF transcription factor genes, *WAXPRODUCTION 1* (*WXP1*) and *WAXPRODUCTION 2* (*WXP2*), cloned from *Medicago sativa*, in *Arabidopsis* resulted in wax accumulation in leaf epidermis [25].

Rapeseed is an oil crop widely planted across the world. It is the largest oil crop in China. Rapeseed plants have a typical cuticle, and the epidermis of leaves and stems is usually covered with wax powder. In a novel dominant glossy mutant (*BnaA.GL*) in *Brassica napus*, the expression of the *BnCER1* gene and other waxy synthesis genes were down-regulated [26]. Over-expression of the *Brassica campestris* lipid transfer protein gene *BraLTP1* in *B. napus* reduced wax deposition in leaves and affected cell division and flower development [27]. The candidate genes controlling the waxy phenotype of cabbage (*Brassica oleracea*) were located on chromosome C08. Genome-wide analysis of the coding and non-coding RNAs in the cuticular wax synthesis process in cabbage revealed that *CER1* and *CER4* genes were down-regulated in the waxless mutant [28]. The waxless trait has been used as a morphological marker in crossbreeding. The presence or absence of wax powder on the surface of plants can be used as a morphological marker in hybrid seed production [29]. Therefore, the wax powder on the surface of plants is of great importance. Bulked segregant analysis (BSA) is a fast and simple method for mapping target traits. The method has the advantage that it does not require the investigation of individual progenies, and the progenies of two opposite traits are pooled. This method was established and a downy mildew resistance gene was mapped in lettuce in a 25 cM chromosomal region [30]. Early BSA applications were based on molecular markers such as RFLP, RAPD, and SSR [31,32]. With the wide application of high-throughput sequencing technology, a large number of SNPs and Indel markers generated by chips, RNAseq, and genome-wide resequencing facilitated the use of BSA in rapidly mapping and identifying important crop traits and genes, such as the peanut branching habit [33], citrus polyembryonic loci [34], Asian pear skin color [35], and watermelon dwarfism gene [36].

In the present study, *Brassica napus* L. materials without wax powder were found. Waxless mutant plants showed significant reduction or absence of wax powder on leaves and stems. To find out whether the wax powder trait is dominant or recessive, and whether the trait is controlled by a single gene or multiple genes, we performed genetic analyses. Moreover, in order to map the genes related to wax powder development in *B. napus*, BSA-based genome-wide resequencing was performed. The resequencing data was analyzed and the candidate regions associated with wax powder trait were localized to chromosomes.
The SNPs were found between the DNA sequences of waxy plants and waxless plants and the corresponding candidate genes were found. qRT-PCR analysis was conducted to explore the expression patterns of these candidate genes. Meanwhile, GO and KEGG pathway enrichment analyses were performed for gene function prediction. The results would provide insights for exploring the genes involved in wax powder development.

2. Materials and Methods

2.1. Plant Materials

In the current study, *B. napus* male sterile line P202001 was used as the maternal parent and the temporary maintainer line P202002 was used as the male parent, and their hybrid offspring was male sterile line (Figure 1). The male sterile line was further hybridized with the waxless restorer line P202003 to obtain F1. F1 plants were self-inbred to obtain F2. In the F2 population, the ratio of waxy plants to waxless plants was 15:1, and several waxy plants were selected and self-inbred to obtain F3. According to the genetic laws and the phenotypic situation, four kinds of groups appeared in F3. In group I, all the plants were waxy plants. In group II, the ratio of waxy plants to waxless plants was 15:1. In group III, the ratio of waxy plants to waxless plants was 3:1. In group IV, all the plants were waxless plants. Several waxy plants from group III were selected and self-inbred to obtain F4. In the F4 population, the ratio of waxy plants to waxless plants was 3:1. Several waxy plants were selected and self-inbred to obtain F5. Based on the genetic laws and the phenotypic situation, F5 was divided into two groups. In group I, all the plants were waxy plants. In group II, the ratio of waxy plants to waxless plants was 3:1. Several waxy plants from group II were selected and self-inbred to obtain F6. In the F6 population, two kinds of groups occurred. Group I was numbered ‘17-4107’, and all plants in group I had wax powder. Group II was numbered ‘17-4105’, which contained waxy plants and waxless plants with a ratio of 3:1. All these plant materials were planted in the experimental farm of Zhuanghang comprehensive experimental station of Shanghai Academy of Agricultural Sciences. And all the crossings/selfings were conducted in the farm. During seedling stage, leaves of 50 waxless plants were, respectively, harvested from the ‘17-4105’ group for the construction of DNA pool of waxless plants. Meanwhile, leaves of 50 waxy plants were, respectively, harvested from the ‘17-4107’ group for the construction of DNA pool of waxy plants. In addition, leaves of 20 plants were, respectively, harvested from parents P202001, P202002, and P202003 for the construction of three parental DNA pools. All the leaf samples were quickly frozen in liquid nitrogen and stored at −80 °C.

2.2. Observation of Wax Powder Trait on Waxy Plants and Waxless Plants

The wax powder trait of waxy plants and waxless plants was observed in the flowering and carob stages. Fresh leaves from the waxy plant and waxless plants were fixed on specimen holders with glue. The samples were transferred to a preparation chamber under vacuum for coating (SD-900, BoYuan, Beijing, China). Then, the wax powder layer on the surface of the leaves was scanned and photographed using scanning electron microscope (TM4000plus, Hitachi, Tokyo, Japan).

2.3. Sample Pooling and Genotyping

Take 0.2 g of leaf tissue from each plant for DNA extraction. The leaf DNAs of 50 waxy plants were mixed in equal amounts to construct a DNA pool of waxy plants. The leaf DNAs of 50 waxless plants were also mixed in equal amounts to construct a DNA pool of waxless plants. In addition, three parental DNA pools were constructed. A TruSeq DNA LT Sample Prep kit was used for the library construction of the two extreme DNA pools and three parent DNA pools. Genome-wide resequencing at 30× coverage was performed using Illumina Hiseq Xten (Shanghai OE Biotech Co., Ltd, Shanghai, China). Quality control of raw sequencing data was performed using Trimomatic v0.36 [37]. Clean reads were aligned and compared to *Brassica napus* genome (http://www.genoscope.cns.fr/brassicanapus/data/Brassica_napus_v4.1.chromosomes.fa.gz, accessed on 29 June 2018)
using BWA v0.7.17 [38]; then, SAMtools v1.6 [39] was used to convert the format of the obtained data. Subsequently, the PCR repeats were removed using the Picard module of GATK v4.0.2.1 (GATK). Based on the alignment of the sample sequence with the reference genome, the SNP and InDel sites in the samples were detected using the SAMtools v1.6 mpileup module with default parameters. SNPs and InDels were annotated using snpEff v4.1 [40].

Figure 1. The genetic analysis chart of wax powder trait.

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2.4. Localization and Prediction of Candidate Genes Associated with Wax Powder Development

Based on the genotyping results, the homozygous polymorphic SNPs between parents were screened. The parents, P202001, P202002, and P202003, were used as references. The frequency of the SNPs (SNP-index) at each polymorphic site of the two progeny pools was calculated according to the method reported by Takagi et al. [41]. For SNPs in progeny, the reads of mutant-type parental origin were determined based on parental genotypes, and the SNP-indexes were calculated. The average distribution of the SNP-index was estimated with 1-Mb window size and 10-kb step and was plotted to generate an SNP-index curve. The peak in the linkage region became distinct after the noise was partially eliminated. The SNP-index plot was generated for the two progeny pools using the absolute value of the ∆SNP-index that was the difference in the SNP-index between the two pools at the segregation position. The 95% and 99% confidence levels were chosen.

Figure 1. The genetic analysis chart of wax powder trait.
as screening thresholds, and areas above the threshold were possible phenotypic linkage regions. By comparing the type of the SNPs in the candidate region with that of the corresponding position of the reference genome, the mutant type of SNPs in parental lines and waxless and waxy pools was estimated. Candidate genes with the same type of SNPs in a parent and the progeny pool with a similar phenotype were selected. The candidate genes were screened primarily through homology-based annotation using NR (Non-Redundant Protein Sequence), GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. The BRAD database (http://brassicadb.org/brad/, accessed on 1 September 2023) and TAIR database (https://www.arabidopsis.org, accessed on 1 September 2023) were used to analyze and predict the functions of candidate genes.

2.5. Expression Analysis of Candidate Genes

At the 12-leaf stage of rapeseed seedlings, the amount of wax powder on the waxy plants was large and the wax powder trait was very easy to be observed, so the waxy and waxless plants could be clearly distinguished. At the 12-leaf stage, the leaves of P202001 and P202003 were sampled and continued on day 7, 14, and 21 after the first sampling. And total RNAs of these leaf samples were extracted using RNeasy Plant Mini Kit (Qigem). The concentration and OD260/OD280 were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNA integrity was analyzed via agarose gel electrophoresis. The RNA was reverse transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, R223-01, Nanjing, China). The reverse transcription reaction procedure included 2 steps. Step 1: A reaction including 0.5 μg total RNA, 2 μL 4× gDNA wiper mix, and 8 μL nuclease-free H2O was prepared and placed at 42 °C for 2 min. Step 2: In this reaction, 2 μL 5× HiScript II Q RT SuperMix IIa was added, and the reaction was incubated at 25 °C for 10 min, 50 °C for 30 min, and 85 °C for 5 min. The total volume of the reaction was 10 μL. After reverse transcription, 90 μL nuclease-free H2O was added, and the solution was stored in a −20 °C freezer. The relative expression levels of the selected candidate genes in the leaves of *B. napus* were analyzed using qRT-PCR. The PCR primers (Table S1) were designed using the software Primer Premier 5 and were synthesized by Shanghai Sunny Biotech Co., Ltd. (Shanghai, China). The amplification reaction was prepared by adding 0.5 μL (about 100 ng) cDNA, 10 μL SYBR Premix Ex Taq II (2×), 0.8 μL 10 μmol·L⁻¹ each of the forward and reverse primers, and ddH2O to a final volume of 20 μL. The amplification was conducted using the real-time PCR thermo cycler Bio-Rad CFX96. The thermo cycles were pre-denaturing at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The qRT-PCR results were analyzed using the Bio-Rad CFX96 Manager, and the threshold line was automatically set using the software. The *B. napus* gene *BnActin7* (GenBank accession number EV086936) was used as an internal reference. The relative expression levels of the target genes were quantified by using the 2⁻ΔΔCt method [42]. Each experiment was conducted in three biological replicates, and the same sample was performed in three technical replicates. The Student’s *t*-test was used to test the probability of significant differences between samples.

3. Results

3.1. Genetic Analysis and Morphological Observation of the Waxless Trait of Rapeseed

In this study, genetic analysis showed that the separation ratio of waxy plants to waxless plants was 15:1 in the F2 population. And in F3, when a pair of genes was homozygous, the separation ratio of waxy powder plants and waxless powder plants was 3:1. The whole genetic analysis indicated that the wax powder formation was controlled by two pairs of genes and the waxy phenotype was dominant over the waxless phenotype (Figure 1). In contrast to waxy plants, waxless plants had no wax on the surface of stalks, leaves, floral buds, and silique (Figure 2A,B). The waxless trait was distinctly visible throughout the growth period. The results of the scanning electron microscopy showed there were abundant and intact wax crystals on the leaf blade of waxy parent P202001.
However, the leaf epidermis of waxless parent P202003 was smooth, without wax crystals (Figure 2C,D).

Figure 2. Plant appearance of waxless P202003, waxy P202001, and the corresponding micro-structure of leaf blade. (A) Plants at the beginning of flowering, waxless P202003 (left) and waxy P202001 (right). (B) Plants in the carob stage, waxless P202003 (left) and waxy P202001 (right). (C) Microstructure of P202003 blade ventral, wax crystals were dense, with high proportion of tubular-like wax crystals under high magnification. The red arrows indicate the wax powder crystal structures, and all similar structures in the figure are wax powder crystal structures. (D) microstructure of P202001 blade ventral. Scale bar = 50 μm.

3.2. RNA-Seq Analysis

To rapidly map the candidate genes associated with the wax powder trait, BSA-Seq analysis was performed. Five DNA pools were constructed, including three parental DNA pools (P202001, P202002 and P202003), waxless plants DNA pool (waxless plants from ‘17-4105’ group), and waxy plants DNA pool (waxy plants from ‘17-4107’ group). These five DNA pools were subjected to whole-genome resequencing. As shown in Table 1, after filtering the raw data, the clean data of each DNA pool ranged from 28,936 to 56,780 million bp. High-quality sequencing data (Q20 ≥ 97.33% and Q30 ≥ 91.97%) were obtained with the GC ≥ 36.37%. The numbers of clean reads ranged from 198.2 to 383.2 million. The clean reads were then mapped to the B. napus reference genome (http://brassicadb.cn/, accessed on 29 June 2018) with a mapping rate of 94.9% to 99.1%. The mean coverage ranged from 29.2× to 51.3×. The mean mapping quality ranged from 42.2 to 43.3. Compared to the B. napus reference genome, a total of 21,826,671 SNPs and 2,630,037 InDel loci were detected in the five pools. The numbers of SNPs and InDels in the upstream and downstream regions of genes and the intergenic regions were higher than those in other regions of chromosomes. The total number of SNPs and InDels detected in different pools was similar (Table 2).
Table 1. The alignment statistics results with the reference genome for all samples.

<table>
<thead>
<tr>
<th>Sequencing Information</th>
<th>17-4105</th>
<th>17-4107</th>
<th>P202001</th>
<th>P202003</th>
<th>P202002</th>
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<td>HQ clean data (bp)</td>
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<td>34,577,724,967</td>
<td>28,936,047,349</td>
<td>37,904,376,134</td>
<td>56,780,469,639</td>
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<td>Q20 (%)</td>
<td>97.33%</td>
<td>97.33%</td>
<td>97.56%</td>
<td>98.70%</td>
<td>98.74%</td>
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<tr>
<td>Q30 (%)</td>
<td>92.01%</td>
<td>91.97%</td>
<td>92.53%</td>
<td>96.00%</td>
<td>96.10%</td>
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<td>GC content (%)</td>
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<td>37.20%</td>
<td>37.82%</td>
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<td>HQ clean reads number and</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>percent (%)</td>
<td>228,591,684</td>
<td>237,268,442</td>
<td>198,207,190</td>
<td>255,719,126</td>
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<td>171,778,467</td>
<td>202,899,761</td>
<td>296,825,463</td>
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<tr>
<td>mapped rate (%)</td>
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<td>(88.16%)</td>
<td>(89.67%)</td>
<td>(93.6%)</td>
<td>(93.83%)</td>
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<td>Mean coverage</td>
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<td>35.7912 ×</td>
<td>29.2245 ×</td>
<td>35.0443 ×</td>
<td>51.3133 ×</td>
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<tr>
<td>Mean mapping quality</td>
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<td>43.2226</td>
<td>43.2545</td>
<td>42.2296</td>
<td>43.0776</td>
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3.3. Screening and Analysis of Candidate Genes

The distribution of the SNP-index on the chromosomes of the waxy and waxless DNA pools was estimated. The absolute value of the difference in the number of SNP-indexes between the two pools, which was |ΔSNP-index|, was calculated. By setting 95% and 99% confidence thresholds, a candidate region between the 590,663 and 1,657,546 bp on chromosome A08 was identified (Figure 3). There were 48 SNPs distributed across 16 gene loci in this region.

To predict the biological functions of the 16 potential candidate genes, GO and KEGG pathway enrichment analyses were performed. The annotated genes were divided into three major functional categories, namely, biological processes (BP), cellular components (CC), and molecular functions (MF, Figure 4). The results showed that BnaA08g01250D, BnaA08g01020D, and BnaA08g02130D were related to “metabolic process (GO:0008152)”; BnaA08g02130D, BnaA08g013300D, and BnaA08g01270D were related to “ATP binding, glucose binding and Ca²⁺ binding activity (GO:0005488)”, respectively; BnaA08g00820D and BnaA08g02130D were associated with biological regulation (GO:0065007); BnaA08g01020D and BnaA08g1000D were annotated as “catalytic activity (GO:0003824)” and BnaA08g00820D was annotated as “regulation of biological process (GO:0050789)”.

Figure 3. Map of the ΔSNP-index of waxless and waxy plants after screening with parental homozygous polymorphic loci. Note: The blue arrow indicates the candidate region (590,663–1,657,546 bp) on chromosome A08 with an association threshold higher than 1%. (1) Green and blue dots both represent the SNP-index or ΔSNP-index. (2) The red line represents the fitted line of SNP-index or ΔSNP-index after window sliding. (3) The purple lines represent the 95% confidence threshold. (4) The orange lines represent the 95% confidence threshold.
### Table 2. Statistics of SNPs and Indels.

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<th>CDS</th>
<th>Synonymous</th>
<th>Upstream</th>
<th>Downstream</th>
<th>Intronic</th>
<th>Intergenic</th>
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<td>10,720</td>
<td>316,979</td>
<td>41,969</td>
<td>608,670</td>
<td>264,950</td>
<td>1,871,755</td>
<td>787,613</td>
<td>102,305</td>
<td>1,087,082</td>
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<td></td>
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<td>336</td>
<td>250</td>
<td>/</td>
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<td>19,694</td>
<td>/</td>
<td>305,119</td>
<td>109,734</td>
<td>18,138</td>
<td>81,927</td>
<td>72</td>
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<td>1,858,996</td>
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<td></td>
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<td>/</td>
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<td>19,657</td>
<td>/</td>
<td>303,705</td>
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<td>561,866</td>
<td>244,017</td>
<td>1,733,726</td>
<td>730,785</td>
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<td></td>
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<td>238</td>
<td>/</td>
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<td>/</td>
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<td>761,545</td>
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<tr>
<td></td>
<td>Indels</td>
<td>340</td>
<td>235</td>
<td>/</td>
<td>9870</td>
<td>19,384</td>
<td>/</td>
<td>295,891</td>
<td>105,899</td>
<td>17,387</td>
<td>78,665</td>
<td>65</td>
<td>527,096</td>
</tr>
<tr>
<td>P202003</td>
<td>SNPs</td>
<td>12,358</td>
<td>10,418</td>
<td>303,309</td>
<td>40,212</td>
<td>583,115</td>
<td>254,081</td>
<td>1,797,291</td>
<td>758,743</td>
<td>97,673</td>
<td>1,065,920</td>
<td>2949</td>
<td>4,342,954</td>
</tr>
<tr>
<td></td>
<td>Indels</td>
<td>344</td>
<td>250</td>
<td>/</td>
<td>9706</td>
<td>18,749</td>
<td>/</td>
<td>287,494</td>
<td>104,172</td>
<td>17,266</td>
<td>79,185</td>
<td>65</td>
<td>516,572</td>
</tr>
</tbody>
</table>
KEGG pathway analysis was conducted to investigate whether these candidate genes were involved in special pathways. For these candidate genes, the most significantly enriched pathway were metabolic pathways, proteasome, pyruvate metabolism, biosynthesis of secondary metabolites, and citrate cycle.

The homologous genes of these 16 candidate genes in Arabidopsis were found and annotated based on the Brassicaceae Database (BRAD, http://brassicadb.cn/#/, accessed on 1 September 2023) and The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org, accessed on 1 September 2023) (Table 3). According to previous reports, BnaA08g00820D, which was homologous to AT1G54200, was likely to regulate the plant biomass, stress tolerance, seed weight, and yield. BnaA08g00890D was likely to regulate reactive oxygen species homeostasis and leaf senescence. BnaA08g01000D encoded alpha5 subunit of 20s proteosome involved in protein degradation and RNA degradation. BnaA08g01010D may refer to 26S proteasome AAA-ATPase subunit RPT1a. BnaA08g01020D was SRF6a gene, which might be involved in stress-related processes, including responses to light, heat, and plant immunity. BnaA08g01030D encoded Calcineurin-like metallophosphoesterase superfamily protein. BnaA08g01070D was ETHE1 gene and encoded a sulfur dioxygenase essential for embryo and endosperm development. BnaA08g01170D was a member of the plant TLP family, with an unknown function. BnaA08g01250D was mMDH1, which encoded a mitochondrial malate dehydrogenase. It was involved in leaf...
respiration and altered photorespiration and plant growth. *BnaA08g01260D* was TCP3, which was involved in heterochronic regulation of leaf differentiation. *BnaA08g01270D* was the ATNCL gene, which encoded a Na\(^+\)/Ca\(^{2+}\) exchanger-like protein that participated in the maintenance of Ca\(^{2+}\) homeostasis. The previous study showed it was involved in salt stress in Arabidopsis. *BnaA08g01330D* encoded putative DUF616 with an unknown function. *BnaA08g01350D* encoded ubiquitin-conjugating enzyme E2. *BnaA08g02130D* was reported to be involved in glucose-ethylene crosstalk. *BnaA08g02150D* and *BnaA08g02160D* were homologous to AT1G50420. Their other names were SCL-3 or SCL3, which refer to regulatory network in Arabidopsis roots.

Table 3. Information on candidate genes identified by BSA-seq.

<table>
<thead>
<tr>
<th>Candidate Genes</th>
<th>Arabidopsis Genes</th>
<th>Functional Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>BnaA08g00820D</em></td>
<td>AT1G54200</td>
<td>Regulation of Big Grain1 in rice and Arabidopsis increases plant biomass, stress tolerance, seed weight, and yield.</td>
<td>[43,44]</td>
</tr>
<tr>
<td><em>BnaA08g00830D</em></td>
<td>AT1G54115</td>
<td>CCX1, a putative cation/Ca(^{2+}) exchanger, participates in regulation of reactive oxygen species homeostasis and leaf senescence.</td>
<td>[45]</td>
</tr>
<tr>
<td><em>BnaA08g01000D</em></td>
<td>AT1G53850</td>
<td>Encodes alpha subunit of 26S proteosome involved in protein degradation and RNA degradation.</td>
<td>[46]</td>
</tr>
<tr>
<td><em>BnaA08g01010D</em></td>
<td>AT1G53750</td>
<td>26S proteasome AAA-ATPase subunit RPT1a (RPT1a) mRNA.</td>
<td>[46,47]</td>
</tr>
<tr>
<td><em>BnaA08g01020D</em></td>
<td>AT1G53730</td>
<td>SRF6. SRF6 has a role in the defense against pathogenic fungi. SRF6 gene may be involved in stress-related processes, including responses to light, and heat. The potato SRF family gene, StLRPK1, is involved in plant immunity.</td>
<td>[48,49]</td>
</tr>
<tr>
<td><em>BnaA08g01030D</em></td>
<td>AT1G53710</td>
<td>Calcineurin-like metallo-phosphoesterase superfamily protein, GPI, anchor biosynthetic process.</td>
<td>[50]</td>
</tr>
<tr>
<td><em>BnaA08g01070D</em></td>
<td>AT1G53470</td>
<td>Other name: ETHE1, ETHE1-LIKE. Arabidopsis ETHE1 encodes a sulfur dioxygenase that is essential for embryo and endosperm development.</td>
<td>[51,52]</td>
</tr>
<tr>
<td><em>BnaA08g01170D</em></td>
<td>AT1G53320</td>
<td>Member of plant TLP family. TLP7 is tethered to the PM but detaches upon stimulus and translocates to the nucleus.</td>
<td>[53]</td>
</tr>
<tr>
<td><em>BnaA08g01250D</em></td>
<td>AT1G53240</td>
<td>mMDH1 encodes a mitochondrial malate dehydrogenase. It is expressed at higher levels than the other mitochondrial isofrom mMDH2 (AT3G15020) according to transcript and proteomic analyses. It has copper-ion-binding and L-malate dehydrogenase activity. In Arabidopsis, mitochondrial malate dehydrogenase reduces leaf respiration and alters photorespiration and plant growth.</td>
<td>[54]</td>
</tr>
<tr>
<td><em>BnaA08g01260D</em></td>
<td>AT1G53230</td>
<td>Other name: TCP3. Encodes a member of a recently identified plant transcription factor family that includes Teosinte branched 1, Cycloidea 1, and proliferating cell nuclear antigen (PCNA) factors, PCFI and 2. Regulated by miR319. Involved in heterochronic regulation of leaf differentiation.</td>
<td>[55]</td>
</tr>
<tr>
<td><em>BnaA08g01270D</em></td>
<td>AT1G53210</td>
<td>Other name: ATNCL. Encodes a Na(^+)/Ca(^{2+}) exchanger-like protein that participates in the maintenance of Ca(^{2+}) homeostasis. It is involved in salt stress in Arabidopsis. ATNCL may be a novel Ca(^{2+}) transporter in higher plants (Wang et al., 2012).</td>
<td>[56]</td>
</tr>
<tr>
<td><em>BnaA08g01330D</em></td>
<td>AT1G53040</td>
<td>rRNA (met) cytidine acetyltransferase, putative (DUF616). Protein of unknown function DUF616.</td>
<td>[57]</td>
</tr>
<tr>
<td><em>BnaA08g01350D</em></td>
<td>AT1G53020</td>
<td>Ubiquitin-conjugating enzyme E2.</td>
<td>[58]</td>
</tr>
<tr>
<td><em>BnaA08g02130D</em></td>
<td>AT1G50460</td>
<td>It has ATP binding, glucose-binding and hexokinase activities and participates in lipid biosynthesis and protein metabolism. Involved in glucose-ethylene crosstalk.</td>
<td>[59]</td>
</tr>
<tr>
<td><em>BnaA08g02150D</em></td>
<td>AT1G50420</td>
<td>Other name: SCARECROW-LIKE 3; SCL-3 or SCL3. SCL3-induced regulatory network in Arabidopsis thaliana roots.</td>
<td>[60]</td>
</tr>
</tbody>
</table>
### 3.4. Specific Expression of Candidate Genes

To explore whether the 16 candidate genes were associated with wax powder development, qRT-PCR was used to detect their expression levels in the leaves of waxy parent P202001 and waxless parent P202003 (Figure 5). The qRT-PCR results showed that the expression levels of *BnaA08g01070D* and *BnaA08g02130D* in the waxy parent P202001 were significantly higher than those in the waxless parent P202003. The expression level of *BnaA08g00890D* in the waxless parent P202003 was significantly higher than that in the waxy parent P202001 (6.15-fold). The expression levels of these three genes were further analyzed at four time points when the wax powder was clearly visible (Figure 6). The expression levels of *BnaA08g01070D* and *BnaA08g02130D* in the leaves of the waxy parent P202001 were consistently higher than in the leaves of the waxless parents P202003. Their expression levels gradually increased, reaching their highest levels at day 14 and decreasing at day 21. *BnaA08g00890D* was up-regulated in the leaves of the waxy parent P202001 at day 0, day 7, and day 14. At day 21, its expression level was highest in the waxless parent, but the lowest in the waxy parent.

![Figure 5](image-url) Expression profile of 16 candidate genes in the leaves of waxless parent P202003 and waxy parent P202001, revealed via qRT-PCR. Data represent means of three replicates ± SD. *, p < 0.05, **, p < 0.01. Student’s t-test.

![Figure 6](image-url) Expression patterns of three candidate genes in the leaves from the waxless and the waxy parents at four time points when the wax powder was clearly visible. P202001, the waxy parent. P202003, the waxless parent. The relative expression levels of *BnaA08g01070D* (A), *BnaA08g02130* (B), and *BnaA08g00890D* (C) by qRT-PCR.
4. Discussion

Wax powder is a mixture of organic compounds, which is widely distributed in the plant epidermis to prevent direct contact between plant tissues and the outside world, thereby preventing strong light radiation, high temperature burning, drought, frost damage, air pollution, mechanical damage, and other abiotic stress injuries [26,61–65]. Wax powder plays an important role in improving the stress resistance of plants. Drought increased the amount and content of cuticular waxes in rapeseed [26,64]. Cuticular wax is a major factor to defend crop plants from extreme ultraviolet (UV) radiation. It is reported that changes in cuticular wax production and gas exchange depend on high UV-B radiation in plants [63]. And cuticular waxes accumulations have an effect on the rate of gas exchange in the leaf surface of canola (Brassica napus) [61]. In addition, it was reported that cuticular wax could increases drought tolerance in Brassica napus [65]. Therefore, cuticular wax production and accumulation is helpful to improve the stress tolerance and indirectly improve crop quality and yield.

The wax powder trait is easy to observe, and waxy plants can be visually distinguished from waxless plants from the seedling stage, and the wax powder trait can be stably inherited under natural conditions, so the wax powder trait can be used as morphological marker in hybrid production. In rapeseed, the recessive sterility or fertility traits can be marked with wax powder trait, and the fertility of plants can be judged by observing the presence or absence of wax powder at the seedling stage, then 50% of the fertile plants can be removed. Using wax powder trait as morphological marker can quickly simplify the two-line hybrid breeding process.

Although wax powder synthesis pathway has been identified and the related genes have been cloned in Arabidopsis, the mechanism of wax powder formation is not well understood in Brassica. Most studies on Brassica have focused on the analysis of genetic models, and there were few in-depth studies [29,66]. Zhang et al. [67] closely mapped the BrWax1 gene in a recessive waxless mutant of Chinese cabbage, which is located in an 86.4 kb region of the A1 linkage group. There were 15 annotated genes in this region, of which Bra013809 was a homologous gene of Arabidopsis CER2, and an insertion mutation was found in its sequence. The expression level of Bra013809 was significantly reduced in the waxless mutant. The candidate gene controlling the cabbage waxy phenotype was located on the C08 chromosome. Genome-wide analysis of the coding and non-coding RNAs in the process of cabbage wax synthesis showed that the CER1 and CER4 genes were down-regulated in the waxless materials [28]. BSA coupled with RNA-Seq and appropriate statistical procedures (bulked segregant RNA-Seq or BSR-Seq) were used to clone the glossy3 (gl3) gene of maize, which was responsible for epicuticular wax accumulation on juvenile leaves [26]. Mutants of the glossy loci exhibit altered accumulation of epicuticular waxes on juvenile leaves. RNA sequencing performed on bulked RNA from blueberry progenies with and without wax power helped in the identification of genes related to the protective waxy coating on blueberry fruit [68].

In the current study, B. napus L. material without wax powder was found. In order to identify the candidate genes involved in wax power development, bulked segregant analysis (BSA) was performed. The results indicated that the 590,663 to 1,657,546 bp region on the chromosome A08 might be associated with wax power trait. There were 48 SNPs distributed across 16 gene loci in this region. Because waxy plants and waxless plants differed only in the presence or absence of wax powder, we reasoned that these genes might be the candidate genes associated with wax powder development. The qRT-PCR analysis revealed that the expression levels of the candidate genes, BnaA08g01070D and BnaA08g02130D, were significantly more up-regulated in the leaves of waxy parent P202001 than that in the leaves of waxless parent P202003, while the expression levels of candidate gene BnaA08g00890D were significantly down-regulated in the leaves of waxy parent P202001. Therefore, BnaA08g01070D, BnaA08g02130D, and BnaA08g00890D might be involved in wax power development.
5. Conclusions

In the present study, *B. napus* L. materials with and without wax powder were observed. Through genetic analysis, it was found that the wax powder trait was controlled by two pairs of genes. In order to identify the candidate genes involved in wax powder development in *B. napus* L., bulked segregant analysis (BSA) was performed. The resequencing results showed that the wax powder trait-associated loci might be located in the region of 590,663~1,657,546 bp on chromosome A08. There were 48 single-nucleotide polymorphisms (SNPs) found in this region between the DNA sequences of waxy plants and waxless plants. And these SNPs were distributed across 16 gene loci. qRT-PCR analysis was conducted for the 16 candidate genes and three genes (*BnaA08g01070D*, *BnaA08g02130D*, and *BnaA08g00890D*) showed significantly differential expression between the leaves from waxy and waxy parents. GO and KEGG pathway enrichment analysis revealed that the *BnaA08g02130D* gene was enriched in lipid biosynthetic or metabolic processes. All the results in our study would provide valuable clues for exploring the genes involved in wax powder development.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13102611/s1. Table S1 qRT-PCR primers in this study.

**Author Contributions:** J.Z. (Junying Zhang), J.Z. (Jifeng Zhu) and X.Z. designed the research and performed the experiments. J.Z. (Junying Zhang) and J.J. analyzed the sequencing data and finished the manuscript. L.Y., Y.L. and W.W. performed the field experiment. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The BSA resequencing data is unavailable due to privacy. We may use the resequencing data for further study.

**Conflicts of Interest:** The authors declare no conflict of interest.

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