RESEARCH ARTICLE

Genome-wide association study and genetic mapping of BhWAX conferring mature fruit cuticular wax in wax gourd

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Abstract

Background: Wax gourd [Benincasa hispida (Thunb) Cogn. (2n = 2x = 24)] is an economically important vegetable crop of genus Benincasa in the Cucurbitaceae family. Fruit is the main consumption organ of wax gourd. The mature fruit cuticular wax (MFCW) is an important trait in breeding programs, which is also of evolutionary significance in wax gourd. However, the genetic architecture of this valuable trait remains unrevealed.

Results: In this study, genetic analysis revealed that the inheritance of MFCW was controlled by a single gene, with MFCW dominant over non-MFCW, and the gene was primarily named as BhWAX. Genome-wide association study (GWAS) highlighted a 1.1 Mb interval on chromosome 9 associated with MFCW in wax gourd germplasm resources. Traditional fine genetic mapping delimited BhWAX to a 0.5 Mb region containing 12 genes. Based on the gene annotation, expression analysis and co-segregation analysis, Bhi09G001428 that encodes a membrane bound O-acyltransferase (MBOAT) was proposed as the candidate gene for BhWAX. Moreover, it was demonstrated that the efficiency of a cleaved amplified polymorphic sequences (CAPS) marker in the determination of MFCW in wax gourd reached 80%.

Conclusions: In closing, the study identified the candidate gene controlling MFCW and provided an efficient molecular marker for the trait in wax gourd for the first time, which will not only be beneficial for functional validation of the gene and marker-assisted breeding of wax gourd, but also lay a foundation for analysis of its evolutionary meaning among cucurbits.

Keywords: Wax gourd, Fruit, Cuticular wax, GWAS, Genetic mapping, MBOAT

Background

In nature, plant surface is covered by cuticles, which consist of non-polymerized cuticular waxes and cutins synthesized in epidermal cells [1]. Cutins are made up of ester bonds linked C16 and C18 fatty acid and their derivatives, as well as some glycerol and phenolic acids [2]. Cuticular waxes are mainly composed of very long chain fatty acids (VLCFAs) with C20-C34 chains and their derivatives, such as alcohols, esters, aldehydes, alkanes and ketones [3]. The composition of cuticular waxes differs between plant species as well as between organs in the same plant.

The synthesis of wax components is relatively not complex and has been extensively studied in model plant Arabidopsis. In plastids, C16- and C18-fatty acids that are synthesized de novo act as precursors, which are hydrolyzed by fatty acyl-acyl carrier protein thioesterase and then transported into the cytoplasm [4]. Then, they are catalyzed into C16 or C18 fatty acyl-CoAs by fatty acid synthase and exported to the endoplasmic reticulum [5]. Later, the C16 or C18 fatty acyl-CoAs are further elongated to very long chain (C24-C36) acyl-CoAs by fatty...
acid elongase (FAE) [3, 6], followed by conversion to primary alcohols and wax esters via alkane pathway and to alkanes and their derivatives via primary alcohol pathway, respectively [3, 6]. Many genes have been identified to be involved in cuticular wax synthesis. A couple of FAE genes including KCS1, KCS2, KCS9, KCS16, KCS20 and KCR1 that play roles in VLCFA elongation are essential for wax biosynthesis [7–11]. Other genes such as CER1, CER1-LIKE1, CER2-like, CER3 and CER26 that modify the structure of VLCFAs are also vital for cuticular wax synthesis [12–16].

In addition, the synthesis of cuticular waxes can also be regulated at the transcriptional, posttranscriptional and postranslational levels [4]. According to reports, WIN1/SHN1, SHN2, SHN3, CFL1, HDG1 and several MYB transcription factors directly or indirectly affect the expression of genes involved in the cuticular wax biosynthesis at the transcriptional level; CER7, WAR3/RDR1 and WAR4/SGS3 regulate cuticular wax synthesis by affecting mRNA stability at the posttranscriptional level. CER9 influences cuticular wax synthesis composition probably by changing the structure of proteins produced during wax synthesis process at the postranslational level.

The inheritance of cuticular wax on the leaf surface of many plant species has been extensively investigated. In some cases, the trait is controlled by quantitative trait loci [17, 18], and in most cases, the genetic architecture of cuticular waxes is dominantly controlled by a single gene [19–23]. Many dominant loci have been successfully mapped using a forward mapping method, and CER2 [23] and GDSL-like lipase/acylhydrolase [22] are considered as candidate genes for wax synthesis. In cucurbits, cuticular waxes exist on the fruit surface of many species and is a prominent appearance quality trait valued in the breeding program. The density of fruit waxes differs in cucumber cultivars. According to the genotype generated by polymorphic SSR markers, the cucumber cultivars without fruit waxes can be distinguished from those with fruit waxes based on the principle analysis [24]. CER1 and WAX2 in cucumber are induced by low temperature, drought, salt stress and ABA, and these two genes influence the biosynthesis of very long chain alkanes, a predominant wax component in cucumber [25, 26]. Moreover, the grafting with pumpkin onto cucumber also affects the biosynthesis of fruit wax esters in cucumber, and this might be regulated by an AP2/ERF-type transcription factor CsWIN1 [27]. Hydrocarbons are the most abundant chemicals, followed by alcohols in the wax of watermelon fruit surface [28]. The waxy phenotype of watermelon is controlled by a single dominant gene, located in a 1.7 Mb physical interval based on BSA-seq, and genes including ECR are probable candidates for the trait [28].

Wax gourd [Benincasa hispida (Thunb) Cogn. (2n = 2x = 24)], the only member of genus Benincasa in the Cucurbitaceae family, is an economically important vegetable crop mainly cultivated in China, India, Japan and many other tropical, subtropical and temperate countries. The same as most of cucurbits, fruit is the main consumption organ of wax gourd. In view of the physiological existence of mature fruit cuticular wax (MFCW), wax gourd can be divided into two subgroups, Fenpi Donggua (wax gourd with MFCW) and Qingpi Donggua (wax gourd without MFCW), which has been confirmed by evolutionary analysis based on the re-sequencing of wax gourd germplasm resources [29]. Apart from the evolutionary significance, the MFCW is also an important trait in the breeding program of wax gourd because of individual preference of consumers. However, until now, the chemical composition as well as the genetic basis for this important trait remains unknown. In this study, a genome-wide association study (GWAS) was firstly performed to localize the major MFCW locus. Thereafter, two wax gourd inbred lines, one with thick MFCW and the other without MFCW, were used to generate populations for genetic analysis and genetic mapping of the gene conferring this trait. The study will not only highlight the genetic basis of MFCW of wax gourd and promote the breeding of wax gourd cultivars with appealing appearance, but also shed light on the evolution basis of cuticular wax on the fruit surface of cucurbits.

Results

Phenotypic characterization of MFCW between P1 and P2

To further characterize the formation of cuticular waxes on the fruit surface of wax gourd, the fruit at different developmental stages of both P1 and P2 were phenotypically observed. It turned out that no MFCW was observed at any developmental stages of P2 and at 0, 5 and 10 DAP of P1. At 20 DAP, MFCW could be easily seen at the fruit pedicel, and the fruit was fully covered with thick MFCW at 40 DAP (Fig. 1).

Genetic analysis results of MFCW in wax gourd

According to genetic analysis results, MFCWs were detected in 43 individuals derived from BC1P1 and 44 out of 85 individuals derived from BC1P2, which did not deviate from 1: 1 segregation ratio (χ2 = 0.053, p = 0.8179). Among 574 F2 individuals tested, 430 exhibited MFCWs while 144 exhibited no MFCWs, fitting with the Mendelian inheritance model controlled by a single dominant gene (χ2 = 0.01, p = 0.9748) (Table 1). Taken together, it could be concluded that the inheritance of the MFCW in wax gourd was controlled by a single dominant gene, which was named as BhWAX.
GWAS results of MFCW in wax gourd
According to the existence of MFCW and re-sequencing data of 146 wax gourd germplasm resources [29], GWAS was performed to decipher the genetic control of MFCW in wax gourd. The significant thresholds for SNP-based GWAS were set at 3.12022E-08 (0.01/320,490) (solid line) and 1.56011E-07 (0.05/320490) (dash line), respectively, where 320,490 SNPs were used for GWAS herein. A clear signal with an interval around 1.1 Mb (from 45,182,409 to 46,295,796 bp) on chromosome 9 was observed to show close correlation with MFCW based on the GWAS result (Fig. 2). Inside this interval, 24 genes (from Bhi09M001405 to Bhi09M001428) were included according to Cucurbit Genomics Database (http://cucurbitgenomics.org/v2/organism/3).

Genetic mapping results and candidate genes identified for BhWAX locus
To rapidly map the BhWAX locus, BSA-seq analysis was performed. Based on the phenotype of F₂ individuals, they were randomly selected to construct F₂-1 pool (30 individuals) and F₂-0 pool (30 individuals), respectively. After the sequencing of the two pools, a total of 28.27, 30.38, 31.58 and 26.98 Gb raw data were generated for P₁, P₂, F₂-1-pool and F₂-0-pool, respectively. After raw data screening, the Q30 was all above 91% and the GC content was all around 37% (Table 2). In addition, a total of 1,451,083 SNPs were identified between two pools and used for BSA-seq analysis based on Δ(SNP-index) method (99% confidence interval). Furthermore, the results also revealed a strong signal peak on chromosome 9 covering about 27.92 Mb (31,580,001 bp—59,500,000 bp), displaying a correlation with BhWAX locus (Fig. 3).

Based on the re-sequencing results of two parental lines, 159 Indel markers from linkage group 9 were developed. Among them, 56 markers were polymorphic and thereafter used for genotyping of 145 F₂ individuals. After the removal markers with ambiguous amplification and distorted markers, the genotypes obtained

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Table 1 Segregation of MFCW trait among six-generation family

| Generation | No. individuals | No. MFCW | No. Non-MFCW | Segregation ratio(with: without) | χ² | p    |
|------------|-----------------|----------|--------------|---------------------------------|----|------|
| P131 (P₁)  | 30              | 30       | 0            | -                               |    |      |
| W3 (P₂)   | 30              | 0        | 30           | -                               |    |      |
| F₁         | 30              | 30       | 0            | -                               |    |      |
| BC₁P₁      | 43              | 43       | 0            | -                               |    |      |
| BC₁P₂      | 85              | 44       | 41           | 1.073:1                         | 0.053 | 0.8179 |
| F₂         | 574             | 430      | 144          | 2.986:1                         | 0.001 | 0.9748 |

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Fig. 1 Fruit phenotype of P131 and W3 at different days after pollination (DAP). The red arrow indicated the cuticle wax around the fruit pedicel of P131 at 20 DAP.
by 18 informative markers were used for genetic mapping of BhWAX locus. Then the BhWAX locus was mapped between markers C9-46.175 and C9-47.164, with a genetic distance of 3.7 cM and 3.0 cM, respectively, corresponding to a physical interval ~0.99 Mb (46,175,172 bp–47,164,043 bp) (Fig. 3). The LOD value was 20.11, which could explain 42.03% of the phenotypic variance.

To further fine map the BhWAX locus, the same large F2 population comprising 2,487 individuals were genotyped using two flanking markers C9-46.175 and C9-47.164 in the spring of 2021. As a result, 46 recombinants were successfully identified and these recombinants were planted in the field until harvest for MFCW trait observation. Based on the genomic difference between two parental lines, 24 markers were further developed to genotype the 46 recombinants. As revealed by the genotyping and phenotyping results of 46 recombinants, the BhWAX locus was delimited to a 0.51 Mb interval (46,175,172 bp–46,685,864 bp) between marker C9-46.175 and maker SNP15 (The representative recombinant events see Fig. 3).

**Bhi09G001428 was the candidate gene for BhWAX locus**

Based on the wax gourd reference genome [Wax gourd (B227) v1 Genome, http://cucurbitgenomics.org/v2/organism/3], totally 12 genes were identified inside the fine mapping region, including 10 with annotated function and the other 2 encoding unknown proteins (Table 3). We then analyzed the expression patterns of 6 genes (Bhi09G001426, Bhi09G001428, Bhi09G001434, Bhi09G001435, Bhi09G001436 and Bhi09G001437) in different tissues and organs that are probably related to the MFCW of wax gourd using qPCR (Fig. 4). No obvious difference existed in the flesh or the peel between P1 and P2 at any time-point tested for Bhi09G001434 and Bhi09G001437. Only considerable expression differences were found in the peel of fruit at 40 DAP between P1 and P2 for Bhi09G001435, and in the peel and the flesh of fruit between P1 and P2 for Bhi09G001436. Regarding Bhi09G001426, marked gene accumulation difference could be found between the fruit peel between P1 and P2 at 10, 20 and 40 DAP. Interestingly, the expression of Bhi09G001428 was relatively higher in all the flesh and peel of P1 than in those of P2 at 10, 20, 40 DAP (Fig. 4). Moreover, Bhi09G001428 also presented a gradually increased accumulation in the fruit peel and the flesh of P1 with the growth of the fruit. Based on the presence pattern of MFCWs in the wax gourd fruit peel, Bhi09G001426 and Bhi09G001428 were primarily considered as candidate genes for BhWAX locus.

One nonsynonymous mutation, C (P2) to T (P1) at 46,222,154 bp, was detected in gene Bhi09G001426. Two nonsynonymous mutation sites, C (P2) to T (P1) at 46,262,292 bp and G (P2) to A (P1) at 46,262,321 bp were detected in gene Bhi09G001428. Based on these

**Table 2 Sequencing statistics of BSA-seq samples**

| Sample | Raw reads | Clean reads | Q30 Percentage (%) | Mapped reads | Mapping rate (%) | Coverage 1X (%) | Coverage 4X (%) | Coverage 10X (%) |
|--------|-----------|-------------|---------------------|--------------|-----------------|----------------|----------------|-----------------|
| P131   | 187,937,372 | 171,855,518 | 91.49               | 171,713,436  | 91.37           | 95.35          | 94.22          | 89.83           |
| W3     | 202,002,394 | 183,065,950 | 91.97               | 182,339,950  | 90.27           | 95.29          | 94.20          | 89.88           |
| F2-1   | 209,992,928 | 192,113,997 | 92.33               | 192,362,254  | 91.60           | 95.78          | 94.90          | 91.14           |
| F2-0   | 158,059,030 | 143,856,847 | 91.08               | 144,321,550  | 91.31           | 95.69          | 94.00          | 86.74           |
three SNP sites, three cleaved amplified polymorphic sequences (CAPS) markers, WAXS1-CAPS, WAXS4-CAPS1 and WAXS4-CAPS2, were developed to test 82 F2 individuals, showing 70.51, 97.44 and 89.86% accuracy, respectively (Table 4). Moreover, the WAXS4-CAPS1 displaying the highest accuracy rate was further tested with 30 wax gourd germplasm resources (Table 5). All the 15 non-MFCW germplasm resources had the P2-like bands, while among 15 MFCW germplasm resources, 12 had the P1-like or heterozygous bands and the other 3 had the P2-like bands (Fig. 5, the original gel see Additional File 2). The accuracy of WAXS4-CAPS1 in detecting the MFCW in wax gourd germplasm resources was 80%. Altogether, we postulated that Bhi09G001428 is the candidate gene for BhWAX conferring the MFCW in wax gourd.

Phylogenetic analysis of BhWAX protein

Bhi09G001428 was predicted to encode a membrane bound O-acyltransferase (MBOAT), which is homologous to AT5G55350 in Arabidopsis. To further dissect the relationship between BhWAX and homologous
proteins from other plant species, a phylogenetic tree was built. The phylogenetic tree indicated that the gene product from dicotyledons formed a subclass, while those from monocotyledons formed another subclass (Fig. 6). The results also manifested that \( \text{BhWAX} \) exhibited a closer relation with \( \text{CmaCh01G011060.1} \) (\( \text{Cucurbita maxima} \)), \( \text{MELO3C013111.2.1} \) (\( \text{Cucumis melo} \)) and \( \text{CsaV3_7G019890.1} \) (\( \text{Cucumis sativus} \)) (Fig. 6), indicating that this \( MBOAT \) gene is conserved among cucurbits.

### Discussion

Plant cuticular waxes play vital roles in protecting plants against low temperature [30], drought [31], water loss [32, 33] and many other abiotic and biotic stresses [34]. Cuticular waxes on the fruit surface could prevent plants from unlimited water loss to maintain fruit quality [35, 36] and from pathogen infection [37], and they are synthesized and change during fruit development [38]. In wax gourd, cuticular waxes are synthesized

### Table 3  Candidate gene information in the mapping interval

| Gene ID       | Start (bp) | End (bp) | Description                           |
|---------------|------------|----------|---------------------------------------|
| Bhi09G001426  | 46,218,704 | 46,222,472 | membrane bound O-acyl transferase     |
| Bhi09G001427  | 46,225,954 | 46,226,163 | Unknown protein                       |
| Bhi09G001428  | 46,261,864 | 46,263,661 | membrane bound O-acyl transferase     |
| Bhi09G001429  | 46,302,760 | 46,303,394 | ABC1 domain-containing protein        |
| Bhi09G001430  | 46,355,542 | 46,355,736 | CCHC-type domain-containing protein    |
| Bhi09G001431  | 46,359,866 | 46,382,186 | Protein ENHANCED DOWNY MILDEW 2       |
| Bhi09G001432  | 46,393,952 | 46,394,107 | Unknown protein                       |
| Bhi09G001433  | 46,396,018 | 46,400,123 | Protein ENHANCED DOWNY MILDEW 2       |
| Bhi09G001434  | 46,517,195 | 46,521,892 | Upstream activation factor subunit spp27 |
| Bhi09G001435  | 46,634,565 | 46,635,510 | AAI domain-containing protein         |
| Bhi09G001436  | 46,658,408 | 46,659,280 | AAI domain-containing protein         |
| Bhi09G001437  | 46,677,870 | 46,685,864 | Sodium/hydrogen exchanger             |

**Fig. 4** Analysis of the expression pattern of 6 genes in the \( \text{BhWAX} \) fine mapping interval
from the pedicel at around 20 DAP, whose thickness is increased with the development of the wax gourd fruit. The MFCW is an important trait that affects the commercial value of wax gourd. In China, wax gourd cultivars without MFCWs are mainly distributed in Southern China, such as Guangdong Province, Guangxi Province and Hainan Province while wax gourd cultivars with MFCW are more popular in the Southwest part, such as Yunnan Province and Sichuan Province. Furthermore, the MFCW is also a domesticated trait. MFCWs are present in wild accessions and landraces, and the cultivated non-MFCW cultivars are more likely bred from those with MFCW [29]. In this study, the GWAS demonstrated a clear signal correlated with the MFCW of wax gourd on chromosome 9. Additionally, signals on chromosome 2 and 6 could also be seen, indicating that the MFCW of wax gourd could be controlled by deferent genes dependent on germplasm resources.

In the present study, the MFCW of wax gourd could be controlled by a single dominant gene BhWAX, which is in accordance with most of previous studies on other plant species [19, 23, 39]. Recently, BSA-seq has been widely used in the rapid and efficient identification of QTL or genes related to specific traits [40–42]. The combination of BSA-seq with fine mapping using 2,487 F2 individuals, the candidate gene for BhWAX was mapped to a 0.51 Mb interval. During the process of fine mapping, no recombination was found between marker SNP1 and SNP15, which could be attributed to the short distance between this region and the centromere of the chromosome. Because of the large genome size of wax gourd (913 Mb) [29], only 12 genes were found in the interval. Among these genes, Bhi09G001426 and Bhi09G001428 had relatively higher expression levels in the fruit peel of wax gourd inbred line with MFCW than in the line without MFCW at 20 and 40 DAP. This expression pattern is consistent with the timing of wax formation on the wax gourd fruit peel. Therefore, we primarily considered these two genes as candidates. Further, CAPS markers were developed based on the nonsynonymous mutation sites inside two genes, and Bhi09G001428 was finally proposed as the candidate gene because of its high efficiency in determination of MFCW trait in both wax gourd F2 population and germplasm resources. Bhi09G001428 encodes a MBOAT gene, also known as Wax Synthase (WS) [43]. In a previous study, a total of 136 MBOAT genes were identified from 15 plant species [44]. MBOAT genes contain MBOAT domains [45] and were firstly identified in Drosophila [46]. However, the function of MBOAT genes remain largely uncharacterized as there are only few relevant reports. The heterologous overexpression of a WS gene from Jojoba greatly increases the wax content in the seed oil [47]. The heterologous overexpression of a sunflower WS gene in Saccharomyces cerevisiae can

| Marker Name | SNP | Restriction Enzyme | Primer sequences (5’–3’) | Product Size (bp) | Accuracy |
|-------------|-----|-------------------|--------------------------|------------------|----------|
| WAXS1-CAPS  | C-T | Hpy99I            | F: TTTCTACGGAAGTTGTTGGG  | 404, 239, 165     | 70.51%   |
|             |     |                   | R: CGCCCTACTCTCTCTCACT   |                  |          |
| WAXS4-CAPS1 | C-T | Aval              | F: TTCAGGAAATGGCCATAGT   | 445, 308, 137     | 97.44%   |
|             |     |                   | R: CCCTTGCTCTGACGCTCTCC  |                  |          |
| WAXS4-CAPS2 | G-A | BstXI             | F: TTCAGGAAATGGCCATAGT   | 445, 271, 174     | 89.86%   |
|             |     |                   | R: CCCTTGCTCTGACGCTCTCC  |                  |          |

**Table 5** MFCW phenotype of 30 wax gourd germplasm resources

| ID | Name | MFCW phenotype | ID | Name | MFCW phenotype |
|----|------|---------------|----|------|---------------|
| 1  | B96  | N             | 16 | P281 | Y             |
| 2  | B98  | N             | 17 | P269 | Y             |
| 3  | B184 | N             | 18 | P262 | Y             |
| 4  | BS232| N             | 19 | P240 | Y             |
| 5  | B235 | N             | 20 | P109 | Y             |
| 6  | B249 | N             | 21 | P264 | Y             |
| 7  | B274 | N             | 22 | P150 | Y             |
| 8  | BNH367| N         | 23 | P257 | Y             |
| 9  | BS372| N             | 24 | P234 | Y             |
| 10 | B430 | N             | 25 | P229 | Y             |
| 11 | B442 | N             | 26 | P234 | Y             |
| 12 | B445 | N             | 27 | F3-2 | Y             |
| 13 | B450 | N             | 28 | F2-2 | Y             |
| 14 | B468 | N             | 29 | F280 | Y             |

N represents wax gourd germplasm resources without MFCW while Y represents those with MFCW.
accumulate C-32 to C-36 wax esters by feeding C16 and C18 fatty alcohols combined with 16:0, 18:0 fatty acyl CoAs [48]. In microalgae, a WS gene exhibits wax synthase activity and diacylglycerol acyltransferase activity, thus promoting triacylglycerol accumulation [43]. Altogether, the above studies indicate that MBOAT genes function in the synthesis of wax ester components, which are also main compounds of plant cuticular waxes. Therefore, it is reasonable for us to propose that Bhi09G001428 act as the candidate conferring the MFCW in wax gourd.

MFCW trait differs among wax gourd germplasm resources, some with thin powder while many other with thick powder, and we speculate that Bhi09G001428 is not the only gene contribute to MFCW trait in wax gourd. Except for the gene on chromosome 9, other loci, for instance on chromosome 6 and chromosome 2, were detected by GWAS (Fig. 2). Therefore, further work are
still needed to be done to explore other genes related to MFCW in wax gourd.

Conclusion
In this study, we found that the MFCW of wax gourd was dominantly controlled by a single gene *BhWAX*. Genetic mapping delimited *BhWAX* to a 0.51 Mb interval on chromosome 9 containing 12 genes. Based on the gene annotations and expression patterns, a MBOAT gene was proposed as the candidate for *BhWAX*. Further, an efficient CAPS marker was developed to determine the MFCW of wax gourd. This study is the first report about the identification of the gene controlling the MFCW and development of the trait-related molecular markers in wax gourd, which will not only contribute to the functional validation of the gene and marker-assisted breeding of wax gourd, but also lay a foundation for analysis of its evolutionary significance among cucurbits.

Methods

Plant materials
Two wax gourd inbred lines P131 (P1, with MFCW) and W3 (P2, without MFCW) were selected as experimental materials and crossed to produce F1 hybrid. In the spring of 2019, F1 underwent self-pollination to produce F2, and backcrossed with P1 and P2 to produce BC1P1 and BC1P2, respectively. The P1, P2, F1, BC1P1, BC1P2 and 574 F2 individuals were planted in the autumn of 2019. Besides, 2,487 F2 individuals were firstly planted in 96-well trays and the recombinants were later transplanted to the field. All plant materials were grown in the experimental field of Vegetable Research Institute, Guangdong Academy of Agricultural Sciences.

Phenotypic data collection
The MFCW of each tested individual was observed visually and scored by three people at the fruit maturity stage [around 50 days post pollination (50 DAP)]. The fruit with cuticular waxes was scored as "1" point while that without was scored as "0" points.

Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) extraction
After collection, young leaf samples of P1, P2, F1 and all F2 individuals were frozen in liquid nitrogen and kept in a -80°C freezer for further use. The DNA was extracted as described in literature [49]. At different developmental stages (0, 5, 10, 20 and 40 DAP), the fruit peel and flesh as well as root, leaf and stem were collected from P1 and P2 for RNA extraction, each with three biological replicates. Subsequently, the RNA was extracted using TransZol Up Plus RNA Kit (TransGen, Beijing, China) according to manufacturer’s instructions.

GWAS
The existence of MFCWs and re-sequencing data of 146 wax gourd germplasm resources were collected from our previous study [29] for GWAS analysis. A mixed linear model (MLM) was applied for relevant signal detection. *p* value indicating the correlation between each single nucleotide polymorphism (SNP) and the MFCW was calculated with TASSEL v5.0 [50]. Finally, the Manhattan plots were graphed using CMplot [51].

Bulked segregant analysis (BSA) combined with sequencing (BSA-seq) analysis
For the bulked segregant analysis (BSA), two bulks, F2-1 and F2-0, were constructed by pooling equal quantities of genomic DNAs from 30 F2 plants with cuticular wax and 30 F2 plants without cuticular waxes, respectively. The high-throughput genome sequencing data of two bulks and two parental lines based on pair-end libraries were sequenced using HiSeq X10 (Illumina Inc., San Diego, CA, USA) and NGS platforms (Genedenovo, Guangzhou, China).

Genetic mapping of *BhWAX* locus
According to BSA-seq analysis, 159 InDel sites from chromosome 9 were selected for developing InDel markers. The InDel markers were then validated using P1, P2 and F1, and polymorphic markers were used to genotype 145 F2 individuals (Detailed marker information is shown in Additional File 1). Next, linkage map was prepared using JoinMap4.0, and gene mapping was conducted using QTL IciMapping Version 4.2 [52]. Later, 2,487 F2 individuals were genotyped by two flanking markers C9-46.175 and C9-47.164. Inside the primary gene mapping interval, 24 SNP sites were further selected for recombination screening. Briefly, primers were designed to amplify fragment containing the SNP sites, and the amplicons were sent for sequencing to obtain the SNP information of the recombinants (Detailed marker information is shown in Additional File 1). Based on the genotype and MFCW trait of the recombinants, the final candidate region of *BhWAX* was confirmed.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis
QRT-PCR analysis was performed using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus, Takara, Kyoto, Japan) kit according to manufacturer’s instructions. The reaction was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad) containing 50 ng of cDNAs, 100 nM of each primer in a reaction system (10 μL). Three biological replicates and three technological replicates were set for each
sample. With the wax gourd UBQ gene as internal control, the expression of genes was calculated using $2^{-\Delta \Delta C_t}$. Primers used in this analysis are listed in Additional File 1.

**Abbreviations**

BSA: Bulked segregant analysis; CAPS: Cleaved Amplified Polymorphic Sequences; DAP: Days after pollination; GWAS: Genome wide association study; MBOAT: Membrane bound O-acyl transferases; MFCW: Mature fruit cuticle wax.

**Supplementary Information**

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**Authors’ contributions**

JY and BJ conceived and designed the experiments. JY, FC, PS and YQ performed experiments. WL and DX provided experimental materials. JY and FC analyzed the data. JY wrote the manuscript. JY and BJ revised the manuscript. All authors have read and approved the manuscript.

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**Availability of data and materials**

The re-sequencing data and MFCW trait of wax gourd germplasm resources generated and analyzed in the study are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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