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Full Length Article

Transcriptome Analysis of *Brassica napus* Wax-Deficient Mutant Revealed the Dynamic Regulation of Leaf Wax Biosynthesis is Associated with *Basic pentacysteine 6*

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Abstract

Wax is a protective layer through which plants directly contact the environment and it plays an important role in plant responses to biotic and abiotic stresses. The biosynthesis of wax is a dynamic process that occurs over different growth stages. Although wax biosynthesis has been well studied, there are limited reports on how plants regulate wax synthesis at different developmental stages. In this study, we identified a wax-deficient mutant (*wad*) in rapeseed (*Brassica napus L.*). The *wad* true leaf was shiny at the young stage (*wad-y*) but recovered at the mature stage (*wad-m*) and further analyses indicated that wax biosynthesis was blocked in *wad-y* leaves. The gene expression patterns of wild-type (WT) during the young (WT-y) and mature (WT-m) stages, *wad-y* and *wad-m* were monitored using RNA sequencing. The Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation showed that differentially expressed genes (DEGs) were gathered in the fatty acid elongation pathway. These genes were down-regulated in *wad-y* and returned to normal levels in *wad-m*, indicating that the changes in fatty acid elongation genes' expression lead to dynamic changes in wax. Moreover, a transcription factor-enrichment analysis showed that *BASIC PENTACYSTEINE 6* (*BPC6*) is the key regulator affecting fatty acid elongation. Taken together, our study showed *BPC6* potentially affects the biosynthesis of wax during different developmental stages by regulating the elongation of fatty acid chains. This study will provide new insights into the regulation of wax biosynthesis during different developmental stages of rapeseed.

Keywords: Wax recovery; Gene expression; Fatty acid elongation; Transcriptional factors

Introduction

Rapeseed (*Brassica napus L.*) is an important oil crops worldwide (Liu *et al.*, 2010). During its growth, rapeseed often faces various biological and non-biological stresses, such as infections and drought (Nishiyama *et al.*, 2013). Wax forms a protective layer through which plants directly contact the environment, and it helps plants to defend against pathogens, pests and ultraviolet rays (Franke *et al.*, 2005). As a hydrophobic layer, wax can reduce the retention of water droplets in leaves to avoid the deposition of dust or other air pollutants (Lu *et al.*, 2016). In addition, wax can also reduce the loss of non-stomatal water and improve the drought resistance of plants (Baker, 1982; Asaph *et al.*, 2004). Changes in wax components may affect the development of epidermal or pollen cells, which may lead to the adhesion of leaves or stems and decreases in pollen fertility (Sieber *et al.*, 2000; Koch *et al.*, 2009; Yoshimi *et al.*, 2013).

The biosynthesis of wax is closely related to fatty acid elongation. In the endoplasmic reticulum, very-long-chain fatty acids (VLCFAs, >18 carbon) are enzymatically converted into the precursors of cuticular waxes (Frédéric *et al.*, 2009). Carbon chains of 16:0- or 18:0-CoA esters are elongated by β-ketoacyl-CoA synthase (KCS), β-ketoacyl-CoA reductase , 3-hydroxyacyl-CoA dehydratase and enoyl-CoA reductase (Haslam and Kunst, 2013) . After this four-step reaction, 16:0- and 18:0-CoA esters are elongated by the addition of a two-carbon unit to the carboxyl terminus and the product continues to enter this four-step reaction to generate VLCFA wax precursors between 20 and 34 carbons in length (Lessire *et al.*, 1989). Then VLCFA-CoAs are enzymatically converted into cuticular waxes through two biological pathways: the acyl reduction pathway for the production of primary alcohols and wax crystals and the alkane formation pathway for the production of aldehydes, alkanes, secondary alcohols and

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ketones (Wang *et al.*, 2017). In the acyl reduction pathway, VLCFA-acyl CoAs are catalyzed by alcohol-forming fatty acyl-CoA reductase to form C24:0–C30:0 primary alcohols (Rowland *et al.*, 2006). C24:0- C24:0–C30:0 primary alcohols and 16:0 CoA are subsequently catalyzed by wax synthase/acyl-CoA:diacylglycerol acyltransferase to form wax esters (Lai *et al.*, 2007; Li *et al.*, 2008). In the alkane-forming pathway, VLCFA-acyl CoAs are catalyzed by aldehyde-forming fatty acyl-CoA reductase to form fatty aldehydes, which are catalyzed by *ECERIFERUM1* to synthesize alkanes (Sakuradani *et al.*, 2013). Alkanes are subsequently catalyzed by midchain alkane hydroxylase 1 to form secondary alcohols that are further oxidized to ketones (Greer *et al.*, 2007).

Wax biosynthesis can also be regulated by some transcription factors (TFs). AP2/EREBP -type TFs *SHN1/WIN1*, *SHN2* and *SHN3*, as well as MYB-type TFs *MYB30*, *MYB41* and *MYB96*, are involved in wax biosynthesis (Aharoni *et al.*, 2004; Seo and Park, 2011). The overexpression of *SHN1/WIN1*, *SHN2* or *SHN3* upregulates many wax biosynthetic genes and causes significant increases in all cuticular wax components (Broun *et al.*, 2004). BASIC PENTACYSTEINE (BPC) is a plant-specific TF family, having seven members (*BPC1–7*) (Meister *et al.*, 2010). BPCs can regulate many hormones, such as ethylene, abscisic acid and cytokinin (Shanks *et al.*, 2018). A correlation between BPCs and wax biosynthesis has not been reported.

The biosynthesis of wax is a dynamic process, having different stages (Rhee and Post-Beittenmiller, 1998). Although increasing numbers of genes controlling cuticular wax biosynthesis have been identified and the associated biosynthetic processes have been uncovered (Yeats and Rose, 2013; Wang *et al.*, 2018), the mechanisms behind its dynamic changes are still unknown. Here, we revealed that fatty acid elongation pathway is responsible for wax's dynamic changes. The expression level of *KCS2*, *KCS1*, *PAS2*, *KCR1*, *ECR*, *KCS6* and *KCS9* play a key role in the regulation of fatty acid elongation. More importantly, we discovered TF *BPC6* affects the biosynthesis of wax during different developmental stages by regulating the expression level of these genes. Collectively, these findings suggest a mechanism whereby *BPC6* regulates wax's dynamic changes by regulating the elongation of fatty acid chains, provide new insights into the regulation of wax biosynthesis during different rapeseed developmental stages.

Materials and Methods

Plant Materials and Growth Conditions

Rapeseeds ('Ning You 12') were grown in a growth room, with a 16-h light at 22°C and 8-h dark at 20°C cycle. After the seedlings had grown for a month, they were transplanted to the experimental fields at Zhenjiang Institute of Agricultural Sciences in Hilly Area of Jiangsu Province Zhenjiang, China. At the experimental field, the plants grew in natural environment from November to June next year. After rapeseed grew 20 days, the true leaves of WT and *wad* in the same growth state were used for scanning electron microscopy, determination of wax contents and total fatty acid, analyses of transcriptome and RT-PCR. After rapeseed bolting stage, leaves of WT and *wad* in the same growth state were also picked for analyses of transcriptome and RT-PCR.

Scanning Electron Microscopy

The methods were performed as described by Chen *et al.* (2011) with slight modifications. Briefly, the leaves were cut into 0.5 cm^2 and the samples were placed in a 2.5% aqueous glutaraldehyde solution at 4°C overnight. The solution was then poured off and the samples were 2 rinsed three times with 0.1 mol/L phosphate buffer (pH 7.0) for 18 min each time. The leaf samples were fixed with 1% citric acid solution for 60–120 min. The citrate fixative was then removed, and the samples were rinsed three times with 0.1 mol/L phosphate buffer (pH 7.0) for 18 min each time. Leaf samples were dehydrated with different concentrations (50%, 70%, 80%, 90% and 95%, respectively) of ethanol, with each treatment lasting 12 min, followed by two 20 min treatments with 100% ethanol. Leaf samples were then treated with a mixture of isoamyl acetate and ethanol (1:1) for 30 min and then soaked in 100% isoamyl acetate for 60–120 min. The processed leaf samples were observed using a scanning electron microscope (XL30 ESEM, Philips, Netherlands).

Determination of Wax Contents

After the leaf samples were collected, the area of each blade was measured using a portable area meter (LI-3000C, LI-COR Biosciences, USA). The amount of wax was analyzed using a method adapted from Owen *et al.* (2006). Leaves were immersed in chloroform for 30 s to remove epi- and intracuticular waxes. 20 μg n-tetracosane was added to each sample to create an internal reference standard. Then wax samples were evaporated under nitrogen gas, dissolved in 50 mL of N, O-bis trifluoroacetamide with 1% trimethylchlorosi-lane (TMCS, Pierce, U.S.A.) and derivatized at 80°C for 90 min. Derivated samples in the GC chromatographic bottle (2 mL, Agilent corporation) were evaporated under nitrogen gas with nitrogen and then add 1 mL chloroform to dissolve the sample. The samples were dissolved in 1 mL chloroform, filtered by an organic phase filter (SCAA-103, Whatman, U.K.) and blown dry with nitrogen to 500 μL. The samples were analyzed by Gas phase - mass spectrometer detector (GCMS-QP2010 Ultra, SHIMADZU, Japan) under conditions followed by Owen *et al.* (2006).

Determination of Total Fatty Acid Profiles

The amount of fatty acids was analyzed using a method adapted from Chen *et al.* (2011). Leaves were heated at 80°C for 1.5 h in 1 mL of methanol containing 5% H_2SO_4 (v/v). Then sample were quenched 10 min in isopropanol at 85°C. Fatty acid methyl esters were extracted two times with 2 mL hexane and the solvent were evaporated to dryness under a stream of nitrogen. After evaporation of the solvents under nitrogen gas, fatty acid methyl esters were redissolved in hexane and analyzed by GC (5973C MSD, Agilent, U.S.A.) under conditions allowing the separation of regular and VLCFAs.

RNA Extract and Transcriptome Analysis

Three biological replicates (WT-y(1-3), WT-m(1-3), *wad-y*(1-3), *wad-m*(1-3)) were sent directly to the Biomarker Technology Company (Beijing, China) for RNA extraction and transcriptome sequencing. Sequencing data were analyzed on the BMKcloud (http://www.biocloud.net/) and included DEGs analysis, KEGG enrichment, expression pattern analyses and protein–protein interactions analyses. DEGs were selected using the criteria that the absolute value of the log2 | fold changes |≥1 and FDR < 0.05.

TF Enrichment Analysis

TF enrichment analysis was based on the Plant TF database (http://planttfdb.cbi.pku.edu.cn/index.php). The transcriptional regulations used here are identified from literature and ChIP-seq data, or inferred by combining TF binding motifs and regulatory elements data.

Quantitative RT-PCR Analysis

RNA for transcriptome sequencing was returned to our laboratory from the Biomarker Technology Company (Beijing, China). A total of 500 ng of RNA was used to synthesize cDNA with a PrimeScript™RT Kit (TaKaRa, Japan). The cDNA was diluted 10 times as the template. The reaction solution contained SYBR® PremixExTaq™II (Roche, Switzerland) and was conducted in a Bio-Rad CFX96TM Real-Time System (Applied Systems, U.S.A.). Three replicates were performed per sample.

Results

The *B. napus wad* Mutant is Wax Deficient during the Young Stage

Under normal conditions, after the true leaves grew, the leaves of *wad*-y were glossy (Fig. 1A). After bolting, the wax on the leaf' surfaces of *wad-m* showed a visible recovery (Fig. 1B). The WT-y leaf surface was covered by dense irregular wax crystals, while the leaf surface of *wad-y* was nearly devoid of wax crystals (Fig. 1C).

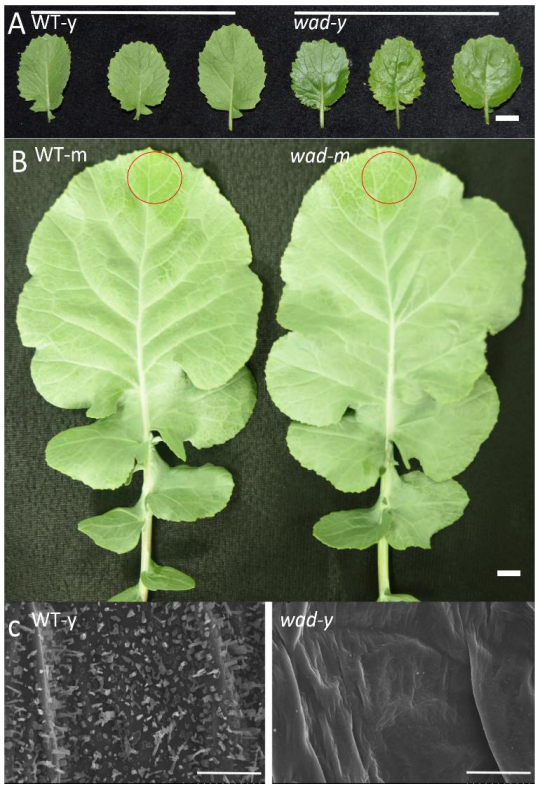

Fig. 1: Morphology of leaves of WT and *wad*
A: Leaves of WT-y and *wad-y*, the leaves is glossy in *wad-y*, bar=1 CM. **B:** Leaf of WT-m and *wad-m*, the red circle points to the place where the wax powder is removed to highlight the surrounding area that is covered by wax powder, bar=1 CM. **C:** Scanning electron micrographs of leaf cuticular waxes in WT-y and *wad-y*. WT-y leaf was covered by dense irregular wax crystals, and *wad-y* leaf were not covered by wax crystals, bar=10 μm

The Contents of Cuticular Wax and Fatty Acids in *wad-y* were Altered

Compared with WT-y, the total wax content on the *wad-y* leaf surface was significantly reduced by nearly 60% (Fig. 2A). The aldehyde, alkane and primary alcohol contents were reduced by 64%, 61% and 53%, respectively (Fig. 2B). C26 and C28 aldehydes, C27, C29 and C31 alkanes, and C26, C28 and C30 primary alcohols were significantly reduced. Interestingly, C22 primary alcohols accumulated in *wad* leaves (Fig. 2C).

C10–C26 fatty acids were detected in WT-y and *wad-y*. Compared with WT-y, C24 and C26 were significantly reduced in *wad-y* (Fig. 2D).

KEGG Enrichment Analysis Indicated the Involvement of the Fatty Acid Elongation Pathway

To investigate pathways that resulted in the dynamic wax changes in *wad*, a KEGG enrichment analysis was carried

out on DEGs in WT-y VS *wad-y*, *wad-y* VS *wad-m*, WT-y VS WT-m and WT-m VS *wad-m* comparisons (Fig. 3). DEGs in WT-y VS *wad-y* were clustered in the fatty acid elongation pathway. DEGs in *wad-y* VS *wad-m*, like those in WT-y VS WT-m, were mainly concentrated in the pathways required for plant growth and development, such as amino acid biosynthesis, but the DEGs in *wad-y* VS *wad-m* were concentrated in the fatty acid elongation pathway. Moreover, unlike DEGs in WT-y VS *wad-y* and *wad-y* VS *wad-m*, DEGs in WT-m VS *wad-m* were no longer enriched in the fatty acid elongation pathway.

Some DEGs that Clustered in the Fatty Acid Elongation Pathway Showed Similar Expression Patterns

A Venn diagram for DEGs involved in fatty acid elongation was constructed (Fig. 4A). The intersection of DEGs in the WT-y VS *wad-y* and *wad-y* VS *wad-m*, revealed that the expression levels of 11 genes were exclusively changed in WT-y VS *wad-y* and *wad-y* VS *wad-m*. These 11 genes were annotated as *KCS2*, *KCS2*, *KCS1*, *PAS2*, *KCR1*, *PAS2*, *KCR1*, *ECR*, *KCS6*, *ECR* and *KCS9* (Table 1).

Interestingly, these 11 genes had consistent expression patterns. Their expression levels were lower than WT levels in *wad-y*, but they all returned to WT levels in *wad-m* (Fig. 4B).

BPC6 may be the Key Regulator Affecting Fatty Acid Elongation in *wad*

The 11 genes were predicted to have no protein-protein interactions. TF enrichment analysis showed that TF *BPC6* (threshold p-value < 0.001) regulated these 11genes (Fig. 5).

Real-time Quantitative PCR Verification

Twenty DEGs related to fatty acid elongation were selected randomly for a quantitative RT-PCR assessment (Fig. S1). The results of the qRT-PCR were basically consistent with the results of the transcriptome sequencing, which indicated that the RNA sequencing results were reliable.

Discussion

Most wax-deficient mutants exhibit a significant glossy phenotype after germination and cannot recover during the developmental process. Mutants such as *pas1*, *kcs2* and *kcs20* exhibit glossy phenotypes throughout their life cycles (Lee *et al.*, 2010; Roudier *et al.*, 2010). Although some *MYB* TFs involved in the regulation of cuticle development are related to environmental stresses and/or developmental stages (Yoshimi *et al.*, 2013), such mutants with a wax recovery at a specific developmental stage have not been previously documented. There may be other mechanisms that regulate wax's dynamic changes.

In *wad-y*, a significant decrease was seen in the total wax content on the leaf surface, and this decrease was

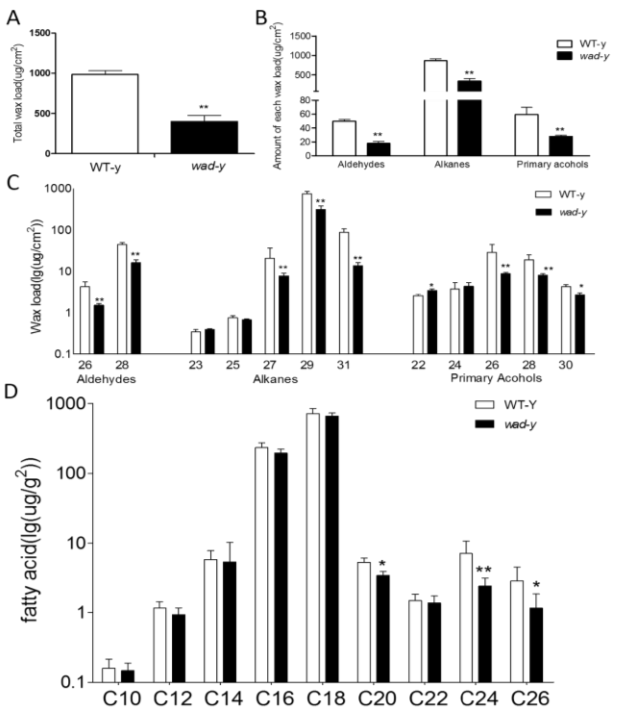

Fig. 2: Compositional analysis of leaf cuticular waxes in WT-y and *wad-y*

A, Total amounts of wax in leaves. B, Different kinds of wax in leaves. C, Content of each wax in leaves. D, Content of each fatty acids in leaves. T-test, $**p < 0.01$, $*p < 0.05$

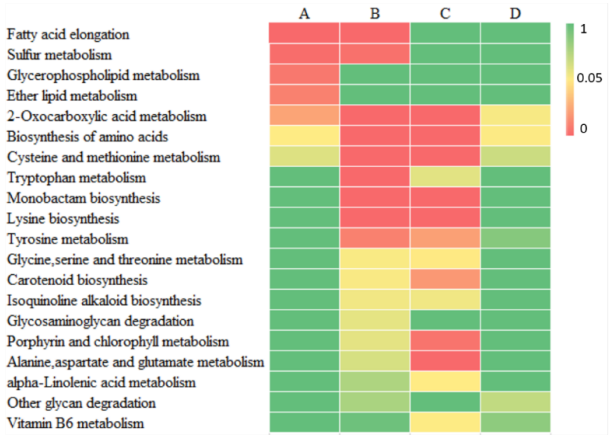

Fig. 3: KEGG enrichment analysis of DEGs in A WT-y VS *wad-y*, B *wad-y* VS *wad-m*, C WT-y VS WT-m, D *wad-m* VS WT-m

distributed among various kinds of wax (Fig. 2). The amount of cuticular wax can be altered by regulating the expression levels of genes involved in fatty acid elongation and wax biosynthesis pathways (Mao *et al.*, 2012).

The complete loss of genes involved in the wax biosynthesis pathway always results in a decrease in one kind of wax (Costaglioli *et al.*, 2005). The blockage of fatty acid elongation often results in the reduction or

Table 1: The annotation of DEGs gathered in the fatty acid elongation

GENE ID	ID in *Arabidopsis thaliana*	Annotation
BnaC05g02350D	AT1G04220	KCS2
BnaA10g02480D	AT1G04220	KCS2
BnaA10g00700D	AT1G01120	KCS1
BnaA03g02950D	AT5G10480	PAS2
BnaA07g26670D	AT1G67730	KCR1
BnaC09g46070D	AT5G10480	PAS2
BnaC06g28830D	AT1G67730	KCR1
BnaA09g34930D	AT3G55360	ECR
BnaA09g28510D	AT1G25450	KCS6/CUT1
BnaC08g26140D	AT3G55360	ECR
BnaC07g05570D	AT2G16280	KCS9

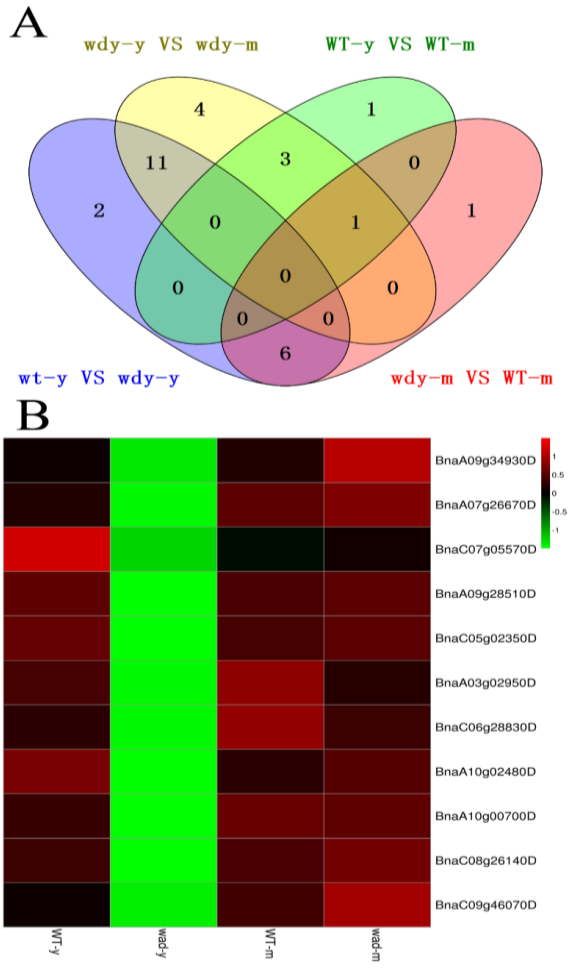

Fig. 4: Venn diagram and expression patterns of DEGs involved in fatty acid elongation. **A**: the venn diagram of DEGs involved in fatty acid elongation. **B:** expression patterns of 11 DEGs involved in fatty acid elongation

accumulation of wax having specific carbon-chain lengths (Hooker *et al.*, 2002). We speculated that fatty acid elongation pathway is responsible for the dynamic wax changes in *wad*. Supporting this notion, we discovered that C24 and C26 fatty acids were significantly reduced in *wad-y*. Moreover, KEGG enrichment analysis revealed that DEGs gathered in fatty acid elongation pathway play a crucial role in the dynamic wax changes.

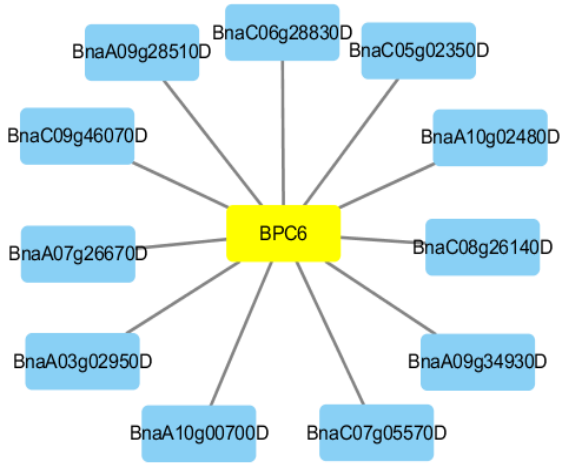

Fig. 5: The results of TF enrichment analysis for DEGs in fatty acid elongation. The result showed that BPC6 may target all of these genes

Fatty acid elongation pathway was regulated by 11 DEGs including *KCS1*, *KCS6* and *KCS9*. And to our surprise, these 11 genes had consistent expression patterns. The changes in expression of these genes affect the elongation of fatty acids and the biosynthesis of waxes (Millar *et al.*, 1999; Lee *et al.*, 2010; Haslam and Kunst, 2013; Kim *et al.*, 2013). *KCS1* and *KCS6* are involved in the fatty acid elongation of C24 to C26. The complete loss of *KCS1* expression results in a decrease of up to 80% in the levels of C26 to C30 fatty acids and in a decrease in the biosynthesis of waxes (Todd *et al.*, 2010). *KCS6* plays a key role in the production of VLCFA precursors that are essential for the synthesis of cuticular wax (Millar *et al.*, 1999). *KCS9* is involved in the elongation of C22 to C24, and *kcs9* knockout mutants exhibit significant reductions in C24 wax but an accumulation of C20 and C22 wax (Kim *et al.*, 2013). Our results are consistent with previous studies. The low expression levels of these genes in *wad-y* resulted in a decrease in wax having carbon chains > C24 and the recovery of their expression levels in *wad-m* resulted in a recovery in wax.

Our data suggested that *BPC6* is a key regulator of these 11 DEGs. *BPC6* is a member of BPCs, which is a poorly characterized plant-specific TF family (Simonini and Kater, 2014). BPCs are regulators of *INNER NO OUTER* and *SEEDSTICK*, which are involved in ovule development (Rebecca *et al.*, 2003; Meister *et al.*, 2010). BPCs are also involved in seed development through the regulation of the *LEAFY COTYLEDON 2* gene (Berger and Dubreucq, 2012). Moreover, BPCs control the floral transition by regulating *SHORT VEGETATIVE PHASE* (Sara *et al.*, 2012). *BPC1–3* can regulate *SHOOTMERISTEMLESS* to establish and

maintain the meristem (Simonini and Kater, 2014). These findings showed BPCs play various roles during different developmental stages (Hecker *et al.*, 2015). *BPC6* is widely expressed in plants (Monfared *et al.*, 2011), only has a known role in some organs, such as ovules and seeds, while its functions in the leaf and stem are still unknown. Based on its spatial distribution, *BPC6* is likely to participate in fatty acid elongation. While we have determined that *BPC6* is associated with fatty acid elongation at the transcriptional level, the details of how *BPC6* regulates fatty acid elongation needs to be investigated in future works.

Cuticular wax plays crucial roles in protecting plants from various biological and non-biological stresses. Although there are many studies about the wax biosynthesis pathway, the biosynthese the mechanisms behind its dynamic changes are still unknown. In this paper, we show that fatty acid elongation pathway is responsible for wax's dynamic changes and *BPC6* is the key regulator in this dynamic process. These findings provide new insights into the regulation of wax biosynthesis and a valuable target for breeding stress-tolerant rapeseed.

Conclusion

A novel *B. napus* wax-deficient mutant that shows a wax recovery phenotype at specific developmental stages was found. Based on the analysis of transcriptomic data of this mutant, a mechanism whereby *BPC6* regulates wax's dynamic changes by regulating the elongation of fatty acid chains was presented.

Acknowledgements

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