A combination of genome-wide association study and transcriptome analysis in leaf epidermis identifies candidate genes involved in cuticular wax biosynthesis in *Brassica napus*

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**Abstract**

**Background:** *Brassica napus* L. is one of the most important oil crops in the world. However, climate-change-induced environmental stresses negatively impact on its yield and quality. Cuticular waxes are known to protect plants from various abiotic/biotic stresses. Dissecting the genetic and biochemical basis underlying cuticular waxes is important to breed cultivars with improved stress tolerance.

**Results:** Here a genome-wide association study (GWAS) of 192 *B. napus* cultivars and inbred lines was used to identify single-nucleotide polymorphisms (SNPs) associated with leaf waxes. A total of 202 SNPs was found to be significantly associated with 31 wax traits including total wax coverage and the amounts of wax classes and wax compounds. Next, epidermal peels from leaves of both high-wax load (HW) and low-wax load (LW) lines were isolated and used to analyze transcript profiles of all GWAS-identified genes. Consequently, 147 SNPs were revealed to have differential expressions between HW and LW lines, among which 344 SNP corresponding genes exhibited up-regulated while 448 exhibited down-regulated expressions in LW when compared to those in HW. According to the gene annotation information, some differentially expressed genes were classified into plant acyl lipid metabolism, including fatty acid-related pathways, wax and cutin biosynthesis pathway and wax secretion. Some genes involved in cell wall formation and stress responses have also been identified.

**Conclusions:** Combination of GWAS with transcriptomic analysis revealed a number of directly or indirectly wax-related genes and their associated SNPs. These results could provide clues for further validation of SNPs for marker-assisted breeding and provide new insights into the genetic control of wax biosynthesis and improving stress tolerance of *B. napus*.

**Keywords:** *Brassica napus* L., Cuticular wax, Genome-wide association study, RNA-seq, Single nucleotide polymorphism
Background

Brassica napus L. (2n = 38, genome AACC) is an allotetraploid crop evolved from natural hybridization between two diploid progenitor species, Brassica rapa (AA, 2n = 20) and Brassica oleracea (CC, 2n = 18), followed by chromosome doubling about ~7500 years ago [1]. It is a globally important oil crop which contributes edible oil, biofuels, and industrial compounds such as plasticizers and stabilizer for plastics, lubricants, and surfactants [2]. However, unfavorable environmental factors induced by climate changes such as drought and high temperature, severely influence its yields and qualities [3, 4]. For stabilizing or enlarging oilseed supply, it is important to improve stress tolerance of B. napus. The aerial parts of plants are covered with cuticular wax, a mixture of hydrophobic compounds. Both increased cuticular wax deposition and improved tolerance to water deficiency stress were observed in some transgenic crops with the overexpression of wax-associated genes [5–8], suggesting that this hydrophobic layer could protect plants from abiotic stresses.

Cuticular waxes are consisted of very long chain fatty acids (VLCFAs) and their derivatives such as alcohols, aldehydes, alkanes, ketones, and wax esters [9, 10]. The biosynthesis of these aliphatic wax components involves C_{16−C_{18}} fatty acids synthesis in the plastid, C_{16−C_{18}} fatty acid elongation in the endoplasmic reticulum (ER), and subsequent modification via either the decarbonylation or the acyl reduction pathway [11].

Many genes involved in cuticular wax biosynthesis and its regulation have been identified in Arabidopsis. However, the genetic basis of cuticular waxes in B. napus has still not been fully understood. The wax load on B. napus leaves is considerably higher in comparison to that on Arabidopsis leaves. However, the wax composition patterns are similar in these two plant species, both consisting of fatty acids, aldehydes, alkanes (predominant compounds), primary alcohols, secondary alcohols, ketones, and esters. Characterization of a novel dominant glossy mutant BnaAGL revealed that the suppression of the CER1 (a putative aldehyde decarboxylase gene involving in the biosynthesis of alkane) and other wax-related genes such as MAH1 (a midchain alkane hydroxylase gene involving in the biosynthesis of alkane) and other wax-related genes in B. napus accessions were used to perform GWAS to detect genes potentially related to cuticular wax biosynthesis. To further aid identification of wax related genes and explore molecular mechanism of wax biosynthesis, expression profiles of all GWAS-identified genes were determined by transcriptome of the leaf epidermis from high- and low-wax load B. napus lines. This study first used the GWAS tool and the epidermis transcriptome to identify candidate genes related to B. napus wax traits. Our results provided insight into the genetic regulation of B. napus cuticular wax metabolism; therefore, laid a foundation for genetic improvement of B. napus stress tolerance by wax modification.

Results

Phenotypic variations of leaf cuticular wax

A total of 192 B. napus accessions were used to characterize the leaf wax profiles in 2016 and 2017. The
cuticular wax profiles in this panel were similar to those reported by Tassone et al. [25] and Holloway et al. [28]. The leaf wax was mainly consisted of long-chain fatty acids, aldehydes, alkanes, primary alcohols (1-alcohols), secondary alcohols (2-alcohols), ketones, and alkyl esters. In total 21 predominant compounds were obtained from seven wax classes, such as C_{27} alkane, C_{29} alkane, C_{31} alkane, and C_{29} 2-alcohol, etc. Total wax coverage, amounts of wax classes, and the amount of each predominant wax compound were assessed as single trait. Additionally, the sum of C_{29} alkane, C_{29} ketone and C_{29} 2-alcohol, the three most abundant compounds from same biosynthesis pathway were also assessed as a single trait (Total C_{29}).

Heritability values ranged from 0.60 (C_{28} acid) to 0.84 (C_{27} alkane) for each independent wax trait (Table 2). The total wax coverage ranged from 7.75 to 53.93 μg·cm^{-2} in 2016 (with an average of 27.44 μg·cm^{-2}) and from 4.23 to 44.83 μg·cm^{-2} in 2017 (with an average of 18.65 μg·cm^{-2}).

A two-way ANOVA analysis indicated that most wax traits were influenced by genotype (G), year (Y) and their interactions (G × Y) (P < 0.001), suggesting the indispensable role of environment on wax synthesis regulation (Table 2). Extensive phenotypic variations of these wax traits were observed in two consecutive years (Table 1; Additional file 1: Fig. S1). The sum of products from alkane-forming pathway and the sum of products from alcohol-forming pathway were also characterized as single trait, respectively. A complete list of the 31 wax traits was provided in Table 1. Extensive phenotypic variations of these wax traits were observed in two consecutive years (Table 1; Additional file 1: Fig. S1). The total wax coverage ranged from 7.75 to 53.93 μg·cm^{-2} in 2016 (with an average of 27.44 μg·cm^{-2}) and from 4.23 to 44.83 μg·cm^{-2} in 2017 (with an average of 18.65 μg·cm^{-2}).

High correlation coefficients were observed between C_{29} alkane and C_{29} ketone (r = 0.69) and between C_{29} alkane and C_{29} 2-alcohol (r = 0.67), which were produced from alkane branch pathway, and between 1-alcohol and C_{38} ester (r = 0.87), C_{40} Ester (r = 0.93), and C_{42} ester (r = 0.63), which were produced from alcohol branch pathway (Additional file 2; Table S1). High positive correlation coefficient was also found between the products from alkane-forming pathway and alcohol-forming pathway (r = 0.72), indicating that these wax compositions were not independently regulated (Additional file 2; Table S1).

Population structure and relative kinship of the association panel
A subset of 4623 SNPs with missing data < 0.2 and MAF > 0.2, which distributed evenly across the entire B. napus genome, was selected for population structure and relative kinship analysis (K). Population structure analysis can provide information about the optimal number of subgroups (i.e. the optimal K value) and the proportion of each subgroup in each accession (i.e. Q matrix), which is useful to select the Q matrix corresponding to the optimal K value in the next association analysis, so as to control the false positives caused by the population structure. A clustering inference performed with possible clusters (K) from 1 to 10 showed that the most significant change in likelihood occurred when K increased from 2 to 4 (Fig. 1a), and the highest Δk-value was observed at k = 4 (Fig. 1b). Based on the Δk method described by Evanno et al. [29], the 192 accessions could be divided into four major sub-populations, which were designated as P1, P2, P3, and P4 (Additional file 3: Table S2). The analysis of genetic relatedness revealed that 55.8% of the pairwise kinships were equal to 0, and 69% of them ranged from 0 to 0.05 (Fig. 1d), suggesting that most of the accessions in this panel have no or weak kinship, which might be attributed to the broad ranging collections of the genotypes. The results of genetic relatedness analysis would be used in GWAS model as random effect covariate matrix (K matrix) to avoid the false positives in the next association analysis.

The Linkage disequilibrium (LD) decay rate was measured as the chromosomal distance at which the average pairwise correlation coefficient (r²) between all pairs of SNP markers dropped to half of its maximum value. The genome-wide LD decay of A and C subgenome for 192 rapeseed lines were shown in Fig. 1e. The LD of A subgenome decayed faster than that of the C subgenome. The average distance for A subgenome was 500 kb, and for C subgenome was 1600 kb, where r² decayed to 0.1.

Association mapping in B. napus for wax traits
To evaluate the effects of population structure (Q, PCA) and kinship (K), six models, including Q, PCA, K, PCA + K, Q + K and naïve (without controlling for Q, PC and K), were separately performed association analysis with the 31 wax traits (Additional file 5; Fig. S2). According to the P values from six models, the population structure and kinship can be corrected effectively by the linear mixed model such as PCA + K, Q + K and naïve when performing GWAS. Eventually, the PCA + K model was selected to perform association mapping for C_{28} acid, C_{26} alkane, C_{20} alkane, C_{29} 2-alcohol, C_{29} ketone, and C_{38} ester, while Q + K model for the remaining 25 wax traits for controlling population structure in GWAS. Thus, a total of 202 significantly associated SNPs for 31 wax traits were identified in a genome-wide scan (P < 2.95E-05) (Fig. 2; Additional file 5; Fig. S3; Additional file 6: Table S3). Among these SNPs, 18 were co-associated with multiple wax traits (Additional file 6: Table S3). For example, Bn-A01-p6380934, Bn-A05-p2030789, and Bn-
Table 1 Phenotypic variations of leaf cuticular wax in the association panel of *Brassica napus*

| Traits      | Year | Min (μg·cm⁻²) | Max (μg·cm⁻²) | Mean ± SD (μg·cm⁻²) | CV (%) |
|-------------|------|---------------|---------------|---------------------|--------|
| Total wax   | 2016 | 7.75          | 53.93         | 27.44 ± 7.75        | 28.24  |
|             | 2017 | 4.23          | 44.83         | 18.65 ± 5.55        | 29.76  |
| Acids       | 2016 | 0.15          | 2.08          | 0.44 ± 0.27         | 60.19  |
|             | 2017 | 0.05          | 0.96          | 0.18 ± 0.11         | 60.01  |
| Aldehydes   | 2016 | 0.22          | 2.16          | 0.73 ± 0.34         | 46.63  |
|             | 2017 | 0.06          | 1.75          | 0.50 ± 0.28         | 55.29  |
| Alkanes     | 2016 | 4.39          | 31.78         | 14.32 ± 4.39        | 30.65  |
|             | 2017 | 2.69          | 31.82         | 11.71 ± 3.98        | 34.02  |
| 2-Alcolols  | 2016 | 1.37          | 9.34          | 3.48 ± 1.37         | 39.51  |
|             | 2017 | 0.26          | 4.59          | 1.60 ± 0.65         | 40.49  |
| C29 Ketone  | 2016 | 1.99          | 14.70         | 6.39 ± 1.99         | 31.08  |
|             | 2017 | 0.14          | 7.93          | 3.90 ± 1.45         | 37.27  |
| 1-Alcohols  | 2016 | 0.35          | 2.63          | 1.15 ± 0.45         | 39.14  |
|             | 2017 | 0.05          | 1.52          | 0.46 ± 0.27         | 58.59  |
| Esters      | 2016 | 0.16          | 2.29          | 0.93 ± 0.46         | 49.79  |
|             | 2017 | 0.08          | 0.78          | 0.31 ± 0.11         | 34.83  |
| C26 Acid    | 2016 | 0.05          | 1.25          | 0.28 ± 0.22         | 75.91  |
|             | 2017 | 0.00          | 0.83          | 0.11 ± 0.09         | 79.28  |
| C28 Acid    | 2016 | 0.00          | 0.06          | 0.02 ± 0.01         | 83.15  |
|             | 2017 | 0.00          | 0.03          | 0.01 ± 0.01         | 90.39  |
| C26 Aldehyde| 2016 | 0.01          | 0.07          | 0.02 ± 0.01         | 49.32  |
|             | 2017 | 0.00          | 0.04          | 0.01 ± 0.01         | 42.94  |
| C28 Aldehyde| 2016 | 0.05          | 1.21          | 0.17 ± 0.13         | 80.62  |
|             | 2017 | 0.02          | 0.86          | 0.13 ± 0.12         | 92.38  |
| C30 Aldehyde| 2016 | 0.10          | 0.88          | 0.42 ± 0.19         | 45.37  |
|             | 2017 | 0.02          | 0.67          | 0.28 ± 0.12         | 42.32  |
| C25 Alkane  | 2016 | 0.00          | 0.04          | 0.01 ± 0.01         | 70.91  |
|             | 2017 | 0.00          | 0.04          | 0.01 ± 0.01         | 90.12  |
| C26 Alkane  | 2016 | 0.00          | 0.01          | 0.01 ± 0.00         | 43.70  |
|             | 2017 | 0.00          | 0.02          | 0.00 ± 0.00         | 68.72  |
| C27 Alkane  | 2016 | 0.03          | 0.27          | 0.11 ± 0.05         | 43.09  |
|             | 2017 | 0.03          | 0.25          | 0.10 ± 0.04         | 42.40  |
| C28 Alkane  | 2016 | 0.03          | 0.25          | 0.09 ± 0.03         | 36.08  |
|             | 2017 | 0.01          | 0.17          | 0.06 ± 0.02         | 29.82  |
| C29 Alkane  | 2016 | 4.27          | 30.90         | 10.89 ± 3.07        | 28.19  |
|             | 2017 | 2.23          | 25.52         | 11.30 ± 3.83        | 33.90  |
| C30 Alkane  | 2016 | 0.03          | 0.38          | 0.11 ± 0.06         | 54.70  |
|             | 2017 | 0.04          | 0.38          | 0.13 ± 0.05         | 33.80  |
| C31 Alkane  | 2016 | 0.01          | 0.08          | 0.17 ± 0.15         | 87.60  |
|             | 2017 | 0.00          | 0.58          | 0.36 ± 0.72         | 200.0  |
| C26 2-Alcohol| 2016 | 1.30          | 8.68          | 3.30 ± 1.30         | 39.31  |
|             | 2017 | 0.21          | 4.08          | 1.50 ± 0.65         | 43.49  |
| C31 2-Alcohol| 2016 | 0.01          | 0.11          | 0.04 ± 0.02         | 52.51  |
|             | 2017 | 0.01          | 0.08          | 0.02 ± 0.01         | 56.29  |
scaff_15798_1-p733219 were simultaneously associated with total wax, alkanes, alkane-forming pathway, and 1-alcohol-forming pathway. The marker Bn-A02-p25285941 was closely related to C_{26} 1-alcohol, total 1-alcohols and 1-alcohol-forming pathway. Bn-A08-p16793918 was closely related to C_{38} ester, C_{40} ester, C_{42} ester, and total esters. Bn-A05-p19622826 was closely related to C_{28} aldehyde, C_{29} 2-alcohol, and total 2-alcohols. According to A- and C- subgenome’s LD decay, genes within ~250 kb upstream and downstream to the associated SNPs on A-subgenome and ~800 kb on C-subgenome were selected for identification of candidate genes. No SNP was significantly associated with C_{30} aldehyde.

### Genome-wide expression profiles in *B. napus* epidermis based on RNA-seq data

Next, RNA from epidermis was pooled among high-wax load (HW) lines and low-wax load (LW) lines separately and performed sequencing. The wax load of leaves was 53–70% lower in LW lines when compared to HW lines (Fig. 3b). A reduction in the alkane, 2-alcohol and ketone content was prominent in leaves of LW lines (Fig. 3a and c). By mapping all unique sequences to the *B. napus* genome, a total of 216.49 million mapped reads (258.15 clean reads) were obtained (Additional file 7: Table S4). A high correlation (R^2 > 0.98) was observed among the three biological replicates, suggesting that the sequencing results were reliable (Additional file 8: Fig. S4). Using software DESeq [30] and FDR < 0.001 and absolute fold change ≥4 as the criteria, a total of 6966 differentially expressed genes (DEGs) between HW and LW lines were detected (Additional file 9: Fig. S5). Among the DEGs, 4608 genes were down-regulated while 2358 genes were up-regulated in LW when compared to HW (Fig. 4a). Since the epidermal samples used in this study possibly contained all epidermal cell types, thus cell type-specific genes, like guard cell-specific genes, could also be included in the DEGs. Among 490 DEGs identified as encoding transcription factors (TFs), 152 were up-regulated in LW, while 338 were down-regulated (Additional file 10: Table S5). Additionally, 20 TFs were only expressed in LW, while 109 TFs only in HW. Among these differentially expressed TFs, 86 belonged to MYB type, 44 belonged to zinc finger type, 40 belonged to AP2/ERF, and the others (Additional file 10: Table S5). About 70% of the MYB, WRKY, ERF and zinc finger type genes were down-regulated in LW, of which 21 MYBs, 7 WRKYs, 3 ERFs and 7 zinc finger types were not expressed in LW. Some orthologs of well-characterized Arabidopsis genes related to wax regulation were identified, such as *MYB16* and *MYB30* (Additional file 10: Table S5).

### Table 1 Phenotypic variations of leaf cuticular wax in the association panel of *Brassica napus* (Continued)

| Traits          | Year | Min (μg·cm^{-2}) | Max (μg·cm^{-2}) | Mean ± SD (μg·cm^{-2}) | CV (%) |
|-----------------|------|------------------|------------------|------------------------|--------|
| C_{26} 1-Alcohol| 2016 | 0.19             | 1.74             | 0.85 ± 0.33            | 38.85  |
|                 | 2017 | 0.03             | 0.91             | 0.34 ± 0.20            | 58.09  |
| C_{28} 2-Alcohol| 2016 | 0.03             | 0.93             | 0.30 ± 0.13            | 44.32  |
|                 | 2017 | 0.00             | 0.61             | 0.13 ± 0.10            | 79.04  |
| C_{38} Ester    | 2016 | 0.00             | 0.09             | 0.02 ± 0.02            | 99.82  |
|                 | 2017 | 0.00             | 0.08             | 0.01 ± 0.01            | 99.42  |
| C_{40} Ester    | 2016 | 0.01             | 0.38             | 0.15 ± 0.09            | 59.26  |
|                 | 2017 | 0.01             | 0.18             | 0.06 ± 0.03            | 42.75  |
| C_{42} Ester    | 2016 | 0.13             | 1.34             | 0.59 ± 0.31            | 53.05  |
|                 | 2017 | 0.03             | 0.39             | 0.18 ± 0.06            | 35.83  |
| C_{44} Ester    | 2016 | 0.01             | 0.50             | 0.16 ± 0.11            | 67.07  |
|                 | 2017 | 0.01             | 0.18             | 0.06 ± 0.02            | 38.36  |
| Total C_{29}    | 2016 | 6.83             | 47.31            | 23.51 ± 6.83           | 29.04  |
|                 | 2017 | 3.10             | 35.06            | 18.43 ± 4.63           | 28.17  |
| Alkane Pathway  | 2016 | 7.00             | 48.63            | 24.19 ± 7.00           | 28.95  |
|                 | 2017 | 3.61             | 41.76            | 17.19 ± 5.12           | 29.80  |
| 1-Alcohol Pathway| 2016 | 0.51             | 4.56             | 2.08 ± 0.70            | 33.73  |
|                 | 2017 | 0.24             | 1.97             | 0.78 ± 0.32            | 40.73  |

Note: Total C_{29}, the sum of C_{29} Alkane, C_{29} Ketone and C_{29} 2-Alcohol; Alkane Pathway, the sum of products from alkane-forming pathway; 1-Alcohol Pathway, the sum of products from alcohol-forming pathway.
Table 2 ANOVA analysis of wax traits in the association panel

| Trait        | Source | SS        | df  | Mean Square | P-value | H²  |
|--------------|--------|-----------|-----|-------------|---------|-----|
| Total wax    | G      | 37,981.09 | 191 | 197.82      | 2.41E-57 | 0.70 |
|              | Y      | 22,267.34 | 1   | 22,267.34   | 2.80E-93 |     |
|              | G x Y  | 29,597.49 | 191 | 154.15      | 1.88E-40 |     |
|              | Error  | 30,801.61 | 772 | 39.90       |         |     |
| Acids        | G      | 24.44     | 185 | 0.13        | 9.60E-13 | 0.63 |
|              | Y      | 13.94     | 1   | 13.94       | 9.40E-45 |     |
|              | G x Y  | 25.00     | 185 | 0.14        | 1.93E-13 |     |
|              | Error  | 45.94     | 744 | 0.06        |         |     |
| Aldehydes    | G      | 83.74     | 191 | 0.44        | 5.53E-99 | 0.69 |
|              | Y      | 3.27      | 1   | 3.27        | 2.58E-14 |     |
|              | G x Y  | 72.66     | 191 | 0.38        | 1.51E-85 |     |
|              | Error  | 41.92     | 772 | 0.05        |         |     |
| Alkanes      | G      | 13,825.27 | 191 | 72.01       | 9.11E-118| 0.68 |
|              | Y      | 1215.46   | 1   | 1215.46     | 5.41E-34 |     |
|              | G x Y  | 12,841.18 | 191 | 66.88       | 4.86E-110|     |
|              | Error  | 5758.86   | 772 | 7.46        |         |     |
| 2-Alcohols   | G      | 806.35    | 191 | 4.20        | 4.14E-47 | 0.65 |
|              | Y      | 762.84    | 1   | 762.84      | 4.74E-119|     |
|              | G x Y  | 789.47    | 191 | 4.11        | 1.04E-45 |     |
|              | Error  | 756.20    | 772 | 0.98        |         |     |
| C29 Ketone   | G      | 2208.71   | 191 | 11.97       | 2.72E-79 | 0.72 |
|              | Y      | 1551.63   | 1   | 1551.63     | 8.69E-126|     |
|              | G x Y  | 1718.17   | 191 | 8.95        | 7.71E-56 |     |
|              | Error  | 1422.16   | 772 | 1.84        |         |     |
| 1-Alcohols   | G      | 120.84    | 191 | 0.63        | 8.39E-103| 0.77 |
|              | Y      | 109.31    | 1   | 109.31      | 2.14E-179|     |
|              | G x Y  | 68.46     | 191 | 0.36        | 6.62E-54 |     |
|              | Error  | 58.20     | 772 | 0.08        |         |     |
| Esters       | G      | 89.35     | 191 | 0.47        | 2.15E-12 | 0.66 |
|              | Y      | 112.97    | 1   | 112.97      | 1.12E-86 |     |
|              | G x Y  | 76.70     | 191 | 0.40        | 2.90E-08 |     |
|              | Error  | 172.07    | 772 | 0.22        |         |     |
| C26 Acid     | G      | 49.20     | 191 | 0.26        | 2.14E-27 | 0.66 |
|              | Y      | 7.43      | 1   | 7.43        | 5.83E-20 |     |
|              | G x Y  | 45.93     | 191 | 0.24        | 3.83E-24 |     |
|              | Error  | 64.88     | 772 | 0.08        |         |     |
| C28 Acid     | G      | 0.09      | 191 | 0.00        | 4.12E-03 | 0.60 |
|              | Y      | 0.00      | 1   | 0.00        | 1.28E-03 |     |
|              | G x Y  | 0.10      | 191 | 0.00        | 1.83E-04 |     |
|              | Error  | 0.28      | 772 | 0.00        |         |     |
| C29 Aldehyde | G      | 0.09      | 191 | 0.00        | 1.19E-35 | 0.66 |
|              | Y      | 0.01      | 1   | 0.01        | 2.83E-19 |     |
|              | G x Y  | 0.08      | 191 | 0.00        | 1.67E-31 |     |
|              | Error  | 0.10      | 772 | 0.00        |         |     |
| Trait        | Source | SS    | df  | Mean Square | P-value | H²  |
|--------------|--------|-------|-----|-------------|---------|-----|
| C₂₈ Aldehyde | G      | 24.02 | 191 | 0.13        | 1.40E-77 | 0.66|
|              | Y      | 0.22  | 1   | 0.22        | 9.19E-04 |     |
|              | G x Y  | 23.11 | 191 | 0.12        | 2.93E-74 |     |
|              | Error  | 15.16 | 772 | 0.02        |         |     |
| C₃₀ Aldehyde | G      | 22.21 | 191 | 0.12        | 9.30E-89 | 0.72|
|              | Y      | 4.42  | 1   | 4.42        | 3.55E-53 |     |
|              | G x Y  | 15.96 | 191 | 0.08        | 1.01E-60 |     |
|              | Error  | 12.37 | 772 | 0.02        |         |     |
| C₂₅ Alkane   | G      | 0.05  | 191 | 0.00        | 9.51E-69 | 0.67|
|              | Y      | 0.01  | 1   | 0.01        | 2.04E-48 |     |
|              | G x Y  | 0.05  | 191 | 0.00        | 1.82E-63 |     |
|              | Error  | 0.04  | 772 | 0.00        |         |     |
| C₂₆ Alkane   | G      | 0.15  | 191 | 0.00        | 4.45E-75 | 0.66|
|              | Y      | 0.00  | 1   | 0.00        | 2.64E-03 |     |
|              | G x Y  | 0.14  | 191 | 0.00        | 8.02E-73 |     |
|              | Error  | 0.10  | 772 | 0.00        |         |     |
| C₂₇ Alkane   | G      | 1.91  | 191 | 0.01        | 1.63E-60 | 0.84|
|              | Y      | 0.04  | 1   | 0.04        | 5.81E-15 |     |
|              | G x Y  | 0.71  | 191 | 0.00        | 1.86E-61 |     |
|              | Error  | 0.55  | 772 | 0.00        |         |     |
| C₂₈ Alkane   | G      | 0.44  | 191 | 0.00        | 2.96E-87 | 0.70|
|              | Y      | 0.16  | 1   | 0.16        | 1.75E-83 |     |
|              | G x Y  | 0.36  | 191 | 0.00        | 5.02E-69 |     |
|              | Error  | 0.25  | 772 | 0.00        |         |     |
| C₂₉ Alkane   | G      | 10,644.50 | 191 | 55.44      | 2.43E-108 | 0.69|
|              | Y      | 2092.91 | 1   | 2092.91    | 4.11E-62 |     |
|              | G x Y  | 9158.35 | 191 | 47.70      | 1.77E-93 |     |
|              | Error  | 4853.36 | 772 | 6.29       |         |     |
| C₃₀ Alkane   | G      | 1.98  | 191 | 0.01        | 4.04E-18 | 0.67|
|              | Y      | 0.50  | 1   | 0.50        | 4.05E-26 |     |
|              | G x Y  | 1.70  | 191 | 0.01        | 7.19E-13 |     |
|              | Error  | 3.23  | 772 | 0.00        |         |     |
| C₃₁ Alkane   | G      | 922.16 | 191 | 4.80       | 6.50E-73 | 0.65|
|              | Y      | 117.85 | 1   | 117.85     | 2.73E-31 |     |
|              | G x Y  | 927.06 | 191 | 4.83       | 2.32E-73 |     |
|              | Error  | 614.41 | 772 | 0.80       |         |     |
| C₂₉ 2-Alcohol| G      | 713.85 | 191 | 3.72       | 6.55E-44 | 0.65|
|              | Y      | 724.19 | 1   | 724.19     | 7.97E-121|     |
|              | G x Y  | 708.50 | 191 | 3.69       | 1.98E-43 |     |
|              | Error  | 703.01 | 772 | 0.91       |         |     |
| C₃₁ 2-Alcohol| G      | 0.63  | 191 | 0.00       | 4.93E-11 | 0.65|
|              | Y      | 0.10  | 1   | 0.10       | 6.53E-15 |     |
|              | G x Y  | 0.62  | 191 | 0.00       | 1.87E-10 |     |
|              | Error  | 1.28  | 772 | 0.00       |         |     |
It is reported that the overexpression of MYB30 in transgenic Arabidopsis plants promoted the production of cuticular wax [31], while MYB16 functioned as a major regulator of cuticle formation in vegetative organs [32]. Our results indicated that MYB family could play a role in the wax regulation of B. napus.

Table 2 ANOVA analysis of wax traits in the association panel (Continued)

| Trait         | Source | SS   | df  | Mean Square | P-value  | H²  |
|---------------|--------|------|-----|-------------|----------|-----|
| C26 1-Alcohol | G      | 65.90| 191 | 0.34        | 1.52E-92 | 0.77|
|               | Y      | 63.95| 1   | 63.95       | 1.43E-175|     |
|               | G x Y  | 37.33| 191 | 0.19        | 1.27E-46 |     |
|               | Error  | 35.27| 772 | 0.05        |          |     |
| C28 2-Alcohol | G      | 10.48| 191 | 0.05        | 1.23E-97 | 0.75|
|               | Y      | 6.04 | 1   | 6.04        | 2.29E-129|     |
|               | G x Y  | 6.50 | 191 | 0.03        | 1.29E-56 |     |
|               | Error  | 5.32 | 772 | 0.01        |          |     |
| C38 Ester     | G      | 0.68 | 191 | 0.00        | 1.21E-03 | 0.61|
|               | Y      | 0.05 | 1   | 0.05        | 1.14E-05 |     |
|               | G x Y  | 0.70 | 191 | 0.00        | 3.81E-04 |     |
|               | Error  | 1.95 | 772 | 0.00        |          |     |
| C40 Ester     | G      | 3.08 | 191 | 0.02        | 1.78E-10 | 0.65|
|               | Y      | 2.05 | 1   | 2.05        | 4.47E-49 |     |
|               | G x Y  | 2.74 | 191 | 0.01        | 1.32E-07 |     |
|               | Error  | 6.33 | 772 | 0.01        |          |     |
| C42 Ester     | G      | 34.74| 191 | 0.18        | 2.05E-11 | 0.66|
|               | Y      | 49.55| 1   | 49.55       | 1.10E-92 |     |
|               | G x Y  | 30.50| 191 | 0.16        | 5.09E-08 |     |
|               | Error  | 69.13| 772 | 0.09        |          |     |
| C44 Ester     | G      | 11.38| 191 | 0.06        | 1.29E-02 | 0.62|
|               | Y      | 3.74 | 1   | 3.74        | 2.01E-18 |     |
|               | G x Y  | 10.81| 191 | 0.06        | 3.97E-02 |     |
|               | Error  | 35.79| 772 | 0.05        |          |     |
| Total C29     | G      | 26,185.62| 191 | 136.38  | 1.60E-104| 0.69|
|               | Y      | 12,555.16| 1  | 12,555.16  | 2.20E-119|     |
|               | G x Y  | 22,094.52| 191 | 115.08  | 4.14E-88 |     |
|               | Error  | 12,396.99| 772 | 16.06    |          |     |
| Alkane Pathway| G      | 31,529.40| 191 | 164.22  | 3.32E-57 | 0.70|
|               | Y      | 14,543.28| 1  | 14,543.28  | 2.02E-77 |     |
|               | G x Y  | 24,847.46| 191 | 129.41  | 5.01E-41 |     |
|               | Error  | 25,617.74| 772 | 33.18    |          |     |
| 1-Alcohol Pathway| G    | 460.79| 191 | 2.40   | 1.14E-13 | 0.71|
|                  | Y    | 706.92| 1   | 706.92   | 2.30E-103|     |
|                  | G x Y| 299.06| 191 | 1.56   | 8.35E-04 |     |
|                  | Error| 852.10| 772 | 1.10    |          |     |

Note: G and Y indicate genotype and year, respectively, and G x Y indicate interaction of G and Y. Total C29, the sum of C29 Alkane, C29 Ketone and C29 2-Alcohol; Alkane Pathway, the sum of products from alkane-forming pathway; 1-Alcohol Pathway, the sum of products from alcohol-forming pathway.

Functional classification of DEGs in the B. napus epidermis
To monitor the difference of gene expression pattern between LW and HW lines, Gene Ontology (GO) enrichment analysis was conducted (Additional file 11: Fig. S6). Significantly overrepresented top GO terms of
DEGs between HW and LW were enriched in response to stress, cell wall, and transcription factor activity, etc. (Additional file 12: Fig. S7). Among KEGG significantly enriched pathways, 12 DEGs were annotated in fatty acid elongation (ko00062) (Fig. 4b) and 14 DEGs were annotated in wax, and cutin and suberin biosynthesis (ko00073) (Fig. 4c and d). In the most cases, more than one DEG was assigned to the same enzyme in KEGG pathway.

Identification of candidate genes
For decreasing false positive error, the expression profile of candidate gene regions on A- and C- subgenome were determined by transcriptome of leaf epidermis from HW and LW lines. Totally, 792 GWAS-identified genes, which associated 147 GWAS-identified SNPs, were revealed to have differential expression between HW and LW lines, including 344 up-regulated genes and 448 down-regulated genes in LW when compared to those in HW (Fig. 4a; Additional file 6: Table S3). KEGG pathway analysis showed that some differentially expressed GWAS-identified genes enriched in fatty acid elongation, wax biosynthesis, and cutin and suberin biosynthesis pathway (Fig. 4b, c and d). Proposed wax-related genes were listed in Table 3, including some reported A. thaliana orthologous genes. For example, BnaA10g00700D, BnaC09g16050D and BnaC09g51620D were annotated as KCS1, CER1 and MAHI, which were mainly involved
in VLCFAs biosynthesis, alkane biosynthesis, and secondary alcohol and ketone biosynthesis, respectively (Fig. 4b and c; Table 3) [13, 33, 34].

To further validate the efficiency of RNA-seq analysis, expression profiles of 10 genes that were commonly identified by GWAS and RNAseq were detected by qRT-PCR. The results showed that the expression changes in these 10 genes from LW and HW were similar to those based on RNAseq analysis (Fig. 5), suggesting the reliability of the RNA-seq data.

**Fig. 2** Manhattan plots of GWAS results showing significant SNPs associated with total wax and 7 wax components in rapeseed diversity panel. X-axis shows the distribution of SNPs across 19 chromosomes while Y-axis shows Bonferroni corrections threshold. The black dashed horizontal line depicts the uniform significance threshold \(-\log_{10}(P) = 4.5\)
Discussion

*B. napus* is an allotetraploid crop with complex genome structure, which imposes a huge challenge to genome-wide SNP discovery. In the present study, a total of 192 lines were genotyped with the Brassica 60 K SNP array, and the variations in cuticular wax were investigated for two consecutive years. A total of 202 significantly associated SNPs for 31 wax traits were identified in GWAS (Additional file 6: Table S3). These SNP corresponding genes within the LD decay range were selected for identification of the candidates. Considering significant limitations in existing methods for the genome-wide identification of genes, such as false positive and negative results remaining in GWAS [35], we further analyzed the transcriptome of epidermal cells in *B. napus* leaves from HW and LW lines. By integrating GWAS with transcriptomic data, 73% GWAS-identified SNPs, including 792 genes, were revealed to have differential expression between HW and LW lines (Fig. 4a). Although it was impossible to clearly say that these genes were associated with wax traits, they should nevertheless be considered potential wax-related genes.

Based on GO analysis, organic substance metabolic process, cellular metabolic process, primary metabolic process, single-organism cellular process and response to stimulus enriched most of the related genes. A number of genes enriching in cellular process and metabolic process suggested that a complex polygenic network was involved in wax production or deposition in *B. napus*. Cuticular wax is synthesized in the plastids of epidermal
cells with the de novo \( C_{16} \) and \( C_{18} \) fatty acyl-acyl carrier proteins (ACPs) synthesis. \textit{BnaC09g10800D}, the candidate gene in the LD range of the SNP Bn-scaff_17487_1-p812141, encoded ortholog of Arabidopsis \textit{KAS III} [36] and was potentially involved in the de novo fatty acid synthesis. Before being exported to the ER, \( C_{16} \) and \( C_{18} \) fatty acids were released from ACPs by fatty acyl–ACP thioesterases (FaTA and FaTB) and subsequently esterified to Coenzyme A (CoA) by long-chain acyl-CoA synthetases (LACS) [37]. The acyl-CoA forms of these fatty acids are then elongated to wax precursors of VLCFAs by fatty acid elongase (FAE) complex in the ER. In this study, \textit{BnaA05g23790D}, \textit{BnaC06g43550D} and \textit{BnaA07g08340D}, locating within the LD range of marker Bn-A05-p19622826, Bn-scaff_16485_1-p747170 and Bn-A07-p6765464, separately, encoded FaTA which was potentially involved in the release of \( C_{18} \) fatty acid from ACP [38]. \textit{BnaA10g00700D}, \textit{BnaA10g02480D} and \textit{BnaA10g00380D}, the SNP Bn-A10-p4786596 corresponding genes, were annotated as genes encoding KCS1, KCS2 and Fig. 4 Differentially expressed genes between high-wax load (HW) lines and low-wax load (LW) lines. a The number of differentially expressed genes between HW lines and LW lines. b, c and d Annotation map of KEGG pathway of differentially expressed genes involved in fatty acid elongation, wax biosynthesis, and cutin and suberin biosynthesis, respectively. Green represents down-regulation, red represents up-regulation, and blue means up- and down-regulation co-existed in LW lines compared to HW lines. The number of differentially expressed genes encoding a specific enzyme was noted in italics next to the colored box.
| SNP            | Candidate genes in LD interval (number of DEGs) | Wax-related DEGs* | Log2FCa | Arabidopsis orthologs** | Gene description |
|----------------|-----------------------------------------------|------------------|----------|------------------------|-----------------|
| Bn-A01-p6380934 | BnaA01g11230D-BnaA01g12160D (6)               |                  |          |                        |                 |
| Bn-A01-p12504370 | BnaA01g03740D-BnaA01g36700D (92)             |                  |          |                        |                 |
| Bn-A05-p1299570 | BnaA05g02080D-BnaA05g36070D (57)              |                  |          |                        |                 |
| Bn-A05-p2030789 | BnaA05g03470D-BnaA05g36470D (47)             |                  |          |                        |                 |
| Bn-A08-p1924416 | BnaA08g23200D- BnaA08g23820D (6)              |                  |          |                        |                 |
| Bn-A08-p25285941| BnaA08g32000D-BnaA08g32920D (12)             |                  |          |                        |                 |
| Bn-A05-p19622826| BnaA05g23200D-BnaA05g24000D (2)               |                  |          |                        |                 |
| Bn-A01-p1566936 | BnaA10g02020D-BnaA10g28040D (50)             |                  |          |                        |                 |
| Bn-A05-p3188319 | BnaA10g06770D-BnaA05g07650D (3)               |                  |          |                        |                 |
| Bn-A02-p5156551 | BnaA10g03090D-BnaA05g31670D (1)               |                  |          |                        |                 |
| Bn-A05-p23445454| BnaA10g03240D-BnaA05g32450D (15)             |                  |          |                        |                 |
| Bn-A07-p6765464 | BnaA10g08250D-BnaA07g08960D (7)               |                  |          |                        |                 |
| Bn-A08-p14906652| BnaA10g09180D-BnaA08g16770D (19)             |                  |          |                        |                 |
| Bn-A09-p30763709| BnaA10g04180D-BnaA09g11120D (4)               |                  |          |                        |                 |
| Bn-A09-p32945202| BnaA10g04430D-BnaA09g45200D (1)               |                  |          |                        |                 |
| Bn-A10-p4786596 | BnaA10g00010D-BnaA10g28040D (50)             |                  |          |                        |                 |
| Bn-A11-p15096523| BnaA11g01210D-BnaA10g24090D (18)             |                  |          |                        |                 |
| Bn-A03-p12996010| BnaA10g02460D- BnaA03g25500D (12)            |                  |          |                        |                 |
| Bn-A03-p18766028| BnaA10g03580D-BnaA03g36690D (2)              |                  |          |                        |                 |
| Bn-A06-p600029  | BnaA06g00610D- BnaA06g38370D (76)             |                  |          |                        |                 |
| Bn-A08-p4273091 | BnaA08g01000D-BnaA08g04440D (3)               |                  |          |                        |                 |
| Bn-A07-p1657613 | BnaA07g01820D-BnaA07g39090D (31)             |                  |          |                        |                 |

**Table 3 Proposed most likely genes for wax traits by combined GWAS and RNAseq**

**SNP Candidate genes in LD interval**

**Wax-related DEGs**

**Log2FC**

**Arabidopsis orthologs**

**Gene description**
CYP86A4 in KEGG pathway, which catalyze the biosynthesis of VLCFAs and cutin (Fig. 4b and d) [13, 39, 40]. Overexpression of BnaA10g00700D/BnKCS1–2 in B. napus can promote the production of cuticular wax [14]. BnaA01g13470D and BnaA05g31340D, two genes in LD to the SNP Bna-scaffold_18636_1-p11498 and Bn-scaffold_20270_1-p1172081, respectively, encoded orthologs of Arabidopsis LACS4 and LACS6, respectively [41, 42], and were potentially involved in the activation from fatty acid to CoA thioesters.

Following elongation, VLC-acyl-CoAs are modified via either the acyl-reduction or the decarbonylation pathway. In the acyl-reduction pathway, fatty acyl-CoA reductase (FAR) catalyzes fatty acyl-CoAs into primary alcohols [10, 11], and wax ester synthase/acyl-CoA: diacylglycerol acyltransferase 1 (WSD1) catalyzes primary alcohols and fatty acid into wax esters [43]. In the decarbonylation pathway, VLC-acyl-CoAs are catalyzed into alkanes by an ER-localized CER1, CER3 and cytochrome B5 complex [33]. It was recently reported that the CER1 homolog, CER1-LIKE1, was also involved in alkane formation [44]. Subsequently, alkanes are oxidized into secondary alcohols and ketones by the mid-chain alkane hydroxylase 1 (MAH1) [34]. In this study, some DEGs which are homologous to the reported Arabidopsis genes involved in surface lipid biosynthesis were identified within LD blocks. For example, BnaA02g05700D located in LD to the marker SNP Bna-A02-p5516551 and encoded ortholog of Arabidopsis ALCFAR1 (Fig. 4c) [45]. BnaA02g15790D, located in LD to the SNP Bna-A02-p7004091, were annotated as WSD1-like. BnaC09g16505D, the SNP Bn-scaffold_20836_1-p125625 corresponding gene, was annotated as Arabidopsis orthologous CER1 in KEGG pathway (Fig. 4c). Overexpression of BnaC09g16505D/BnCER1–2 in B. napus can promote the biosynthesis of alkane [14], BnaA05g06830D, located in LD to the SNP Bn-A05-p4055839, were annotated as CER1-like2 in KEGG pathway. BnaC09g16520D, located in LD to the SNP Bn-scaffold_17526_1-p1726345, encoded ortholog of Arabidopsis MAH1 (Fig. 4c) [34]. BnaC09g10340D, located in LD to the SNP Bn-scaffold_17487_1-p812141, encoded ortholog of Arabidopsis ALE2, which was involved in cuticle development [46]. Wax components synthesized in the ER need to be exported to the extracellular matrix. In the current study, some genes were identified to function in lipid transportation and maintaining ER morphology. For

### Table 3

| SNP                  | Candidate genes in LD interval (number of DEGs) | Wax-related DEGs* | Log2FCa Arabidopsis orthologies** | Gene description                                      |
|----------------------|-----------------------------------------------|-------------------|----------------------------------|------------------------------------------------------|
| Bn-A04-p14642152    | BnaA04g18420D- BnaA04g20360D (3)              | BnaA04g19180D     | −2.76                            | ATG33150** peroxisomal 3-ketoacyl-CoA thiolase (KAT2/FED1) |
| Bn-scaffold_17386_1-p884077 | BnaC01g15540D- BnaC01g18260D (7)             | ATG33380**        | −2.61                            | ATG34770** Fatty Acid 2-hydroxylase (FADH1)            |
| Bn-scaffold_15918_1-p77704 | BnaC02g38670D- BnaC02g39660D (22)           | BnaC01g17900D     | −2.23                            | ATG425970** phosphatidylethanolamine decarboxylase 3 (PSD3) |
| Bn-scaffold_22728_1-p703468 | BnaC03g11180D- BnaC03g78270D (16)         | BnaC03g12050D     | 3.37                             | ATG59320** Lipid transfer protein type 1 (LTP3)        |
| Bn-scaffold_16888_1-p1834154 | BnaC04g00010D- BnaC04g56960D (14)      | BnaC03g79100D     | −5.35                            | ATG411850** Phospholipase D &gamma                  |
| Bn-scaffold_16485_1-p747170 | BnaC06g02420D- BnaC06g43990D (4)            | BnaC03g73050D     | −2.56                            | ATG411840** Phospholipase D &gamma                  |
| Bn-scaffold_17516_1-p1726345 | BnaC09g00020D- BnaC09g02480D (39)         | BnaC04g2420D      | 2.15                             | ATG33380** Phospholipase D &gamma                  |
| Bn-scaffold_17487_1-p812141 | BnaC09g09990D- BnaC09g11020D (19)          | BnaC04g29660D     | 2.57                             | ATG56290** Acyl carrier protein (ACP)                 |
| Bn-scaffold_20836_1-p125625 | BnaC09g15380D- BnaC09g16870D (1)          | BnaC04g24210D     | 4.59                             | ATG51380** salt tolerance homologue                  |

* Thresholds for significantly differential expression between high-wax load (HW) and low-wax load (LW) lines were set to false discovery rate (FDR) < 0.001 and fold change ≥ 4. Positive and negative Log2FC values indicate up and down regulation of gene expression in LW when compared to HW. N indicates not expressed in LW and I indicates not expressed in HW
** indicates the genes annotated in Fatty acid elongation pathway, wax, and cutin and suberin biosynthesis pathway in Fig. 4b, c and d

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## References

1. Jin, et al. BMC Plant Biology (2020) 20:458.
example, BnaA07g08720D, located in LD to the SNP Bn-A07-p6765464, is orthologous to Arabidopsis LTPG1 which is participated in the output of cuticular wax [47]; BnaC04g45800D, locating in LD to the marker Bn-scaff_16888_1-p1834154, is orthologous to B. rapa LTP1, which is involved in wax production or deposition as a lipid transfer protein member [48]. BnaA08g17990D, locating in LD to Bn-A08-p16793918, is orthologous to Arabidopsis ERD2 which mediates the transport of soluble ER-localized proteins containing a C-terminal K/HDEL signal [49].

Although the cuticle is usually considered independently from the epidermal cell wall underlining polysaccharide, the cuticle and the cell wall are structurally related (the cuticular layer protrude deeply into cell wall) and have some overlapping functions with each other [50]. Several DEGs related to cell wall were also observed in this study. For example, BnaA05g32440D and BnaA05g32300D, locating in LD to the SNP Bn-A05-p2344545, encoded orthologs of Arabidopsis xylan biosynthesis gene AXY9 and cellulose synthase-like D3 (CSLD3), respectively, which were involved in cell wall formation [51, 52].

Some stress-responsive related genes have also been identified, such as BnaC04g11110D, which potentially encoding orthologs of Arabidopsis bZIP63, WRKY74 and bHLH105/ILR3 which were involved in modulating responses to starvation, salt tolerance and multiple stress responses [55–57], were also obtained in this study. Plant cuticular wax has been associated with improved plant stress tolerance. In this study, some stress-responsive related genes have been identified by a combined GWAS-RNAseq approach, suggesting that cuticular wax biosynthesis directly or indirectly related with the response to environmental stimulus. However, it remains unknown whether these genes have overlapping effects on wax biosynthesis besides regulating plant responses to stresses. Further studies are required to explore the precise roles of these identified genes.

In this study, some orthologs of well-characterized Arabidopsis wax-related genes could be identified by epidermis transcriptome, such as BnaC02g37590D (orthologous to AtMYB16) and BnaC02g05990D (orthologous to AtMYB30) (Additional file 10: Table S5) [31, 32], whereas they couldn't be identified by GWAS, implying that the role of these genes in wax production might act through variable expressions. Our results showed that combined GWAS and the epidermis transcriptome sequencing analysis could increase the efficiency to detect genes associated with cuticular wax biosynthesis and to prioritize likely candidates, though some wax-related genes that did not vary in gene transcription but had an impact on enzyme function/activity might be overlooked.

Conclusions
This study first used the GWAS tool and the epidermis transcriptome to identify candidate genes associated
with *B. napus* wax traits. A total of 202 SNPs were found to be significantly associated with 31 wax traits. Furthermore, 792 GWAS-identified genes and their associated 147 SNPs were revealed to have differential expressions between HW and LW lines, including 344 up-regulated genes and 448 down-regulated genes in LW when compared to those in HW. These identified SNPs could provide clues for further exploration and validation for marker-assisted breeding, and the proposed wax-related genes could provide new insights into the genetic control of wax metabolism and improving stress tolerance of *B. napus*.

**Methods**

**Plant material and experimental design**

A total of 192 *B. napus* accessions classified as spring, semi-winter and winter types, were used for an association analysis in this study (Additional file 3: Table S2). All the accessions were provided by the Chongqing Rapeseed Engineering & Technology Research Center, Southwest University, Chongqing, China. Most of these accessions were derived from research institutions in China, with the remaining introduced from Germany, Denmark, and Canada. The experiments were conducted at Xiema Experimental Station (29°45′ N, 106°22′38E), Beibei, Chongqing, China. The *B. napus* accessions were seeded in randomized complete blocks with three replicates in September, 2016 and 2017. Each accession was planted in two rows, 40 cm between rows, and 20 cm between plants. Routine field management was carried out. Nine weeks after planting (late December, pre-flowering stages for all accessions) uniform and fully expanded leaf samples from all plots were collected for wax trait investigation. The average month temperature in September, October, November and December in 2016 and 2017 were 22.9 and 23.3 °C, 18.9 and 17.4 °C, 12.7 and 13.3 °C, and 9.4 and 8.3 °C, respectively; whereas the month rainfall were 95.7 and 71.9 mm, 96.9 and 178.4 mm, 89.7 and 25.5 mm, and 17.1 and 6.9 mm, respectively.

**Cuticular wax analysis**

The cuticular wax composition of leaves from all accessions was determined as described by Wang et al. [8] with some modifications. Three leaves (third leaf from the top) were sampled from three plants in each replicate for each accession. The sampling was finished within three days. Leaf cuticular waxes were extracted in chloroform containing 10 μg tetracosane (Sigma Aldrich, Missouri, USA) as an internal standard. Then the extracts were derivatized with 50 μL N.O–bis (trimethylsilyl) trifluoroacetamide (BSTFA) and 50 μL pyridine. The derivatized extracts were dried and re-dissolved in chloroform for GC-FID analysis. The wax compound was identified and quantified by mass spectrums, internal standard and leaf area. Leaf areas were determined using ImageJ software (http://rsb.info.nih.gov/ij/).

**Statistical analysis**

Wax traits was investigated for three replicates in 2016 and 2017 (Additional file 13: Table S6). Each wax trait of each accession was defined as the average of the three replicates in the same year. For each wax trait, best linear unbiased predictors (BLUP) were estimated for each line across the two years based on a linear model using the lme4 package [58]. The BLUP values were used as phenotypes for the association analysis. The broad-sense heritability was calculated as $H^2 = \delta_e^2/ (\delta_e^2 + \delta_g^2/n + \delta_{e}^{2}/nr)$, where $\delta_e^2$ is the genetic variance, $\delta_{ge}^2$ is the interaction variance of the genotype with year, $\delta_e^2$ is the error variance, n is the number of years and r is the number of replicates within a year.

**SNP genotyping, filtering and in silico mapping of SNPs**

SNP genotyping was performed using the Brassica 60 K Illumina SNP array, and SNP data were analyzed using Illumina GenomeStudio genotyping software. SNPs with call frequencies < 0.8 or MAF < 0.05 were excluded in the study. After processing, 31,846 SNP sequences were aligned with the genome sequences of *B. napus*, with an E-value cut-off of <1E-10. The blast-hits with a minimum E value and a maximum score were selected for further analysis.

**Population structure, relative kinship and disequilibrium (LD) analysis**

A subset of 4623 SNPs distributed evenly across the entire genome (missing data < 0.2, MAF > 0.2, and unique position on chromosome) was selected for population structure and relative kinship analysis (K). The model-based program STRUCTURE 2.3.4 software was used to estimate the population structure (Q) with a Bayesian Markov Chain Monte Carlo model (MCMC) [59]. Five independent runs were performed with a K-value (the putative number of genetic groups) from 1 to 10. Both the length of burning period and the number of MCMC replications after burning were set to 100,000 iterations under the “admixture model”. The optimal K value was determined by the log probability of the data [LnP(D)] in the STRUCTURE output and a statistic $\Delta K$ based on the rate of change of LnP(D) between successive k values as described by Evanno et al. [29]. The results of replicate run from STRUCTURE were integrated to acquire a Q matrix with the CLUMPP software [60] and graphically displayed using DISTRACT software [61]. The relative kinship matrix of the natural population was calculated using TASSEL 5.2.1 [62]. All negative values between two accessions were set to 0. The linkage disequilibrium (LD) between pair-wise SNPs (with MAF ≥
 Genome-wide association studies

Trait–SNP association analysis was separately performed in six models including Q (controlling for population structure), PCA (controlling for principal component), K (controlling for kinship), PCA + K (controlling for both principal component and kinship), Q + K (controlling for both population structure and kinship), and naïve (without controlling for population structure and kinship) model. The naïve, Q and PCA models were performed using a general linear model (GLM); the K, Q + K and P + K models were performed using a mixed linear model (MLM). Both GLM and MLM were implemented in TASSEL 5.2.1 [62]. For each trait, the optimal model was selected based on the distribution of the \( -\log_{10}(P) \) values of each SNP against the expected value in a Quantile-quantile (Q-Q) plot [63]. A uniform threshold for the significant SNPs-trait association was set to \( -\log_{10}(P) = 4.50 \) and a Manhattan plot was generated in the R package qqman [64]. Genes within \( \sim 250 \) kb upstream and downstream to the associated SNPs on A-subgenome and \( \sim 800 \) kb on C-subgenome were selected for identification of candidates.

Transcriptome sequencing and identification of differentially expressed genes

Three high-wax load lines (HW) and three low-wax load lines (LW) were selected from the GWAS population for transcriptome sequencing, including Zhongshuang11, Shilijia, and Yangyou6 (HW) and SWU68, Tonglinghuaye, and Shengguang77 (LW). Leaf epidermis was collected from HW and LW lines, separately, with three biological replicates. In each replicate, two independent plants were sampled from each line and pooled together. Epidermal peels were manually dissected from leaves as a thin transparent film. Total RNA were extracted from peel samples for sequencing and quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Sequencing library preparation and sequencing reactions were conducted at the Biomarker Technologies Corporation (Beijing, China). Sequencing libraries were constructed using NEBNext UltraTM RNA Library Prep Kit (NEB, USA). Subsequently, these libraries were sequenced on an Illumina platform and paired-end reads were generated.

Raw reads were transformed into clean reads after removing reads containing adapter, reads containing poly-N and low-quality reads. These clean reads were then mapped to the \( B. \ napus \) reference genome sequence using Hisat2 tools soft. Quantification of gene expression levels were estimated by fragments per kilobase of transcript per million fragments mapped. Genes with significantly differential expression between HW and LW were identified based on the following criteria: false discovery rate (FDR) < 0.001 and absolute fold change ≥4. Transcription factor prediction was performed using BMKCloud (www.biocloud.net).

GO and KEGG enrichment analysis of differentially expressed genes (DEGs)

To assess the biological significance of DEGs, GO enrichment analysis was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution [65], which can adjust for gene length bias in DEGs. Statistically significant GO terms were obtained based on Kolmogorov-Smirnov like test. KEGG pathway enrichment analysis was performed by using the KOBAS software [66].

Validation of DEGs by qRT-PCR

According to the results of combining GWAS with RNA-seq analysis, ten candidate genes were selected for qRT-PCR analysis. The primers for qRT-PCR are listed in Additional file 14: Table S7, and the \( B. \ napus \) actin 7 gene (\( BnACT7 \)) was used as the internal control. The total RNA was the same as used for RNA sequencing. The qRT-PCR assay was performed on a Bio-Rad CFX96 Real-Time PCR Detection System using the SYBR Premix Ex TaqII (Takara, Beijing, China). The relative gene expression levels were calculated using the \( 2^{-\Delta\Delta Ct} \) method. Three independent biological replicates, each with two technical replicates were analyzed for HW and LW, respectively.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-020-02675-y.

Additional file 1: Figure S1. Histogram of wax traits investigated from 2016 to 2017.
Additional file 2: Table S1. Correlations among measured wax traits.
Additional file 3: Table S2. The information of 192 \( B. \ napus \) accessions and population structure used in this study.
Additional file 4: Figure S2. Quantile-quantile (QQ) plots from association analysis using six methods for 31 wax traits.
Additional file 5: Figure S3. Manhattan plots of GWAS results showing significant SNPs associated with 24 wax compounds in \( B. \ napus \) diversity panel.
Additional file 6: Table S3. Summary of SNPs significantly associated with wax traits.
Additional file 7: Table S4. Summary of RNA-Seq reads.
Additional file 8: Figure S4. The correlation between three biological replicates among high wax-load lines and low wax-load lines.
Additional file 9: Figure S5. Gene cluster that are differentially expressed in the \( B. \ napus \) epidermis with high wax coverage (HW) and low wax coverage (LW).
The raw RNA-sequencing data from availability of data and materials analysis, and the manuscript preparation. Foundation of China (31771694) and the Chongqing basic and advanced funding manuscript. commented on the manuscript. All authors read and approved the final manuscript. SZ, SJ, and YW worked on the phenotyping, and performed the genotyping YN and JL have made substantial contributions to conception and design. Received: 8 April 2020 Accepted: 24 September 2020 The authors declare no competing of interests. Competing interests We thank Dr. Y. J. Guo at Southwest University for GC–MS analysis. Authors’ contributions YN and JL have made substantial contributions to conception and design. SZ, SJ, and YW worked on the phenotyping, and performed the genotyping and bioinformatics analysis. SJ and YL worked on transcriptome sequencing analysis. YN analyzed all the data and wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript. Funding This work was financially supported by the National Natural Science Foundation of China (31771694) and the Chongqing basic and advanced research project (cstc2018jyjA60857). Authors declare that none of the funding bodies have any role in the research design, the data collection and analysis, and the manuscript preparation. Availability of data and materials The raw RNA-sequencing data from B. napus leaf epidermis were deposited in the NCBI under SRA accession number PRJNA602672 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA602672). Ethics approval and consent to participate Not applicable. Consent for publication Not applicable. Competing interests The authors declare no competing of interests. References 1. Chalhoub B, Denoeud F, Liu S, Parkin IAP, Tang H, Wang X, Chiquet J, Belcmam H, Tong C, et al. Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. Science. 2014;345:950–3. 2. 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