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SHORT COMMUNICATION

Targeted mutations of *BnPAP2* lead to a yellow seed coat in *Brassica napus* L.

Wei Huang[1, 2], Ruyu Jiao[2], Hongtao Cheng[3], Shengli Cai[3], Jia Liu[3], Qiong Hu[3], Lili Liu[1], Bao Li[1], Tonghua Wang[1], Mei Li[1], Dawei Zhang[2#], Mingli Yan[1, 2#]

[1] *Hunan Research Center of Heterosis Utilization in Rapeseed/Crop Research Institute, Hunan Academy of Agricultural Sciences, Changsha 410125, China*

[2] *Hunan Key Laboratory of Economic Crops Genetic Improvement and Integrated Utilization/School of Life and Health Science, Hunan University of Science and Technology, Xiangtan 411201, China*

[3] *Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture and Rural Affairs/Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan 430062, China*

Abstract

The yellow seed trait is preferred by breeders for its potential to improve the seed quality and commercial value of *Brassica napus*. In the present study, we produced yellow seed mutants using a CRISPR/Cas9 system when the two *BnPAP2* homologs were knocked out. Histochemical staining of the seed coat demonstrated that proanthocyanidin accumulation was significantly reduced in the *pap2* double mutants and decreased specifically in the endothelial and palisade layer cells of the seed coat. Transcriptomic and metabolite profiling analysis suggested that disruption of the *BnPAP2* genes could reduce the expression of structural and regulated genes in the phenylpropanoid and flavonoid biosynthetic pathways. The broad suppression of these genes might hinder proanthocyanidin accumulation during seed development, and thereby causing the yellow seed trait in *B. napus*. These results indicate that *BnPAP2* might play a vital role in the regulatory network controlling proanthocyanidin accumulation.

Keywords: yellow seed, *BnPAP2*, proanthocyanidins, CRISPR/Cas9

1. Introduction

Brassica napus L. is an important cultivated oilseed crop in China, which accounts for about 20% of production worldwide (Hu *et al.* 2017). Yellow-seeded *B. napus* has a thinner seed coat with a lower proanthocyanidin content and greater oil and protein contents than black seeds under the same genetic background (Chao *et al.* 2022). Thus, yellow seeds have been preferred by breeders to improve the seed quality in *Brassica* crops (Yan *et al.* 2011; Liu L *et al.* 2017; Rong e*t al.* 2022; Zhu *et al.* 2021).

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Wei Huang, E-mail: 2646213662@qq.com; #Correspondence Dawei Zhang, Mobile: +86-19173213360, E-mail: zhangdawei@hnust.edu.cn; Mingli Yan, Tel: +86-731-84691287, E-mail: ymljack@126.com

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The formation of yellow seeds in *Arabidopsis* is due to the accumulation of less proanthocyanidins (PAs) in the innermost cell layer of the seed coat (Lepiniec *et al.* 2006). The PA biosynthetic pathway has been well characterized, and is known to be regulated by at least four MBW ternary complexes formed by MYB123/TT2 (R2R3-MYB), TT8 (transparent testa 8, basic helix-loop-helix) and TTG1 (transparent testa glabra1, WD40 protein) (Xu *et al.* 2014). For example, the TT2-TT8-TTG1 complex plays a vital role in controlling the expression of *DFR* (*dihydroflavonol 4-reductase*), *ANS* (*anthocyanidin synthase*), and *TT19* (*transparent testa 19*) in the seed coat (Xu *et al.* 2015). In *B. napus*, the yellow seed trait is associated with the low expression or functional loss of the proanthocyanidin biosynthetic genes, such as *BnTTG1*, *BnTT1*, and *BnTT8* (Qu *et al.* 2013; Hong *et al.* 2017). Recently, multi-omics analyses revealed that *BrMYB113* and *BnTT8s* play key roles in determining the seed color by modulating lignin biosynthesis in *B. rapa* and *B. napus*, respectively (Zhang *et al.* 2022; Zhao *et al.* 2022). Meanwhile, targeted mutation of either the *BnTT8* or *BnTT2* homologs using a CRISPR/Cas9 system could create a stable yellow seed trait in *B. napus* (Xie *et al.* 2020; Zhai *et al.* 2020).

As an MYB transcription factor that forms the MBW complex, PAP2 (production of anthocyanin pigment 2) has long been recognized as a trigger that accelerates the accumulation of anthocyanin pigments in vegetative tissues (Xu *et al.* 2015; Zhang *et al.* 2020). The up-regulation of *BnPAP2* and its target genes has been identified as the key to promoting anthocyanin accumulation in *B. napus* leaves and stem bark (He *et al.* 2021; Fu *et al.* 2022). Ectopic expression of the *Orychophragmus violaceus PAP2* gene produces red anthers and red petals in *B. napus* (Fu *et al.* 2018). Moreover, transcriptional activation by insertions in the promoter region of *BnaA07.PAP2* could launch the whole anthocyanin pathway and produce new apricot and pink colors in *B. napus* flowers (Ye *et al.* 2022).

PA and anthocyanin biosynthesis share the flavonoid pathway, which is regulated by the MBW complex in a tissue-specific manner (Xu *et al.* 2015). However, while the critical role of *BnPAP2* in anthocyanin biosynthesis has been well explored, its role in the regulation of PA biosynthesis remains unclear. Therefore, we targeted the knockout of two *BnPAP2* homologs using the CRISPR/Cas9 system and produced a yellow seed trait in the mutants of *B. napus*. Further analysis confirmed that the mutation of the *BnPAP2* homologs significantly reduced the contents of PAs through the downregulation of genes involved in the flavonoid biosynthetic pathway.

2. Materials and methods

2.1. Plant materials

The *B. napus* cultivar Zhongshuang 6 (wild type (WT)) with a black seed color was used for genetic transformation. Both Zhongshuang 6 and the transgenic lines were grown in the greenhouse (16/8 h of light/dark at 22°C) at the Oil Crops Research Institute, Chinese Academy of Agricultural Sciences in Wuhan.

2.2. Cloning and sequence analysis of *BnPAP2*

Total DNA was extracted using the CTAB method. Full-length DNA sequences encoding *BnPAP2.A07* (*BnaA07G0287000ZS*) and *BnPAP2.C06* (*BnaC06G0329100ZS*) were amplified through PCR using gene-specific primers (Appendix A). The amplified PCR products were cloned into the pMDTM19-T vector (TaKaRa, Japan) and sequenced *via* Sanger sequencing.

2.3. Construction of the CRISPR/Cas9 vector and plant transformation

Two sgRNAs sequences were designed to target the *BnPAP2.A07* and *BnPAP2.C06* genes using CRISPR-P 2.0 (Liu H *et al.* 2017). The expression cassettes with target sequences were integrated into the pKSE401 CRISPR/Cas9 vector and then transformed into Zhongshuang 6 using *Agrobacterium*-mediated hypocotyl as described previously (Cheng *et al.* 2021). Total DNA was extracted from wild-type Zhongshuang 6 and the transgenic lines, and plasmid specific-primers were designed to screen the transgenic plants (Appendix A). The PCR products of *BnPAP2.A07* and *BnPAP2.C06* in the transgenic lines were transformed into *E. coli* for further Sanger sequencing.

2.4. Seed pigmentation observations

The seeds of Zhongshuang 6 and the transgenic lines were collected and stained with vanillin and DMACA as described previously (Yan *et al.* 2011). Developing seeds at 21 and 28 days after flowering (DAF) were harvested for microscopic analysis using Safranin O and Fast Green staining (Zhai *et al.* 2020).

2.5. Metabolite profiling analysis

Flavonoids were extracted from seeds of the double mutants and WT with three biological replicates. Metabolite identification and quantification were performed

using a UPLC-MS/MS System at Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China). The sample extraction was done essentially as described previously (Xie *et al.* 2020).

2.6. RNA-seq analysis

Seed coat tissues were collected and immediately frozen in liquid nitrogen with three biological replicates at 14, 21, and 28 DAF. The RNA extraction, cDNA library construction, sequencing, read filtering and mapping, differentially expressed gene identification, and KEGG pathway enrichment was performed as described previously (He *et al.* 2021). The accession number(s) of the transcriptomic and metabolite profiling analysis can be found at https://ngdc.cncb.ac.cn/gsa/, CRA009226.

3. Results

3.1. CRISPR/Cas9-targeted mutations in *BnPAP2* disrupt PA deposition and cause a yellow seed coat in *B. napus*

To check for putative mutation sites in the target genes, *BnPAP2.A07* and *BnPAP2.C06* were cloned and sequenced in Zhongshuang 6 which was used for genetic transformation (Appendix B). Phylogenetic analysis showed that BnPAP2.A07 and BnPAP2.C06 were clustered with PAP2 in *B. rapa* and *B. oleracea*, respectively (Appendix C). To generate Cas9-induced knockout mutations of *BnPAP2*, two specific target sites were designed in the second and third exons of *BnPAP2.A07* and *BnPAP2.C06* (Fig. 1-A). We ultimately obtained 50 independent transgenic lines of the T_0 generation through *Agrobacterium tumefaciens*-mediated transformation, and the mutations of *BnPAP2.A07* and *BnPAP2.C06* were verified by sequencing the genomic regions flanking the target sites. Three distinct homozygous double mutant lines (*pap2-6*, *pap2-38*, and *pap2-49*) were isolated in the T_1 generation. The single-base insertion (–1 bp) or deletion (+1 bp) at the target sites within *BnPAP2* were predicted to cause frameshifts, and the 12-base deletion (–12 bp) could cause the deletion of four amino acids in the protein (Fig. 1-B).

The *BnPAP2* mutants (*pap2-38* and *pap2-49*) exhibited yellow seed color compared with the WT black seed color at the mature stage (Fig. 1-C). Using DMACA and vanillin staining to visualize the PA accumulation in the seed coat of the WT and mutants, we found that the pigmentation of flavonoids was reduced in *pap2-38* and *pap2-49* compared with the WT during seed development (Fig. 1-D and E). Transverse sections of seeds showed that the red-stained PAs were deposited in the endothelial and palisade layer cells in the WT, but only slightly accumulated in part of the cells in mutants (Fig. 1-F).

3.2. Targeted mutations in *BnPAP2* repress the expression of flavonoid biosynthesis genes and change the flavonoid composition in the seeds

To assess the changes in flavonoid biosynthesis gene expression and composition after the targeted mutation of *BnPAP2*, transcriptomic analysis of seed coats at 14, 21, and 28 DAF (Appendix D), as well as metabolite profiling analysis of mature seeds (Appendix E) were performed for the WT and *pap2-49*, respectively (Fig. 2). The flavonoid biosynthesis pathway was significantly enriched in both differentially expressed genes (Fig. 2-A) and differentially accumulated metabolites (Fig. 2-B) between the double mutant and its WT control. Annotation of the differentially expressed genes involved in phenylpropanoid and flavonoid biosynthetic pathway showed that the majority of these genes (92.1%, 31/34) were suppressed to varying extents during seed development in the mutant as compared with the WT (Fig. 2-C). Besides the late biosynthetic genes, such as *DFR*, *LDOX* (*leucoanthocyanidin dioxygenase*), and *TT19*, which were directly regulated by PAP2, the early biosynthetic and regulated genes, including *PAL* (phenylalanine ammonia-lyase), *C4H* (cinnamic acid 4-hydroxylase) and *TT8*, were also downregulated in the *pap2* mutant. Metabolite profiling analysis also found that most flavonoid-related metabolites (86/99), such as naringenin and procyanidin A6, were lower in mutant seeds than in the WT (Fig. 2-D; Appendix F). These findings indicated that the disruption of *BnPAP2* repressed the expression of flavonoid biosynthesis pathway genes and subsequently hindered PA accumulation during seed development in *B. napus*, which was consistent with the yellow seed phenotype in the mutants (Fig. 2-E).

4. Discussion

Recent studies have shown that targeted mutation of either the *BnTT8* or *BnTT2* homologs can completely block PA deposition in the seed coat and create a stable yellow seed trait in *B. napus* (Xie *et al.* 2020; Zhai *et al.* 2020). In the present study, we found that CRISPR/Cas9-targeted mutations in *BnPAP2* could disrupt PA deposition and subsequently cause a yellow seed coat in *B. napus* (Fig. 1). PAs are the prominent pigments that are specifically accumulated in the inner integument

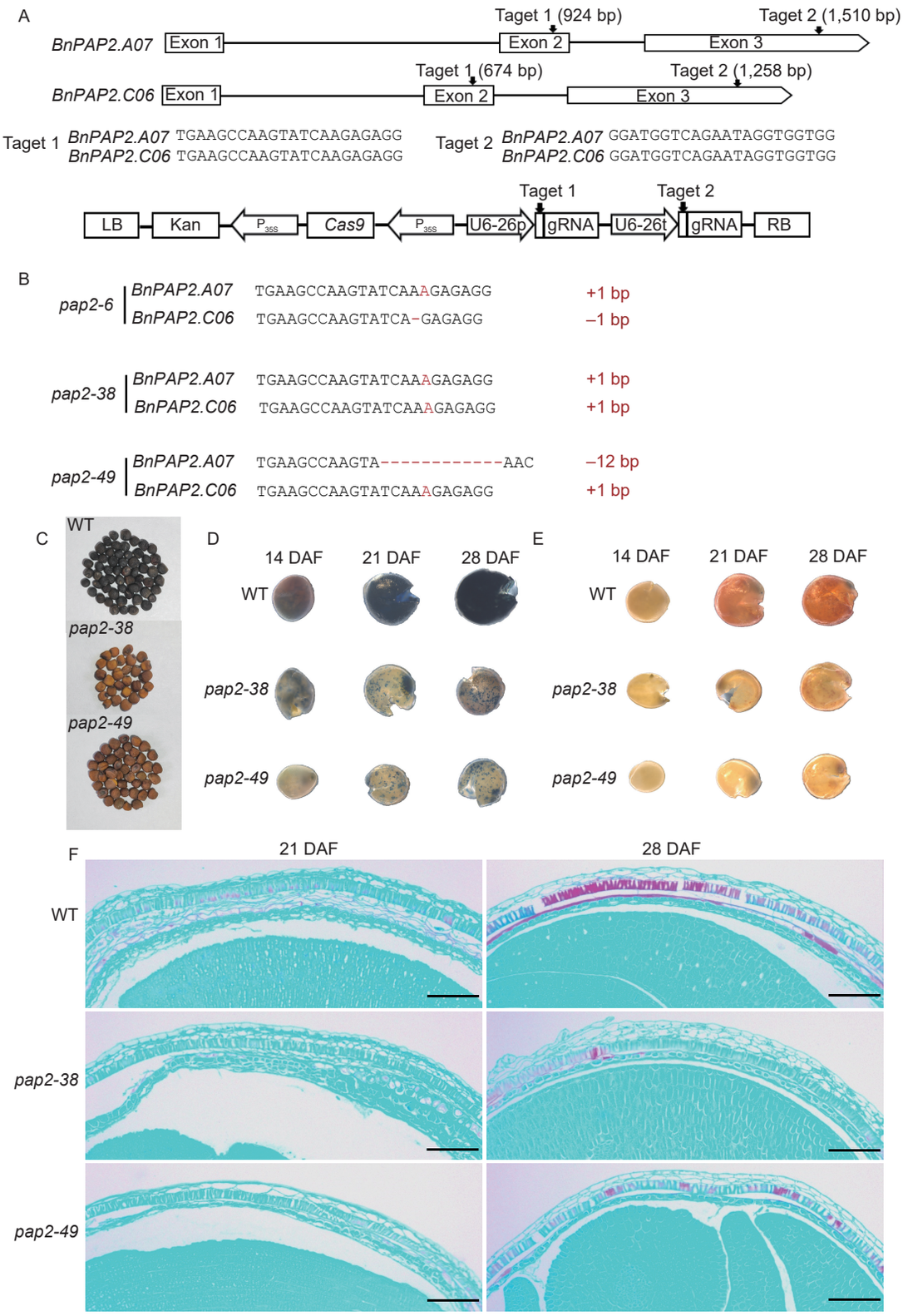

Fig. 1 Creation and phenotypes of the *BnPAP2* knockout lines. A, two target sites of the two exons of the *BnPAP2* homologs and vector structure of the CRISPR/Cas9 hosting two sgRNA expression cassettes. The vertical arrow in the gene model indicates the target site. B, sequencing at the mutation sites of the *BnPAP2* homologs in the T_1 generation. Nucleotide indels in the three homozygous mutations are marked in red, with details labeled at the right. C, seed colors of wild type (WT) and the *BnPAP2* mutants. Seed coat from the WT and *BnPAP2* mutants after DMACA (D) and vanillin (E) staining. F, microscopy of Safranin O and Fast Green stained seeds at 21 and 28 days after flowering (DAF). Bars, 50 μm.

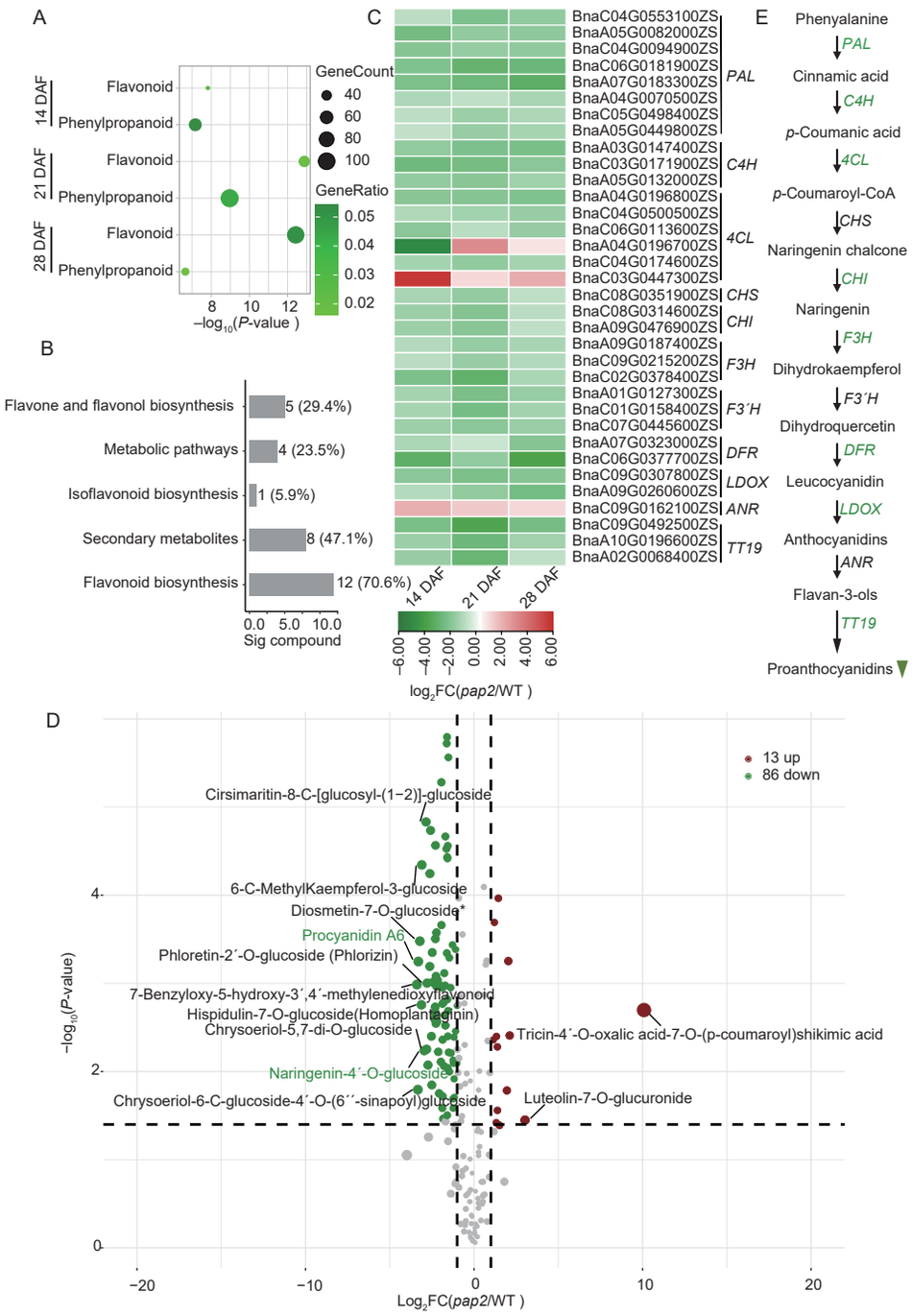

Fig. 2 Transcriptomic and metabolite profiling analysis of *BnPAP2* knockout lines. A, KEGG enrichment of differentially expressed genes in seed coats between the *pap2* mutant and wild type (WT) at different developmental stages. Phenylpropanoid synthesis and flavonoid synthesis are interrelated, mainly through the pathway of chalcone for entering the flavonoid pathway (Lepiniec *et al.* 2006). The *x*-axis indicates the $-\log_{10}$(*P*-value), and the circle indicates the number of genes enriched in that item. B, KEGG enrichment of differentially accumulated metabolites in seeds between the *pap2* mutant and WT at the mature stages. The number of metabolites in each item is marked at the top of the bar chart. KEGG pathway enrichment was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) databases as described by Ye *et al.* (2022). C, heatmap of differentially expressed genes involved in the phenylpropanoid and flavonoid biosynthetic pathway. The red bands indicate high gene expression and the green bands indicate low gene expression in the mutant. *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamic acid 4-hydroxylase; *4CL*, 4-coumarate coenzyme A ligase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone 3-hydroxylase; *F3′H*, flavonoid 3′-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *LDOX*, leucoanthocyanidin dioxygenase; *ANR*, anthocyanidin reductase; *TT19*, transparent testa 19. D, differentially accumulated metabolites in seeds between the *pap2* mutant and WT. The red points indicate significantly higher accumulation of metabolites and the green points indicate significantly lower accumulation of metabolites in the mutant. E, annotation of differentially expressed genes in the flavonoid and phenylpropanoid pathways.

of the seed coat, which confer a dark color in the seeds through their oxidation during maturation (Xu *et al.* 2015). Herein, DMACA and vanillin, as well as Safranin O and Fast Green staining of seed coats, demonstrated that PA accumulation was significantly lower in the *pap2* double mutants and decreased specifically in the endothelial and palisade layer cells of the seed coat (Fig. 1-D–F). Although the blocking of PA deposition in *pap2* mutants was not as complete as in *tt8* and *tt2*, the obvious differences in phenotype and histochemical staining still suggest a critical role for *BnPAP2* in controlling the accumulation of PAs in the seed coat.

In *Brassica*, the ternary MBW complex formed by TT2–TT8–TTG1 is necessary for the temporal and spatial regulation of flavonoid biosynthesis structural genes in seeds (Padmaja *et al.* 2013; Xie *et al.* 2020; Zhai *et al.* 2020; Hu *et al.* 2022). Although PAP2 is also part of complex forming the MBW ternary, it has long been considered as a core component that accelerates the accumulation of anthocyanins in vegetative tissues rather than PAs in seeds in both *Brassica* (Fu *et al.* 2018; Ye *et al.* 2022) and *Arabidopsis* (Lepiniec *et al.* 2006; Xu *et al.* 2014, 2015). In this study, a transcriptomic analysis of *pap2* mutants revealed that the disruption of *Bn PAP2* genes could reduce not only the expression of structural genes in the phenylpropanoid and flavonoid biosynthetic pathways but also the expression of core-regulated genes, such as *TT8* (Fig. 2-C). The broad suppression of phenylpropanoid and flavonoid biosynthesis genes in *pap2* mutants suggested that BnPAP2 is a key regulator of the MBW complex in controlling PA accumulation in seeds (Fig. 2-D and E).

5. Conclusion

Collectively, our results demonstrate that *BnPAP2* might play a vital role in the regulatory network controlling PA accumulation. The knockout of *BnPAP2* could suppress gene expression in the PA pathway, and reduce PA accumulation in the endothelial and palisade layer cells, resulting in a yellow seed trait in *B. napus*.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendices associated with this paper are available on https://doi.org/10.1016/j.jia.2023.05.001

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