Rapeseed (*Brassica napus*) Mitogen-Activated Protein Kinase 1 Enhances Shading Tolerance by Regulating the Photosynthesis Capability of Photosystem II

Zhen Wang\(^1,2,\ast\), Miao Liu\(^3,\ast\), Mengnan Yao\(^4,\ast\), Xiaoli Zhang\(^1\), Cunmin Qu\(^1,2,\ast\), Hai Du\(^1,2,\ast\), Kun Lu\(^1,2,\ast\), Jiana Li\(^1,2,\ast\), Lijuan Wei\(^1,2,\ast\) and Ying Liang\(^1,2,\ast\)

\(^1\)Engineering Research Center for Rapeseed, College of Agronomy and Biotechnology, Southwest University, Chongqing, China, \(^2\)Engineering Research Center of South Upland Agriculture of Ministry of Education, Academy of Agricultural Sciences, Chongqing, China, \(^3\)Key Laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), Institute of Agro-Bioengineering College, Guizhou University, Guiyang, China, \(^4\)Jiangsu Yanjiang Institute of Agricultural Sciences, Nantong, China

\(\ast\)Correspondence: Ying Liang

yliang@swu.edu.cn

Lijuan Wei

lijuan525888@163.com

\(\ast\)These authors have contributed equally to this work

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Rapeseed (*Brassica napus*) is the third-largest source of vegetable oil in the world with an edible, medicinal, and ornamental value. However, insufficient light or high planting density directly affects its growth, development, yield, and quality. Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that play key roles in regulating the responses to biotic and abiotic stresses in plants. In this study, we found that the promoter of *BnaMAPK1* contained several light-responsive elements (including the AT1-motif, G-Box, and TCT-motif), consistent with its shading stress-induced upregulation. Compared with the wild type under shading stress, *BnaMAPK1-* overexpressing plants showed higher light capture efficiency and carbon assimilation capacity, enhancing their shading tolerance. Using RNA sequencing, we systematically investigated the function of *BnaMAPK1* in shading stress on photosynthetic structure, Calvin cycle, and light-driven electron transport. Notably, numerous genes encoding light-harvesting chlorophyll a/b-binding proteins (BnaLHCBS) in photosystem II-light-harvesting complex (LHC) II supercomplex were significantly downregulated in the *BnaMAPK1-* overexpressing lines relative to the wild type under shading stress. Compared with the wild type under shading stress, *BnaMAPK1-* overexpressing plants showed higher light capture efficiency and carbon assimilation capacity, enhancing their shading tolerance. Using RNA sequencing, we systematically investigated the function of *BnaMAPK1* in shading stress on photosynthetic structure, Calvin cycle, and light-driven electron transport. Notably, numerous genes encoding light-harvesting chlorophyll a/b-binding proteins (BnaLHCBS) in photosystem II-light-harvesting complex (LHC) II supercomplex were significantly downregulated in the *BnaMAPK1-* overexpressing lines relative to the wild type under shading stress. Combining RNA sequencing and yeast library screening, a candidate interaction partner of BnaMAPK1 regulating in shading stress, BnaLHCB3, was obtained. Moreover, yeast two-hybrid and split-luciferase complementation assays confirmed the physical interaction relationship between BnaLHCB3 and BnaMAPK1, suggesting that BnaMAPK1 may involve in stabilizing the photosystem II–LHC II supercomplex. Taken together, our results demonstrate that *BnaMAPK1* positively regulates photosynthesis capability to respond to shading stress in rapeseed, possibly by controlling antenna proteins complex in photosystem II, and could provide valuable information for further breeding for rapeseed stress tolerance.

Keywords: *BnaMAPK1*, shading stress, photosynthesis, photosystem II, *Brassica napus*
INTRODUCTION

Rapeseed (Brassica napus) is grown across the globe in various climatic conditions, from boreal to subtropical climates. It is utilized as leafy vegetable for human consumption, and also used for flower ornamental plant, oil production, biofuel, and animal feed (Confortin et al., 2019). The production of rapeseed is highly influenced by biotic and abiotic stresses, such as Sclerotinia sclerotiorum, Peronospora parasitica, light, temperature, drought, and salinity, all of which can drastically decrease yields (Raza et al., 2020; He et al., 2021; Raza, 2021); therefore, identifying stress-tolerance genes and understanding the related genetic network will provide a theoretical basis for developing new cultivars in rapeseed.

Plants can adapt to the complex environments by regulating gene expression and signal transduction pathways to advantageously alter their physiology and development. Mitogen-activated protein kinase (MAPK) cascades are highly conserved signal transduction pathways present in all eukaryotes (Cristina et al., 2010). A canonical MAPK network is composed of MAPK kinase kinases (MAPKKKs), MAPK kinases (MKKs), and MAPKs (Ichimura et al., 2002). The phosphorylated MAPKs in turn phosphorylate various downstream substrates, which are involved in the regulation of a wide variety of stress responses (Ichimura et al., 2002; Jonak et al., 2002; Pitzschke et al., 2009; Cristina et al., 2010; Zhang et al., 2018). MAPKs can be classified into four subfamilies (A–D), with subfamily C containing four members (MAPK1, MAPK2, MAPK7, and MAPK14) (Ichimura et al., 2002), which mainly play a role in abiotic stress responses. AtMAPK1, AtMAPK2, and PsMAPK2 in pea (Pisum sativum) are all transcriptionally activated in response to wounding, jasmonic acid, and hydrogen peroxide (Ortiz-Masia et al., 2007, 2008). The four subfamily C MAPKs in Arabidopsis are activated by AtMKK3 to regulate the ABA signaling pathway (Danquah et al., 2015). It is likely that the MAPKs play varying roles in different stress responses (Teige et al., 2004).

Among several stress factors, light plays a vital role as it provides energy for photosynthesis and determines the growth, development, and morphogenesis of plants (Ruberti et al., 2012; Zoratti et al., 2014; Viallet-Chabrand et al., 2017). Insufficient light or shade condition is a pervasive abiotic stress in plant breeding and cultivation due to light blockage from intercropping, high planting density, horticulture facilities, cloud, rain, and snow. In rapeseed, shading stress is not conducive to flowering stage, when nutrients from the stem are also used for reproductive growth in the inflorescence (Li et al., 2013; Lu et al., 2017), with the leaves being the most important until the flowering stage, when nutrients from the stem are also used for reproductive growth in the inflorescence (Li et al., 2013; Wang et al., 2016; Lu et al., 2017). At maturity, photosynthesis in the siliques facilitates the biosynthesis of proteins and lipids to store in the seeds (Elahi et al., 2016; Wang et al., 2016; Zafar et al., 2019). The reduction of photosynthesis under shading stress is therefore a major problem for rapeseed leaf and flower development and biomass accumulation, and overcoming this issue would be of great importance for breeding rapeseed with high edible, medicinal, and ornamental value.

In order to adapt to a shady environment, plants have evolved many strategies to increase their photosynthetic rate, including enhancing the stability of the photosystem I (PS I) and PS II complexes, the transcription, translation, and post-translational modification of photosynthesis-related genes and proteins (Walters, 2005). In higher plants, the light-harvesting complexes (LHCs) are divided into LHC I and LHC II groups, serving as PS I and PS II antennae, respectively (Klimmek et al., 2005, 2006). LHC II proteins are divided into four types (LHC II a–d), of which LHC II b is the major type. The three LHC II b proteins are encoded by the highly similar genes LHCB1, LHCB2, and LHCB3, and probably form homo- or hetero-trimers (Creppin and Caffarri, 2018). The minor LHC II proteins associated with PS II, CP29 (LHC II a), CP26 (LHC II c), and CP24 (LHC II d), are encoded by LHCB4, LHCB5, and LHCB6, respectively (Caffarri et al., 2009; Kirilovsky and Büchel, 2019). In the PS II complex, the inner antennae CP43 (PsbC) and CP47 (PsbB) bind to the D1 (PsBA) and D2 (PsBD) subunits to form core polypeptides, which then associate to the LHC II complex to form the PS II–LHC II supercomplex, playing a crucial role in capturing light in photosynthesis (Standfuss and Kühnbrandt, 2004).

The shading response affects the dynamic balancing of light transmission and distribution between PS I and PS II, which modulates the stability of the LHC II by the phosphorylation/dephosphorylation of protein kinases and phosphatases, increasing the light-harvesting area, and enhancing the efficiency with which light energy is used (Tikkanen and Aro, 2012; Rantala et al., 2020). Reversible and differential phosphorylation are dependent on the serine/threonine protein kinases 7 (STN7), STN8, and the thylakoid-associated phosphatase of 38-kD/protein phosphatase 1 (TAP38/PPH1) playing important roles in the phosphorylation of LHC II (Pribil et al., 2010; Tikkanen and Aro, 2012; Rantala et al., 2016, 2020). In addition, Zhang’s lab discovers that another type of serine/threonine protein kinases, AtMAPK3 and AtMAPK6, can rapidly downregulate various components in the PS II–LHC II supercomplex to participate in the hypersensitive response cell death in Arabidopsis (Su et al., 2018). In rapeseed, however, the effects of limited light and the molecular mechanisms of the shade response, especially the cross-link between MAPKs and LHCBS, remain undercharacterized and poorly understood.

Here, we isolated the promoter of BnaMAPK1 (ProBnaMAPK1) and identified a series of light-responsive cis-acting elements, and tested the effect of shading on BnaMAPK1 expression. Using wild-type (WT) and transgenic BnaMAPK1-overexpressing (BnaMAPK1-OE) rapeseed, the mechanisms of the BnaMAPK1 response to shading stress were, respectively, investigated on photosynthetic structure (pigment and enzyme system and photosynthesis-related complex), Calvin cycle, and light-driven electron transport through the examination of a combination of photosynthetic traits, RNA sequencing (RNA-Seq), and protein–protein interactions. This research aims to characterize the function of BnaMAPK1 under shady conditions,
MATERIALS AND METHODS

Plant Materials and Growth Conditions
The black-seed doubled haploid inbred B. napus cultivar Zhongyou821 (WT group), three BnaMAPK1-OE T3 lines (OE group), and tobacco (Nicotiana benthamiana) used in this study were obtained from Southwest University in China. The complete coding sequence of BnaMAPK1 (BnaA06g06010D) under the control the CaMV 35S promoter was transformed into Zhongyou821 to create OE materials with Basta resistance, as described previously (Weng et al., 2014). The rapeseed and tobacco seeds were soaked in Petri dishes and stratified at 4°C for 2–3 days. The seeds were then sown in nutritious soil in a growth chamber (PGR15, Controlled Environments, Winnipeg, MB, Canada), with a 16-h light (25°C)/8-h dark (20°C) photoperiod, 75% humidity, and an 800 μmol·m−2·s−1 light intensity. The photosynthetic photon flux density (PPFD) was measured using a quantum radiometer/photometer (LI-185B; LI-COR Biosciences, Lincoln, NE, United States).

Photosynthetic Light-Response Curve Analysis and Shading Treatment
For the photosynthetic light-response curve measurements, three healthy and mature leaves from the center of each 3–4-week-old Zhongyou821 seedling at the six-leaf stage (with four expand true leaves and two bud leaves) were selected and marked as fixed measured leaves. Curves were measured 10 times for each marked leaf, so that 30 times were measured for each seedling, with three seedlings used for each light intensity. During the measurement, the CO2 concentration was set to the atmospheric CO2 concentration, the flow rate of air in the measuring chamber was about 500 μmol·s−1, the temperature of the leaf chamber was 25°C±1°C, and the relative humidity was 75%±5%. For every measurement, the photosynthetically active radiation (PAR) was set at 1,400, 1,200, 1,000, 800, 500, 400, 300, 200, 100, 50, 20, and 0 μmol·m−2·s−1 by portable photosynthetic system (LI-6400; LI-COR Biosciences) to measure the net photosynthetic rate (Pn) under different light intensities (Li et al., 2019). For each PAR, the measurement time was controlled to 4 min and Pn was stabilized and recorded automatically by the instrument. The light saturation point (LSP), light compensation point (LCP), dark respiration rate (Rd), maximum net photosynthetic rate (Pnmax), and apparent quantum efficiency (AQE) were estimated based on the trend of the light-response curve by the modified rectangular hyperbola model (Ye et al., 2013).

Based on the photosynthetic light-response curve, a light intensity of 300 μmol·m−2·s−1 was selected to perform the shading treatment on the WT and three independent BnaMAPK1-OE lines (OE-1, OE-2, and OE-3) at the six-leaf stage. These plants were exposed to the shading treatment for 0, 7, 14, 21, and 28 days to investigate their photosynthetic traits under this stress. For the analysis of BnaMAPK1 expression, the leaves of the WT seedlings were, respectively, collected after 0, 1.5, 3, 6, 9, 12, and 15 h of the shading treatment and used for RNA extraction. Leaves were also collected after 0 and 12 h of treatment for RNA-Seq. These leaves samples were transferred into RNase-free microfuge tubes and immediately placed into liquid nitrogen. Three biological duplicates were performed for each experiment.

Photosynthetic Trait Measurements
For the measurements of the physiological and chlorophyll fluorescence traits, WT and BnaMAPK1-OE leaves after 0 and 28 days of shading stress were measured using a LI-6400 portable photosynthetic system, with the same settings as were used for the light-response curve (Zhao et al., 2015; Pan et al., 2018). Each leaf was tested 10 times, and the average values after 0 and 28 days of the shading treatment were calculated. The biochemical traits include the Pn, stomatal conductance (Gs), and intercellular CO2 concentration (Ci), and transpiration rate (Tr). For the chlorophyll fluorescence measurements, all plants were dark-adapted in advance for at least 3 h. The minimal fluorescence (F0), photochemical quenching coefficient (qP), non-photochemical quenching coefficient (qN), and maximum photochemical efficiency of PS II (Fv/Fm) were measured in each leaf and the average values were calculated (Li et al., 2020).

For the measurements of the biochemical traits, the relative content of chlorophyll (SPAD value) was determined in the WT and BnaMAPK1-OE by means of a chlorophyll meter (SPAD-502; Konica Minolta, Tokyo, Japan). Each leaf was marked at 10 representative points, and the average SPAD values after 0, 7, 14, 21, and 28 days of the shading treatment were calculated (Li et al., 2020). The Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity was assayed as described in the protocol of the plant Rubisco enzyme-linked immunosorbent assay kit (Cusabio Biotech, Wuhan, China). Fresh leaf material (0.5g) was obtained from the WT and BnaMAPK1-OE seedlings after 0, 7, 14, 21, and 28 days of the shading treatment. Using the slightly modified enzyme extraction method of Xu et al., (2020), the material was homogenized with 6ml of cooled extraction buffer containing 100 mM Tris–HCl (pH 7.8), 10 mM MgCl2, 1 mM EDTA, 20 mM β-hydroxy-1-ethanethiol, and 2% (m/v) polyvinyl pyrrolidone at 0°C–4°C. The product was centrifuged at 14,000×g for 20 min at 4°C, and the supernatant was used for Rubisco activity measurement.

RNA Isolation, cDNA Biosynthesis, and Real-Time Quantitative PCR Analyses
To identify the relative expression levels of BnaMAPK1 during shading stress, Zhongyou821 seedling leaf samples were harvested after 0, 1.5, 3, 6, 9, 12, and 15 h of the shading treatment. Total RNA was extracted from non-stressed and shade-stressed rapeseed seedling leaves using the RNAprep pure plant kit.
RNA-Seq Analysis

The RNA-Seq analysis was performed on WT and BnaMAPK1-OE rapeseed plants after 0 and 12 h of shading stress. The leaves, stems, and roots of seedlings were collected as mixed samples for total RNA extraction. The RNAs of three OE lines were extracted, respectively, and mixed with equal quality by three biological replicates, named as OE-Rep1, OE-Rep2, and OE-Rep3. Libraries were constructed and sequenced at Beijing Biomarker Technology (Beijing, China). An Illumina HiSeq X 10 sequencing platform was used to generate paired-end reads. Sequencing data has been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) database with the accession BioProject ID: PRJNA680826.

The raw data were first processed to perform quality control to obtain clean reads which were then mapped to the B. napus reference genome sequence with Hisat2 tools (Kim et al., 2015). The gene expression levels were estimated using the fragments per kilobase of transcript per million fragments mapped (FPKM) method (Mortazavi et al., 2008). A differential expression analysis of the control/shading-treated plants and the BnaMAPK1-OE/WT groups was performed using DESeq (Wang et al., 2009). The following pairwise comparisons were performed: BnaMAPK1-OE samples with WT samples under the control (0h_OE vs. WT) and shading treatments (12h_OE vs. WT), and WT plants (WT_12h vs. 0h) or BnaMAPK1-OE plants (OE_12h vs. 0h) subjected to the control and stress conditions. Differentially expressed genes (DEGs) were defined as having a FPKM value > 1 for each of the three biological replicates, an absolute fold change (FC) value ≥ 2, and false discovery rate (FDR) < 0.05. The enrichment analysis of Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathways of the DEGs in the 12h_OE vs. WT group was implemented by GOseq R packages with a \( p_{\text{adj}} < 0.05 \) (Young et al., 2010) and KOBAS software with a \( q_{\text{value}} < 0.05 \) (Mao et al., 2005), respectively. qRT-PCR was performed to verify the RNA-Seq data (primers are listed in Supplementary Table S1).

RESULTS

BnaMAPK1 Can Be Induced by Shading Stress in Rapeseed

To identify the putative cis-acting regulatory elements in the promoter of BnaMAPK1 in the Zhongyou821 rapeseed line, a 1,389-bp sequence upstream of the start codon was obtained using a PCR amplification and cloned based on the promoter of BnaA06g06010D from the Genoscope database. A bioinformatics analysis of the promoter sequence using the PlantCARE online tools (Lescot et al., 2002) revealed the presence of cis-acting elements that were shown to regulate gene expression in response to stress in plants. Several light-responsive elements were also identified, including the AT1-motif (ATTAA(TT/TA)C), G-box (CACGTC), and TCT-motif (TCTTAC), suggesting that BnaMAPK1 may be involved in responding to light stress. The detected putative light-responsive...
BnaMAPK1 Responds to Shading Stress and Improves Shading Tolerance

To evaluate the role of BnaMAPK1 in protecting the photosynthetic apparatus from shading stress, we produced BnaMAPK1-OE lines of rapeseed. In the three overexpression independent lines (OE-1, OE-2, and OE-3), the relative expression levels of BnaMAPK1 were significantly higher than in WT rapeseed (Supplementary Figure S1C). To investigate how the rapeseed plants were affected under shading stress, the physiological traits of WT and BnaMAPK1-OE rapeseed were determined. Under normal light conditions (Control, 0 days), the Pn, Gs, and Ci displayed no significant differences between BnaMAPK1-OE and WT rapeseed, although the Tr values of the BnaMAPK1-OE-1 and BnaMAPK1-OE-3 lines were significantly higher than that of the WT (p-value < 0.05). When the plants were exposed to the shading treatment for 28 days, the Pn of both the BnaMAPK1-OE and WT lines decreased markedly, although the decrease was greater in the WT than in the BnaMAPK1-OE plants (p-value < 0.01) (Figure 1A), suggesting that overexpressing BnaMAPK1 could alleviate the decrease in photosynthetic rate caused by shading stress. In addition, the observed changes in Gs, Ci, and Tr were similar to Pn, with significantly greater decreases observed in the WT than in the transgenic plants under shading stress (Figures 1B–D). These data indicated that overexpressing BnaMAPK1 enhances the gas exchange ability of rapeseed to improve its photosynthetic rate under long-term low-light environments.

To observe the energy dissipation, the chlorophyll fluorescence characteristics of F0, qP, qN, and Fv/Fm were measured. When plants were exposed to shading stress for 28 days, the F0 and qN increased in both the WT and BnaMAPK1-OE rapeseed relative to the normal light condition, with these traits remaining significantly lower in the BnaMAPK1-OE lines than in the WT under the shaded condition (p-value < 0.01) (Figures 2A,C). This suggests that overexpressing BnaMAPK1 enhances the light capture efficiency to improve plants’ sensitivity to light. Additionally, after 28 days of shading, qP and Fv/Fm decreased in the WT and BnaMAPK1-OE plants, indicating a decrease in the rate of light energy conversion. Both qP and Fv/Fm remained higher in the BnaMAPK1-OE lines than in the WT plants under shading stress, demonstrating that the overexpression of BnaMAPK1 could effectively maintain the transmission of light in PS II to regulate photosynthetic ability during shading stress (Figures 2B,D).

Additionally, the biochemical characteristics of WT and BnaMAPK1-OE rapeseed were also assessed under shading stress. As shown in Figure 1E, the relative chlorophyll contents of the BnaMAPK1-OE lines were all significantly higher (p-value < 0.01) than the WT after 14 days of shading stress (BnaMAPK1-OE plants: 61.52, 52.01, and 50.66; WT: 44.20, 30.35, and 29.33 after 14, 24, and 28 days of shading). This demonstrated that overexpressing BnaMAPK1 led to the retention of more chlorophyll, increasing the light energy utilization under shading stress in rapeseed. Rubisco is a key enzyme for photosynthesis, and was therefore investigated next. The activities of Rubisco in the three BnaMAPK1-OE lines were significantly higher than in the WT plants under normal light condition, while the shading treatments decreased the Rubisco activity of both the BnaMAPK1-OE and WT lines, although the BnaMAPK1-OE lines remained markedly higher than that of the WT (Figure 2E). These findings suggest that overexpressing BnaMAPK1 could improve Rubisco activity, which would affect the net photosynthetic rate. Our data therefore confirmed that BnaMAPK1 positively regulates light capture efficiency and carbon assimilation capacity to improve shading tolerance in rapeseed, which was consistent with the results of the promoter cis-acting element analysis.

Identification of DEGs Under Shading Stress Using RNA-Seq

To gain insight into the regulatory mechanisms by which BnaMAPK1 is involved in shading stress, an RNA-Seq analysis was performed. The RNA used for the RNA-Seq was extracted from the leaves of the WT and BnaMAPK1-OE plants under normal light conditions and after 12 h of the shading treatment. Through pairwise comparisons, a total of 3,225, 3,000, 7,163, and 5,558 DEGs were, respectively, identified between the BnaMAPK1-OE and WT plants under normal light conditions (0h_OE vs. WT), BnaMAPK1-OE and WT under shading stress (12h_OE vs. WT), WT under normal light conditions and shading stress (WT_12h vs. 0h), and BnaMAPK1-OE under normal light conditions and shading stress (OE_12h vs. 0h), with a FDR < 0.05 and a [Log(FC)] threshold > 1 (Figures 3A,B). In the 0h_OE vs. WT, 12h_OE vs. WT, WT_12h vs. 0h, and OE_12h vs. 0h groups, 1,904, 1,346, 3,213, and 2,302 DEGs were upregulated and 1,321, 1,654, 3,950, and 3,256 were downregulated in the shade treatment/overexpression line relative to the normal light condition/WT (Figure 3B). Moreover, using [Log(FC)] > 3 as a more stringent cutoff, Figure 3C shows the 164 DEGs (accounting for 5.47% of all DEGs) in the 12h_OE vs. WT comparison, of which 85 were upregulated and 79 were downregulated.

elements and their functions are listed in Supplementary Table S2.

Using a quantum radiometer/photometer, the Pn of Zhongyou821 rapeseed was measured to investigate the light-response curve. Before the PAR reached 800 μmol·m⁻²·s⁻¹, the Pn increased markedly, although little change was observed when the PAR was between 300 and 400 μmol·m⁻²·s⁻¹ (Supplementary Figure S1A). Using the Ye Zi-Piao model to fit the light-response curve, the fitted result was similar to the actual measured value, with 800 μmol·m⁻²·s⁻¹ set as the light saturation point and 300 μmol·m⁻²·s⁻¹ as the shading stress light intensity (Supplementary Table S3). To detect the response patterns of BnaMAPK1 expression, the plants were subjected to 15 h of shading stress at 300 μmol·m⁻²·s⁻¹. A qRT-PCR analysis showed that BnaMAPK1 was upregulated after 3 h, reached a peak at 12 h, then decreased, indicating that BnaMAPK1 was induced by shading stress (Supplementary Figure S1B).
GO Enrichment Analysis of Photosynthesis-Related DEGs

A GO enrichment analysis was performed on the 3,000 DEGs in the 12h_OE vs. WT group to annotate their functions into three main categories: molecular function (MF), cellular component (CC), and biological process (BP). Using a `p.adjust < 0.05`, a total of 570 GO pathways were assigned to the 3,000 DEGs that responded to shading stress, with 19, 83, and 468 in the MF, CC, and BP categories, respectively. Within GO-MF, "oxidoreductase activity (GO:0016491)," "oxidoreductase activity, acting on single donors with incorporation of molecular oxygen (GO:0016701)," and "cobalt ion binding (GO:0050897)" were the most dominant enriched terms. Within GO-CC, the significantly enriched terms were related to "cytoplasmic part (GO:0044444)," "cytoplasm (GO:0005737)," and "plastid (GO:0009536)." Within GO-BP, the majority of the enriched GO terms were involved in "small molecule metabolic process (GO:0044281)," "oxoacid metabolic process (GO:0043436)," and "organic acid metabolic process (GO:0006082)." The eight most significantly overrepresented GO terms in each category are shown in Figure 4A.

Given that the overexpression of BnaMAPK1 improves photosynthesis, we focused on the GO-BP category differences between the BnaMAPK1-OE and WT plants that were implicated...
in the photosynthesis pathways, and identified 12 enriched GO pathways, including “response to light stimulus (GO:0009416),” “photosynthesis, light harvesting in PS II (GO:0009769),” and “response to light intensity (GO:0009642)” (Figure 4B; Supplementary Table S4). In these 12 photosynthesis-related GO-BP pathways, 276 DEGs were enriched in the 12h_OE vs. WT group, comprising 120 upregulated genes and 156 downregulated genes (Supplementary Table S5). According to the annotation, we found that the expression of Rubisco activase (RCA; BnaA03g18710D), RAC-like 3 (RAC; BnaA03g39270D), Early light-inducible protein (ELIP1; BnaCmg37300D), and Thioredoxin F2 (TRXF2; BnaC02g06570D) were significantly upregulated in BnaMAPK1-OE plants under shading stress, all of which encode the core subunits of PS I and PS II. We also observed that the key component of the light-harvesting antenna complex gene in PS II, Light-harvesting complex photosystem II 4.2 (LHCB4.2; BnaA05g29390D), was significantly downregulated in the BnaMAPK1-OE plants compared with the WT under shaded conditions (Supplementary Table S5). Moreover, 19 DEGs were identified in this comparison using a |Log₂FC| > 3 cutoff, of which 12 were upregulated and seven were downregulated (Figure 4C; Supplementary Table S5). These data indicated that BnaMAPK1 may participate in photosynthesis by regulating the transcription of the

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**FIGURE 2** | Effect of shading stress on the chlorophyll fluorescence photosynthetic traits and Rubisco activity of WT and BnaMAPK1-OE transgenic rapeseed.
(A) Minimal fluorescence (F₀). (B) Photochemical quenching coefficient (qP). (C) Non-photochemical quenching coefficient (qN). (D) Maximum photochemical efficiency of PS II (Fv/Fm). (E) Rubisco activity. The chlorophyll fluorescence parameters (F₀, qP, qN, and Fv/Fm) were measured under control and shading conditions at 0 and 28 days each, and the Rubisco activity at 0, 7, 14, 21, and 28 days each. Vertical bars are means of three replicates ± SD (three plants of each line, three leaves of each plant, and 10 times of each leaf were measured); asterisk and double asterisk indicate p-value < 0.05 and p-value < 0.01.
photosystem complex subunits to improve the shading tolerance of rapeseed.

**KEGG Pathway Enrichment Analysis of the Photosynthesis-Related DEGs**

For an exploration of the complex biological functions of the DEGs, the significantly enriched pathways were identified using KEGG analyses. The DEGs involved in these pathways are listed in Supplementary Table S3. The 3,000 DEGs from the 12h_OE vs. WT comparison were subjected to this KEGG characterization of their biological behaviors. In total, 18 KEGG pathways were obtained using a \( q \)-value < 0.05 (Supplementary Table S6). The top 10 KEGG pathways with the highest representation of DEGs are shown in Figure 5A, including “peroxisome (ko04146),” “valine, leucine and isoleucine degradation (ko00280),” “carbon metabolism (ko01200),” and “photosynthesis-antenna proteins (ko00196).” These data further confirmed the regulatory role of BnaMAPK1 in the response to shading stress.

Based on the BnaMAPK1-mediated regulation of shading stress, we focused our analysis on 13 DEGs related to the “photosynthesis-antenna proteins” KEGG pathway, which includes genes related to PS II, such as LHCb. Based on their annotation, we found no obvious difference in the expression levels of genes encoding the PS I–LHC I supercomplex in the BnaMAPK1-OE plants compared with the WT under shading stress, while these 13 DEGs encoding LHCb subunits that function in the PS II–LHC II supercomplex were all significantly downregulated, especially LHCb2.1 (BnaC03g44110D, BnaA03g37990D), LHCb2.3 (BnaC07g24660D, BnaC09g01520D, BnaA06g31910D), LHCb3 (BnaA10g07350D), LHCb4.2 (BnaA05g29390D), and LHCb6...
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These data indicated that the expression of genes encoding LHCB subunits of the PS II–LHC II supercomplex was regulated by BnaMAPK1 during shading stress.

qRT-PCR Validation of the DEGs in the “Photosynthesis-Antenna Proteins” Pathway

To verify the authenticity of the expression levels obtained from the RNA-Seq data, the expression of nine selected LHCB genes were subjected to qRT-PCR analysis. LHCB1.3 (BnaA04g20150), LHCB1.4 (BnaC03g18980, BnaA05g09410, BnaA03g15830, BnaA05g09380), LHCB2.3 (BnaC09g01520), LHCB3 (BnaA10g07350), LHCB4.2 (BnaA05g29390), and LHCB6 (BnaC08g38660) were selected for validation, and BnACT7 (BnaC02g00690D) was used as the internal control. The RNA templates for the qRT-PCR were obtained from 3 to 4-week-old mixed samples (leaf, stem, and root) of WT and BnaMAPK1-OE plants subjected to 0 or 12 h of the shading treatment. As shown in Figure 5D and Supplementary Figure S2, the changes in transcript expression revealed using RT-PCR were identical to those acquired using RNA-Seq. Collectively, the above expression results suggest that the RNA-Seq data were credible and that BnaMAPK1 responds to shading stress by negatively regulating the LHCB genes in the PS II–LHC II supercomplex.
Verification of the Physical Interaction of BnaLHC3 With BnaMAPK1 Using Y2H and Split-LUC System

Our previous study revealed that a subunit of the PS II–LHC II supercomplex, BnaLHC3 (BnaA10g07350D), is a candidate interaction partner of BnaMAPK1, as determined using Y2H library screening assays (Wang et al., 2020). To further explore whether BnaLHC3 was regulated by BnaMAPK1, the full-length coding sequences of BnaLHC3 and BnaMAPK1 were inserted into the pGADT7 and pGBKT7 vectors, respectively (Figure 6A). The prey and bait constructs were cotransformed into yeast to explore the point-to-point protein interactions. The Y2Hgold strain expressing BnaLHC3 and BnaMAPK1 grew well on the SD–Ade–His–Leu–Trp medium (Figure 6C), indicating that BnaLHC3 could interact with BnaMAPK1 in yeast cells.

To further elucidate the physical interaction of BnaLHC3 and BnaMAPK1, tobacco-based firefly luciferase complementation assays were performed. BnaLHC3 was inserted into the N-terminal of LUC, while BnaMAPK1 was linked to the C-terminal of LUC (Figure 6B). The coexpression of the
nLUC-BnaLHC3 and cLUC-BnaMAPK1 constructs showed a strong ability to rescue the luciferase enzyme activity (\(p\)-value < 0.01) (Figures 6D,E). Taken together, these results suggested that BnaLHC3 and BnaMAPK1 interact in vivo, which might affect the stability of the PS II–LHC II supercomplex to further regulate shading tolerance in rapeseed.

**DISCUSSION**

Shade is widespread in nature, affecting many plants throughout their lifecycle. Plants respond on cellular and molecular levels to adapt to limited light. In our study, the AT1-motif, Box 4, G-box, GAG-motif, Sp1, TCT-motif, and As-2-box elements were identified in the promoter of BnaMAPK1 (Supplementary Table S2), all of which are involved in the light-mediated transcriptional regulation of light-regulated genes in potato (Solanum tuberosum), maize (Zea mays), and Arabidopsis thaliana (Shariatipour and Heidari, 2020). Notably, a G-box in a promoter can bind tightly to phosphorylated proteins to negatively regulate the transcriptional activity of the LHC II genes to further respond to light in a variety of plants. This suggested that BnaMAPK1 may play an important role in regulating photosynthesis in response to changing light levels.

**BnaMAPK1 Regulates Light Capture Efficiency of the Photosynthetic Apparatus in Xanthophyll Cycle and Photosynthetic Pigment System**

Previous studies show that the photosynthetic capability of shade-tolerant rapeseed is increased to extend the vegetative phase under a light-restricted environment (Brunel-Muguet et al., 2013), as well as optimized light capture and utilization through increased photosynthetic structures, primary reactions, and carbon assimilation in plants (Niinemets, 2010). Since the shading-induced expression pattern of BnaMAPK1 was consistent with the cis-acting elements analysis of its promoter, we examined the photosynthetic structure of WT and BnaMAPK1-OE rapeseed under shade stress. In plants, Pn is the most important indicator for evaluating photosynthetic ability, especially under an artificial environment when it is provided with a constant light quality and intensity (Shengxin et al., 2016). Our data showed that the gas exchange values (Pn, Gs, Ci, and Tr) of both WT and BnaMAPK1-OE rapeseed decreased in the shading treatment, with the values in BnaMAPK1-OE higher than those in the WT (Figures 1A–D). Previously, however, silencing Nicotiana attenuata NaMAPK4 (the homolog of Arabidopsis AtMAPK4) greatly enhances stomatal conductance, transpiration, and the photosynthetic rate in an ABA-dependent manner (Hettenhausen et al., 2012). Under shading stress, the decrease in Gs and Ci caused the decrease of Tr, which in turn caused the decrease of Pn, suggesting that BnaMAPK1 plays a positive role in regulating the photosynthetic rate, which might be inconsistent with the mechanism by which AtMAPK4 functions. In terms of energy dissipation, the trends of qP and Fv/Fm were similar to the gas exchange values, while the F0 and qN values of the WT and BnaMAPK1-OE plants increased after the shading treatment, with the WT displaying the higher values (Figures 2A–D). Photochemical damage is reflected in either an increase in F0 or decrease in the ratio of Fv/Fm under both shade stress and high-light conditions (Qiyuan and Ibrahim, 2016), implying that shading stress may limit the activity of the light reaction center and increase the energy dissipation in rapeseed. Our photosynthetic parameters demonstrated that BnaMAPK1-OErapeseed maintained low heat dissipation to improve light capture efficiency to further enhance its photosynthetic rate under shading.

Xanthophylls play essential roles in both light absorption and photoprotection, acting as accessory pigments and structural elements of the antennae to stabilize the LHC II complex (Ballottari et al., 2012). In higher plants, the xanthophyll cycle is closely related to photosynthesis and photoinhibition under a shaded environment. This cycle mainly involves thermal energy dissipation, for which zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) are key catalyzing enzymes (Hieber et al., 2000). To gain a better understanding of how BnaMAPK1 enhances photosynthetic capability under shading stress, GO pathway and related DEG analyses were performed in RNA-Seq. Our data showed that the expression of Zeaxanthin epoxidase 1 (ABA1/ZEP; BnaC09g07550D) and violaxanthin de-epoxidase-related protein (VDE; BnaCng13890D) were upregulated in transgenic rapeseed after the shading treatment (Supplementary Table S5), which was consistent with its photosynthetic traits. These data suggested that BnaMAPK1 functions in the xanthophyll cycle of the photosynthetic apparatus to regulate energy dissipation and improve the light sensitivity of PS II.

Light affects the biosynthesis of the photosynthetic pigments, which in turn affects photosynthesis itself and plays an important role in plant growth and development. One such pigment, chlorophyll, absorbs and transmits light energy for photosynthesis (Sims and Gamon, 2002; Gao et al., 2020). In the present study, the relative chlorophyll contents of both WT and BnaMAPK1-OE were found to decrease under the shading treatment, but a long-term (14, 21, and 28-d) shading treatment resulted in significantly higher relative chlorophyll contents in BnaMAPK1-OE rapeseed compared with the WT (Figure 1E). Notably, three Chlorophyllase 1 (CLH1; BnaA02g18330D, BnaA06g13830D, and BnaC05g15260D) genes, which are involved in chlorophyll degradation in PS II (Tian et al., 2021), were differentially expressed in the two genotypes, and were shown to be downregulated in the transgenic plants under shading stress. Here, the expression level of BnaELIP1 (BnaCmg37300D), which belongs to the LHC-like protein family and is a target gene for controlling chlorophyll accumulation to enhance tolerance to high light and shading stresses (Heddam and Adamska, 2002), was upregulated in BnaMAPK1-OE rapeseed under the shading treatment (Supplementary Table S5). In Arabidopsis and Chlamydomonas reinhardtii, ELIP1 associates with LHCB antennae to function in the xanthophyll cycle, carbon assimilation, and chlorophyll accumulation (Stress et al., 2006; Lee et al., 2020). These findings indicated that the
overexpression of BnaMAPK1 reduces the damage caused to the photosynthetic pigments by shading stress, maintaining photosynthetic capability.

**BnaMAPK1 Regulates Carbon Assimilation in Photosynthetic Enzyme System and Calvin Cycle in PS II**

In addition to the photoreaction and pigment systems, the enzyme system is also an important component of photosynthesis. Rubisco is the major enzyme involved in carbon assimilation and the limiting factor of photosynthetic efficiency and productivity (Mitra and Baldwin, 2008). Lower levels of Rubisco, ATP synthase, and PS II activities, as well as less electron transport and CO$_2$ consumption, are observed in shade-grown barley (Hordeum vulgare) leaves (Zivcak et al., 2014). By contrast, our photosynthetic measurements showed that the Rubisco activity in BnaMAPK1-OE rapeseed was significantly higher than the WT under both normal light and shade, which was positively correlated with the Pn values (Figure 2E). These findings are consistent with those of Liang et al., who report that shade-tolerant rapeseed has a higher photosynthetic net rate and chlorophyll content than the WT, as well as more stable Rubisco activity under shading stress (Liang and Li, 2004). Our results demonstrated that the photosynthetic enzyme system was less affected by shading in the BnaMAPK1-OE plants compared with WT rapeseed.

The Calvin cycle is responsible for photosynthetic carbon assimilation in plants. Relevant studies on shady environments reveal that the abundances of Rubisco, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and F-type ATPase, which are involved in the Calvin cycle, electron transport, and carbon assimilation, respectively, are reduced, suppressing photosynthesis (Zivcak et al., 2014; Liu et al., 2020). Rubisco catalyzes the first step of photosynthetic carbon assimilation and is a rate-limiting enzyme for photosynthetic efficiency and productivity in the Calvin cycle, in which Rubisco activity is regulated by a second enzyme, RCA (Portis, 2003; Bracher et al., 2017). Compared with the WT under shading stress, our RNA-Seq analysis showed that the expression level of BnaRCA (BnaA03g18710D) was upregulated in the BnaMAPK1-OE lines, which was consistent with the finding that overexpressing BnaMAPK1 improved the Rubisco activity of shaded rapeseed. Moreover, previous studies report that GAPDH is involved in carbon assimilation and limited the regeneration of ribulose-1,5-bisphosphate (RuBP) in the Calvin cycle (Suzuki et al., 2021). We also found that Glyceraldehyde-3-phosphate dehydrogenase C1 (BnaGAPC1; BnaA09g46260D and BnaC08g40330D) and BnaGAPC2 (BnaA05g33200D), encoding GAPDH, were all significantly downregulated in the BnaMAPK1-OE lines, indicating that BnaMAPK1 may play key roles in the Calvin cycle by accelerating RuBP carboxylation and limiting carbon assimilation. We therefore propose that the overexpression of BnaMAPK1 is beneficial to shade-stressed rapeseed because it improves the efficiency of light energy conversion and allows for decreased energy dissipation during the carbon assimilation in PS II in a shaded environment.

**BnaMAPK1 Interacts With BnaLHCB3 to Participate in Photosynthesis-Antenna Proteins Pathway**

Enhancing the photosynthetic rate is critical for increasing crop yields to meet the rising demands for food (Zhu et al., 2010; Long et al., 2015). The GO pathway analysis of the BnaMAPK1-OE lines in this study showed a significant enrichment in not only genes associated with the light reaction and harvesting in photosynthesis, but also those involved in plastoquinone biosynthesis (Figure 4; Supplementary Table S4). Of the DEGs, Phytene desaturation 1 (BnaPDS1; BnaA09g49870D, BnaA10g04310D, BnaC05g04530D, and BnaC08g44820D) and Ferredoxin 3 (BnaFD3; BnaA07g13260D, BnaC04g16810D) were downregulated in BnaMAPK1-OE rapeseed compared with the WT under shading stress. AtPDS1 was previously reported to function in plastoquinone biosynthesis, where it affects the availability of the electron carrier plastoquinone by controlling p-hydroxyphenylpyruvate dioxygenase activity during the light response in Arabidopsis (Qin et al., 2007). In higher plants, FD3 is a 2Fe2S plant-type iron–sulfur protein and associates with other ferredoxins to respond to a limitation in PS I acceptors (Voss et al., 2011). In the photoreaction process, ferredoxins act as electron acceptors of the photoreaction terminal, mediating electron transfer between PS I and carbon assimilation, and regulating electron reflow in photosynthetic phosphorylation (Fan et al., 2019). Electrons can be recycled from reduced ferredoxin at PS I to plastoquinone at PS II in the light-dependent reactions of photosynthesis (Munekage et al., 2004); therefore, based on the qP values presented here, it seems probable that BnaMAPK1 influences the Calvin cycle and the xanthophyll cycle to further regulate electron transport to adapt to the shade, but is not highly likely to directly respond to an acceptor limitation at PS I or PS II in rapeseed.

Further mining our transcriptomic data, we conducted a KEGG pathway enrichment analysis to identify the potential genes and pathways involved in photosynthesis in the DEGs. We identified the enrichment of an important KEGG pathway, the photosynthesis-associated antenna proteins pathway (Figure 5; Supplementary Table S6). In higher plants and green algae, the chlorophyll-binding subunits of PS I and PS II are internal antennae; LHCs act as peripheral antennae that enable a more efficient absorption of light energy and rapidly transfer energy to the reaction center to improve the photosynthetic rate (Papadatos et al., 2017). Zhang's lab discovers that AtMAPK3/AtMAPK6 activation can rapidly downregulate the PS II–LHC II supercomplex to inhibit photosynthesis (Su et al., 2018); however, combining the RNA-Seq and RT-PCR data, we identified an interesting group of DEGs encoding photosynthesis-associated antenna proteins in PS II, the BnaLHCBs, which are essential for photoprotection in both high-light and low-light stresses (Ballottari et al., 2012). These genes were significantly downregulated in the BnaMAPK1-OE
rapeseed, including \textit{BnaLHCB1}, \textit{BnaLHCB2}, \textit{BnaLHCB3}, \textit{BnaLHCB4}, and \textit{BnaLHCB6} (Figure 5; Supplementary Figure S2). In \textit{Arabidopsis}, six \textit{lhhcb} single mutants, \textit{lhhcb}1–6, are investigated to show that each AtLHCB plays a specific role in photosynthesis and ABA signaling (Xu et al., 2012). Each of the five \textit{BnaLHCBs} showed similar shading-sensitive expression patterns in our study, suggesting that all \textit{BnaLHCBs} were necessary for building the antenna complex and keeping it intact. It is tempting to speculate that \textit{BnaMAPK1} plays an important role in regulating the stability of the core molecular complex of the PS II antenna machinery, and that these \textit{BnaLHCBs} might be the interacting partners of \textit{BnaMAPK1} in regulating photosynthesis to improve the shade tolerance of rapeseed.

A previous proteomics analysis reports that differentially expressed proteins were also mapped to the photosynthesis-associated antenna proteins pathway; \textit{GmLHCB}1–6 (except \textit{GmLHCB3}) are significantly differentially abundant in soybean plants subjected to shading stress (Fan et al., 2019). Shading stress also induces PS state transitions (state 1–state 2 transitions), which maximize the efficiency of light harvesting at low light intensities (Mikko et al., 2006). However, LHCB3 affects the excitation energy transfer, the macrostructure of PS II, and the state transitions in \textit{Arabidopsis} (Adamiec et al., 2015), and confers continuous-light tolerance and enhances yields in tomato (\textit{Solanum lycopersicum}) and oil palm (Velez-Ramirez et al., 2014; Neoh et al., 2019). We previously use Y2H library screening assays to show that \textit{BnaLHCB3} is a candidate interacting partner of \textit{BnaMAPK1} (Wang et al., 2020). In the present study, we used point-to-point Y2H and split-LUC results to validate this interaction relationship between \textit{BnaMAPK1} and \textit{BnaLHCB3} (Figure 6), suggesting the function of \textit{BnaMAPK1} in stabilizing the PS II–LHC II supercomplex under shading stress in rapeseed.

Taken together, our findings provide useful insights into the mechanisms underlying the rapeseed shading response through the exploration of \textit{BnaMAPK1}, which is mainly involved in PS II-associated processes. This gene is particularly important in regulating the efficiency of the xanthophyll cycle and chlorophyll-mediated light capture and energy dissipation, controlling the Calvin cycle-mediated carbon assimilation, and stabilizing the PS II–LHC II supercomplex to improve photosynthesis capacity (Figure 7). This study provides important evidence for the molecular mechanisms underlying the shading response of the MAPK cascades, and is potentially relevant for use in molecular breeding strategies.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA680826/, PRJNA680826.
AUTHOR CONTRIBUTIONS
YL, LW, KL, and JL designed the research and supervised the project. ZW, ML, MY, and XZ performed the experiments. ZW, CQ, and HD analyzed the data. ZW and ML drafted the manuscript. ZW, LW, KL, and YL revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.902989/full?supplementary-material

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