

Brape1 regulates male fertility by influencing the tapetum and pollen wall in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Research Article

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Abstract

Key Message The mutation of *Brape1* causes male sterility in Chinese cabbage and has been validated by RNAi.

The male-sterile line of Chinese cabbage represents an ideal resource to resolve challenges in hybrid seed production by exploiting heterosis. However, the molecular basis for the relationship between the tapetum, pollen wall and male sterility in Chinese cabbage has not been fully elucidated. Here, a male-sterile mutant, *M5007*, was obtained from the Chinese cabbage 'FT' via ethyl methanesulfonate mutagenesis. The tapetum in *M5007* degraded prematurely, with pollens exhibiting an abnormal concave morphology and a defective exine structure. MutMap sequencing and Kompetitive Allele-Specific PCR sequencing identified *BraA09g013200.3.5C* as the candidate gene, encoding an acyl-CoA synthetase, involved in pollen exine formation. According to the abnormal pollen exine in *M5007*, the gene was named *Brape1*. BrAPE1 and Brape1 were subcellularly localized to the endoplasmic reticulum. qRT-PCR revealed that *Brape1* was specifically expressed in buds, but not in flowers, leaves, roots, or stems. The activity of acyl-CoA synthetase was significantly attenuated in *M5007* relative to 'FT'. RNA interference for *Brape1* resulted male sterility, with a significant reduction in *Brape1* expression levels. Yeast two-hybrid screening of BrAPE1 identified two interacting proteins, BrRGP1 and BrRGP2. The interaction between BrAPE1 and BrRGP1/BrRGP2 was validated through yeast two hybrid screening, luciferase complementation imaging, and pull-down experiments. Transcriptome analysis revealed that the *Brape1* mutation affected genes associated with tapetum development and meiosis, while the absence of pollen likely influenced pollen tube gene expression. This study provided new insights into the regulatory mechanisms of male sterility in Chinese cabbage and offered valuable genetic resources for hybrid breeding in Chinese cabbage.

Introduction

Male sterility in plants results from abnormal development of male organs or pollen, leading to an inability to produce functional pollen. This phenomenon plays a pivotal role in hybrid breeding. The utilization of male sterile lines facilitated hybrid breeding efficiency, thereby contributing to the maintenance or enhancement of crop genetic diversity (Duvick 2005). By exploiting male sterility, it is possible to produce hybrid crops with heterosis on a large scale, thereby improving yield, disease resistance, and adaptability. Studies on male sterility in plants have not only contributed to a deeper understanding of the regulatory mechanisms of plant reproduction, but also provided a rich reference of genetic resources and innovative technological tools for modern agricultural breeding.

The tapetum is located around the microsporocytes, providing nutritional support and essential materials for pollen grain development, while also contributing to pollen wall formation. Aberrations in tapetal function often led to disruptions in pollen development, leading to male sterility (Wu et al. 2000). Mutations in the promoter region of the rice *SDGMS* gene led to aberrant programmed cell death (PCD) in tapetal cells, resulting in dominant male sterility (Xu et al. 2023). Overexpression of *SiOASTL1* in

sesame delayed tapetum PCD, also resulting in male sterility (Zhou et al. 2024). Abnormal tapetum development has been reported to cause male sterility in multiple species. A male sterility-associated gene, *CIATM1*, was cloned from watermelon, and its mutation led to abnormal tapetum development and subsequent male sterility (Zhang et al. 2021). Abnormal tapetum development was observed in the tomato male sterile mutant *ms32*, caused by a mutation in the *Solyc01g081100* gene (Liu et al. 2019). Research on *Arabidopsis* has identified multiple crucial genes involved in tapetum development, including *MS1* and *DYT*, that modulate tapetum cell differentiation and lipid transport via transcriptional regulatory networks (Paxson-Sowders et al. 2001; Wei et al. 2006). The *Bnms3* mutant of *Brassica napus* exhibited anther developmental defects, specifically including impaired transition of the tapetum to a secretory type, reduced callase enzyme activity, and abnormal pollen wall biosynthesis (Zhou et al. 2012). In cabbage, the abnormal degradation of the tapetum in the *Ms-cd1* dominant genic male-sterile *DGMS01-20* plants prevented the degradation of the pollen mother cell wall, leading to the failure of microspore release and ultimately affecting normal pollen wall formation (Han et al. 2023). Multiple male sterile mutants were obtained from Chinese cabbage through ethyl methanesulfonate (EMS) mutagenesis, and these mutants exhibited abnormal tapetum development (Dong et al. 2022; Xu et al. 2024; Zhao et al. 2022; Zou et al. 2023). To date, the molecular mechanisms governing tapetum development and male sterility in Chinese cabbage have not been fully elucidated.

Reversibly glycosylated polypeptides (RGPs), a class of highly conserved, plant-specific proteins. RGPs in rice were found to be involved in the interconversion between UDP-L-arabinopyranose (UDP-Arap) and UDP-L-arabinofuranose (UDP-Araf) (Konishi et al. 2007). L-arabinose (L-Ara) is a component of the cell wall, which includes pectin I and II, extensins, and arabinogalactan proteins. The *RGP* gene in cotton was highly expressed not only during fiber development but also during the stages of primary cell wall elongation and secondary cell wall thickening (Zhao and Liu 2002). The *rgp1* and *rgp2* double mutants in *Arabidopsis* resulted in male gametophyte lethality, while plants with single gene disruptions showed no obvious phenotype, indicating that the *RGP* family is functionally redundant. The double mutants exhibited pollen defects and abnormal pollen morphology. The mutant pollen grains displayed aberrant vacuoles and irregular cell membranes, leading to the disintegration of the pollen structure during the first stage of pollen meiosis (meiosis I) (Drakakaki et al. 2006). RGP is present in both dicotyledonous and monocotyledonous plants, but absent in non-plant species, indicating that it is a plant-specific functional protein.

Sporopollenin, the primary component of the outer walls of plant spores and pollen, exhibited unique chemical inertness and a complex structure, providing a protective barrier against environmental stresses and contributing to species-specific surface ornamentation (Blackmore et al. 2007). In recent years, several key enzymes involved in sporopollenin biosynthesis had been identified, with acyl-CoA synthetase (ACOS) family members playing a central role in activating fatty acid precursors (de Azevedo Souza et al. 2009). In *Arabidopsis*, *ACOS5* functioned as a crucial enzyme in this pathway, catalyzing the activation of medium- and long-chain fatty acids into acyl-CoA derivatives, which served as substrates for polyketide synthases and tetraketide α -pyrone reductases (Kim et al. 2010). *ACOS5* localized to the endoplasmic reticulum of tapetal cells and formed a metabolic complex with PKSB and TKPR1 to

regulate sporopollenin biosynthesis (Lallemant et al. 2013). The *acos5* mutant exhibited abnormal tapetal development, defective sporopollenin deposition, complete loss of the pollen exine, and male sterility (de Azevedo Souza et al. 2009). The function of ACOS appeared highly conserved across plants, as *Oryza sativa OsACOS12*, sharing 63.9% amino acid identity with *Arabidopsis ACOS5*, was essential for sporopollenin precursor synthesis, and its mutation resulted in pollen wall defects and complete male sterility (Li et al. 2016). Furthermore, *OsACOS12* expression driven by its native promoter partially restored fertility in *Arabidopsis acos5* mutants, suggesting functional conservation of ACOS between monocots and dicots (Li et al. 2016).

This study isolated a male sterile mutant, *M5007*, and characterized the function of the causal gene *Brape1* (named for its phenotype of abnormal pollen exine). The tapetum of *M5007* degraded prematurely, and the pollens exhibited abnormal concave morphology and defective outer wall structures. Based on MutMap sequencing and Kompetitive Allele-Specific PCR analysis, *Brape1* was identified as the candidate gene encoding an acyl-CoA synthetase expressed in the tapetum. *Brape1*-RNA interference (RNAi) plant exhibited male sterility. *Brape1* was localized to the endoplasmic reticulum and was specifically expressed in buds. The activity of acyl-CoA synthetase was significantly attenuated in *M5007* relative to 'FT'. The interaction between BrAPE1 and BrRGP1/BrRGP2 was validated through yeast two-hybrid assays, luciferase complementation imaging, and pull-down experiments. Transcriptome analysis revealed that the *Brape1* mutation led to lower expression levels of genes associated with pollen tube development, meiosis, and the tapetum. This study highlighted the essential role of *Brape1* in tapetum development, providing new insights into the molecular mechanisms of pollen wall formation during Chinese cabbage anther development.

Materials and methods

Plant materials

Double haploid 'FT' of Chinese cabbage derived from isolated microspore culture (Huang et al. 2016), was used as the wild-type. The male-sterile mutant *M5007* was generated by EMS mutagenesis of 'FT'. F₁ and F₂ populations were developed using 'FT' and *M5007* for genetic analysis. The F₂ population was used for gene mapping and KASP genotyping.

Pollen viability assay

Pollen from 'FT' and *M5007* were placed on microscope slides for examination. A 50 µL drop of 0.2% 2,3,5-triphenyltetrazolium chloride was applied to the pollen, a coverslip was added, and the slides were allowed to stand for 30 minutes. Observation was performed using a fluorescent inverted microscope (OLYMPUS DP80, Tokyo, Japan).

Observation of floral organs

During the reproductive growth stage of 'FT' and *M5007*, floral organs (buds, sepals short, stamens, petals, long stamens, and pistils) were dissected using forceps and observed under a stereomicroscope (Nikon SMZ800, Japan).

Scanning electron microscopy (SEM) observation

Anthers from 'FT' and *M5007* were dissected and placed in a vial containing a 1:1 mixture of 1% osmium tetroxide and 2.5% glutaraldehyde. After vacuum infiltration, the anthers were completely submerged in the fixative and stored at 4°C. The anthers were then rinsed in 0.2 M phosphate buffer for 15 min, followed by passage through a graded ethanol series (50%, 70%, 80%, 90%, and 100%). After dehydration in a graded tert-butanol series (50%, 70%, 80%, 90%, and 100%), the samples were mounted on conductive double-sided adhesive tape and sputter-coated with gold. The morphology of the microspores was observed using a SEM (Hitachi Regulus8100, Hitachi, Tokyo, Japan).

Paraffin section observation

Buds of 'FT' and *M5007* were fixed in formalin-acetic acid-alcohol for 12 h, followed by dehydration through a graded ethanol series (50–100%). After clearing in a xylene-ethanol series (3:1, 1:1, 1:3), buds were soaked in paraffin-xylene mixtures and encased in paraffin wax. Sections were cut using a microtome (Leica RM2016, Germany). The anther sections were stained with safranin and fast green, and then observed under an optical microscope (ECLIPSE 80i, Nikon). The specific procedures were based on prior methodologies (Tan et al. 2019).

Transmission electron microscope (TEM) observation

An equal volume mixture of paraformaldehyde and glutaraldehyde was used to fix the buds. The samples were then transferred to osmium tetroxide fixative for 7 hours of fixation. Subsequently, dehydration was performed by immersing the samples in a graded series of ethanol solutions, followed by a final dehydration step in acetone. Next, the samples were infiltrated with a mixture of acetone and 812 embedding medium at 37°C, then placed in embedding molds and left overnight in a 37°C oven. Finally, the molds were transferred to a 60°C oven for polymerization over 48 hours, after which the resin blocks were ready for use. Ultrathin sections (~ 60–80 nm thick) were prepared and collected on 150-mesh copper grids. The sections were then stained and observed under a transmission electron microscope (Hitachi HT7800) for image acquisition. Specific TEM operations were conducted following established methods from previous studies (Ji et al. 2017).

Genetic analysis

Genetic analysis was performed using F_1 and F_2 populations derived from a cross between 'FT' and *M5007*. The numbers of male fertile and sterile plants in both the F_1 and F_2 generations were recorded

and statistically analyzed. The chi-square (χ^2) test was employed to analyze the segregation ratio in the F_2 population.

MutMap analysis

DNA from the parental line 'FT' was extracted as the wild-type parental pool. DNA samples from 58 F_2 generation male-sterile plants were mixed equally to construct the male-sterile bulk. After quality control, the DNA was re-sequenced using the NovaSeq 6000 platform (Illumina, San Diego, CA, USA). The raw data were filtered using fastp (v0.20.0) to obtain high-quality reads. Subsequently, the filtered data were aligned to the reference genome using Burrows-Wheeler Aligner (0.7.12-r1039) (Li and Durbin 2010). The GATK toolkit was employed to extract INDELs and SNPs (McKenna et al. 2010). The SNP-index distribution along the chromosomes of Chinese cabbage was plotted using a sliding window approach. The 95th percentile was chosen to define the candidate region, and gene functional annotation within these regions was conducted with ANNOVAR (Wang et al. 2010).

KASP genotyping

Two 'FT' plants and 72 F_2 generation male-sterile plants were selected for KASP analysis to further screen candidate genes. SNPs within the candidate interval were chosen for primer design, and the thermal cycling was established according to a previous study (Xi et al. 2018).

Cloning and sequence analysis of the candidate gene

Primers were designed based on the reference genome sequence to amplify the full-length and cDNA of the candidate gene in 'FT' and the male-sterile mutant *M5007*. The experimental procedure was conducted according to previously established methods (Liu et al. 2021).

Sequence alignment and phylogenetic analysis of the BrAPE1 protein

The SMART database was employed to identify protein domains within the BrAPE1 amino acid sequence. SwissModel (Waterhouse et al. 2018) was employed to predict the three-dimensional structure of BrAPE1. A phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates in MEGA6.0 software (Tamura et al. 2013). Multiple sequence alignment of protein sequences was performed using DNAMAN v6.0 (Lynnon Biosoft, San Ramon, CA, USA).

Subcellular localization of BrAPE1 and Brape1

The BrAPE1 and Brape1 cDNA were amplified by PCR and cloned into the pCAMV35S vector carrying GFP, and subsequently verified by sequencing. The resulting construct was introduced into *Agrobacterium tumefaciens* GV3101. 20 microliters of AS (acetosyringone) solution (50 mM) and 1 mL of *Agrobacterium* culture containing the vector were added to 100 mL of liquid LB medium supplemented with kanamycin and rifampicin. The culture was incubated in the dark at 28°C with

shaking at 200 rpm/min. When the OD600 reached 0.5–0.6, the *Agrobacterium* culture was collected and centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in an infiltration solution containing 10 mM MgCl₂, 10 mM MES, and 15 μL AS (pH = 5.6). The OD600 of the suspension was adjusted to approximately 0.8 and left to stand at room temperature for 2–3 h. A 1 mL syringe with the needle removed was used to infiltrate the bacterial suspension into the underside of tobacco leaves, allowing the liquid to spread throughout the entire leaf. Confocal laser scanning microscopy (Leica Microsystems, Wetzlar, Germany) was employed to visualize the fluorescence signals in tobacco leaf tissues after 24 hours of dark and light treatments. GFP fluorescence was detected at 496–540 nm.

qRT-PCR

RNA was extracted from the flowers, leaves, roots, buds, and stems of 'FT' and *M5007*, and subsequently reverse-transcribed into cDNA. The relative expression levels of target genes were determined by qRT-PCR, with *actin* (*BraA10g027990.3C*) as an internal control. The $2^{-\Delta\Delta C_t}$ method was used for data analysis (Livak and Schmittgen 2001).

Enzyme activity analysis

The activities of acyl-CoA synthetase were measured with the plant ELISA Kit. Anthers (1 g) from both *M5007* and 'FT' were homogenized in pre-chilled phosphate-buffered saline (PBS; pH 7.2). The homogenate was then centrifuged at 12,000 × g for 10 min at 4°C to obtain the supernatant for subsequent analysis. Supernatants were processed following the manufacturer's protocol and the absorbance was measured at 450 nm.

Construction of the *Brape1* RNAi vector and transformation into Chinese cabbage

Cloned the 257 bp region (1299–1555 bp) of the *Brape1* coding sequence and ligated it into the pBWA(V)BS vector using the ClonExpress II One Step Cloning Kit (Vazyme, Jiangsu, China). Within the T-DNA region of the vector, a double-stranded RNA (dsRNA) expression module is designed, typically constructed as a hairpin RNA structure. This structure is composed of the sense and antisense strands of the target gene, with a short intron inserted between them to enhance the formation of the hairpin RNA and the RNAi effect. Subsequently, Chinese cabbage 'FT' was transformed via *Agrobacterium*-mediated transformation, as previously described (Su et al. 2021).

Determination of UDP-arabinose mutase (UAM) activity

Solutions containing 20 ng of 'FT' and *M5007* UAM proteins were reacted with 2 mM UDP-arabinofuranose or 2 mM UDP-arabinopyranose in 50 mM Mes/KOH buffer (pH 6.5, 10 mL), containing 5 mM MnCl₂ and 10 mM 2-aminobenzamide-labeled arabinoheptaose, at 25°C for 30 min. The reaction was terminated by heating at 100°C for 5 min, followed by centrifugation to collect the supernatant. The products were analyzed using normal-phase high-performance liquid chromatography coupled with fluorescence detection (excitation wavelength: 330 nm, emission wavelength: 420 nm), as described in the literature (Konishi et al. 2006).

Transcriptome analysis

RNA was extracted from 'FT' and *M5007* inflorescences, with three biological replicates. The quality of RNA samples was determined by Qubit 2.0 fluorometry. The insert size distribution was evaluated using a Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The library concentration was precisely quantified using qRT-PCR to ensure library quality. Following quality assessment, the libraries were pooled according to the required sequencing depth for Illumina sequencing. Subsequently, the raw data were processed according to previously established protocols (Yan et al. 2013). HISAT2 was employed to map the filtered reads to the reference genome, resulting in a precise alignment (Mortazavi et al. 2008). Reads aligning to the start and end regions of each gene on the reference genome were counted to quantify gene expression. Low-quality reads (mapping quality < 10), unpaired reads, and multi-mapping reads were removed. The featureCounts tool from the subread software package was used for this analysis (Liao et al. 2014). To identify genes whose expression levels changed significantly under different conditions, analysis of DEGs was conducted. Subsequently, KEGG pathway enrichment analysis was conducted on the DEGs using clusterProfiler (G et al. 2012).

Y2H library screening

After constructing the bait vector, it was transformed into Y2HGold competent yeast cells for self-activation and toxicity testing. The Y2H library screening was performed using the mating method. The detailed procedures followed the instructions provided in the GAL4-based Y2H screening kit (mating method), product code YH2000 (Clontech, Mountain View, USA).

Y2H interaction validation

Using the ClonExpress II One Step Cloning Kit (Vazyme, Jiangsu, China), BrAPE1 was ligated into pGBKT7 using the EcoRI and Sall restriction enzymes to generate pGBKT7-BrAPE1, while BrRGP1 and BrRGP2 were ligated into pGADT7 using the EcoRI and BamHI restriction enzymes to obtain pGADT7-BrRGP1 and pGADT7-BrRGP2, respectively. The pGBKT7-BrAPE1 construct was co-transformed with pGADT7-BrRGP1 and pGADT7-BrRGP2 into Y2H competent cells, separately. Positive controls included pGADT7-T and pGBKT7-53, while negative controls consisted of pGADT7-T and pGBKT7-Lam. The transformation products were plated on DDO plates, and individual colonies were cultured in SD/Leu/Trp liquid medium. After shaking culture, the cells were collected and resuspended in 0.9% NaCl. Serial dilutions were spotted at four gradients onto DDO and QDO/X plates. The plates were incubated at 30°C for 3–5 days, and colony growth patterns were observed.

Luciferase complementation imaging assays

Using the ClonExpress II One Step Cloning Kit (Vazyme, Jiangsu, China), the CDS of BrAPE1 was ligated into the pCambia1300-nLUC vector using the KpnI and Sall restriction enzymes, while the CDS of BrRGP1 and BrRGP2 were cloned into the pCambia1200-cLUC vector using the KpnI and PstI restriction enzymes. These constructs were co-expressed in tobacco leaves via agroinfiltration. After a 24-hour dark treatment, the leaves were exposed to light for 24 hours. Luciferase activity was then measured using a

NightShade LB 985 system (Berthold Technologies, Bad Wildbad, Germany) after treatment with potassium luciferin.

GST pull-down assay

The CDS of BrAPE1 was cloned into the pGEX-4T vector, while the CDS of BrRGP1 and BrRGP2 were cloned into the PET28a vector. The *Escherichia coli* BL21 expression system was used to produce and purify GST, GST-BrAPE1, His-BrRGP1, and His-BrRGP2. Subsequently, pull-down assays were performed, and both input and pull-down samples were analyzed using western blotting. The detailed experimental procedures were adapted from a previous study (Li et al. 2017).

Results

Creation of the male sterile mutant M5007 in Chinese Cabbage

Ethyl methanesulfonate (EMS) mutagenesis of Chinese cabbage cultivar 'FT' resulted in the creation of a male sterile mutant, *M5007*. Phenotypic observations conducted during the full flowering stage revealed that *M5007* exhibited stamen abortion (Fig. 1a). Compared with 'FT', mutant *M5007* had no pollen grains on the surface of the anthers (Fig. 1b). 'FT' produced viable pollen, whereas *M5007* did not (Fig. 1c). *M5007* had shorter long stamens, short stamens, buds and petals than 'FT' (Fig. 1d-g, i), with no difference observed in pistils and sepals (Fig. 1h) (Table S1).

Genetic analysis was conducted using F_1 and F_2 populations. All F_1 plants exhibited male fertility. In the F_2 population, 178 were male sterile and 527 plants were male fertile, resulting in a ratio of 3:1 ($\chi^2 = 0.02$) (Table S2). These results indicated that the male sterility phenotype of *M5007* was controlled by a single recessive nuclear gene.

Premature degradation of the tapetum and abnormal exine structure of microspores resulted in male sterility in M5007

Paraffin sections were used to observe the anthers of *M5007* and 'FT', clarifying the male sterility characteristics from a cytological perspective. Using the *Arabidopsis* anther development classification system as a reference (Sanders et al. 1999), five developmental stages were selected for analysis in 'FT' and *M5007*. There were no differences between 'FT' and *M5007* in anther developmental stages S7 and S8 (Fig. 2a1, b1, a2, b2). At stage S9, tapetal cells in wild-type anthers were arranged neatly and regularly, with normal morphology, while tapetal cells in mutant *M5007* began to abnormally disintegrate (Fig. 2c1, c2). During stages S10 and S11, tapetal degeneration continued in *M5007*, and the wild-type microspores had clear contours and developed normally, whereas the microspores in *M5007* exhibited abnormal morphology (Fig. 2d1, d2). At stage S12, the wild-type anther locule dehisced, and pollen grains developed normally, while the anther locule of *M5007* was abnormally crescent-shaped, and no mature pollen grains were formed (Fig. 2e1, e2).

Scanning electron microscopy (SEM) revealed that pollens of 'FT' were spherical and had intact reticulate exines (Fig. 2f), while those of *M5007* were abnormally concave with aberrant exine structures (Fig. 2g). Transmission electron microscopy (TEM) further confirmed these observations. In 'FT', the pollens possessed intact exines with distinct columellae and tectums (Fig. 2h1, h2), whereas in *M5007*, the exine structure was abnormal with disorganized bacula and tectums, and the nexine exhibited disorganization (Fig. 2i1, i2). Collectively, the male sterility in mutant *M5007* was attributed to premature degradation of the tapetum and abnormal pollen exine development.

Identification of the male sterility mutant gene

A modified MutMap approach was employed to identify candidate gene. After filtering the sequencing data, a total of 175 935 758 (95.25%) and 179 301 404 (95.87%) high-quality reads were generated from pooled DNA of 'FT' and F₂ male-sterile plants, respectively. Of these reads, 99.04% and 99.21% were mapped to the reference genome. A total of 733 454 SNPs were detected and filtered. Subsequently, the SNP-index was calculated, and their chromosomal distribution was visualized (Fig. 3a). A candidate region associated with male sterility in *M5007* was identified by targeting an interval with a SNP-index > 95th percentile, which was located on chromosome A09 between 8 880 000 and 12 240 000 bp (Fig. 3a). Within this candidate region, 57 SNPs were identified, and two SNPs with an SNP-index of 1 were located in the exons of genes, namely SNP 7,454,274 and SNP 7,929,510. SNP 7,454,274 was a non-synonymous mutation, while SNP 7,929,510 was a synonymous mutation.

Kompetitive Allele-Specific PCR (KASP) genotyping was conducted on two 'FT' plants and 72 F₂ male sterile plants to validate the candidate SNPs. SNP 7,454,274 co-segregated with the mutant phenotype, with all 'FT' plants having the G:G genotype and all mutant plants having the A:A genotype. For SNP 7,929,510, one G:A genotype was identified in the sterile plants (Fig. 3b). SNP 7,454,274 was located in the gene *BraA09g013200.3.5C*, whose *Arabidopsis* homolog is *AT1G62940*, encoding an acyl-CoA synthetase. This gene is expressed in the tapetum and is involved in the formation of the pollen exine (de Azevedo Souza et al. 2009).

Cloning results of *BraA09g013200.3.5C* indicated that its full-length cDNA is 7 269 bp, containing 14 exons (Fig. 3c). A SNP at position 4 007 resulted in a nonsynonymous substitution, changing glycine (Gly) to aspartic (Asn) acid in the encoded protein (Fig. 3d). Therefore, *BraA09g013200.3.5C* was predicted to be the candidate gene for male sterility in mutant *M5007*, and was named *Brape1* because of the abnormal pollen exine phenotype in *M5007*.

Structural and phylogenetic analysis of BrAPE1

BrAPE1 is a 1 482-amino acid protein with a predicted molecular weight of 162.5 kDa and a theoretical isoelectric point of 4.36. It contains two transmembrane domains (Fig. 4a). Mutations in BrAPE1 resulted in significant conformational changes, as evidenced by structural comparisons of the wild-type protein and its mutant variants (Fig. 4b, c). Phylogenetic analysis elucidated the evolutionary relationship

among BrEMS1 homologs in different species, indicating a high degree of homology among *Brassicaceae* crops (Fig. 4d).

Expression pattern and enzymatic activity analysis of Brape1

Subcellular localization and quantitative fluorescence analysis were performed to investigate the expression pattern of *Brape1*. BrAPE1-GFP and Brape1-GFP were transiently expressed in *Nicotiana benthamiana*. Both BrAPE1-GFP and Brape1-GFP were observed to co-localize with the endoplasmic reticulum marker (HDELmCherry) (Fig. 5a), indicating that BrAPE1 is localized to the endoplasmic reticulum and the mutation does not affect its subcellular localization.

Quantitative real-time PCR (qRT-PCR) was performed to evaluate the relative transcription levels of *Brape1* in different organs of 'FT' and *M5007*. The results showed that *Brape1* was exclusively expressed in buds of both *M5007* and 'FT', but was not detected in flowers, leaves, roots, or stems. Furthermore, there was no significant difference in the relative expression levels of *Brape1* between 'FT' and *M5007* buds (Fig. 5b). qRT-PCR analysis of *Brape1* expression at different developmental stages of 'FT' buds showed that its expression was low at stages S7-8, peaked at S9, decreased at S10-11, and was nearly undetectable at S12 (Fig. 5c).

To determine whether a single-base mutation in *M5007* affected the activity of BrAPE1 enzyme, an enzyme-linked immunosorbent assay (ELISA) was performed. The results indicated that the activity of acyl-CoA synthetase was significantly attenuated in *M5007* relative to 'FT' (Fig. 5d), suggesting that SNP4007 in *Brape1* is a key regulatory site for this enzyme.

Interference with Brape1 expression results in male sterility

To verify the function of the candidate gene *Brape1*, RNAi-mediated transformation was conducted in 'FT'. In the T₀ generation, a positive transgenic plant, *Brape1*-RNAi-7, was identified, exhibiting evident stamen abortion (Fig. 6a). Aside from the stamens, no significant differences were observed in other floral organs, plant height, stem diameter, or leaf size. Observation of the whole flowers of 'FT' and RNAi-7 revealed that, compared with 'FT', the anther surface of RNAi-7 lacked pollen grains (Fig. 6b). Pollen viability analysis of 'FT' and RNAi-7 showed that 'FT' produced viable pollen grains, whereas RNAi-7 resulted in complete pollen sterility (Fig. 6c). In RNAi-7, *Brape1* expression was significantly suppressed compared to the control (Fig. 6d).

BrAPE1 interacts with BrRGP1 and BrRGP2

To delve deeper into the role of BrAPE1 in the molecular mechanisms of in Chinese cabbage male sterility, a yeast two-hybrid (Y2H) library screening assay was designed. Given that the BrAPE1 protein, possessing two transmembrane domains and extracellular N- and C-termini, lacks a signal peptide, a 780 bp fragment encompassing the mutation site was subcloned into the pGBKT7 vector (C-terminal truncation) for nuclear library screening. Auto-activation assays showed that the positive control combination (pGBKT7-53 + pGADT7-T) grew and turned blue on both DDO/X and QDO/X/A medium,

indicating the success of the positive control experiment. The negative control combination (pGBKT7Lam + pGADT7T) grew as white colonies on DDO/X plate without turning blue, while it did not survive on QDO/X/A medium, indicating the success of the negative control experiment. The experimental group pGBKT7-BrAPE1 + pGADT7 grew on DDO/X medium but did not grow on QDO/X/A medium, suggesting that the pGBKT7-CDS plasmid was successfully transformed into the yeast strain and there was no auto-activation phenomenon on QDO/X/A, suggesting that the screening experiment was feasible (Fig. 7a). A Y2H library screening was performed using pGBKT7-BrAPE1 as the bait against a Chinese cabbage cDNA library. A total of 64 positive clones grew blue on both QDO/X/A and TDO/X selective media (Fig. 7b). Sequencing of all positive clones followed by alignment to the reference genome identified a total of 47 proteins (Table S3).

The positive clones included BrRGP1 (BraA03g031230.3.5C) and BrRGP2 (BraA03g007040.3.5C), which encode UDP-arabinose mutases that interconvert UDP-L-arabinopyranose and UDP-L-arabinofuranose. The *Arabidopsis* homologs *rgp1/rgp2* double mutant exhibited a male gametophytic lethal phenotype (Drakakaki et al. 2006). Thus, BrRGP1 and BrRGP2 were selected for further interaction studies. A Y2H assay demonstrated that pGBKT7-BrAPE1 and pGADT7-BrRGP1 co-transformation resulted in normal yeast growth on QDO/X plate, similar to the positive control, while no growth was observed in the negative control (Fig. 8a), indicating an interaction between BrAPE1 and BrRGP1 *in vitro*. This interaction was further supported by luciferase complementation imaging (LCI), where strong luminescence was observed in tobacco leaves co-expressing cLUC-BrAPE1 and BrRGP1-nLUC, but not in controls with empty vectors (Fig. 8b), suggesting that BrAPE1 and BrRGP1 also interact *in planta*. Additionally, a GST pull-down assay revealed the presence of BrRGP1 in the GST-BrAPE1 complex, but not in the GST control, confirming a specific interaction between BrAPE1 and BrRGP1 proteins (Fig. 8c).

The interaction between BrAPE1 and BrRGP2 was investigated using a Y2H assay. Normal yeast growth on DDO plates confirmed successful transformation. On QDO/X plate, the combination of pGBKT7-BrAPE1 and pGADT7-BrRGP2 showed normal growth, similar to the positive control, while the negative control showed no growth (Fig. 9a), indicating an *in vitro* interaction between BrAPE1 and BrRGP2. LCI further supported this interaction, with strong luminescence observed in tobacco leaves co-expressing cLUC-BrAPE1 and BrRGP2-nLUC, but no signal in the empty vector controls (Fig. 9b), suggesting an *in planta* interaction. Additionally, GST pull-down assays detected BrRGP2 in the GST-BrAPE1 complex, but not in the GST control, confirming a specific interaction between BrAPE1 and BrRGP2 (Fig. 9c).

To elucidate the molecular mechanism of the interaction between BrAPE1 and BrRGP1/2, this study compared the UAM activity between 'FT' and *M5007* through enzymatic assays. The results revealed a significant reduction in UAM activity in the *M5007* compared to the 'FT' (Fig. 10), indicating that the loss of *Brape1* function directly impairs UAM catalytic efficiency. This finding suggests that BrAPE1 may regulate the stability or catalytic function of the RGP protein complex through its interaction with BrRGP1/2, thereby maintaining efficient interconversion between UDP-L-arabinopyranose (UDP-Arap) and UDP-L-arabinofuranose (UDP-Araf).

Mutations in *Brape1* affect the expression of genes related to the tapetum, meiosis, and pollen tubes

A total of 136,535,602 and 130,621,328 clean reads were obtained for 'FT' and *M5007*, respectively, with 88.88% and 87.92% mapped to the reference genome for gene expression analysis. A total of 1,071 differentially expressed genes (DEGs) were identified ($\text{padi} \leq 0.05$, $\log^2\text{FoldChange} \geq 1$), with 281 upregulated and 790 downregulated DEGs in *M5007* compared with 'FT'.

Among the DEGs related to male sterility were genes associated with the tapetum, meiosis, and pollen tubes. The male sterility gene *BCP1* (*BraA07g012900.3.5C*), whose encoded protein acts on the tapetum and microspores, was significantly downregulated in *M5007* (Fig. 11). The meiosis-related gene *MS5* (*BraA09g025330.3.5C*), which encodes a tetratricopeptide repeat protein required for cell cycle exit after meiosis II, was also significantly downregulated in *M5007* (Fig. 11a). Among the DEGs associated with pollen tube function, *PRK4* (*BraA01g034430.3.5C* and *BraA03g039700.3.5C*), *LRX10* (*BraA03g059520.3.5C*), and *MDIS1* (*BraA06g043620.3.5C*) were all significantly downregulated (Fig. 11a). These findings suggest that *Brape1* mutation affected genes associated with tapetum development and meiosis, while the absence of pollen likely influenced pollen tube gene expression in Chinese cabbage.

KEGG pathway enrichment analysis of the DEGs revealed significant enrichment in several metabolic pathways, including α -linolenic acid metabolism (brp00592), ascorbate and aldarate metabolism (brp00053), cyanamide metabolism (brp00460), ether lipid metabolism (brp00565), glycerophospholipid metabolism (brp00564), inositol phosphate metabolism (brp00562), linoleic acid metabolism (brp00591), nitrogen metabolism (brp00910), taurine and hypotaurine metabolism (brp00430), and sucrose and starch metabolism (brp00500) (Fig. 11b). Therefore, the mutations in the male sterile mutant *M5007* also impacted multiple metabolic pathways.

qRT-PCR results showed that the relative expression trends of *BCP1* (*BraA07g012900.3.5C*), *MS5* (*BraA09g025330.3.5C*), *PRK4* (*BraA01g034430.3.5C* and *BraA03g039700.3.5C*), *LRX10* (*BraA03g059520.3.5C*) and *MDIS1* (*BraA06g043620.3.5C*) in 'FT' and *M5007* were consistent with the transcriptome data (Fig. 12), and the trends in RNAi-7 plants were also in agreement with the transcriptome data (Fig. 13), confirming the accuracy and reliability of the transcriptome analysis.

Discussion

Understanding the molecular mechanisms underlying male sterility and developing practical male sterility systems are important to utilize hybrid vigor and commercial hybrid seed production in Chinese cabbage. This study identified a male sterile mutant, *M5007*, and validated the function of causative gene, *Brape1*. Loss of *Brape1* function resulted in premature degradation of the tapetum and abnormal pollen morphology. The activity of acyl-CoA synthetase was significantly attenuated in *M5007* relative to 'FT'. *BrAPE1* interacted with *BrRGP1/BrRGP2* and was involved in regulating the expression of genes related to tapetum development, meiosis, and pollen tube growth.

The causes of pollen abortion in Chinese cabbage include abnormalities in male gametophyte, microsporogenesis development, and the processes of tapetal layer development and degeneration. Anther abortion in the Chinese cabbage GMS line 'AB01' was caused by abnormal aggregation of pollen mother cells and excessive proliferation of tapetum (Zhou et al. 2017). In the Chinese cabbage mutant *ftms*, abnormal microspore development and tapetum degeneration resulted in male sterility (Tan et al. 2019). Cytological observations of mutant *msm2* indicated that delayed programmed cell death of tapetum led to pollen abortion (Dong et al. 2022). The male sterility phenotype of the *Bcajh97-01A* mutant was attributed to the premature programmed cell death of tapetum (Shen et al. 2019). The complete male sterility of the *ftms1* mutant was caused by the abnormal enlargement of tapetum (Zhao et al. 2022). Thus, male sterility in Chinese cabbage frequently involves a disrupted tapetum. Herein, the male-sterile mutant *M5007* of Chinese cabbage exhibited premature tapetum degradation, abnormal concave pollen morphology, and exine structural defects.

Acyl-CoA synthetase was involved in the synthesis of fatty acyl-CoA. Acyl-CoA was also a crucial precursor required for the biosynthesis of sporopollenin and the lipidic layer in the endoplasmic reticulum of the tapetum (de Azevedo Souza et al. 2009). *AtACOS5* encoded acyl-CoA synthetase5, which specifically participated in the biosynthesis of sporopollenin precursors, with substrates including fatty acids (C6–C18), hydroxy fatty acids, and hydroxycinnamic acids (de Azevedo Souza et al. 2009). Premature termination of *Osacos12* translation in rice resulted in male sterility, and the introduction of a vector carrying *Osacos12* driven by the *ACOS5* promoter into *Arabidopsis acos5* mutants partially restored male fertility (Li et al. 2016). RNAi lines of the tobacco *NtACOS1* gene exhibited male sterility, with no production of mature pollen grains (Wang et al. 2013). CRISPR/Cas9-mediated homozygous double mutants of *CjACOS5a* and *CjACOS5b* in *Cryptomeria japonica* exhibited a lack of pollen in male cones (Nishiguchi et al. 2023). Thus, mutations in various acyl-CoA synthetase genes are related to male sterility. Recent studies revealed that the transcriptional regulation and protein–protein interactions of acyl-CoA synthetases played a critical role in male fertility. For instance, the transcription factor WRI1, a member of the AP2/EREBP family, bound to AW-box elements in the promoters of lipid biosynthetic genes-including acyl-CoA synthetases-thereby promoting the synthesis of fatty acyl-CoA, a precursor essential for the formation of sporopollenin in the pollen exine (Baud et al. 2009; Kong et al. 2019). The transcription factor MS188 in *Arabidopsis* directly activated the expression of *ACOS5* (Li et al. 2019). MS188 also bound to the promoters of *OsACOS12* and *PpACOS6* and activated the genes driven by these promoters (Li et al. 2019). In this study, a male-sterile mutant were obtained through EMS mutagenesis, and the sterility was caused by a point mutation in *Brape1*, which encodes an acyl-CoA synthetase. *Brape1*-RNAi plant exhibited male sterility, and the reduced expression of *Brape1* confirmed its functional role.

RGP played a crucial role in cell wall formation. L-arabinose is an important component of the cell wall (Scheller and Ulvskov 2010). The interconversion between L-arabinopyranose and L-arabinofuranose is crucial for the incorporation of L-arabinose into plant cell wall polysaccharides. The *PtRGP3* and *PtRGP6* genes in *Populus trichocarpa* play critical roles in the biosynthesis of secondary cell walls (Sun et al. 2020). Enzymes involved in cell wall modification were crucial for successful pollen development

(Schnurr et al. 2006). The high expression levels of the *RGP* gene in cotton during secondary cell wall thickening, primary cell wall elongation, and fiber development indicated its essential role in cell wall biosynthesis (Zhao and Liu 2002). During pollen meiosis and microspore development in *Arabidopsis*, *RGP1* and *RGP2* were essential for maintenance of vacuole integrity and proper cell division. The *RGP1/2* double mutant displayed abnormal pollen morphology, with alterations in vacuole and cell membrane structures, resulting in the disintegration of pollen structures during meiosis I (Drakakaki et al. 2006). In this study, BrRGP1 and BrRGP2 were identified as interacting proteins of BrAPE1 through a Y2H library screening. The interactions were further confirmed by Y2H, LCI, and pull-down, suggesting that BrRGP1 and BrRGP2 may play a role in stamen development in Chinese cabbage. This study provided an experimental foundation for further exploration of the roles of BrRGP1 and BrRGP2 in the mechanism of male sterility in Chinese cabbage. Our findings contributed to uncovering the molecular basis of male sterility, thereby facilitating the development of gene regulation and improvement strategies targeting male sterility in plant breeding.

Conclusions

This study identified the key gene *Brape1* in the male-sterile line *M5007* of Chinese cabbage, encoding an endoplasmic reticulum-localized fatty acyl-CoA synthetase. Its loss-of-function causes premature tapetum degradation and abnormal pollen wall formation. The gene participated in cell wall formation in collaboration with its interacting proteins BrRGP1 and BrRG2. The study revealed a new molecular mechanism for the synergistic regulation of male sterility by the tapetum and pollen wall.

Declarations

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Contributions

CL: Writing – original draft. NW: Data curation, Writing – review & editing. LW, SR, CX and YZ: Methodology, Writing – review & editing. CT and HF: Methodology, Writing – review & editing. LY: Writing – review & editing. ZL: Writing – review & editing.

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Figures

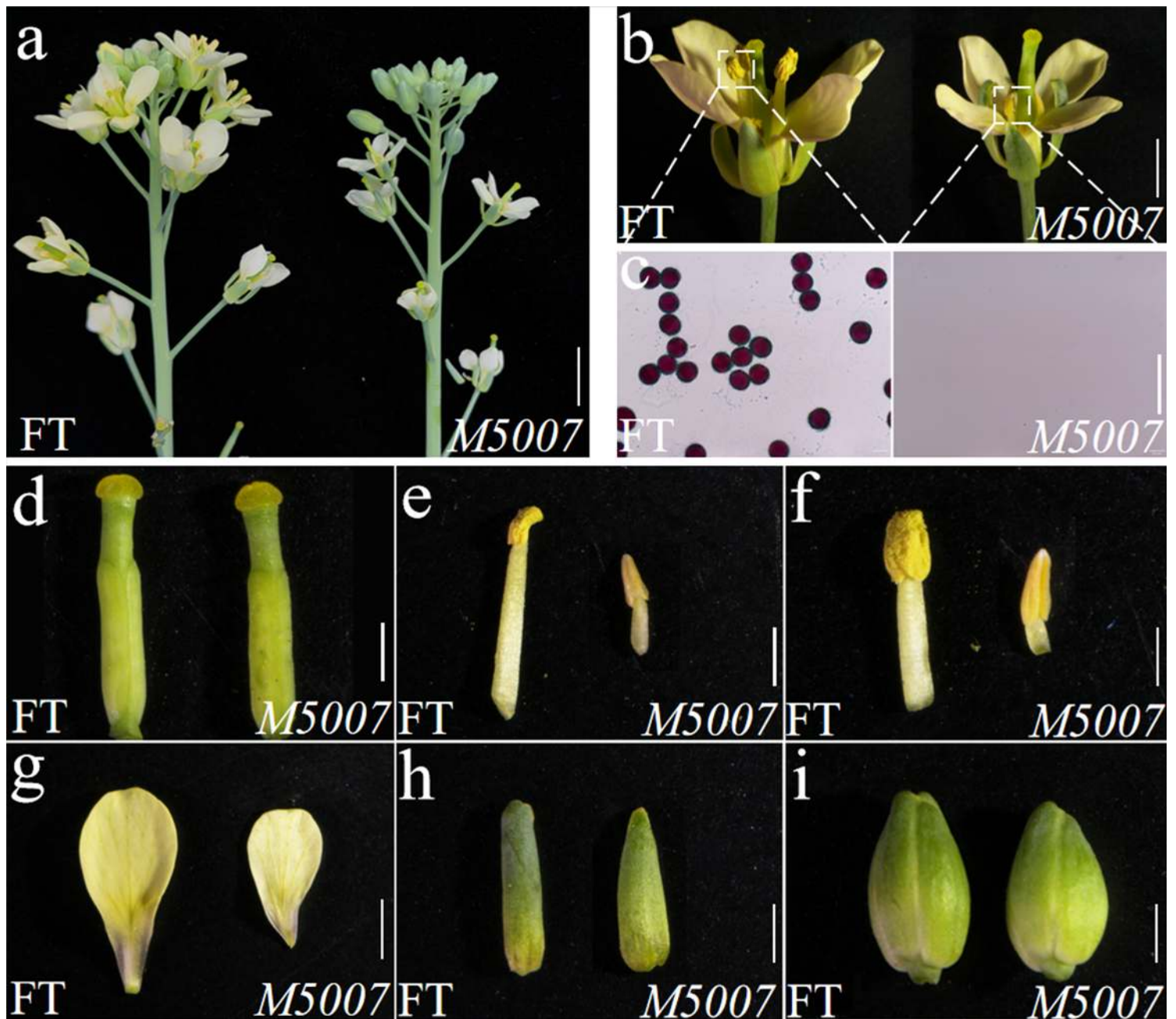


Figure 1

Stamen phenotypic analysis of 'FT' and *M5007*. (a) The inflorescence of 'FT' and *M5007*. Bar, 1 cm. (b) The whole flowers. Bar, 3 mm. (c) Pollen viability. Bar, 50 μm. (d) Pistils. (e) Long stamens. (f) Short stamens. (g) Petals. (h) Sepals. (i) Buds. Bars, 3mm.

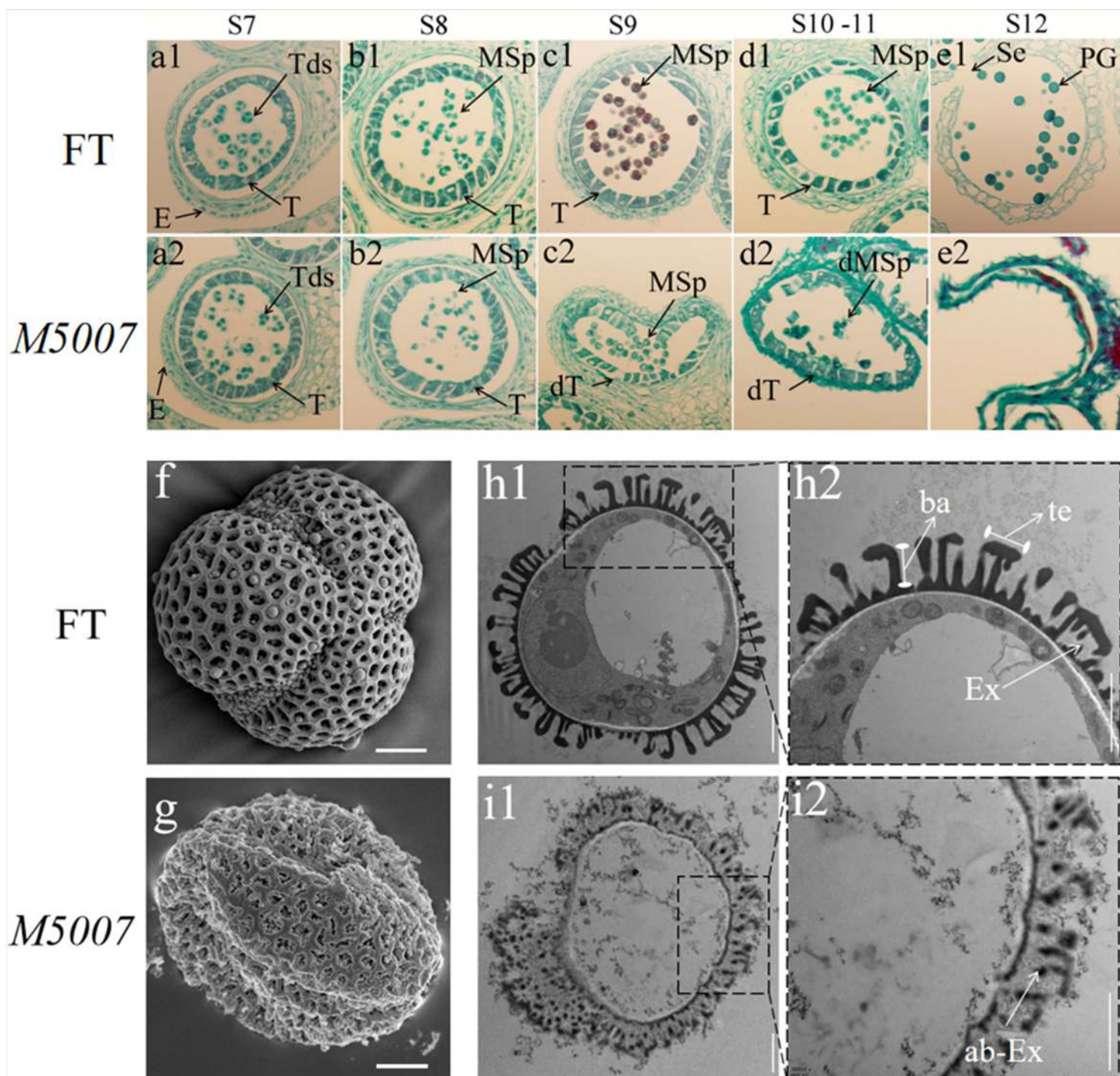


Figure 2

Cytological observation of buds in 'FT' and *M5007*. (a1-e1) Paraffin section observation of anther in 'FT'. (a2-e2) Paraffin section observation of anther in *M5007*. E: epidermis; T: tapetal layer; Tds: tetrad; dT: degraded tapetal layer; Msp: microspore; dMSp: degraded tapetal layer; PG: pollen grain; Se: septum. (f) The morphology of pollens from 'FT' was investigated using SEM. Bar: 30 μ m. (g) The morphology of pollens from *M5007* was investigated using SEM. Bar: 30 μ m. (h) Observation of pollens from 'FT' using transmission electron microscopy. (h1) Pollen. (h2) Localized magnification. (i) Observation of pollens

from *M5007* using transmission electron microscopy. (i1) Pollen of *M5007*. (i2) Localized magnification of pollen in *M5007*. ab-Ex: abnormal exine; te: tectum; ba: bacula; Ex: exine. Bar: 1 μ m.

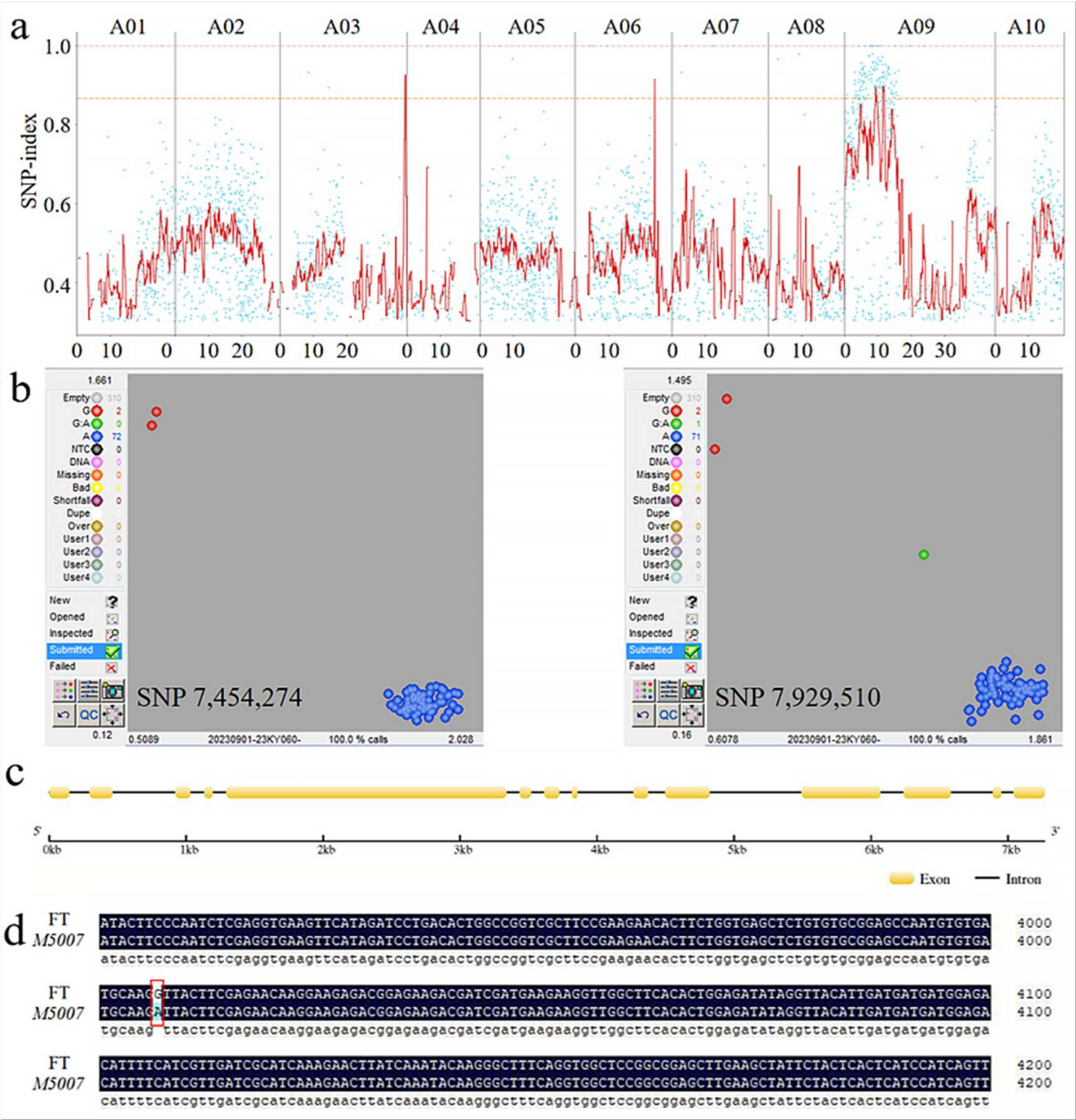


Figure 3

Identification of the mutated gene in *M5007*. (a) SNP-index plot. The 95th percentile corresponded to the orange line. (b) KASP genotyping. (c) The distribution of introns and exons in gene *BraA09g013200.3.5C*.

(d) The partial sequence of *BraA09g013200.3.5C* was compared between 'FT' and *M5007*. The mutation site was in red.

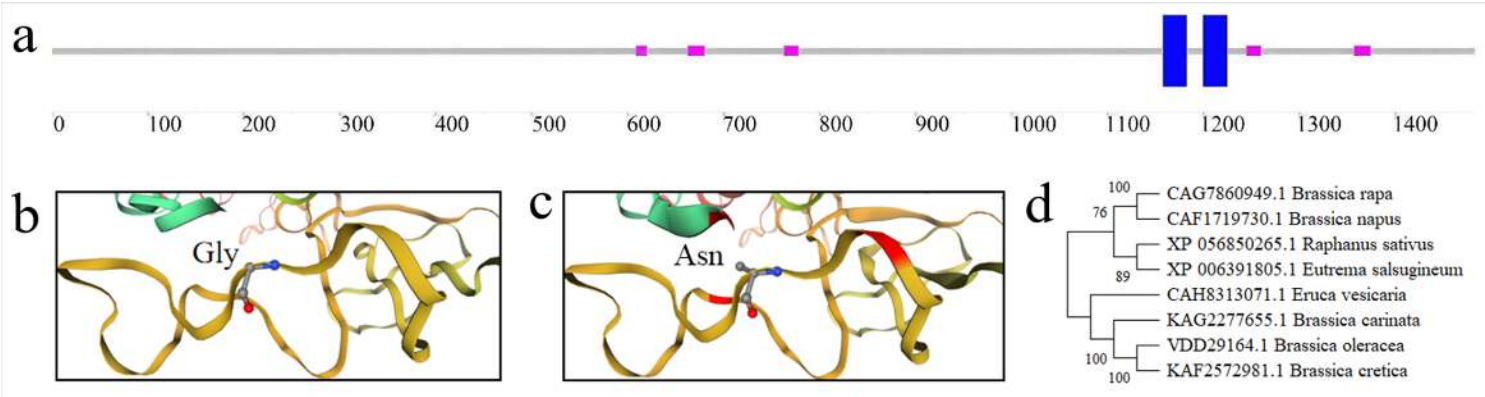


Figure 4

Protein analysis of BrAPE1. (a) Protein domain analysis of BrAPE1. The blue box represented the transmembrane domain. (b) The local three-dimensional structure of BrAPE1 protein around the mutation site. (c) The local three-dimensional structure of Brape1 protein around the mutation site. (d) The phylogenetic tree of BrAPE1.

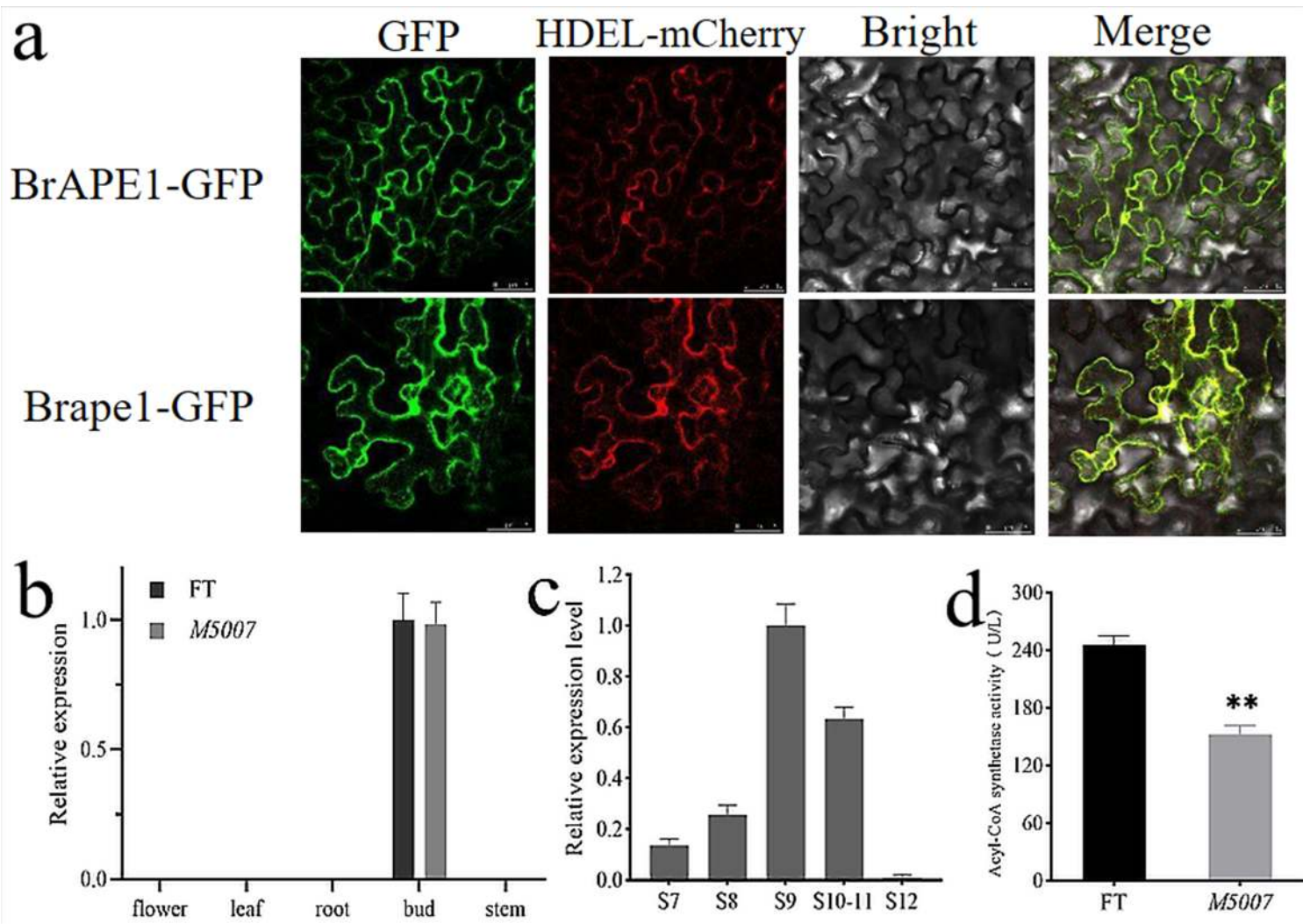


Figure 5

Expression analysis of *Brape1* in *M5007* and 'FT'. (a) The subcellular localization of BrAPE1 and Brape1. The fluorescent part in the image was the area where the endoplasmic reticulum was located. (b) qRT-PCR analysis of *Brape1* in different organs of 'FT' and *M5007*. (c) qRT-PCR analysis of *Brape1* expression at different developmental stages of 'FT' buds. (d) Determination of acyl-CoA synthetase activity in 'FT' and *M5007*.

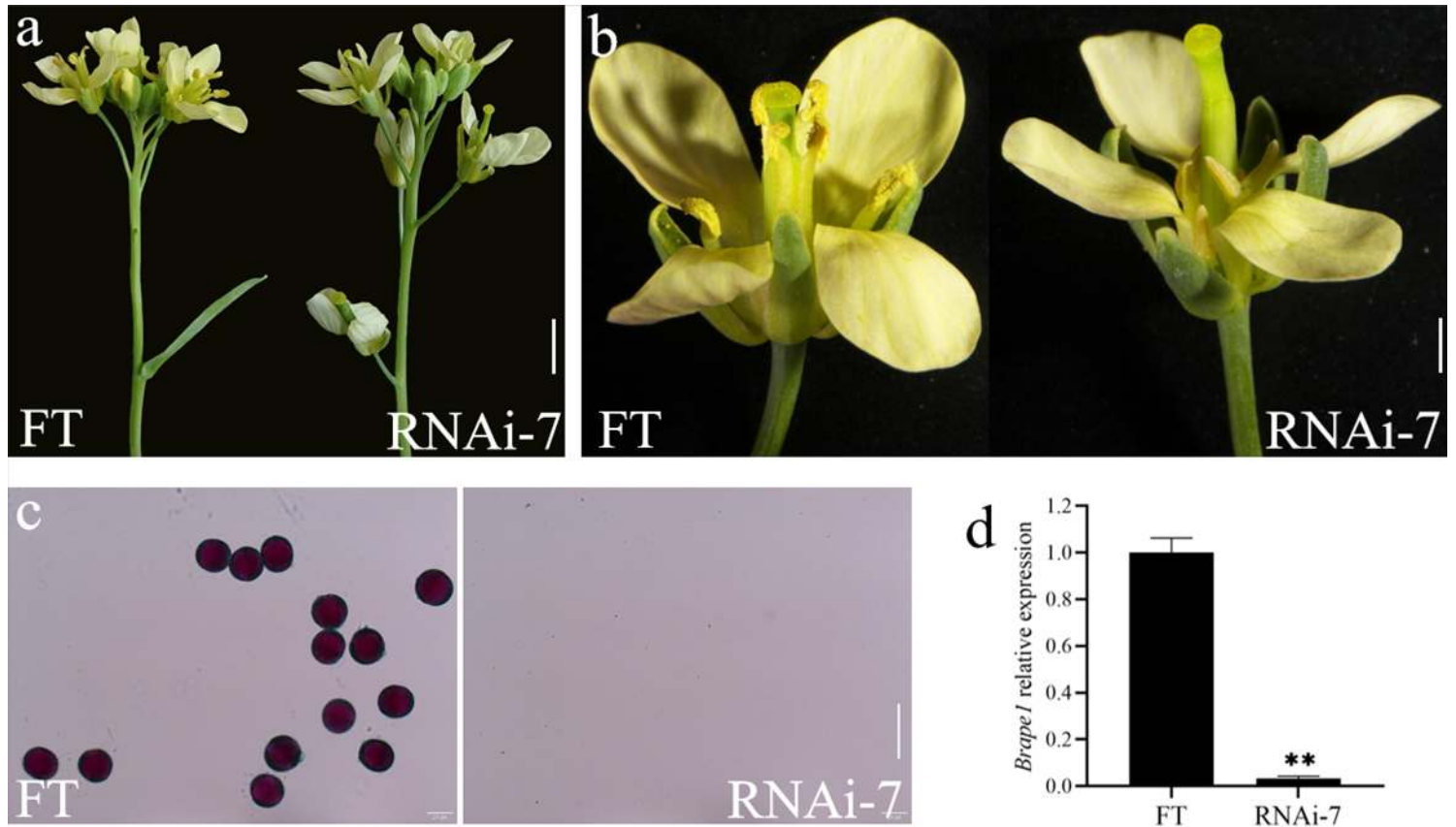


Figure 6

Phenotypic identification and expression analysis of *Brape1*-RNAi-7 plants. (a) Flowering period of 'FT' and RNAi-7. Bar: 8 mm. (b) Whole flower morphology of 'FT' and RNAi-7. Bar: 2 mm. (c) Pollen viability. Bar, 40 μ m. (d) Analysis of relative *Brape1* expression levels in 'FT' and RNAi-7.

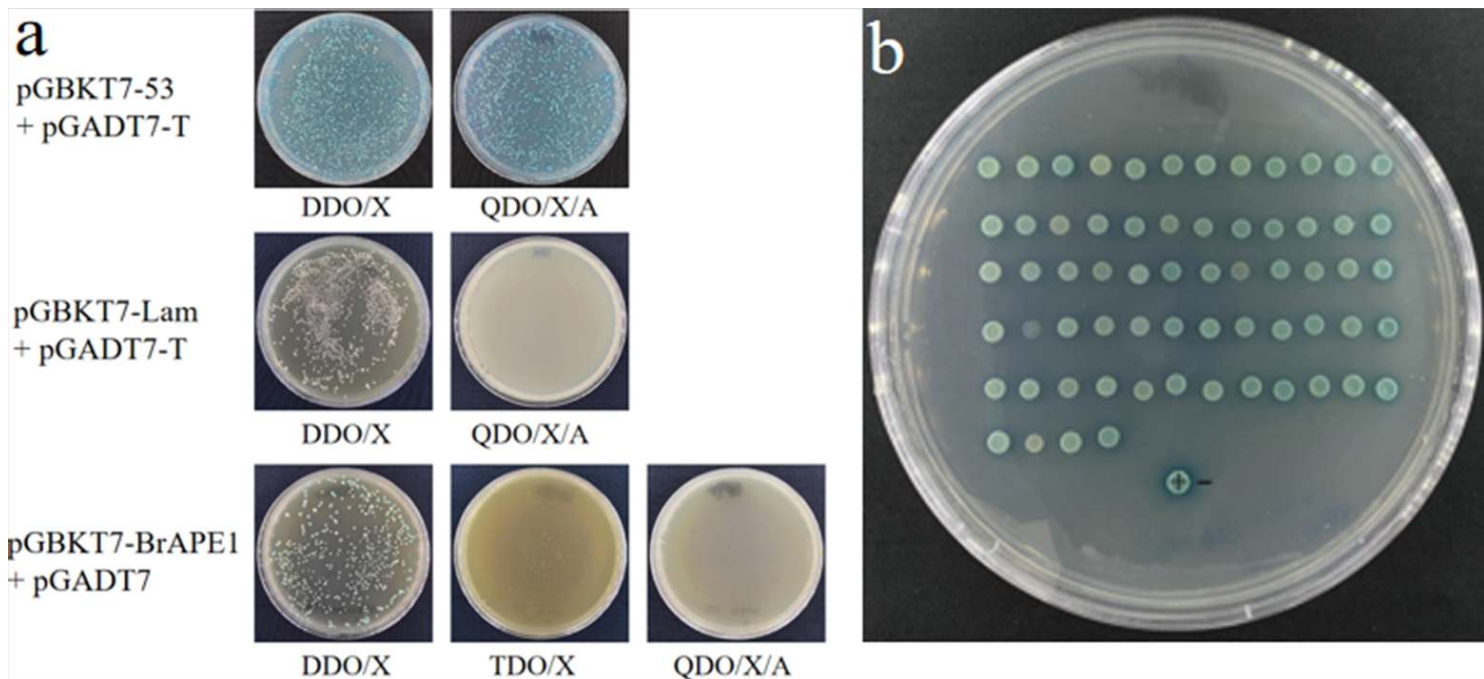


Figure 7

The Y2H screening library for the BrAPE1 protein. (a) Self-activation assay of BrAPE1 protein. (b) Positive clones on the secondary screening plate. A '+' on the blue colonies indicated a positive control. A '-' denoted a negative control.

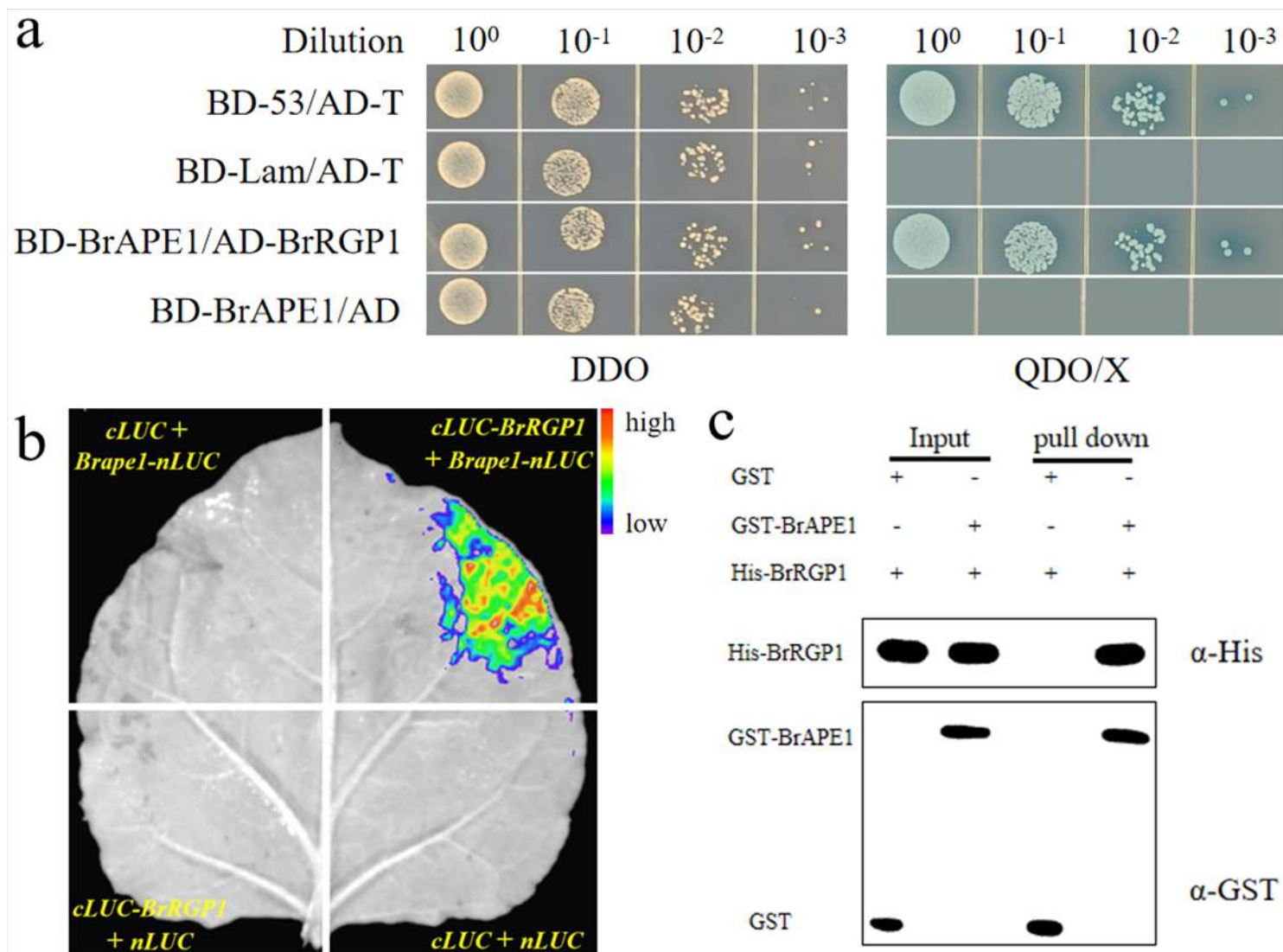


Figure 8

Interaction between BrAPE1 and BrRGP1. (a) Y2H point-to-point assay between BrAPE1 and BrRGP1. (b) LCI assay of BrAPE1 and BrRGP1. (c) GST pull-down assay of BrAPE1 and BrRGP1.

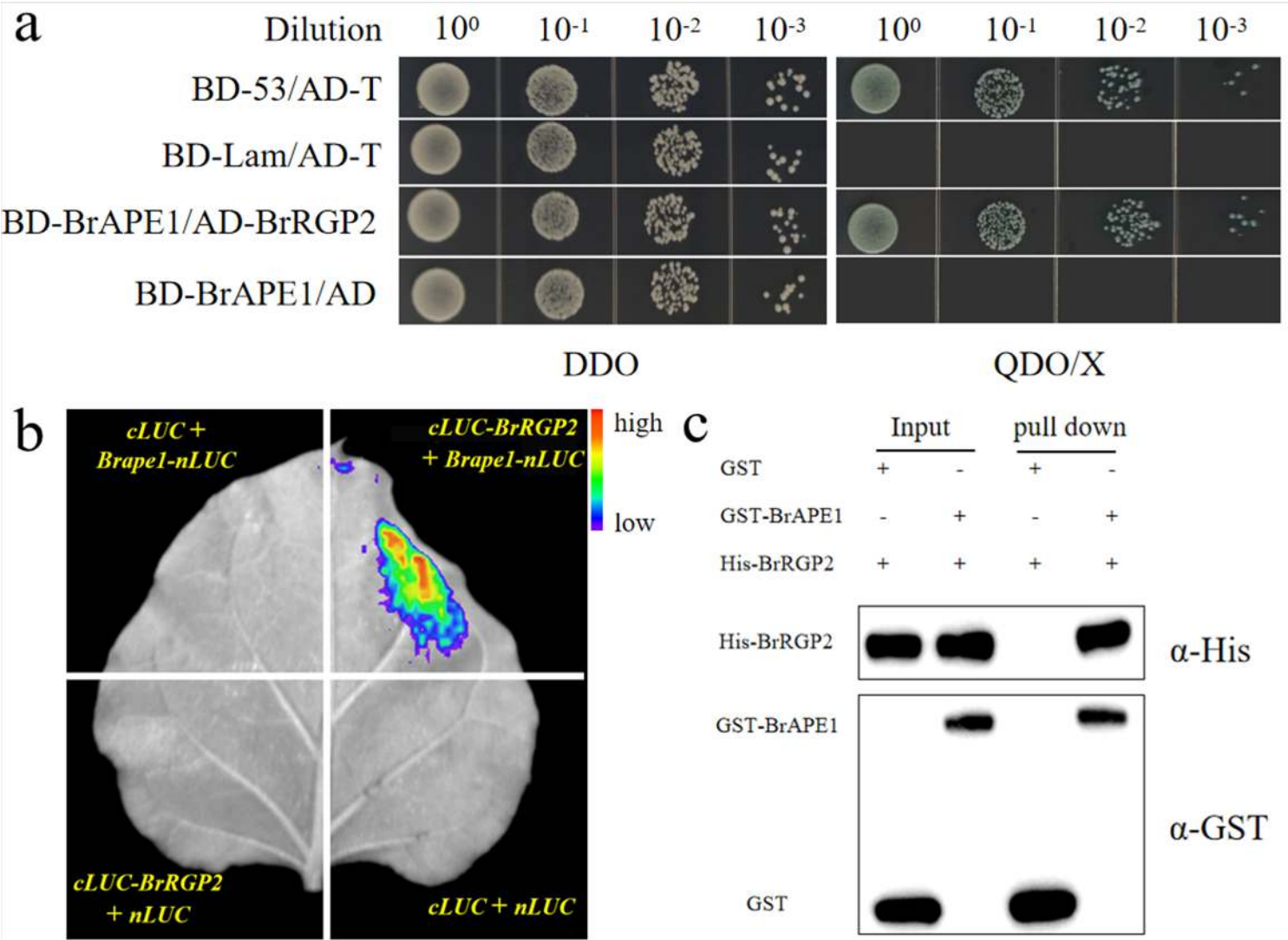


Figure 9

Interaction between BrAPE1 and BrRGP2. (a) Y2H point-to-point assay between BrAPE1 and BrRGP2. (b) LCI assay of BrAPE1 and BrRGP2. (c) GST pull-down assay of BrAPE1 and BrRGP2.

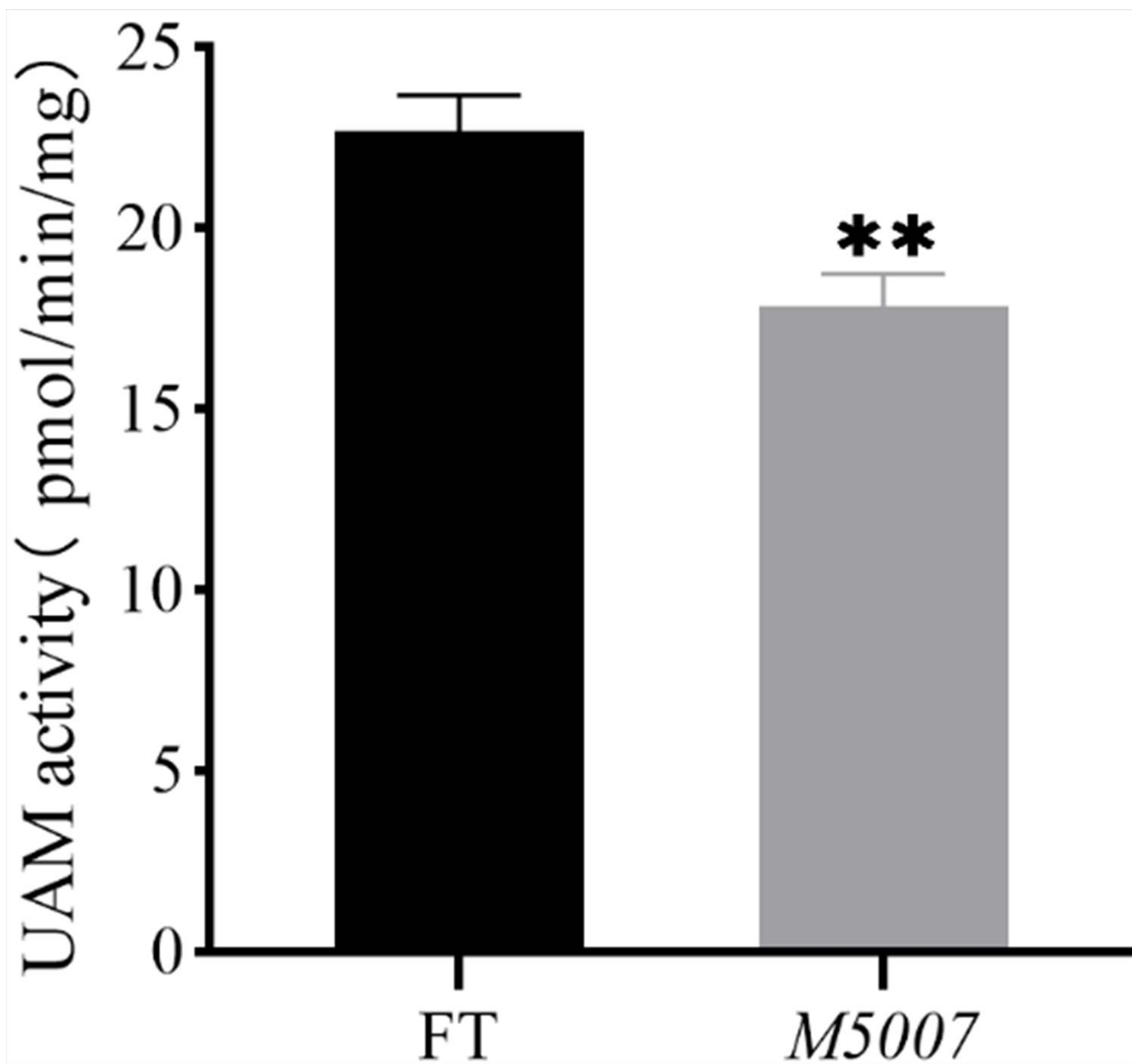


Figure 10

UAM activity in 'FT' and *M5007*.

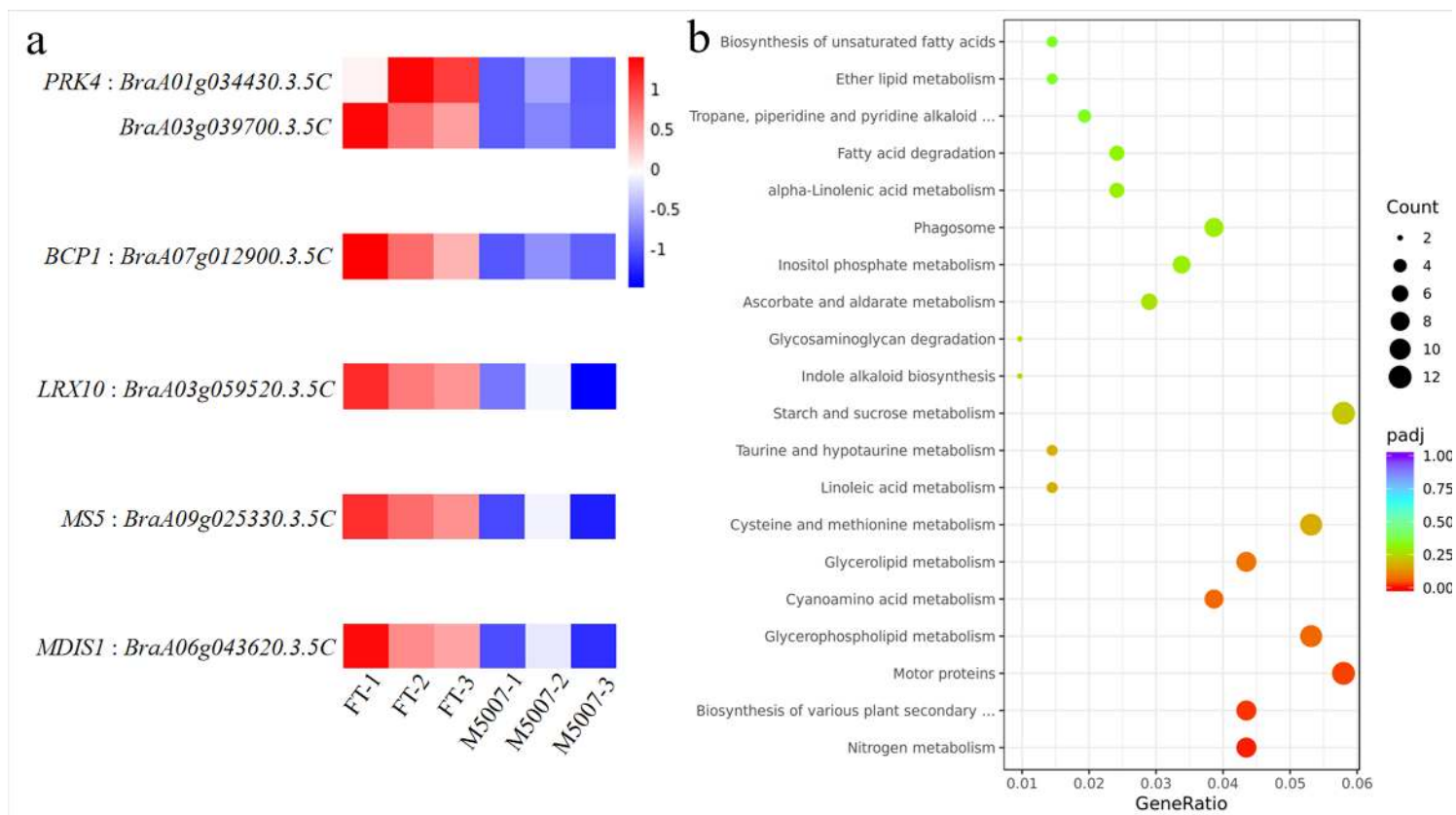


Figure 11

Analysis of selected DEGs and KEGG pathways in the transcriptome. (a) DEGs related to stamen development. (b) Pathway analysis of DEGs using KEGG.

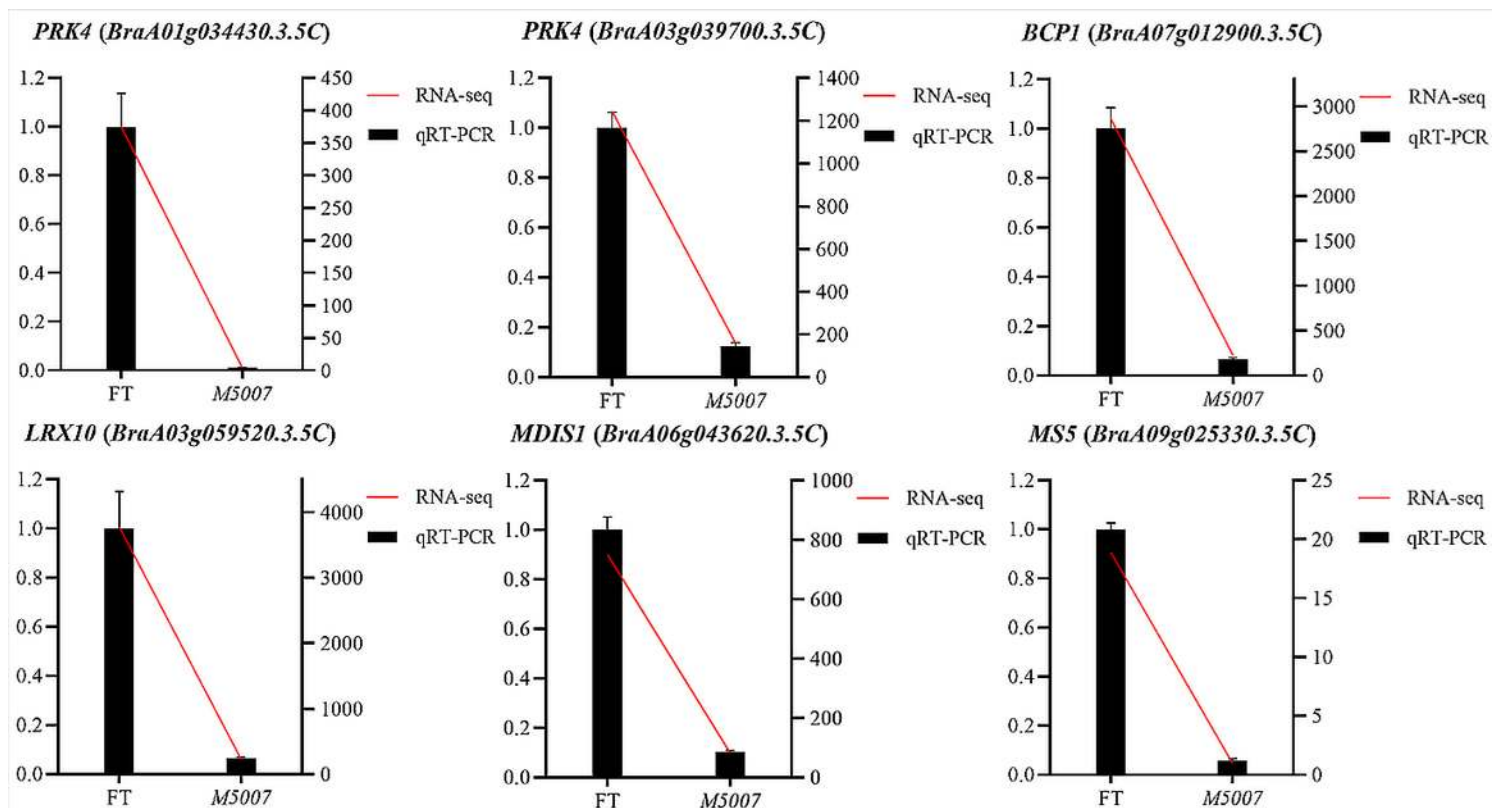


Figure 12

qRT-PCR analysis of 6 DEGs in 'FT' and *M5007*. The left y-axis represented the relative expression level, and the right y-axis represented the FPKM values.

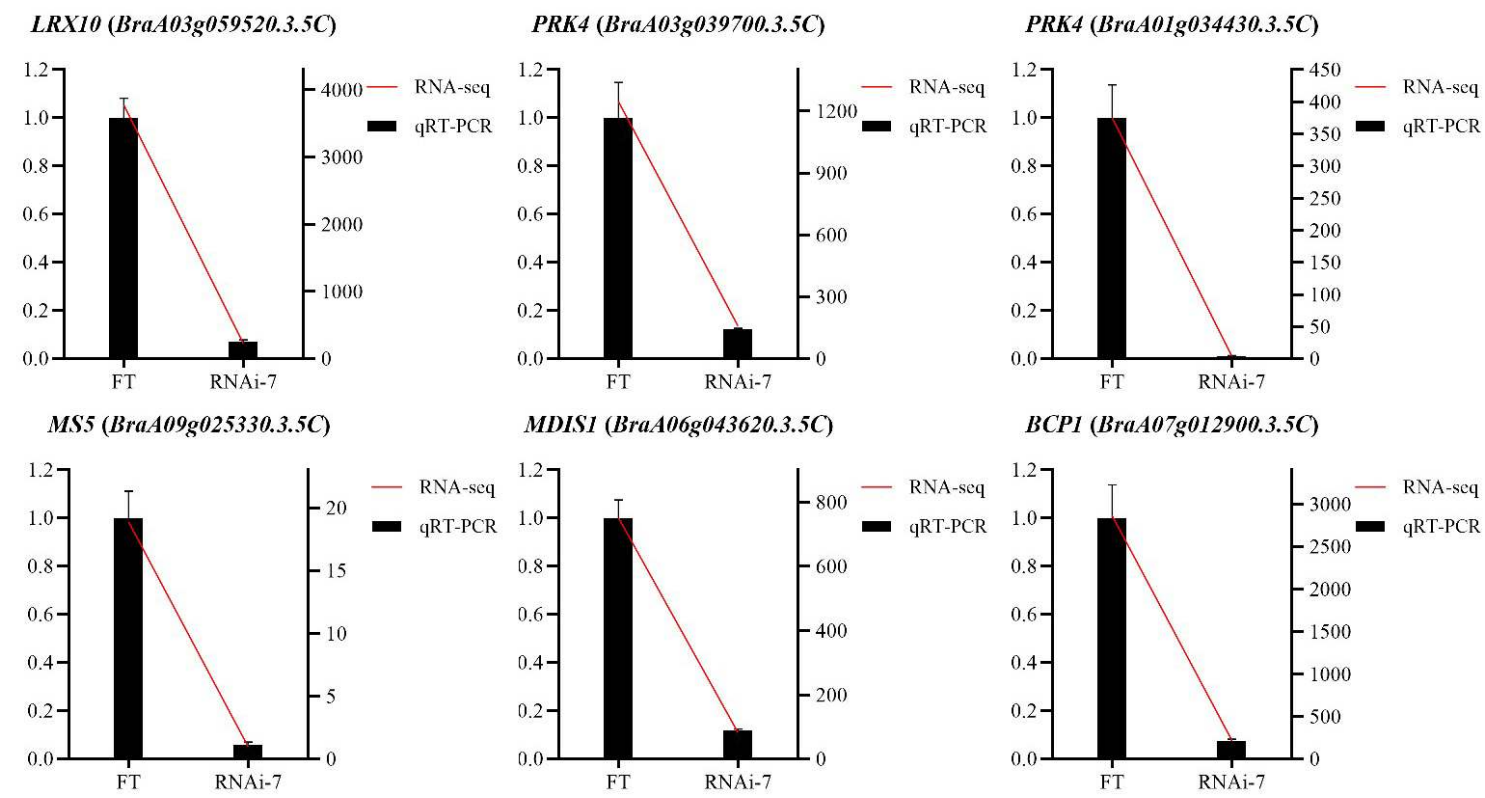


Figure 13

qRT-PCR analysis of 6 DEGs in 'FT' and RNAi-7. The left y-axis represented the relative expression level, and the right y-axis represented the FPKM values.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarytable.docx](#)