Article

Inactivation of BoORP3a, an oxysterol-binding protein, causes a low wax phenotype in ornamental kale

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Abstract

Identifying genes associated with wax deposition may contribute to the genetic improvement of ornamental kale. Here, we characterized a candidate gene for wax contents, BoORP3a, encoding an oxysterol-binding protein. We sequenced the BoORP3a gene and coding sequence from the high-wax line S0835 and the low-wax line F0819, which revealed 12 single nucleotide polymorphisms between the two lines, of which six caused five amino acids substitutions. BoORP3a appeared to be relatively well conserved in Brassicaceae, as determined by a phylogenetic analysis, and localized to the endoplasmic reticulum and the nucleus. To confirm the role of BoORP3a in wax deposition, we generated three orp3a mutants in a high-wax kale background via CRISPR/Cas9-mediated genome editing. Importantly, all three mutants exhibited lower wax contents and glossy leaves. Overall, these data suggest that BoORP3a may participate in cuticular wax deposition in ornamental kale.

Introduction

Wax located outside the cuticle or within the cuticular matrix plays many roles in supporting plant growth and survival in various environments. As a hydrophobic barrier, the cuticular wax primarily reduces transpirational water loss and thus enhances drought tolerance [1]. The cuticular wax also provides a physical barrier that protects plants against microbial infections, insect attacks, and damage from ultraviolet (UV) light. In addition, the cuticular wax layer is waterproof and self-cleaning to prevent the deposition of dust and other pollutants [1,2]. Cuticular wax has been shown to affect plant development, pigmentation, and fertility [3]. The cuticular wax is composed of very-long-chain fatty acids (VLCFAs) and their derivatives, alcohols, alkanes, alkenes, aldehydes, esters, ketones, triterpenoids, and sterols [4]. The biosynthesis of cuticular wax takes place within epidermal cells [5]. The C16 and C18 fatty acids begin to form in plastids, followed by fatty acid elongation into VLCFAs mediated by the fatty acid elongase complex [6]. VLCFAs are subsequently modified via the alkane- and alcohol-forming pathways [7], while the primary alcohol and wax ester are derived from the alcohol-forming pathway. The aldehydes, alkanes, secondary alcohols, and ketones are produced by the alkane-forming pathway [8]. These wax components are collectively exported to the cuticular wax layer by members of the ATP binding cassette (ABC) transporter and glycosylphosphatidylinositol-anchored lipid transfer protein (LTPG) families [9, 10]. Several genes involved in wax biosynthesis, export, and regulation have been identified in Arabidopsis (Arabidopsis thaliana), such as ECERIFERUM1 (CER1)/CER3/CER4–7/CER10, CURLY FLAG LEAF1 (CFL1), FATTY ACYL-ACP THIOESTERASE B (FATB), WAX SYNTHASE/ACYL-CoA:DIACYLGLYCEROL ACYLTRANSFERASE1 (WSD1), ABOG11, and LTPG1 [3]. However, the molecular mechanisms linking these and other genes with cuticular wax transport are poorly understood.

Sterols are vital cellular components that contribute to the maintenance of membrane integrity, metabolism related to membrane properties, secretory trafficking events, as well as the biosynthesis of wax, cellulose, callose, lignin, and the phytohormone brassinosteroids [4,11,12]. Sterol biosynthesis occurs in the endoplasmic reticulum (ER) but later mainly accumulates in the plasma membrane (PM) [13]. Oxysterol-binding proteins (OSBPs) and OSBP-related proteins (ORPs) transport and regulate the metabolism of sterols and phospholipids [14]. ORPs also bind to various lipids, such as ergosterol and cholesterol [13]. Arabidopsis ORP3a, a sterol-binding protein, may participate in the export of sterols from the ER and their circulation between the ER and the Golgi [13,15]. Based on the function of ORP3a, we speculate that ORP3a may play a role in the deposition of cuticular wax.

Ornamental kale (Brassica oleracea var. acephala) is used not only as a leafy vegetable but also as an ornamental crop, owing to its nutritional value (rich in glucosinolates, phenolic compounds, and carotenoids) and attractive and colorful leaves [16,17]. The cuticular wax covering ornamental kale organs may enhance plant tolerance to environmental stresses and changes. However, this agronomic trait also significantly influences the edible and ornamental properties of ornamental kale. Indeed, wax was reported to affect fruit postharvest quality and storage capacity [18]. Brassica plants covered with cuticular wax are usually glaucous, while wax-free mutant plants typically have a
glossy and green color [19]. Thus, exploring how wax forms and is deposited on various organs is critical in ornamental kale to modulate its visual appeal and nutritional value. To date, no study has reported on wax deposition genes in ornamental kale. Our previous study constructed a segregating population between S0835 and F0819 [16]. We exploited bulked segregant analysis combined with next generation sequencing (BSA-seq) to identify the candidate gene(s) responsible for the difference in leaf shape and wax contents between these two inbred lines. Based on the functional annotation of genes from the candidate region, we selected BoORP3a (Bo9g184810), encoding an oxysterol-binding protein, as a high-confidence wax deposition candidate. In the present study, we compared the wax deposition and distribution patterns in two ornamental kale inbred lines with contrasting wax phenotypes by scanning electron microscopy and measured their wax contents by colorimetry. We also cloned the BoORP3a candidate gene for wax deposition and determined the subcellular localization of its encoded protein. Finally, we validated the function of BoORP3a in wax deposition by generating genome-edited mutants via clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)-associated nuclease 9 (Cas9). The results presented here will contribute to our understanding of the functions associated with the ORP family in ornamental kale.

**Results**

**Characterization of cuticular wax in two ornamental kale inbred lines**

We determined the differences in cuticular wax contents and pattern between two ornamental kale inbred lines (Fig. 1a) by scanning electron microscopy (SEM) observations of the leaf surface and quantification of wax contents by colorimetry. We measured the lightness value of leaves before and after wax removal and calculated the lightness difference as an indicator of wax contents (Fig. 1b). Compared to the inbred line S0835, F0819 was characterized by a lower wax content on its leaves (P < 0.01). In the S0835 inbred line, the leaf surface was densely and evenly covered with wax crystals, which was in sharp contrast to the leaf surface for F0819, which exhibited sparse wax crystals, in agreement with the lightness difference results (Fig. 1c and d). However, we observed no apparent differences in wax morphology between the two inbred lines. These results indicate that these two inbred lines differ only in their cuticular wax contents, with S0835 having a high wax content.

**Phylogenetic and sequence analysis of BoORP3a**

In our previous study, a smooth-leaved inbred line (S0835) and a feathered-leaved inbred line (F0819) were used as parental lines to construct the segregating population for BSA-seq and fine mapping [16]. The genetic analysis showed that the feathered-leaved trait was controlled by a semi-dominant gene. Furthermore, we observed the smooth-leaved inbred line always showed more cuticular wax content, while the feathered-leaved inbred line always showed lower cuticular wax content, so it was speculated that these two traits showed linkage. The leaf shape gene was mapped to a 374.532-kb interval and 38 genes were identified, that these two traits showed linkage. The leaf shape gene was mapped to a 374.532-kb interval and 38 genes were identified, indicating that the ORP3a genes experienced purifying selection. The Ka/Ks ratio of most paralogous gene pairs was < 1 (Table S2), indicating that the ORP3a genes experienced purifying selection. Furthermore, 26 pairs with a Ka/Ks ratio greater than 1 are under positive selection. We aligned the full-length, coding sequence and encoded amino acid sequence of BoORP3a from the two inbred lines. The full-length genomic region (from the translation start site to the stop codon) of BoORP3a was 2,180 bp in length in S0835 and 2,185 bp in F0819 (Fig. S4). The BoORP3a coding sequence was of identical length in the two inbred lines at 1,362 bp and encoded a protein of 453 amino acids (Fig. 2b; Fig. S5). The comparison of the two genomic sequences revealed 20 single nucleotide polymorphisms (SNPs) and a 5-bp deletion in S0835 (Fig. S3). The deletion did not affect the coding sequence, but 12 of the 20 SNPs did, of which six caused amino acid substitutions, N28T, E51D, N159I, E404D, and Q409P from S0835 to F0819 (Fig. S4, Fig. 2b and 2c). The protein encoded by both inbred lines contained a conserved domain belonging to the oxysterol-binding protein superfamily from amino acids 70 to 422 (Fig. 2c). Three of the five amino acid substitutions were within this conserved domain. These results indicate that the polymorphisms at BoORP3a between the inbred lines S0835 and F0819 might affect BoORP3a function and contribute to the change in wax contents.

**Subcellular localization of BoORP3a**

We investigated the subcellular localization of BoORP3a by transiently infiltrating a construct encoding a fusion between BoORP3a and green fluorescent protein (GFP) into the leaves of *Nicotiana benthamiana* plants, together with a fluorescent marker for the ER. We detected the fusion protein in the ER and the nucleus (Fig. 3).

**CRISPR/Cas9-mediated mutagenesis of BoORP3a in ornamental kale**

To confirm the function of BoORP3a in ornamental kale, we employed CRISPR/Cas9-mediated gene editing to introduce mutations in this gene in the S0835 background (high-wax line). We obtained 40 kanamycin-resistant primary transformant (T0) plants, which we then subjected to PCR genotyping and sequencing. To this end, we amplified and sequenced the genomic region covering the target site of the sgRNAs from each transgenic plant (Fig. 4b). We identified three transfectants with substitutions and deletion mutations within BoORP3a (Fig. 4d). Of the three mutants, only *orp3a-1* was homozygous, with a 1-bp mutation at target site 2, resulting in a P173Q amino acid substitution (Fig. 4d; Fig. S6, see online supplementary material). We detected a 1-bp deletion at target site 1 in *orp3a-21*, resulting in the frameshift mutation after 22 amino acids (Fig. 4d; Fig. S5, see online supplementary material). In *orp3a-34*, we identified two adjacent substitutions at target site 1, resulting in a N22F amino acid substitution (Fig. 4d; Fig. S5, see online supplementary material). As illustrated in Fig. 4a, we observed a marked difference in appearance between the wild-type (WT) and *orp3a* mutant (T0) plants. Indeed, all three
mutants had glossy green leaves, which is typical of low-wax germplasm, compared to the WT, with orp3a-21 being the most glossy. The lightness difference of WT plants was 4.3-, 5.8-, and 3.5-fold higher than in homozygous orp3a-1, orp3a-21, and orp3a-34 mutants, respectively (Fig. 4c). These results indicate that the loss of BoORP3a function leads to a decrease in the cuticular wax contents of ornamental kale.

**Design and validation of a CAPS marker**

We designed a pair of primers to amplify a region of BoORP3a harboring a non-synonymous SNP between ornamental kale inbred lines S0835 and F0819. We then amplified genomic DNA from the two inbred lines, wax-free Chinese cabbage, and another ornamental kale with high wax (Sunrise). After enzymatic digestion with NcoI, agarose gel electrophoresis showed two bands (254 and 137 bp) for Chinese cabbage and the inbred line F0819, indicating the presence of the restriction site. By contrast, the PCR ampli-cons from the high-wax ornamental kale lines S0835 and Sunrise remained undigested (391 bp) (Fig. 5c). These results provide further evidence that BoORP3a is related to wax deposition.

**Discussion**

Wax contents in plants are essential for hybridization breeding and commercial application. Recently, the accumulation of wax in Brassica vegetables has become a hot research topic [20, 21]. Ornamental kale is a popular vegetable and decorative plant whose cuticular wax contents affect its economic value. In this study, we established the presence of multiple SNPs in BoORP3a (Bo9g184810) between the high-wax inbred line S0835 and the low-wax line F0819. We knocked out BoORP3a in the high-wax line S0835 via genome editing, which resulted in lower cuticular wax contents in all three mutants isolated, as evidenced by colorimetry and the glossy phenotype of their leaf epidermis. Overall, our results demonstrate that the oxysterol-binding protein BoORP3a plays a role in the deposition of cuticular wax in ornamental kale.

Cuticular wax plays a critical role in protecting terrestrial plants against biotic and abiotic stresses [22]. The ultrastructure, composition, and contents of cuticular wax vary between species, organ type, developmental stage, and environmental conditions. Generally, in comparison to the old leaves, wax deposition was often lower in the young leaves in ornamental kale, which may be related to expression levels of wax biosynthetic genes, leaf positions and growth environments [23]. Some wax biosynthetic genes such as BoLACS1.4 and BoKCS1.1 were found to be highly expressed in young leaves, while BoKCR1.1 and BoCER3.4 were highly expressed in old leaves of cabbage [23]. In A. thaliana, AtORP3a (AT5G02100) showed a higher expression level in young leaves compared to old leaves [11]. In this study, we observed the structure of cuticular wax in the inbred lines S0835 and F0819 by SEM. We observed many irregular platelet-shaped wax crystals and a few tubular structures that were densely distributed on the leaf surface of ornamental kale plants, thus exhibiting slight differences with the cuticular wax crystals seen in cabbage [19]. Importantly, the contents, but not the morphology, of cuticular wax differed between the two inbred lines.

Many wax-deficient mutants have been described, most of them in Arabidopsis. Several of the causal genes have been identified and participate in wax biosynthesis, transport and export. For example, CER2, CER3, CER6, CER10, 3-KETOACYL-COA SYNTHASE1 (KCS1), KCS2, BETA-KETOACYL REDUCTASE1 (KCR1), PASSICCINO2 (PAS2), and WSD1 are involved in wax biosynthesis.
Figure 2. Sequencing analysis of BoORP3a. a Phylogenetic tree of ORP3a-related proteins across the indicated cruciferous species. b Amino acid sequence and (c) gene structure analysis of the BoORP3a locus in the two inbred lines.

[1]. CERS and WHITE-BROWN COMPLEX HOMOLOG PROTEIN11 (WBC11) are ABC transporters and participate in wax transport with the lipid transfer protein LTPG [24–26]. In addition, MYB and APETALA2 (AP2)/ETHYLENE RESPONSE FACTOR (ERF) transcription factors have also been reported to directly or indirectly regulate wax biosynthesis [26, 27]. In contrast to wax biosynthesis, wax transport within the plasma membrane is not fully understood. Cuticular wax biosynthesis requires a large pool of lipids exported from epidermal cells to the plant surface [23]. The main two hypothetical mechanisms for transport of lipids from the ER to the plasma membrane are as follows: (i) Lipids move directly from the ER to the plasma membrane [28], and (ii) lipids travel to and through the Golgi apparatus from the ER and then move to the plasma membrane [29]. Therefore, the proteins involved in lipid transport between the ER, Golgi apparatus, and the plasma membrane may also relate to cuticular wax deposition.

Sterols play an essential part in plant growth and development and are a component of wax [4, 30]. Sterols are synthesized in the ER and are then rapidly transported via the Golgi to the plasma membrane [15]. OBPs and ORPs can bind various lipids and have multiple functions, such as sensing lipids and regulating cellular sterol distribution [13, 31]. For instance, PiORP1 may be involved in Pollen receptor-like kinase1 (PRK1) signaling during pollen development and growth in Petunia inflata, and this protein localizes to the plasma membrane of petunia pollen tubes [32]. However, little is known about the function of other plant ORPs. Arabidopsis ORP3a has been reported to cycle and transport sterols between the ER and the Golgi apparatus and might also transport other lipids [13, 33]. Therefore, we speculated that BoORP3a might...
Figure 3. Subcellular localization of BoORP3a. GFP, green fluorescent protein; 35S:eGFP, N. benthamiana leaves expressing eGFP alone; 35S:BoORP3a-eGFP, N. benthamiana leaves expressing BoORP3a-eGFP. White arrows indicate endoplasmic reticulum; green arrow indicates the nucleus. Scale bars = 50 μm.

Figure 4. Characterization of CRISPR/Cas9-induced orp3a mutants. a Representative images of wild-type (WT) and orp3a mutant plants. b PCR amplification of genomic DNA isolated from orp3a mutant plants with primers flanking the sgRNA target sites. c Lightness difference in WT and orp3a mutant plants. Data are means ± SD (n = 5). Asterisks indicate statistically significant differences (P < 0.01). d Schematic diagram of the BoORP3a locus and mutation analysis of the three orp3a mutants created by CRISPR/Cas9-mediated genome editing. Substitutions and deletions are highlighted in yellow.

function as a lipid transfer protein during wax transport. Unlike the gene mentioned above, the role of BoORP3a in wax deposition is largely unknown. Sequencing of BoORP3a from the two inbred lines revealed five amino acid substitutions in the low-wax line F0819 compared to the high-wax line S0835. ORP3a appeared relatively conserved among Brassicaceae. The Arabidopsis
Figure 5. Validation of a CAPS marker associated with variation in wax deposition. a Partial sequence alignment of BoORP3a with the polymorphism between S0835 and F0819 (red letter) resulting in the introduction of a NcoI restriction site in the F0819 inbred line. b Representative images of Chinese cabbage ‘Green Star 70’ and ornamental kale ‘Sunrise’. c Results of PCR amplicons from Green Star 70, Sunrise, S0835, and F0819 after NcoI digest. Two bands indicate that the amplified products were digested. d Lightness difference for Green Star 70 and Sunrise. Data are means ± SD (n = 5). Asterisks indicate statistically significant differences (P < 0.01).

Materials and methods

Plant materials and growth conditions

Seeds for the ornamental kale (B. oleracea var. acerifolia) high-wax (S0835) and low-wax (F0819) inbred lines (Fig. 1a) were obtained from the germplasm nursery of Shenyang Agricultural University and grown in a greenhouse in Shenyang, China, in 2020. The glossy and glaucous phenotypes could be observed visually at the rosette stage (about 2 months after sowing). At the rosette stage, 15 leaves of each inbred line were collected from five plants (three leaves per plant) for scanning electron microscopy (SEM) observations and determination of wax contents.

SEM observations

Leaf samples were cut into about 3 × 5-mm pieces. After being fixed in specimen holders, each tissue fragment was frozen in liquid nitrogen and coated with gold particles in a preparation chamber. The wax deposition and distribution patterns were observed from the leaf surfaces of the two inbred lines with a scanning electron microscope (Regulus 8100, Hitachi, Japan).

Determination of wax contents

The wax contents of leaf surfaces were evaluated with a colorimeter (CR-10 Plus, Konica Minolta, Japan). The L (lightness) index was used to evaluate the lightness change of the leaf surface as previously described [38]. The L values were measured before (L1) and after (L2) the gentle removal of cuticular wax and used to calculate the difference, ΔL = L1 – L2, which represents wax contents.
Five biological replicates were examined for each genotype. The data in Figures 1b, 4c, and 5d are presented as means ± standard deviation (SD). SPSS software (version 22.0, SPSS Inc., Chicago, IL, USA) was used to perform a one-way analysis of variance (ANOVA) and LSD, with P < 0.05 considered a significant difference.

Cloning and sequence analysis of BoORP3a
Genomic DNA extraction of all plant materials in this study was carried out using the cetyltrimethylammonium bromide (CTAB) method [39]. DNA integrity was analysed on 1% (w/v) agarose gels.

Primers specific for the BoORP3a gene (Bo9g184810) were designed according to the sequence obtained from the B. oleracea reference genome (http://plantsensembl.org/Brassica_oleracea/Info/Index) (Table S1, see online supplementary material). The BoORP3a gene was then amplified from the DNA extracted above. The amplification conditions included a 3-min denaturation at 98°C, followed by 35 cycles of 95°C for 10 s, 50°C for 30 s, and 72°C for 2 min, then 72°C for 7 min and hold at 12°C. The PCR products were cloned into the pEASY-Blunt cloning vector (Transgen Biotech, China) and transformed into Escherichia coli strain DH5α. Positive transformants were sequenced by TSINGKE Biotechnology Company (Beijing, China). Each positive clone was sequenced at least twice.

The deduced amino acid sequences and conserved domains for BoORP3a were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). Sequence alignment was performed with DNAMAN 6.0 software (Lynnon Biosoft, USA). Synteny information and homologous protein sequences of ORP3a among cruciferous species were obtained from the BRAD database (http://brassicadb.cn/) using BLAST and Syntenic Gene @ Subgenomes tools [40]. These sequences were used to construct a phylogenetic tree by the Maximum Likelihood method with the MEGA 7.0 software. The nonsynonymous substitution rate (Ka) and the nonsynonymous substitution rate (Ks) between paralogous gene pairs were calculated using the Kumar method implemented in MEGA 7.0 [41]. As a rule, neutral evolution is defined by a Ka/Ks value of one, a value higher than one indicates positive selection, while a value lower than one indicates purifying (negative) selection [42].

Subcellular localization of BoORP3a
Total RNA was extracted using RNAiso reagent (TaKaRa Shuzo Co. Ltd, Japan) according to the manufacturer’s instructions. RNA integrity and purity were assessed on 1% (w/v) agarose gels with a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). First-strand cDNA synthesis was conducted with the AMV First Strand cDNA Synthesis Kit (Shanghai Sangon Biotechnology Co., Ltd.).

The pCAMBIA1302-BoORP3a-eGFP expression vector was constructed using the subcellular localization primers to amplify the BoORP3a coding sequence (Table S1, see online supplementary material). The resulting plasmid was transformed into Agrobacterium (Agrobacterium tumefaciens) strain GV3101. Agrobacteria harboring the 3SS:eGFP or 35S:BoORP3a-eGFP plasmids were then infiltrated into the leaves of 30-day-old Nicotiana benthamiana plants. An endoplasmic reticulum-specific marker (ER-rk) was co-infiltrated with the BoORP3a-eGFP or eGFP constructs. N. benthamiana plants were then cultivated in the dark for 18 h, followed by return to light for 24 h. GFP fluorescence was observed at an excitation wavelength of 488 nm and an emission wavelength of 510 nm with a confocal microscope (Leica TCS SP82400301, Germany) as described previously [43].

Vector construction and genetic transformation
The knockout in BoORP3a was generated by CRISPR/Cas9-mediated genome editing as described previously [44]. Sequence-specific single guide RNAs (sgRNAs) were selected according to an online tool (http://crispor.tefor.net/), and the corresponding primers were synthesized (Table S1, see online supplementary material) and introduced into the pCBC-DT1T2 vector to produce a sgRNA expression cassette. The sgRNA expression cassette was cloned into the pHSE401 expression vector using the restriction enzyme BsaI and T4 DNA Ligase. The resulting plasmid was introduced into Agrobacterium strain GV3101.

The genetic transformation of the high-wax line (S0835) was performed as described previously [45]. The S0835 seeds were sterilized and sown on Murashige and Skoog (MS) medium (pH 5.8) for 4–5 d and then cotyledons were cultivated in pre-cultivation medium [MS + 1.0 mg/L 6-benzylaminopurine (6-BA) + 0.1 mg/L 1-naphthaleneacetic acid (NAA)] + 200 μmol/L acetoxyringone (AS), pH = 5.8] for 2 d in the dark. The cotyledons were subsequently infected with Agrobacteria cell suspensions (OD600 = 0.8) for 5 min and then transferred to co-cultivation medium [MS + 1.0 mg/L 6-benzylaminopurine (6-BA) + 0.1 mg/L 1-naphthaleneacetic acid (NAA)] + 200 μmol/L acetoxyringone (AS), pH = 5.8] for 2 d. Afterwards, the transformed cotyledons were transferred to a delay culture medium (MS + 1.0 mg/L 6-BA + 0.1 mg/L NAA + 300 mg/L cefotaxime, pH = 5.8) and selection medium (MS + 1.0 mg/L 6-BA + 0.1 mg/L NAA + 10 mg/L kanamycin + 300 mg/L cefotaxime, pH = 5.8) in turn under a 16-h light/8-h dark photoperiod. After about 3 weeks, resistant buds were excised and transferred to the rooting culture medium (MS + 0.1 mg/L NAA + 15 mg/L kanamycin + 300 mg/L cefotaxime, pH = 5.8) to obtain transgenic ornamental kale plants (Fig. S1, see online supplementary material).

The transgenic plants were confirmed by comparing gene sequences and phenotypes. Genomic DNA from individual transgenic plantlets was extracted by the CTAB protocol [39]. ORP3a-CRISPR-test-F/R-specific primers flanking the target sites sequence (Table S1, see online supplementary material) were designed to amplify a fragment of about 400 bp. The amplicons were cloned and sequenced as described above.

Design of a Cleaved Amplified Polymorphic Sequence (CAPS) marker
To verify that BoORP3a is associated with cuticular wax deposition, a CAPS marker was designed based on the detected variation between the two inbred lines (Fig. 2c and 5a). The fragments were amplified with the primer pair CAPS-F, 5'-ACGTCTACGTCCTGATAGATAC-3' and CAPS-R, 5'-TTAAGGAAGG TATCTTTGGAAC-3' (annealing temperature 54°C). The amplicons were digested with NcoI enzyme for 30 min at 37°C before being separated on a 2% (w/v) agarose gel. The ornamental kale cultivar Sunrise (high-wax contents) and the Chinese cabbage (B. rapa spp. pekinensis) cultivar Green Star 70 (wax-free) were used to test the CAPS marker (Fig. 5b and d).

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Author contributions
S.Z.: conceptionalization, investigation, writing – original draft. F.Z. and Z.L.: conceptualization, investigation, formal analysis, writing – review and editing. X.F. and Y.L.: investigation, software. P.Z.: funding acquisition, project administration.

Data availability
The data that support the results are included in this article and its supplementary materials. Other relevant materials are available from the corresponding author upon reasonable request.

Conflict of interest
The authors declare that there is no conflict of interests regarding the publication of this article.

Supplementary data
Supplementary data is available at Horticulture Research online.

References
1. Lewandowska M, Keyl A, Feussner I. Wax biosynthesis in response to danger: its regulation upon abiotic and biotic stress. New Phytol. 2020;227:698–713.
2. Liu N, Chen J, Wang T et al. Overexpression of WAX INDUCER1/SHINE1 gene enhances wax accumulation under osmotic stress and oil synthesis in Brassica napus. Int J Mol Sci. 2019;20:4435.
3. Liu ZZ, Fang Z, Zhuang M et al. Fine-mapping and analysis of Cgl1, a gene conferring glossy trait in cabbage (Brassica oleracea L. var. capitata). Plant Sci. 2017;261:239.
4. Shaheenuzzamm M, Wang Z. Research advances on cuticular waxes biosynthesis in crops: a review. Int J Agric Biol. 2019;21:911.
5. Nguyen VNT, Lee SB, Suh MC et al. OsABCG9 is an important ABC transporter of cuticular wax deposition in rice. Front Plant Sci. 2018;9:960.
6. Lee SB, Jung SJ, Go YS et al. Two Arabidopsis 3-ketoacyl CoA synthase genes, KCS20 and KCS2/DAISY, are functionally redundant in cuticular wax and root suberin biosynthesis, but differentially controlled by osmotic stress. Plant J. 2009;60:462–75.
7. Park CS, Go YS, Suh MC. Cuticular wax biosynthesis is positively regulated by WRINKLED4, an AP2/ERF-type transcription factor, in Arabidopsis stems. Plant J. 2016;88:257–70.
8. Bernard A, Joubes J. Arabidopsis cuticular waxes: advances in synthesis, export and regulation. Prog Lipid Res. 2013;52:110–29.
9. Zhang F, Wang R, Yang X et al. The ZnR3-MYB transcription factor AtMYB49 modulates salt tolerance in Arabidopsis by modulating the cuticle formation and antioxidative defence. Plant Cell Environment. 2020;43:1925–43.
10. Debono A, Yeats TH, Rose JKC et al. Arabidopsis LTPG is a glycosylphosphatidylinositol-anchored lipid transfer protein required for export of lipids to the plant surface. Plant Cell. 2009;21:1230–8.
11. Umate P. Oxysterol binding proteins (OSBPs) and their encoding genes in arabidopsis and rice. Steroids. 2011;76:524–9.
12. Peng L, Kawagoe Y, Hogan P, Delmer D. Sitosterol-beta-glucoside as primer for cellulose synthesis in plants. Science. 2002;295:147–50.
13. Saravanan RS, Slabaugh E, Singh VR et al. The targeting of the oxysterol-binding protein ORP3a to the endoplasmic reticulum relies on the plant VAP39 homolog PVA12. Plant J. 2009;58:817–30.
14. Pietrangelo A, Ridgway ND. Bridging the molecular and biological functions of the oxysterol-binding protein family. Cell Mol Life Sci. 2018;75:3079–98.
15. Boutté Y, Grebe M. Cellular processes relying on sterol function in plants. Curr Opin Plant Biol. 2009;12:705–13.
16. Feng X, Li X, Yang X, Zhu P. Fine mapping and identification of the leaf shape gene BoFL in ornamental kale. Theor Appl Genet. 2020;133:1303–12.
17. Reda T, Thavarajah P, Polomski R et al. Reaching the highest shelf: a review of organic production, nutritional quality, and shelf life of kale (Brassica oleracea var. acephala). Plants, People, Planet. 2021;3:308–18.
18. Chai YF, Li A, Chit Wai S et al. Cuticular wax composition changes of 10 apple cultivars during postharvest storage. Food Chem. 2020;324:126903.
19. Liu DM, Tang J, Liu Z et al. Fine mapping of BoGL1, a gene controlling the glossy green trait in cabbage (Brassica oleracea L. var. capitata). Mol Breed. 2017;37:69.
20. Ji JL, Cao W, Tong L et al. Identification and validation of an ECERIFERUM2-LIKE gene controlling cuticular wax biosynthesis in cabbage (Brassica oleracea L. var. capitata L.). Theor Appl Genet. 2021;134:4055–66.
21. Yang YQ, Pu Y, Yin X et al. A splice variant of BrrWSD1 in turnip (Brassica rapa var. rapa) and its possible role in wax ester synthesis under drought stress. J Agric Food Chem. 2019;67:11077–88.
22. Suh MC, Go YS. DEWAX-mediated transcriptional repression of cuticular wax biosynthesis in Arabidopsis thaliana. Plant Signal Behav. 2014;9:1666–80.
23. Laila R, Robin AHK, Yang K et al. Developmental and genotypic variation in leaf wax content and composition, and in expression of wax biosynthetic genes in Brassica oleracea var. capitata. Front Plant Sci. 2017;7:1972.
24. Pighin JA, Zheng H, Balakshin LJ et al. Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant J. 2010;52:485–98.
25. See PJ, Lee SB, Suh MC et al. The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in Arabidopsis. Plant Cell. 2011;23:1138–52.
26. Abaroni A, Dixit S, Jetter R et al. The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. Plant Cell. 2004;16:2463–80.
27. Samuels L, McFarlane HE. Plant cell wall secretion and lipid traffic at membrane contact sites of the cell cortex. Protoplasma. 2012;249:19–23.
28. McFarlane HE, Watanabe Y, Yang W et al. Golgi- and trans-Golgi network-mediated vesicle trafficking is required for wax secretion from epidermal cells. Plant Physiol. 2014;164:1250–60.
29. Li J. Brassinosteroids signal through two receptor-like kinases. Curr Opin Plant Biol. 2003;6:494–9.
30. Yan D, Ollkonen VM. Characteristics of oxysterol binding proteins. Int Rev Cytol. 2008;265:253–85.
31. Skirpan AL, Dowd PE, Sjajic P et al. Identification and characterization of PiORP1, a Petunia oxysterol-binding-protein related protein involved in receptor-kinase mediated signaling in pollen, and analysis of the ORP gene family in Arabidopsis. Plant Mol Biol. 2006;61:553–65.
32. Im YJ, Raychaudhuri S, Prinz WA, Hurley JH. Structural mechanism for sterol sensing and transport by OSBP-related proteins. Nature. 2005;437:154–8.
34. Lee S, Wang PY, Jeong Y et al. Sterol-dependent nuclear import of ORP1S promotes LXR regulated trans-activation of apoE. Exp Cell Res. 2012;318:2128–42.
35. Murovec J, Guček K, Bohanec B et al. DNA-free genome editing of Brassica oleracea and B. rapa protoplasts using CRISPR-Cas9 ribonucleoprotein complexes. Front Plant Sci. 2018;9:1594.
36. Sun B, Jiang M, Zheng H et al. Color-related chlorophyll and carotenoid concentrations of Chinese kale can be altered through CRISPR/Cas9 targeted editing of the carotenoid isomerase gene BoaCRTISO. Hortic Res. 2020;7:161.
37. Sun Q, Lin L, Liu D et al. CRISPR/Cas9-mediated multiplex genome editing of the BrwRKY11 and BrwRKY70 genes in Brassica napus L. Int J Mol Sci. 2018;19:2716.
38. Liu Q, WEI M, SHEN Q et al. Effects of different rootstocks on bloom formation and absorption and distribution of silicon in grafted cucumber. Acta Hortic. 2012;897:897–904.
39. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980;8:4321–6.
40. Chen HX et al. BRAD V3.0: an upgraded Brassicaceae database. Nucleic Acids Res. 2022;50:D1432–41.
41. Tamura K, Stecher G, Peterson D et al. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.
42. Xu LL, Xin Q, Mingyue Z, Shaoling Z. Genome-wide analysis of aluminum-activated malate transporter family genes in six rosaceae species, and expression analysis and functional characterization on malate accumulation in Chinese white pear. Plant Sci. 2018;24:431–42.
43. Deng CP, Shi M, Fu R et al. ABA-responsive transcription factor bZIP1 is involved in modulating biosynthesis of phenolic acids and tanshinones in salvia miltiorrhiza. J Exp Bot. 2020;71:5948–62.
44. Ma X, Zhang Q, Zhu Q et al. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant. 2015;8:1274–84.
45. Feng X, Zhang Y, Wang H et al. The dihydroflavonol 4-reductase BoDFR1 drives anthocyanin accumulation in pink-leaved ornamental kale. Theor Appl Genet. 2021;134:159–69.