frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 April 2018
doi: 10.3389/fpls.2018.00309

Fine Mapping and Candidate Gene Identification for Wax Biosynthesis Locus, *BoWax1* in *Brassica oleracea* L. var. *capitata*

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *20 June 2017*
Accepted: *22 February 2018*
Published: *30 April 2018*

Citation:
Liu D, Dong X, Liu Z, Tang J, Zhuang M, Zhang Y, Lv H, Liu Y, Li Z, Fang Z and Yang L (2018) Fine Mapping and Candidate Gene Identification for Wax Biosynthesis Locus, BoWax1 in Brassica oleracea L. var. capitata. Front. Plant Sci. 9:309. doi: 10.3389/fpls.2018.00309

Cuticular waxes play important roles in plant protection against various biotic and abiotic environmental stresses. The cuticular wax covering gives normal cabbage a glaucous appearance, but the appearance of waxless mutant is glossy green. Based on the present study, inheritance of the glossy green character of mutant HUAYOU2 follows a simple recessive pattern. Genetic analysis of an F_2 population comprising 808 recessive individuals derived from HUAYOU2 (P_1, maternal parent) and M36 (P_2, paternal parent) revealed that a single recessive locus, *BoWax1* (*Brassica oleracea Wax 1*), controls glossy green trait in *B. oleracea*. This locus was mapped to a region of 158.5 kb on chromosome C01. Based on nucleotide sequence analysis, *Bol013612* was identified as the candidate gene for *BoWax1*. Sequencing results demonstrated that there is a deletion mutation of two nucleotides in the cDNA of *Bol013612* of HUAYOU2, which may account for its glossy green trait. These results lay the foundation for functional analysis of *BoWax1* and may accelerate research on wax metabolism in cabbage.

Keywords: cabbage, glossy green mutant, inheritance, gene mapping, candidate gene

INTRODUCTION

The aerial epidermis of terrestrial plants is coated with a cuticle that consists of cutin and cuticular wax (Jetter et al., 2007). Cutin is composed of modified fatty acids and provides mechanical strength to the surface layer (Kerstiens, 1996; Beisson et al., 2012) The cuticular wax, which embeds in and covers the cutin polymer, is composed of very long chain fatty acids (VLCFAs) and their derivatives, including primary and secondary alcohols, alkanes, aldehydes, ketones, and wax esters (Rashotte et al., 1997; Lee and Suh, 2015). Together with cutin, cuticular wax plays an important role in the protection of plants from damage caused by environmental stresses, such as water deficiency (Kerstiens, 1996; Mawlong et al., 2015), insect attacks (Stork, 1980), pathogen infection (Wang et al., 2006), and UV irradiance (Shepherd and Wynne Griffiths, 2006).

Wax composition and content can vary according to species, ontogeny, and environment (Jenks and Ashworth, 1999; Laila et al., 2016). As noted previously, the major components of wax are VLCFAs and their derivatives, including alkanes, aldehydes, primary and secondary alcohols, ketones, and esters (Riederer and Müller, 2006; Bernard and Joubès, 2013). C16 and C18 acyl-CoAs

are precursors in the biosynthesis of C20–C34 VLCFA-CoAs, which are formed by the addition of C2 units derived from malonyl-CoA by the enzymes of the fatty acid elongase (FAE) complex in the endoplasmic reticulum (ER) (Kunst and Samuels, 2009; Li-Beisson et al., 2013). The FAE complex consists of β-ketoacyl-CoA synthase (KCS), β-ketoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD), and *trans*-2,3-enoyl-CoA reductase (ECR), The FAE complex catalyzes four consecutive enzymatic reactions, resulting in a two-carbon extension of the acyl chain during each elongation cycle (Kunst and Samuels, 2009; Li-Beisson et al., 2013). The produced VLCFA-CoAs produced by the FAE complex are modified by two distinct pathways: an alkane-forming pathway and an alcohol-forming pathway. Most of the wax components, including aldehydes, alkanes, secondary alcohols, and ketones, are biosynthesized in the alkane-forming pathway, while the products of the alcohol-forming pathway, including primary alcohols and wax esters, account for only 10–15% of the total wax in *Arabidopsis* stems (Rowland et al., 2006).

Many genes associated with cuticular wax metabolism and its regulation in *Arabidopsis* and other plants have been identified with the help of waxless mutants. These genes include the FAE-related enzymes *KCS1*, *KCS2*, *KCS6*, *KCS9*, *KCS20*, and *KCR1* (Millar and Kunst, 1997; Millar et al., 1999; Todd et al., 1999; Fiebig et al., 2000; Pruitt et al., 2000; Hooker et al., 2002; Beaudoin and Kunst, 2009; Franke et al., 2009; Lee et al., 2009; Kim et al., 2013) and enzymes involved in the modification of VLCFA-CoAs, such as *CER1*, *CER3*, *MAH1*, *CER4*, and *WSD1* (Rowland et al., 2006, 2007; Greer et al., 2007; Li et al., 2008; Bourdenx et al., 2011), which play various roles in wax biosynthesis. *CER4* encodes an alcohol-forming fatty acyl-CoA reductase (FAR) that was reported to be responsible for the synthesis of primary alcohols in the epidermis of aerial tissues and in roots (Rowland et al., 2006).

Several genes implicated in wax biosynthesis of *Brassica* species have been reported in the recent studies. For example, the *BrWax1* gene in *Brassica rapa* was mapped to linkage group A01, and *Bra013809* was predicted to be the candidate gene for *BrWax1* (Zhang et al., 2013). The *BnaA.GL* gene in *Brassica napus* was finely mapped to a linkage group close to the end of chromosome A9 (Pu et al., 2013). In cabbage, the *BoGL1* gene was delimited to the end of chromosome C08 by a flanking marker, SSRC08–76, at a genetic distance of 0.2 cM (Liu D.et al., 2017). Although several genes in the alkane-forming pathway in *Brassica* species were isolated (Zhang et al., 2013; Liu Z. et al., 2017), no study concerning primary alcohol-forming pathway genes has been reported. In the present study, the *BoWax1* gene was mapped to chromosome C01, and *Bol013612* was identified as the candidate *BoWax1* gene. Based on the lower expression level of *Bol013612* and the differences in its sequence in the glossy green mutant HUAYOU2, the *Bol013612* gene was predicted to participate in wax biosynthesis in the plant through the alcohol-forming pathway. These results provide insight into the elucidation of the alcohol-forming pathway and the molecular mechanism of wax biosynthesis in *Brassica* species.

MATERIALS AND METHODS

Plant Materials

The plant materials in this study were provided by our team named the Cabbage and Broccoli Research Group, Institute of Vegetables and Flowers (IVF), Chinese Academy of Agricultural Sciences (CAAS) and were grown in the greenhouse in Changping (39°54′ N, 116°13′ E, Beijing, China).

Five glossy green cabbage mutants have been reported previously (Tang et al., 2015). HUAYOU2 is also a cabbage mutant with a glossy green appearance. The mutant plant HUAYOU2 was initially discovered from the highly inbred line HUA2, which was used as the wild type (WT) line in this research. HUAYOU2 (P_1, maternal parent) was crossed with M36 (P_2, paternal parent), an inbred line of Chinese kale (*Brassica oleracea* var. *alboglabra*) with a glaucous appearance, to generate the F_1, F_2, BC_1P_1, and BC_1P_2 populations for inheritance and mapping studies. The BC_1P_1 and BC_1P_2 populations were created by backcrossing the F_1 plant (maternal parent) with HUAYOU2 (paternal parent) and M36 (paternal parent), respectively. The same F_1 plant was used as the female parent in the backcrosses and was also used to obtain the F_2 population.

SEM Analysis

Scanning electron microscopy (SEM) was employed to study the surfaces of the leaves of HUAYOU2 and WT plants. Fresh leaves from five-leaf-stage plants were fixed overnight in 2% glutaraldehyde, mounted on specimen stubs using double-sided tape and coated with gold particles in a SEMPrep2 sputter coater (Nanotech, England, United Kingdom). The phenotype was analyzed by SEM (S-4800, Hitachi, Japan) with a secondary electron detector at high voltage (10 kV).

DNA Isolation and PCR Amplification

Genomic DNA was extracted from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1990). The concentration of each sample of genomic DNA was measured spectrophotometrically and adjusted to approximately 30 ng/μl.

Polymerase chain reaction (PCR) was carried out in a volume of 20 μl containing 2 μl of 10× PCR buffer (including Mg^{2+}), 1.6 μl of dNTPs (2.5 mM each), 0.4 μl of Taq DNA polymerase (2.5 U/μl), 0.8 μl of forward primer (10 μM), 0.8 μl of reverse primer (10 μM), 2 μl of template DNA, and 10.4 μl of ddH_2O. PCR was conducted on a Bio-Rad (United States) iCycler thermocycler as follows: initial denaturation step at 94°C for 5 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 5 min.

Inheritance Analysis and Mapping Strategy

The glossy and glaucous phenotypes in plants were identified visually and recorded at the five-leaf stage when the difference in appearance could be easily distinguished. Segregation ratios of the F_2 and BC_1P_1 populations were analyzed with the Chi-square (χ^2) test. Two DNA pools were constructed by mixing equal

TABLE 1 | Sequences of primers used in this study.

Primer name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
BolD000046	CACAAAATCATTAGGCCAAC	AAAAGACAGTGCCTTCCTAA
C01gSSR127	GGCGTGAGACAGTCCAAT	TGAGAACCTTGCTTACAAAC
C01gSSR129	ATGCATCATGTTCGTTACTG	TCACTGGTAAATGATGGGTT
C01gSSR132	CAAAACATGAGACCAAGACC	GCTGGATAGCATTCTAAAGG
C01gSSR135	CACAACTTATTCGCTGACAA	CACTCTTATTACGTGCTCCA
C01gSSR139	TTAAGGGATTTTGGACAGG	GAAGGATATACTGTGGTGGC
C01gSSR148	TGGCGGGGACACCCTCCAAAA	TGCACTGCGTGCTAGACTATC
C01gSSR150	AGAATTCAAGTCTTTGCGAG	AAACGAGGGTTGTTTTCTTGC
Bol013612g1	ACTAGGCATAATGTGTGCG	CACGACAGTCATCAGAAGC
Bol013612g2	GTTTGGACTTGTAGTGCTTCTG	GATTATCTATGGGTGGTAACGG
Bol013612g3	AGACGAGGTTTGTCCCTTC	CATACTCTTCTAAAGCACCCG
Bol013612g4	TATCAACACACTGGGCGTC	ACACGAACCACCTTACCTTC
Bol013612g5	GGCTCACCAGGTTCTATTCC	CCCAACTTCCCAATCAGAC
Bol013612c1	CTCGCTCATACGTACATAC	TTTAAATAGCAACATCCC
qPCR-013612	AGCCTCTCCTGAAACCATC	CGGACGAATCAACACAAG
qPCR-actin-Bol	CCTCCGTCTTGACCTTGC	GTCTCCATCTCCTGCTCGT

amounts of DNA from 12 glossy green and 12 glaucous F_2 individuals. Then, 2000 pairs of simple sequence repeat (SSR) primers in the laboratory were used to successively screen the parental lines and the DNA pools. Primers that demonstrated polymorphism between the parents were subsequently used to screen the two pools. The linkage relationship between the target gene and the polymorphic primers was confirmed in 128 F_2 individuals using JoinMap 4.0 (Van Ooijen, 2006). Linkage between markers and the *BoWax1* gene was determined with the Kosambi mapping function, and a genetic map was constructed using MapDraw 2.1 software (Liu and Meng, 2003; Kosambi, 2016). To finely map the mutant gene, 50 pairs of new primers based on primary mapping results were developed. The new primers were used to analyze the parental lines and recombinants of 808 F_2 recessive individuals. The sequences of all polymorphic markers used in this study are listed in **Table 1**

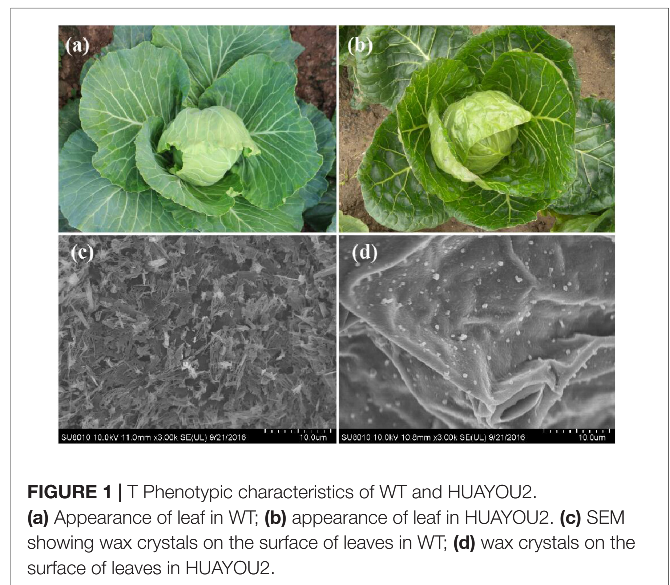

FIGURE 1 | T Phenotypic characteristics of WT and HUAYOU2. **(a)** Appearance of leaf in WT; **(b)** appearance of leaf in HUAYOU2. **(c)** SEM showing wax crystals on the surface of leaves in WT; **(d)** wax crystals on the surface of leaves in HUAYOU2.

Sequence and Expression Analysis of the *Bo13612* Gene

The InterPro annotations for *B. oleracea* and GO annotations for *Arabidopsis thaliana* for all the genes located within the mapped region were obtained from BRAD[1] (Cheng et al., 2011). The expression level of the *Bo13612* gene in WT and HUAYOU2 plants was determined by qPCR. Total RNA was extracted from fresh leaves at the five-leaf stage using the EasyPure® Plant RNA Kit (TRANS) as described by the manufacturer. First-strand cDNAs were synthesized in a 20-μl reaction volume containing approximately 7 μg of RNA and oligo(dT) primers using the TransScript One-Step gDNA Removal and cDNA Synthesis Kit (TRANS). Real-time PCR was performed with ABI SYBR Green on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The β-actin gene was used as an internal control (Okazaki et al., 2007). The $2^{-\Delta\Delta C_t}$ method was used to analyze the data (Livak and Schmittgen, 2001). Three technical replicates were performed in this study. The sequence of the primers for qPCR, qPCR-013612 and qPCR-actin, are listed in **Table 1**. Each sample was tested in triplicate. The sequences of the *Bo13612* gene were amplified using the gDNA and cDNA as templates with the KAPPA HiFi HotStart ReadyMix PCR Kit (Kappa Biosystems). The PCR products were sequenced by BGI TechSolutions, Co., Ltd., and the sequences were aligned using Multalin software[2] (Corpet, 1988).

Primers used to amplify the gDNA sequence were designed using Primer Premier 5 software and date from the *B. oleracea* genome database (BRAD[1]). The primers were named Bol013612g1, Bol013612g2, Bol013612g3, Bol013612g4, and Bol013612g5 (**Table 1**) (Lalitha, 2000). The primer used to

[1]http://brassicadb.org/brad/index.php

[2]http://multalin.toulouse.inra.fr/multalin/multalin.html

amplify the cDNA of the *Bol013612* sequence was designed based on data from the *B. oleracea* transcriptome database[3] and was named *Bol013612*c1 (**Table 1**) (Liu et al., 2014).

RESULTS

The Morphological Appearance Is Altered in HUAYOU2

Regarding the coverage of cuticular wax, the appearance of the leaves in the WT cabbage was glaucous (**Figure 1a**). In contrast to WT, the leaves of HUAYOU2 tended to be glossy green, with little wax on the plant surface (**Figure 1b**). SEM was used to investigate the micro-characteristics and density of the wax crystals on the leaf surface. The results showed that the surface of the WT leaf is covered with compact wax crystals with a scale- and rod-like shape (**Figure 1c**). Unlike the WT, there are very few wax crystals covering the leaf surface of HUAYOU2 leaves, and these wax crystals tended to have a particle-type shaped (**Figure 1d**). This indicates that the glossy phenotype of HUAYOU2 is caused by a decrease in cuticular wax crystals, which is consistent with observations of many wax-deficient mutants in other plants (Pu et al., 2013).

Inheritance Analysis of the Glossy Green Trait in HUAYOU2

The appearance of all 100 F_1 plants from the crosses of HUAYOU2 and M36 was glaucous, indicating that the glaucous trait was dominant over the glossy green trait. Among 3328 F_2 individuals, 2520 had glaucous leaves, and 808 had glossy green leaves, corresponding to a segregation ratio of 3:1 by the Chi-square test. The segregation ratio in the BC_1P_1 population was 1:1 (210 glaucous:222 glossy), and all the BC_1P_2 plants had a glaucous appearance (**Table 2**). These results demonstrated that inheritance of the glossy green trait in HUAYOU2 follows a monogenic recessive pattern.

Fine Genetic and Physical Mapping of the *BoWax1* Gene

A total of 2000 SSR markers available in the laboratory were used to identify polymorphisms in the parental lines (HUAYOU2 and M36) and in the glossy and glaucous DNA bulks. As a result, 114 pairs of primers were identified that showed polymorphism between the parental lines, but only BolD000046 was identified as being polymorphic between two DNA pools. Marker BolD000046 was mapped close to *BoWax1* using 128 segregating F_2 population plants (**Figure 2A**).

To saturate the genetic map containing *BoWax1*, 50 pairs of primers near primer BolD000046 were designed based on the physical position of marker BolD000046. Eight of the 50 newly developed primers showed polymorphism between parental lines HUAYOU2 and M36. The eight SSRs were subsequently screened to investigate recombination using 808 F_2 recessive individuals. Based on information from the polymorphic markers and the recombinants, the genetic distance between the two closest flanking makers, C01gSSR148 and C01gSSR150 (**Figure 2A**),

[3]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42891

TABLE 2 | Segregation of glaucous and glossy traits and the χ^2 goodness-of-fit test of segregation in BC_1P_1 and F_2 populations.

Population	Total	Non-glossy	Glossy	Segregation ratio	χ^2
P_1(HUAYOU2)	20	0	20	–	–
P_2(M36)	20	20	0	–	–
F_1	100	100	0	–	–
F_2	3328	2520	808	3.12:1	0.88
BC_1P_1 (F_1 × HUAYOU2)	432	210	222	0.95:1	0.28
BC_1P_2(F_1 × M36)	200	200	0	–	–

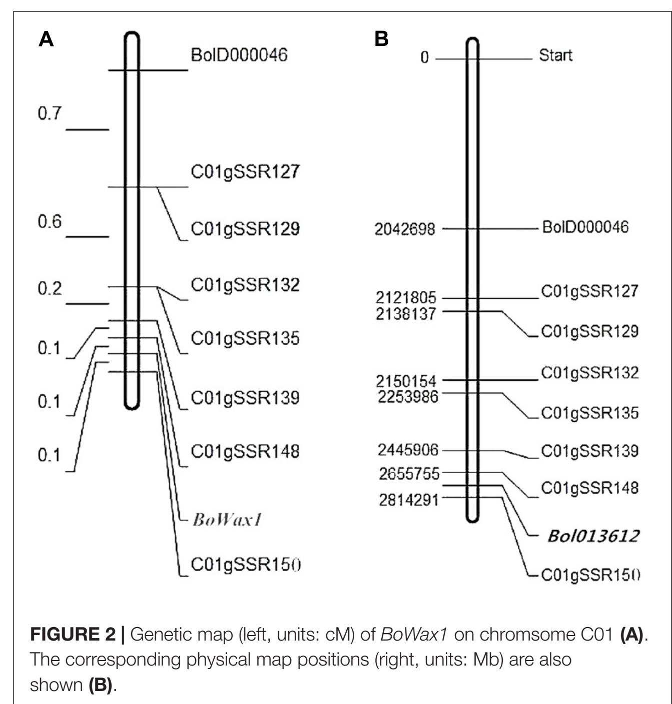

FIGURE 2 | Genetic map (left, units: cM) of *BoWax1* on chromsome C01 **(A)**. The corresponding physical map positions (right, units: Mb) are also shown **(B)**.

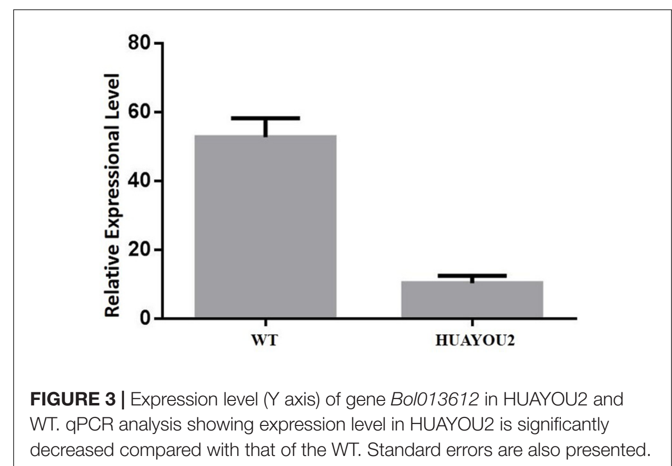

FIGURE 3 | Expression level (Y axis) of gene *Bol013612* in HUAYOU2 and WT. qPCR analysis showing expression level in HUAYOU2 is significantly decreased compared with that of the WT. Standard errors are also presented.

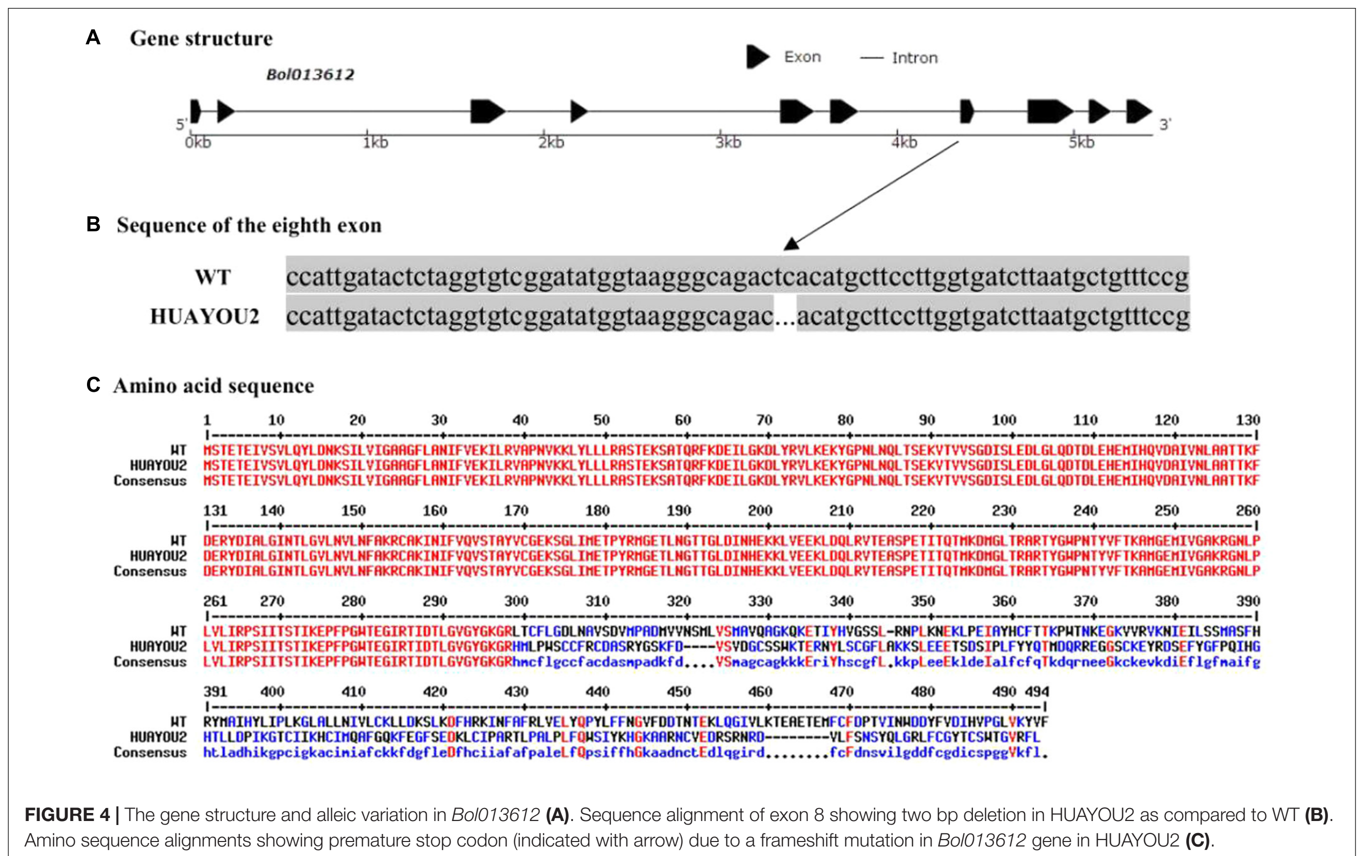

FIGURE 4 | The gene structure and alleic variation in *Bol013612* **(A)**. Sequence alignment of exon 8 showing two bp deletion in HUAYOU2 as compared to WT **(B)**. Amino sequence alignments showing premature stop codon (indicated with arrow) due to a frameshift mutation in *Bol013612* gene in HUAYOU2 **(C)**.

was found to be 0.2 cM. The physical distance between primers C01gSSR148 and C01gSSR150 is 158.5 kb (**Figure 2B**). All of the primers are on chromosome C01 and the order of the markers on the genetic map is consistent with that on the physical map.

Candidate Gene Analysis

Analysis of the genomic region between C01gSSR148 and C01gSSR150 was carried out. Gene annotations in BRAD and alignments with *A. thaliana* showed that gene *Bol013612* contains the conserved FAR domain, which plays important roles in wax biosynthesis and is present in proteins that are responsible for primary alcohols synthesis (Rowland et al., 2006). *At4g3370* is an example of an *Arabidopsis* gene that contains the conserved FAR domain and exhibits the same glossy stem wax phenotype in two mutant lines (cer4-1 and cer4-2). A deletion and/or rearrangement of the promoter region occurs in the cer4-1 mutant and a T-DNA insertion is present in the fourth intron of At4g33790 (at nucleotide 1,960 relative to the start codon) in the cer4-2 mutant (Rowland et al., 2006). According to the homologous analysis results, the *Bol013612* gene was predicted to participate in the alcohol-forming pathway. Aside from *Bol013612*, no other genes in the mapped region were found to be related to plant wax metabolism (Gene analysis and annotations are supplied in Additional file 1: Supplementary 2). The *Bol013612* gene was selected as the candidate for expression analysis using qPCR. The results showed that the expression level of *Bol013612* is significantly decreased compared with that of the WT, implying that transcription of the *Bol013612* gene was affected (**Figure 3**).

The *Bol013612* gene was sequenced to further determine whether the glossy green trait of HUAYOU2 is caused by a mutation in *Bol013612*. The *Bol013612* gene, numbered MG808275 in NCBI database, is 5,448 bp in length, including 10 exons and 9 introns (**Figure 4A**). The The coding sequence (CDS) of *Bol013612* is 1,482 bp in length. The sequencing results showed that there was a deletion of two bases in the eighth exon of HUAYOU2 (sequences of WT and HUAYOU2 are supplied in Additional file 1: Supplementary 1). The deletion mutation was suspected of causing a frameshift mutation in the *Bol013612* gene and a premature stop codon (**Figures 4B,C**). This might disrupt the function of this gene, which may influence the wax biosynthesis and subsequently cause the glossy appearance of HUAYOU2.

DISCUSSION

Waxes are secreted onto the surface or into the interior of cuticles to form the first barrier against various environmental stresses (Millar et al., 1999). Since Dellaert discovered the first waxy cuticle *eceriferum* mutant (*cer*) in *A. thaliana* (Dellaert et al., 1979), many genes related to wax synthesis have been isolated, including wax synthesis-related enzymes (*CER4*, *CER6*, *CER10*, *FATB*, and *GL8*) (Fiebig et al., 2000;

Bonaventure and Ohlrogge, 2003; Dietrich et al., 2005; Zheng et al., 2005; Rowland et al., 2006), wax transporters (*CER5* and *WBC11*) (Pighin and Samuels, 2004; Bird et al., 2007; Panikashvili et al., 2007), and transcription factors (*SHN1*, *MYB30*, *MYB96*, and *WXP1*) (Broun et al., 2004; Zhang et al., 2005; Raffaele et al., 2008; Seo et al., 2011). Several genes implicated in *Brassica* plant wax biosynthesis pathway were reported in recent studies. Though these studies identify the same glossy waxless appearance, the inheritance patterns are different. For example, the waxless trait of the Chinese cabbage mutant 08A235-2 is controlled by a single recessive gene (Zhang et al., 2013), same as that of HUAYOY2 in this study. By contrast, a dominant mutant allele controls the waxless trait in *Brassica napus* mutant 6-1025 (Pu et al., 2013). The glossy appearance of HUAYOU2 in this study is also determined by a single recessive gene, but sequence and functional analysis of the candidate gene revealed that the novel locus for glossiness on chromosome C01 is involved in the primary alcohol-forming pathway, in contrast with the gene *BnaA.GL* in *Brassica napus* (Pu et al., 2013) and the gene *BrWax1* in *Brassica rapa* (Zhang et al., 2013).

C*er4* mutant and cabbage mutant LD10GL are fertile in contrast with the cabbage glossy green mutant 10Q-961, in which only a few seeds were produced after self-pollination (Liu Z. et al., 2017). Enzymes containing the conserved FAR domain usually act on medium- and long-chain fatty acids and have been reported to be involved in the biosynthesis of plant cuticular wax (Rowland et al., 2006). In *Arabidopsis*, FAR has specificity for very-long-chain fatty acids and is present in enzymes responsible for the synthesis of primary alcohols in the epidermal cells of aerial tissues and in the roots (Rowland et al., 2006). *CER4* is one of the genes encoding an FAR domain in *Arabidopsis*. The glossy wax-deficient trait is displayed in *Arabidopsis* mutant *cer4* due to decreases in the production primary alcohols and wax esters.

By studying the cabbage mutant HUAYOU2, *BoWax1* was finely mapped and the target gene was identified. In the F_2 population, gene *BoWax1* was localized to a region of 158.5 kb on cabbage chromosome C01. The sequence variation and abnormal expression of the *Bol013612* gene imply that *Bol013612* may be the target gene that accounts for the glossy green trait of HAUYOU2. These results will help us further elucidate the formation of the glossy trait in HUAYOU2 and accelerate wax biosynthesis studies in cabbage. More research needs to be carried out in subsequent work, such as functional studies of *BoWax1*, investigation of the metabolic mechanism of cabbage wax biosynthesis and identification of other genes involved.

AUTHOR CONTRIBUTIONS

DL, LY, and ZF designed and supervised the study. XD, ZZL, JT, MZ, YZ, HL, YL, and ZSL participated in its design. DL participated in the mapping, RT-PCR, and sequence analysis. JT, ZZL, YL, and ZSL participated in the statistical analysis. DL and LY wrote the manuscript. All the authors discussed the results and contributed to the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was financially supported by grants from the National Natural Science Foundation of China (31672155), the National High Technology Research and Development Program of China (863 Program) (2012AA100101), the Key Projects in the National Science and Technology Pillar Program during the Twelfth Five-Year Plan Period (2012BAD02B01), the Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2013-IVFCAAS), the Modern Agro-Industry Technology Research System (CARS-25-B-01), and the Project of Science and Technology Commission of Beijing Municipality (Z141105002314020-1).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00309/full#supplementary-material

REFERENCES

Beaudoin, F., and Kunst, L. (2009). Functional characterization of the Arabidopsis β-ketoacyl-coenzyme a reductase candidates of the fatty acid elongase. *Plant Physiol.* 150, 1174–1191. doi: 10.1104/pp.109.137497

Beisson, F., Li-Beisson, Y., and Pollard, M. (2012). Solving the puzzles of cutin and suberin polymer biosynthesis. *Curr. Opin. Plant Biol.* 15, 329–337. doi: 10.1016/j.pbi.2012.03.003

Bernard, A., and Joubès, J. (2013). Arabidopsis cuticular waxes: advances in synthesis, export and regulation. *Prog. Lipid Res.* 52, 110–129. doi: 10.1016/j.plipres.2012.10.002

Bird, D., Beisson, F., Brigham, A., Shin, J., Greer, S., Jetter, R., et al. (2007). Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. *Plant J.* 52, 485–498. doi: 10.1111/j.1365-313X.2007.03252.x

Bonaventure, G., and Ohlrogge, J. B. (2003). Disruption of the *FATB* gene in Arabidopsis demonstrates an essential role of saturated fatty acids in plant growth. *Plant Cell* 15, 1020–1033. doi: 10.1105/tpc.008946

Bourdenx, B., Bernard, A., Domergue, F., Pascal, S., Léger, A., Roby, D., et al. (2011). Overexpression of Arabidopsis *ECERIFERUM1* promotes wax very-long-chain alkane biosynthesis and influences plant response to biotic and abiotic stresses. *Plant Physiol.* 156, 29–45. doi: 10.1104/pp.111.172320

Broun, P., Poindexter, P., Osborne, E., Jiang, C. Z., and Riechmann, J. L. (2004). WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4706–4711. doi: 10.1073/pnas.0305574101

Cheng, F., Liu, S., Wu, J., Fang, L., Sun, S., Liu, B., et al. (2011). BRAD, the genetics and genomics database for Brassica plants. *BMC Plant Biol.* 11:136. doi: 10.1186/1471-2229-11-136

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* 16, 10881–10890. doi: 10.1093/nar/16.22.10881

Dellaert, L., Van Es, J., and Koornneef, M. (1979). *Eceriferum* mutants in *Arabidopsis thaliana* (L.) Heynh. II. Phenotypic and genetic analysis. *Arabid. Inf. Serv.* 16, 10–26.

Dietrich, C. R., Perera, M. A., Yandeau-Nelson, M. D., Meeley, R. B., Nikolau, B. J., and Schnable, P. S. (2005). Characterization of two GL8 paralogs reveals that the 3-ketoacyl reductase component of fatty acid elongase is essential for maize

(*Zea mays* L.) development. *Plant J.* 42, 844–861. doi: 10.1111/j.1365-313X.2005.02418.x

Doyle, J. J. (1990). Isolation of plant DNA from fresh tissue. *Focus* 12, 13–15.

Fiebig, A., Mayfield, J. A., Miley, N. L., Chau, S., Fischer, R. L., and Preuss, D. (2000). Alterations in CER6, a gene identical to CUT1, differentially affect long-chain lipid content on the surface of pollen and stems. *Plant Cell* 12, 2001–2008. doi: 10.1105/tpc.12.10.2001

Franke, R., Höfer, R., Briesen, I., Emsermann, M., Efremova, N., Yephremov, A., et al. (2009). The *DAISY* gene from *Arabidopsis* encodes a fatty acid elongase condensing enzyme involved in the biosynthesis of aliphatic suberin in roots and the chalaza-micropyle region of seeds. *Plant J.* 57, 80–95. doi: 10.1111/j.1365-313X.2008.03674.x

Greer, S., Wen, M., Bird, D., Wu, X., Samuels, L., Kunst, L., et al. (2007). The cytochrome P450 enzyme CYP96A15 is the midchain alkane hydroxylase responsible for formation of secondary alcohols and ketones in stem cuticular wax of Arabidopsis. *Plant Physiol.* 145, 653–667. doi: 10.1104/pp.107.107300

Hooker, T. S., Millar, A. A., and Kunst, L. (2002). Significance of the expression of the CER6 condensing enzyme for cuticular wax production in Arabidopsis. *Plant Physiol.* 129, 1568–1580. doi: 10.1104/pp.003707

Jenks, M. A., and Ashworth, E. N. (1999). Plant epicuticular waxes: function, production, and genetics. *Hortic. Rev.* 23, 1–68.

Jetter, R., Kunst, L., and Samuels, A. L. (2007). "Composition of plant cuticular waxes," in *Annual Plant Reviews: Biology of the Plant Cuticle*, Vol. 23, eds M. Riederer and C. Müller (Oxford: Blackwell Publishing Ltd.), 145–181.

Kerstiens, G. (1996). Cuticular water permeability and its physiological significance. *J. Exp. Bot.* 47, 1813–1832. doi: 10.1093/jxb/47.12.1813

Kim, J., Jung, J. H., Lee, S. B., Go, Y. S., Kim, H. J., Cahoon, R., et al. (2013). Arabidopsis 3-ketoacyl-coenzyme a synthase9 is involved in the synthesis of tetracosanoic acids as precursors of cuticular waxes, suberins, sphingolipids, and phospholipids. *Plant Physiol.* 162, 567–580. doi: 10.1104/pp.112.210450

Kosambi, D. D. (2016). *The Estimation of Map Distances from Recombination Values*. Berlin: Springer, 125–130. doi: 10.1007/978-81-322-3676-4_16

Kunst, L., and Samuels, L. (2009). Plant cuticles shine: advances in wax biosynthesis and export. *Curr. Opin. Plant Biol.* 12, 721–727. doi: 10.1016/j.pbi.2009.09.009

Laila, R., Robin, A. H. K., Yang, K., Park, J.-I., Suh, M. C., Kim, J., et al. (2016). Wax content, wax composition and expression of wax biosynthetic genes in *Brassica oleracea* var. *capitata* vary with leaf development and genotype. *Front. Plant Sci.* 7:1972.doi: 10.3389/fpls.2016.01972

Lalitha, S. (2000). Primer premier 5. *Biotech Softw. Internet Rep.* 1, 270–272. doi: 10.1089/152791600459894

Lee, S. B., Go, Y. S., Bae, H.-J., Park, J. H., Cho, S. H., Cho, H. J., et al. (2009). Disruption of glycosylphosphatidylinositol-anchored lipid transfer protein gene altered cuticular lipid composition, increased plastoglobules, and enhanced susceptibility to infection by the fungal pathogen *Alternaria brassicicola*. *Plant Physiol.* 150, 42–54. doi: 10.1104/pp.109.137745

Lee, S. B., and Suh, M. C. (2015). Advances in the understanding of cuticular waxes in *Arabidopsis thaliana* and crop species. *Plant Cell Rep.* 34, 557–572. doi: 10.1007/s00299-015-1772-2

Li, F., Wu, X., Lam, P., Bird, D., Zheng, H., Samuels, L., et al. (2008). Identification of the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in Arabidopsis. *Plant Physiol.* 148, 97–107. doi: 10.1104/pp.108.123471

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., et al. (2013). Acyl-lipid metabolism. *Arabidopsis Book* 11:e0161. doi: 10.1199/tab.0161

Liu, D., Tang, J., Liu, Z., Dong, X., Zhuang, M., Zhang, Y., et al. (2017). Fine mapping of BoGL1, a gene controlling the glossy green trait in cabbage (*Brassica oleracea* L. Var. *capitata*). *Mol. Breed.* 37:69. doi: 10.1007/s11032-017-0674-0

Liu, R. H., and Meng, J. L. (2003). MapDraw: a microsoft excel macro for drawing genetic linkage maps based on given genetic linkage data. *Yi Chuan* 25, 317–321.

Liu, S., Liu, Y., Yang, X., Tong, C., Edwards, D., Parkin, I. A., et al. (2014). The Brassica oleracea genome reveals the asymmetrical evolution of polyploid genomes. *Nat. Commun.* 5:3930. doi: 10.1038/ncomms4930

Liu, Z., Fang, Z., Zhuang, M., Zhang, Y., Lv, H., Liu, Y., et al. (2017). Fine-mapping and analysis of Cgl1, a gene conferring glossy trait in cabbage (*Brassica oleracea* L. var. *capitata*). *Front. Plant Sci.* 8:239. doi: 10.3389/fpls.2017.00239

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Mawlong, I., Ali, K., Srinivasan, R., Rai, R., and Tyagi, A. (2015). Functional validation of a drought-responsive AP2/ERF family transcription factor-encoding gene from rice in Arabidopsis. *Mol. Breed.* 35:163. doi: 10.1007/s11032-015-0290-9

Millar, A. A., Clemens, S., Zachgo, S., Giblin, E. M., Taylor, D. C., and Kunst, L. (1999). CUT1, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *Plant Cell* 11, 825–838. doi: 10.1105/tpc.11.5.825

Millar, A. A., and Kunst, L. (1997). Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *Plant J.* 12, 121–131. doi: 10.1046/j.1365-313X.1997.12010121.x

Okazaki, K., Sakamoto, K., Kikuchi, R., Saito, A., Togashi, E., Kuginuki, Y., et al. (2007). Mapping and characterization of FLC homologs and QTL analysis of flowering time in *Brassica oleracea*. *Theor. Appl. Genet.* 114, 595–608. doi: 10.1007/s00122-006-0460-6

Panikashvili, D., Savaldigoldstein, S., Mandel, T., Yifhar, T., Franke, R. B., Höfer, R., et al. (2007). The Arabidopsis *DESPERADO/AtWBC11* transporter is required for cutin and wax secretion. *Plant Physiol.* 145, 1345–1360. doi: 10.1104/pp.107.105676

Pighin, J. A., and Samuels, A. L. (2004). Plant cuticular lipid export requires an ABC transporter. *Science* 306, 702–704. doi: 10.1126/science.1102331

Pruitt, R. E., Vielle-Calzada, J. P., Ploense, S. E., Grossniklaus, U., and Lolle, S. J. (2000). *FIDDLEHEAD*, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1311–1316. doi: 10.1073/pnas.97.3.1311

Pu, Y., Gao, J., Guo, Y., Liu, T., Zhu, L., Xu, P., et al. (2013). A novel dominant glossy mutation causes suppression of wax biosynthesis pathway and deficiency of cuticular wax in *Brassica napus*. *BMC Plant Biol.* 13:215. doi: 10.1186/1471-2229-13-215

Raffaele, S., Vailleau, F., Léger, A., Joubès, J., Miersch, O., Huard, C., et al. (2008). A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in *Arabidopsis*. *Plant Cell* 20, 752–767. doi: 10.1105/tpc.107.054858

Rashotte, A. M., Jenks, M. A., Nguyen, T. D., and Feldmann, K. A. (1997). Epicuticular wax variation in ecotypes of Arabidopsis thaliana. *Phytochemistry* 45, 251–255. doi: 10.1016/S0031-9422(96)00792-3

Riederer, M., and Müller, C. (2006). *Biology of the Plant Cuticle*. Oxford: Blackwell. doi: 10.1002/9780470988718

Rowland, O., Lee, R., Franke, R., Schreiber, L., and Kunst, L. (2007). The CER3 wax biosynthetic gene from Arabidopsis thaliana is allelic to WAX2/YRE/FLP1. *FEBS Lett.* 581, 3538–3544. doi: 10.1016/j.febslet.2007.06.065

Rowland, O., Zheng, H., Hepworth, S. R., Lam, P., Jetter, R., and Kunst, L. (2006). *CER4* encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. *Plant Physiol.* 142, 866–877. doi: 10.1104/pp.106.086785

Seo, P. J., Lee, S. B., Suh, M. C., Park, M. J., Go, Y. S., and Park, C. M. (2011). The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in *Arabidopsis*. *Plant Cell* 23, 1138–1152. doi: 10.1105/tpc.111.083485

Shepherd, T., and Wynne Griffiths, D. (2006). The effects of stress on plant cuticular waxes. *New Phytol.* 171, 469–499. doi: 10.1111/j.1469-8137.2006.01826.x

Stork, N. E. (1980). Role of waxblooms in preventing attachment to Brassicas by the mustard beetle, *Phaedon cochleariae*. *Entomol. Exp. Appl.* 28, 100–107. doi: 10.1111/j.1570-7458.1980.tb02992.x

Tang, J., Liu, D., Liu, Z., Yang, L., Fang, Z., Liu, Y., et al. (2015). Preliminary study of the characteristics of several glossy cabbage (*Brassica oleracea* var. *capitata* L.) mutants. *Hortic. Plant J.* 1, 93–100.

Todd, J., Postbeittenmiller, D., and Jaworski, J. G. (1999). KCS1 encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. *Plant J. Cell Mol. Biol.* 17, 119–130. doi: 10.1046/j.1365-313X.1999.00352.x

Van Ooijen, J. (2006). *JoinMap 4: Software for the Calculation of Genetic Linkage Maps in Experimental Populations*. Wageningen: Kyazma BV.

Wang, F., Zhang, P., Qiang, S., and Xu, L.-L. (2006). Interaction of plant epicuticular waxes and extracellular esterases of *Curvularia eragrostidis* during infection of *Digitaria sanguinalis* and *Festuca arundinacea* by the fungus. *Int. J. Mol. Sci.* 7, 346–357. doi: 10.3390/i7090346

Zhang, J. Y., Broeckling, C. D., Blancaflor, E. B., Sledge, M. K., Sumner, L. W., and Wang, Z. Y. (2005). Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *Plant J.* 42, 689–707. doi: 10.1111/j.1365-313X.2005.02405.x

Zhang, X., Liu, Z., Wang, P., Wang, Q., Yang, S., and Feng, H. (2013). Fine mapping of BrWax1, a gene controlling cuticular wax biosynthesis in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). *Mol. Breed.* 32, 867–874. doi: 10.1007/s11032-013-9914-0

Zheng, H., Rowland, O., and Kunst, L. (2005). Disruptions of the Arabidopsis Enoyl-CoA reductase gene reveal an essential role for very-long-chain fatty acid synthesis in cell expansion during plant morphogenesis. *Plant Cell* 17, 1467–1481. doi: 10.1105/tpc.104.030155

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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