**ZmPP2C26 Alternative Splicing Variants Negatively Regulate Drought Tolerance in Maize**

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Serine/threonine protein phosphatase 2C (PP2C) dephosphorylates proteins and plays crucial roles in plant growth, development, and stress response. In this study, we characterized a clade B member of maize PP2C family, i.e., ZmPP2C26, that negatively regulated drought tolerance by dephosphorylating ZmMAPK3 and ZmMAPK7 in maize. The ZmPP2C26 gene generated ZmPP2C26L and ZmPP2C26S isoforms through untypical alternative splicing. ZmPP2C26S lost 71 amino acids including an MAPK interaction motif and showed higher phosphatase activity than ZmPP2C26L. ZmPP2C26L directly interacted with, dephosphorylated ZmMAPK3 and ZmMAPK7, and localized in chloroplast and nucleus, but ZmPP2C26S only dephosphorylated ZmMAPK3 and localized in cytosol and nucleus. The expression of ZmPP2C26L and ZmPP2C26 was significantly inhibited by drought stress. Meanwhile, the maize zmpp2c26 mutant exhibited enhancement of drought tolerance with higher root length, root weight, chlorophyll content, and photosynthetic rate compared with wild type. However, overexpression of ZmPP2C26L and ZmPP2C26S significantly decreased drought tolerance in *Arabidopsis* and rice with lower root length, chlorophyll content, and photosynthetic rate. Phosphoproteomic analysis revealed that the ZmPP2C26 protein also altered phosphorylation level of proteins involved in photosynthesis. This study provides insights into understanding the mechanism of PP2C in response to abiotic stress.

**Keywords:** maize, drought stress, protein phosphatase 2C, MAPK, alternative splicing, photosynthesis

**INTRODUCTION**

In plants, numerous proteins will be activated or inactivated via dephosphorylation catalyzed by protein phosphatases (PPs) (Cohen, 1989). Based on their substrate specificity, PPs are mainly classified into three families, namely, serine (Ser)/threonine (Thr)-specific phosphoprotein phosphatase (PPP), metal-dependent protein phosphatase (PPM), and protein tyrosine phosphatase (PTP) (Barford et al., 1998). The PPP and PPM families encode Ser/Thr PP, while PTP family includes tyrosine-specific and dual-specificity phosphatase (Barford et al., 1998). The PP2C of PPM family is a kind of the Mg"⁺- or Mn"⁺-dependent PPs and specifically dephosphorylates...
the phosphorylated Ser/Thr residues of target proteins (Schweighofer et al., 2004; Shi, 2009). The PP2C family is substantially expanded in plants with 80, 90, and 130 members in Arabidopsis, rice, and maize, respectively, and divided into eleven clades of A–K (Xue et al., 2008; Singh et al., 2010; Wang et al., 2018). The clade A members of PP2C act as co-receptors of abscisic acid (ABA), interact with the ABA-receptor protein PYR/PYL/PCAR and SNF1-related protein kinase 2s (SnRK2s) to negatively regulate ABA signaling, and play crucial roles in plant growth, development, and stimuli response (Ma et al., 2009; Park et al., 2009; Komatsu et al., 2013). For instance, AHG1 encoding a PP2C interacts with DELAY OF GERMINATION1 (DOG1) and is impaired by DOG1 to negatively regulate ABA response in seed dormancy and germination (Nishimura et al., 2018). The maize ZmPP2C-A2, ZmPP2C-A6, and ZmPP2C-A10 negatively regulate drought tolerance by mediating ABA signaling (Xiang et al., 2017; He et al., 2019). Tomato SlPP2C3 functions as a negative regulator of ABA signaling to negatively regulate drought tolerance by mediating ABA signaling (Xiang et al., 2017; He et al., 2019). Tomato SlPP2C3 functions as a negative regulator of ABA signaling to negatively regulate drought tolerance, fruit ripening, and glossiness (Liang et al., 2021). In contrast, only few available studies report the functions as a negative regulator of ABA signaling to negatively regulate drought tolerance. Impaired photosynthesis by drought stress was reported for ZmPP2C-A2 knock-out plants that were lacking the ABA transporter AtHKT1;1 activity (Chu et al., 2021). In Arabidopsis, three of six clade B members of PP2C including AP2C1, AP2C3, and PP2C5 are well-elucidated for their function in stomata development, immunity, defense, and K⁺ deficiency response (Schweighofer et al., 2007; Brock et al., 2010; Umbrasaite et al., 2018). The maize ZmPP2C-A2, ZmPP2C-A6, and ZmPP2C-A10 negatively regulate drought tolerance by mediating ABA signaling (Xiang et al., 2017; He et al., 2019). Tomato SlPP2C3 functions as a negative regulator of ABA signaling to negatively regulate drought tolerance, fruit ripening, and glossiness (Liang et al., 2021). In contrast, only few available studies report the function of other clade PP2Cs. A clade G member AtPP2C49 negatively regulates salt tolerance through inhibition of Na⁺ transporter AtHKT1;1 activity (Chu et al., 2021). In Arabidopsis, three of six clade B members of PP2C including AP2C1, AP2C3, and PP2C5 are well-elucidated for their function in stomata development, immunity, defense, and K⁺ deficiency response (Schweighofer et al., 2007; Brock et al., 2010; Umbrasaite et al., 2018). The tobacco B clade NtPP2C2b is found to regulate nicotine biosynthesis (Liu et al., 2021). However, the function of clade B members of PP2C in crops remains unknown.

Alternative splicing (AS) of precursor messenger RNAs (pre-mRNAs) produces more mRNA isoforms from the same pre-mRNA and plays a key role in gene expression and protein diversity (Kelemen et al., 2013). Previous studies show that abundant genes undergo AS to regulate plant growth and stress response, including salt, heat, cold, and drought stress, and photomorphogenesis, flowering, and yield (Farquharson, 2016; Jiang et al., 2017; Qin et al., 2017; Calixto et al., 2018; Gu et al., 2018; Liu et al., 2018; Yu et al., 2018; Li et al., 2019). The AS events likewise can be triggered in different environmental conditions and developmental stages (Liu et al., 2018; Martin et al., 2021). As well known, there are two typical types of intron retention, namely, U2 and U12 type with 5′-GT••••••AG-3′ and 5′-AT••••••AC-3′ splice site, respectively (Sharp and Burge, 1997; Reddy et al., 2013). Intriguingly, two untypical types of AS are recently found in Arabidopsis, containing alternative first exon (AFE) and alternative last exon (ALE) (Zhu et al., 2017). The F72 gene undergoes AFE-type AS and generated two isoforms named as F72S and F72A transcripts to control flowering through regulating their amount during reproductive stage (Qin et al., 2017). Maize is one of the most important crops and is used in food supply, livestock feed, and industries. Its productivity is seriously restricted by drought stress due to its vulnerability to water deficits (Lobell et al., 2014; Sah et al., 2020). Hence, exploring stress-related genes will contribute for facilitating molecular design breeding to improve maize drought tolerance.

The drought-tolerant maize inbred lines 81565/87-1 and drought-sensitive lines 200B/DAN340 were used for gene expression analysis. The four-leaf stage seedlings were treated with 16% (w/v) polyethylene glycol 6000 (PEG-6000). At 0 (blank control), 3, 6, 12, and 24 h of treatment, the seedling of every line was collected and used for total RNA extraction by using RNAiso plus kit (TaKaRa, Japan).

Total RNA samples were reverse-transcribed into cDNA using PrimeScript™ reagent kit (TaKaRa) and used for real-time quantitative PCR (RT-qPCR). The RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing) in the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, United States). The ZmEF1a gene was used as internal control. The information of all primers used in this study is shown in Supplementary Table 1.

**Phosphatase Activity Assay**

The open reading frame (ORF) of ZmPP2C26L/ZmPP2C26S without stop codon was amplified and inserted into pET32a vector to generate His-ZmPP2C26L/His-ZmPP2C26S plasmid, respectively. The reconstructed plasmids were transformed into Escherichia coli strain BL21. The E. coli strain harboring the above plasmid was induced using 0.1 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG) at 16°C for 16 h to express His-ZmPP2C26L/His-ZmPP2C26S protein, which was purified using 6 × His-Tagged Protein Purification Kit (CWBIO, China).

Protein phosphatase activity of ZmPP2C26L/ZmPP2C26S protein was detected as previously reported (Han et al., 2018; Yang et al., 2020). Briefly, 2 mg of His-tagged protein was incubated with 1 mL assay buffer (50 mM Tris–HCl, pH 7.5, 1 mM MgCl₂, 0.5 mM EDTA, and 0.1 g/L BSA) for 30 min at 37°C. Subsequently, 2 mM p-nitrophenyl phosphate (pNPP) was added into the above mixture to produce p-nitrophenol catalyzed by ZmPP2C26L/ZmPP2C26S protein. Then, the absorbance value at wavelength of 405 nm (OD_{405}) of p-nitrophenol was monitored every 2 min. The relative PP activity was calculated according to the curve of OD_{405}.

**MATERIALS AND METHODS**

**Stress Treatment and Expression Analysis**

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Yeast Two-Hybrid, Bimolecular Fluorescence Complementation, and Glutathione-S-Transferase Pull-Down

Yeast two-hybrid (Y2H) assay was conducted using the Matchmaker GAL4 Y2H System (Clontech). The ORF of ZmPP2C26L/ZmPP2C26S was amplified and inserted into prey vector pGADT7 to generate AD-ZmPP2C26L/AD-ZmPP2C26S plasmid, respectively. The ORF of 13 maize PYL genes (e.g., ZmMAPK1-19 and ZmSIMK) was also inserted into pGBK7 for BD-ZmMAPKs, which were kindly provided by Dongtao Ren (China Agriculture University, Chengdu, China). The prey AD plasmid and the bait BD plasmid were cotransformed into Saccharomyces cerevisiae strain Y2H gold by using the yeast transformation kit (Coolaber, Beijing, China). The transformants were cultured on synthetic medium plates (SD medium) lacking Trp and Leu (SD/-Trp/-Leu) at 30°C for 2–3 days, then transferred onto SD/-Trp/-Leu/-His/-Ade plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xα-gal) for blue color development to detect the interaction between ZmPP2C26L/ZmPP2C26S and ZmPYLs/ZmMAPKs. The interaction was further validated by bimolecular fluorescence complementation (BIFC) and glutathione-S-transferase (GST) pull-down assays.

For BIFC assay, the ORF of ZmPP2C26L/ZmPP2C26S was cloned into pSPYNE-35S-nYFP vector generating nYFP-ZmPP2C26L/nYFP-ZmPP2C26S, respectively. The ORF of the ZmMAPK3/ZmMAPK7 gene was inserted into the pSPYNE-35S-cYFP vector generating cYFP-ZmMAPK3/cYFP-ZmMAPK7, respectively. As previously described (Li et al., 2016), the nYFP-ZmPP2C26L/nYFP-ZmPP2C26S and cYFP-ZmMAPK3/cYFP-ZmMAPK7 constructs were cotransformed into maize protoplasts. After 16 h at 28°C, the protoplasts were examined for YFP fluorescence under the confocal laser scanning microscope (ZEISS 800).

For GST pull-down assay, the ORF of ZmMAPK3/ZmMAPK7 was inserted into pGEX-6p-1 vector to create GST-ZmMAPK3/GST-ZmMAPK7, respectively. The GST-tagged protein was induced by 0.1-mM IPTG and purified using Glutathione-Sepharose Resin kit (CW BIO). A total of 2 µg GST-tagged protein was uploaded to Mag-Beads GST Fusion Protein Purification (Sangon Biotech, Shanghai, China) and incubated at room temperature for 2 h. Then, 2 µg of His-tagged protein was added to protein-beads complex for combination. The protein complex was washed down by washing five times with the elution buffer containing 50 mM Tris–HCl and 10 mM reduced glutathion (pH 8.0). The proteins were separated by 12.5% SDS-PAGE and transferred onto the PVDF (polyvinylidene fluoride) membrane by wet transfer at 100 V for 80 min. The membrane was blocked in 2.5% (w/v) non-fat milk powder solution (Coolaber, Beijing, China) for 90 min and incubated with primary antibody (anti-GST/anti-His antibody) for 90 min at room temperature and then with the HRP (horseradish peroxidase)-conjugated Goat Anti-Mouse IgG (Abclonal, Wuhan, China) for 60 min at room temperature. Finally, the signal was visualized using the ChemDoc XRS system (Bio-Rad, Hercules, CA, United States).

Subcellular Localization and Co-localization

For subcellular localization, the ORF of ZmPP2C26L/ZmPP2C26S without stop codon was amplified and independently inserted into pCAMBIA2300-35S-eGFP vector to create 35S-ZmPP2C26L-eGFP/35S-ZmPP2C26S-eGFP plasmid, respectively. The construct was transformed into Agrobacterium tumefaciens strain GV3101 and then used for infiltrating into the leaves of 5-week-old Nicotiana benthamiana. The GFP fluorescence was observed using the confocal laser scanning microscope (ZEISS 800). For co-localization of ZmPP2C26L/ZmPP2C26S and ZmMAPK3/ZmMAPK7, the ORF of ZmMAPK3/ZmMAPK7 was separately cloned into the pCAMBIA1300-35S-mCherry vector to generate 35S-ZmMAPK3-mCherry/35S-ZmMAPK7-mCherry, respectively. The cultures of Agrobacterium carrying 35S-ZmPP2C26L-eGFP/35S-ZmPP2C26S-eGFP and 35S-ZmMAPK3-mCherry/35S-ZmMAPK7-mCherry were co-infiltrated into N. benthamiana leaves. The GFP and mCherry fluorescence were observed by using the confocal laser scanning microscope (ZEISS 800).

Phosphorylation Assay

The phosphorylation assay was performed with minor modification as previously described (Xia et al., 2021). The ORF of ZmMAPK3/ZmMAPK7 was inserted into pCAMBIA1300-35S-3 × HA vector to generate 35S-HA-ZmMAPK3/35S-HA-ZmMAPK7, respectively. The constructs were transformed into maize protoplasts. After transformation, the protoplasts were cultured for 16 h at 28°C. Subsequently, the total protein was extracted using total plant protein extraction kit (Coolaber, Beijing, China) and immunoprecipitated with Anti-HA Affinity Beads (Smart Lifesciences, Changzhou, China) in a rotary mixer for 4 h at 4°C. Then, 1 µg HA-tagged protein mixed with 0.25, 0.5, and 1 µg His-ZmPP2C26L/His-ZmPP2C26S protein, respectively, in the presence of 50 mM ATP and 30 µl kinase buffer (20 mM HEPES [N-[2-hydroxyethyl]piperazine-N'-2-ethane sulfonic acid], 10 mM MgCl2, and 1 mM DTT, pH 7.5), and incubated for 60 min at 30°C. The reaction was stopped by adding SDS-loading buffer and separated by 12.5% phos-tag™ (Wako, Beijing, China) SDS-PAGE and normal SDS-PAGE for immunoblotting using anti-HA and anti-His antibody (Abclonal, Wuhan, China) as previously. The relative density of each band was analyzed using ImageJ software.1

Phenotyping of Transgenic Arabidopsis, Rice, and Maize Mutant

The T-DNA insertion mutant of ZmPP2C26 homolog ap2c1 (SALK_065126) was obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, United States). The 35S-ZmPP2C26L-eGFP/35S-ZmPP2C26S-eGFP construct

1https://imagej.en.softonic.com/
was transformed into ap2c1 and Col-0 wild type (WT) for complementation and overexpression, respectively, by the floral-dip method (Clough and Bent, 1998). The seeds of homozygous lines were screened by 50 mg/L kanamycin on 1/2 MS plates containing 50 µg/ml hygromycin, regenerated, and identified by PCR. The seeds of homozygous lines and WT were germinated for 7 days. Then, 30 seedlings were transplanted into the rectangular plastic pots with mud and grown in greenhouse under a photoperiod of 14 h light/10 h dark at 25°C.

Three-week-old seedlings were subjected to drought treatment by withholding watering for 2 weeks, then re-watered for 3 days and photographed. The survival rate of every line was calculated. Before treatment, the photosynthetic rate of every line was measured. To address whether ZmPP2C26L/ZmPP2C26S participated in ABA and MAPK signaling, Y2H assay was performed to determine the interaction of them with 13 ZmPYLs and 20 ZmMAPKs. The results showed that ZmPP2C26L/ZmPP2C26S did not interact with 13 ZmPYLs (Supplementary Figure 2A). However, on the quadruple dropout (–Leu/–Trp/–His/–Ade/) SD plates with X-α-Gal, the yeast strains cotransformed by AD-ZmPP2C26L and BD-ZmMAPK3/BD-ZmMAPK7, AD-ZmPP2C26S, and BD-ZmMAPK3, as well as positive control (i.e., AD-T and BD-53) could grow well and be stained blue.

RESULTS

ZmPP2C26 Is Subject to Alternative First Exon-Type as Generating Two Variants

During gene cloning, it is found that two AS variants were amplified using a pair of ZmPP2C26 primers and defined as ZmPP2C26L and ZmPP2C26S (Figure 1A). Sequence alignment showed that there was 213 bp retention in the first exon of ZmPP2C26L compared with ZmPP2C26S. The splicing site is 5′-CC●●●●●●GC-3′ and neither U2 nor U12 type, indicating that it is a new AS-type AFE (Figure 1B and Supplementary Figure 1A). Protein sequence alignment showed that 71 amino acids were encoded by 213 bp nucleotides and possessed a highly conserved MAPK interaction motif (KIM motif, [K/R]-(3-4)-X(1-6)-[L/I]-X-[L/I]) (Figure 1C and Supplementary Figure 1B; Schweighofer et al., 2007), implying that ZmPP2C26L might interact with some ZmMAPK members.

Tandem Mass Tag Based Quantitative Phosphoproteomics

Rice fresh shoots of L1, S1, and Nip, as well as maize zmpp2c26 mutant with WT were used for phosphoproteomic analysis with three biological replicates. The total protein of every line was extracted using total plant protein extraction kit (Coolaber, Beijing, China) according to the manufacturer’s instruction and quantified through BCA assay (Smith et al., 1985). For each sample, 200 µg protein solution was mixed with 5 mM DTT and 10 mM iodotyrosine in the dark at room temperature for 15 min, subsequently precipitated by adding six volumes of acetone at -20°C overnight, then centrifuged at 8,000 g for 10 min at 4°C to collect the precipitate, and placed for 3 min at room temperature to volatilize the acetone. After removing supernatant, 100 µl of TEAB (200 mM) was added into tube to re-dissolve the protein. Subsequently, 1/50 of the sample weight of 1 mg/ml trypsin-TPCK was added into solution and digested at 37°C overnight to generate peptides. The peptides were labeled using TMT labeling kit (Thermo Scientific, Waltham, MA, United States) and used for enriching phosphoproteins using the IMAC Phosphopeptide enrichment kit (Thermo Fisher Scientific, United States) according to the manufacturer’s instruction. The enriched peptides were used for LC-MS/MS scan on the Q Exactive HF and EASY-nLC 1200 system (Thermo Scientific, Waltham, MA, United States). ProteomeDiscoverer (version 2.4) was used to search raw data against the sample protein database. A global false discovery rate (FDR) was set to 0.01, and protein groups considered for quantification required at least two peptides. The detailed protocol and parameters were set as described in Supplementary Table 2. The amino acid sequences of differentially phosphorylated proteins were used to analyze their Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and functional enrichment.

Statistical Analysis

All experiments were performed with three replicates. The data were showed as mean ± SD (standard deviation) and analyzed by using Student’s t-test at *P ≤ 0.05 and **P ≤ 0.01.

http://chinamu.jaas.ac.cn/Default.html
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**FIGURE 1** | The splicing variants of ZmPP2C26 via AFE type. (A) The ORF amplification of ZmPP2C26 gene from maize inbred lines 81565, 87-1, 200B, and DAN340. (B) The alternative splicing model of ZmPP2C26. The gray boxes indicate exons. Lines correspond to introns; dotted line represents alternative splicing events; UTR, untranslated regions; TSS, the transcriptional start site; The splicing site is 5′-CC••••••GC-3′. (C) Protein sequence alignment of ZmPP2C26L and ZmPP2C26S. Black boxes indicate conservation domain of protein phosphatase; red line indicates KIM motif; †, basic amino acid; $, hydrophobic amino acids; ◆, active site.

(Figure 2A and Supplementary Figure 2B). The GST pull-down showed that the His-ZmPP2C26L was pulled down by GST-ZmMAPK3/-ZmMAPK7, and His-ZmPP2C26S was only pulled down by GST-ZmMAPK3 (Figure 2B). The BiFC assay further showed that co-expression of nYFP-ZmPP2C26L and cYFP-ZmMAPK3/cYFP-ZmMAPK7 and co-expression of nYFP-ZmPP2C26S and cYFP-ZmMAPK3 in maize protoplasts could produce strong YFP fluorescence signal (Figure 2C). These results confirm that ZmPP2C26L physically interact with ZmMAPK3 and ZmMAPK7, but ZmPP2C26S only interacts with ZmMAPK3 in vitro and in vivo.

**ZmPP2C26 Dephosphorylates ZmMAPK3 and ZmMAPK7**

To test whether ZmPP2C26L/ZmPP2C26S dephosphorylates ZmMAPK3/ZmMAPK7, the dephosphorylation assay was performed in vitro. As shown in Figure 3, only phospho-ZmMAPK3/-ZmMAPK7 was detected in the absence of ZmPP2C26L or ZmPP2C26S on the phos-tag™ SDS-PAGE gel. However, de-phosphorylated ZmMAPK3/ZmMAPK7 was detected when HA-ZmMAPK3/HA-ZmMAPK7 was incubated with His-ZmPP2C26L and HA-ZmMAPK3 was incubated with His-ZmPP2C26S. Notably, the phospho-ZmMAPK3/phospho-ZmMAPK7 proteins were decreased with the increase in ZmPP2C26L/ZmPP2C26S concentration. Furthermore, only dephospho-ZmPP2C26L/-ZmPP2C26S was detected using anti-His when HA-ZmMAPK3/HA-ZmMAPK7 was incubated with His-ZmPP2C26L and HA-ZmMAPK3 was incubated with His-ZmPP2C26S. These results show that ZmPP2C26L dephosphorylates ZmMAPK3 and ZmMAPK7, and ZmPP2C26S dephosphorylates ZmMAPK3, whereas ZmPP2C26L and ZmPP2C26S cannot be phosphorylated by ZmMAPK3 and ZmMAPK7. Likewise, the expression of ZmMAPK3 and ZmMAPK7 gene was dramatically evaluated elevated by drought stress in maize (Supplementary Figure 3).

**Subcellular Localization of ZmPP2C26L/ZmPP2C26S**

To determine the subcellular localization of ZmPP2C26L/ZmPP2C26S, the ORF of them was fused with eGFP under the control of the 35S promoter and introduced into N. benthamiana leaves for transient expression. Confocal laser scanning microscopy showed that the eGFP fluorescence signal was observed in both the cytoplasm and the nucleus from the leaf infiltrated by the empty eGFP vector (control) and ZmPP2C26S-eGFP vector, but in the chloroplast and nucleus from the leaf infiltrated by the ZmPP2C26L-eGFP vector (Figure 4A). Co-localization also showed that ZmPP2C26L co-localized with ZmMAPK3/ZmMAPK7 in the nucleus, and
ZmPP2C26S co-localized with ZmMAPK3 in the cytoplasm and nucleus (Figure 4B).

**ZmPP2C26S Exhibits Higher Phosphatase Activity Than ZmPP2C26L**

Since ZmPP2C26 is a member of PP2C clade B and belongs to PP family (Wang et al., 2018), the phosphatase activity of ZmPP2C26L and ZmPP2C26S was detected using the chromogenic substrate pNPP in vitro phosphatase assays. The PP could catalyze pNPP to produce p-nitrophenol with an absorbance value of OD$_{405}$. After adding the purified His-ZmPP2C26L and His-ZmPP2C26S into the reaction solution, there was a strong OD$_{405}$ values, suggesting that both of them had phosphatase activity. Moreover, the relative phosphatase activity of ZmPP2C26S was significantly higher than ZmPP2C26L (Supplementary Figure 4).

**ZmPP2C26L and ZmPP2C26S Negatively Regulate Drought Tolerance**

The results of RT-qPCR showed that the expression of ZmPP2C26 and ZmPP2C26L was significantly downregulated by drought stress in drought-tolerant lines 81565 and 87-1 and upregulated in drought-sensitive lines 200B and DAN340 (Supplementary Figure 5), which was consist with our previous study (Lu et al., 2020). The data imply that ZmPP2C26 plays a crucial role in regulating drought tolerance.

Hence, ZmPP2C26L and ZmPP2C26S was complemented and overexpressed in Arabidopsis-mutant ap2c1 (AT2G30020,
FIGURE 3 | The dephosphorylation assay in vitro. (A) ZmPP2C26L dephosphorylates ZmMAPK3 and ZmMAPK7. (B) ZmPP2C26S dephosphorylates ZmMAPK3. The phospho-ZmMAPK3/-ZmMAPK7 with HA tag was extracted from maize protoplasts, incubated with His-ZmPP2C26L/His-ZmPP2C26S, separated by 12.5% phos-tag SDS-PAGE gel (top panel) or normal SDS-PAGE gel (middle panel), and detected by using an anti-HA or anti-His antibody. Different amounts of His-ZmPP2C26L/His-ZmPP2C26S were used to dephosphorylate ZmMAPK3 and ZmMAPK7. 1×, 2×, and 4× represent 0.25, 0.5, and 1.0 µg purified His-ZmPP2C26L/-ZmPP2C26S, respectively. + and - denote the presence and absence of the protein in each sample, respectively. The relative intensity of the protein bands was measured using ImageJ, and the lane without His-ZmPP2C26L/His-ZmPP2C26S was set to 1.00.

FIGURE 4 | Subcellular localization and co-localization. (A) The localization of ZmPP2C26L and ZmPP2C26S. (B) Co-localization of ZmPP2C26L and ZmMAPK3/ZmMAPK7 and of ZmPP2C26S and ZmMAPK3. ZmPP2C26L and ZmPP2C26S were separately fused with eGFP. ZmMAPK3 and ZmMAPK7 were separately fused with mCherry. A 4-week-old tobacco (Nicotiana benthamiana) seedling was used for infiltrating using Agro. Tumefaciens cells harboring above plasmids. Scale bar = 50 µm.

Meanwhile, a maize mutant of zmpp2c26 was used for drought tolerance test. As shown in Figure 6, the Mu transposon insertion of zmpp2c26 resulted in knockout of ZmPP2C26 identified by RT-PCR. The zmpp2c26 exhibited drought-tolerant phenotype compared with WT. After drought stress, the survival rate of zmpp2c26 was 87.5% and that of the WT was only 12.5%. The root length and root dry weight of zmpp2c26 were also significantly higher than WT. The chlorophyll content and photosynthetic rate of zmpp2c26 mutant were significantly higher than WT. The above results suggest that ZmPP2C26 negatively modulates drought tolerance and the ZmPP2C26S variant was more sensitive to drought than ZmPP2C26L.

ZmPP2C26 Alters Protein Phosphorylation Level

To uncover the global effects of ZmPP2C26 on protein phosphorylation, we performed TMT-based quantitative
Phosphoproteomic analysis in the ZmPP2C26L-/ZmPP2C26S-overexpressing lines and WT. A total of 3,516 phosphosites and 2,877 phosphopeptides derived from 1,532 phosphoproteins with >1.5-fold change (P < 0.05) were found to form transgenic lines compared with WT (Supplementary Table 3). After evaluation of raw data, clean data with high reliability were screened and used for KEGG analysis. Compared with WT, 48 and 93 phosphopeptides were significantly upregulated and downregulated in the ZmPP2C26L-overexpression lines, respectively. In contrast, 55 and 28 phosphopeptides...
**FIGURE 7** | Phosphoproteomics of transgenic rice identifies a set of ZmPP2C26-affected phosphoproteins. (A) Phosphopeptide abundance for ZmPP2C26L- and ZmPP2C26S-overexpressing lines compared with WT. (B) The GO and KEGG analysis of ZmPP2C26L- and ZmPP2C26S-affected phosphorylation proteins.

**FIGURE 8** | Phosphoproteomics of zmpp2c26 mutant identifies ZmPP2C26-affected phosphoproteins. (A) Phosphopeptide abundance for zmpp2c26 compared with WT. (B) The KEGG analysis of ZmPP2C26-affected upregulated phosphorylation proteins.
were significantly upregulated and downregulated in the ZmPP2C26S-overexpression lines, respectively (Supplementary Table 4). KEGG pathway analysis showed that the ZmPP2C26L-afllicted phosphorylation proteins predominantly enriched in photosynthesis and ZmPP2C26S-afllicted phosphorylation proteins were associated with carbon fixation in photosynthesis and pyruvate metabolism (Figure 7). Among them, 8 upregulated and 12 downregulated phosphoproteins were shared by ZmPP2C26L-OE and ZmPP2C26S-OE lines compared with WT, and the aquaporin PIP2-7 showed the highly increased phosphorylation level (Supplementary Table 5).

Similarly, we analyzed the phosphoproteomic difference of zmpp2c26 compared with WT. The results showed that a total of 671 unique phosphorylation proteins with >1.5-fold change (P < 0.05) were identified in zmpp2c26 (Supplementary Table 6). Also, 324 and 347 phosphoproteins were upregulated and downregulated in zmpp2c26, respectively. As ZmPP2C26 is a PP, the phosphorylation level of its targets should be increased in the zmpp2c26 plants. The KEGG pathway analysis indicated that the upregulated phosphoproteins were enriched in several pathways but mainly involved in photosynthesis (Figure 8). Notably, the phosphorylation level of ZmMAPK3 (accession number: B4FN55) was significantly upregulated in zmpp2c26 mutant (Supplementary Table 6), indicating that ZmPP2C26 involved in photosynthesis and MAPK-mediated signaling pathway.

**DISCUSSION**

Alternative splicing is a critical gene post-transcriptional regulation mechanism for plants in response to surrounding stress, growth, and development (Reddy et al., 2013; Qin et al., 2017). In plants, more than 60% intron-containing genes undergo AS to produce a vast repertoire mRNA isofrom (Marquez et al., 2012; Reddy et al., 2013; Iniguez et al., 2017; Jiang et al., 2017). U2 and U12 are two typical intron retention types of AS (Sharp and Burge, 1997; Reddy et al., 2013). In this study, ZmPP2C26, a maize PP2C clade B member, is found to undergo AS, producing ZmPP2C26L and ZmPP2C26S in different maize germplasm lines (Figure 1). Its splicing site is 5'-CC••••••GC-3' (Figure 1B and Supplementary Figure 1A), which is AFE-type AS and recently also found in Arabidopsis (Wang et al., 2015; Qin et al., 2017; Zhu et al., 2017).

The clade B PP2Cs can recognize MAPK phosphatases and interact with MAPK3, MAPK4, or MAPK6 to regulate stomatal aperture, seed germination, and stress response (Brock et al., 2010; Umbrasaite et al., 2010; Sidonskaya et al., 2016; Shubchynsky et al., 2017). It has been reported that AP2C1 dephosphorylates CBL-interacting protein kinase (CIPK) to regulate K+ deficiency response (Singh et al., 2018). In this study, it is confirmed that 213 bp is retained in first exon of ZmPP2C26L and spliced in ZmPP2C26S, and encodes 71 amino acids including a conserved KIM motif (Figure 1C and Supplementary Figure 1B). However, the ZmPP2C26L physically interacts with ZmMAPK3 and ZmMAPK7 in the nucleus, but ZmPP2C26S only interacts with ZmMAPK3 in both the cytoplasm and the nucleus (Figures 2, 4B and Supplementary Figure 2B), which means that the molecular mechanism of these two variants may be different. Considering ZmMAPK3 and ZmMAPK7 can be phosphorylated by ZmMKK10 (Chang et al., 2017), they are transiently expressed in maize protoplast, immunoprecipitated, and used for dephosphorylation catalyzed by ZmPP2C26. It is expectedly found that ZmPP2C26L dephosphorylates ZmMAPK3 and ZmMAPK7, and ZmPP2C26S dephosphorylates ZmMAPK3, while ZmMAPK3 and ZmMAPK7 cannot phosphorylate ZmPP2C26L and ZmPP2C26S (Figure 3). The ZmPP2C26L/ZmPP2C26S did not interact with 13 PYLs (Supplementary Figure 2), suggesting that ZmPP2C26L and ZmPP2C26S may act on MAPK signaling to regulate downstream signaling in ABA-independent way. Interestingly, ZmPP2C26S lacks KIM motif but interacts with ZmMAPK3, indicating that KIM motif is unnecessary for interaction between ZmMAPKs and other protein. Meanwhile, ZmMAPK3 and ZmMPK7 were found to be upregulated by drought stress (Supplementary Figure 3) and involved in multiple stresses response in previous reports (Wang J. et al., 2010, Wang Y. L. et al., 2010; Wang et al., 2011; Liu et al., 2013). Their homologs, i.e., AtMPK3 and AtMPK6, regulated salt and cold tolerance and hypoxia signaling in Arabidopsis (Li et al., 2017; Yan et al., 2021; Zhou et al., 2021). These studies imply that ZmPP2C26 functions in plants stress response acting on ZmMAPK3/ZmMAPK7.

Since GC content flaking splicing sites is pretty high for primer PP2C26S-F (Supplementary Figure 1), fragment of ZmPP2C26S was not amplified using primers PP2C26S-F/PP2C26S-R for RT-qPCR, so we detected the relative expression level of ZmPP2C26 containing the transcript of ZmPP2C26L and ZmPP2C26S instead of ZmPP2C26S. Under drought stress, the expression...
of ZmPP2C26 and ZmPP2C26L was significantly downregulated by drought stress in drought-tolerant maize and upregulated in drought-sensitive maize (Supplementary Figure 8). The possible cue is due to the inhibition of its promoter by drought (Lu et al., 2020). The results indicate that ZmPP2C26 may play a negative role in regulating drought tolerance. Hence, they are functional validated via ectopic expressing in Arabidopsis, rice, as well as phenotyping maize zmpp2c26 mutant (Figures 5, 6 and Supplementary Figure 6). But the complementation of ZmPP2C26L in Arabidopsis ap2c1 mutant partially restores drought-sensitive phenotype and overexpression of ZmPP2C26L in Arabidopsis WT and rice exhibit much lower hypersensitive to drought tolerance compared with ZmPP2C26S-overexpression lines (Figure 5 and Supplementary Figure 6). These results may be explained by ZmPP2C26S possesses higher activity compared with ZmPP2C26L (Supplementary Figure 4). The mutant of ZmPP2C26 gene increases chlorophyll and photosynthesis rate (Figures 6G,H), which could be explained by affecting the phosphorylation of proteins involved in photosynthesis (Figure 8). However, the downstream of ZmMAPK3- and ZmMAPK7-mediated by ZmPP2C6 needs to be explored in further study. In transgenic rice, ZmPP2C26L affects proteins phosphorylation directly enriched in photosynthesis, but ZmPP2C26S regulates proteins associated with carbon fixation in photosynthesis and pyruvate metabolism (Figure 7), and the phosphorylation of aquaporin PIP2-7 contributes for drought tolerance influenced by ZmPP2C26L and ZmPP2C26S (Jang et al., 2014; Fan et al., 2015).

CONCLUSION

We characterized a clade B type 2C PP, i.e., ZmPP2C26, that undergoes untypical AS to generate two isoforms. ZmPP2C26 directly dephosphorylates ZmMAPK3 and ZmMAPK7 to negatively regulate drought stress and photosynthesis activity (Figure 9). This study provides insights into understanding the mechanism of PP2C in response to abiotic stress.

REFERENCES

Barford, D., Das, A. K., and Egloff, M. P. (1998). The structure and mechanism of protein phosphatases: insights into catalysis and regulation. Annu. Rev. Biophys. Biomol. Struct. 27, 133–164. doi: 10.1146/annurev.biophys.27.1.133
Broek, A. K., Willmann, R., Kolb, D., Greifen, L., Lajunen, H. M., Bethke, G., et al. (2010). The Arabidopsis mitogen-activated protein kinase phosphatase PP2C5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. Plant Physiol. 153, 1098–1111. doi: 10.1104/pp.110.156109
Calixto, C. P. G., Guo, W., James, A. B., Tzioitiou, N. A., Entzone, J. C., Pantner, P. E., et al. (2018). Rapid and dynamic alternative splicing impacts the Arabidopsis cold response transcriptome. Plant Cell 30, 1424–1444. doi: 10.1105/tpc.18.00177
Chang, Y., Yang, H., Ren, D., and Li, Y. (2017). Activation of ZmMKK10, a maize mitogen-activated protein kinase kinase, induces ethylene-dependent cell death. Plant Sci 264, 129–137. doi: 10.1016/j.plantsci.2017.09.012
Chu, M., Chen, P., Meng, S., Xu, P., and Lan, W. (2021). The Arabidopsis phosphatase PP2C49 negatively regulates salt tolerance through inhibition of AtHKT1;1. J. Integr. Plant Biol. 63, 528–542. doi: 10.1111/jipb.13008
Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743. doi: 10.1046/j.1365-313x.1998.00343.x
Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. 58, 453–508.
Fan, W., Li, J., Jia, J., Wang, F., Cao, C., Hu, J., et al. (2015). Pyrabactin regulates root hydraulic properties in maize seedlings by affecting PIP aquaporins in a phosphorylation-dependent manner. Plant Physiol. Biochem. 94, 28–34. doi: 10.1016/j.plaphy.2015.05.005
Farquharson, K. L. (2016). Metabolic Signaling regulates alternative splicing during photomorphogenesis. Plant Cell 28:2697. doi: 10.1105/tpc.16.00842
Gu, J., Xia, Z., Luo, Y., Jiang, X., Qian, B., Xie, H., et al. (2018). Spliceosomal protein U1A is involved in alternative splicing and salt stress tolerance in Arabidopsis thaliana. Nucleic Acids Res. 46, 1777–1792. doi: 10.1093/nar/gks1229
Han, L., Li, J., Jin, M., and Su, Y. (2018). Functional analysis of a type 2C protein phosphatase gene from Ammospatthamus mongolicus. Gene 653, 29–42. doi: 10.1016/j.gene.2018.02.015
He, Z., Wu, J., Sun, X., and Dai, M. (2019). The maize clade A PP2C phosphatases play critical roles in multiple abiotic stress responses. Int. J. Mol. Sci. 20:3573. doi: 10.3390/ijms20143573

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FL performed most of the experiments and drafted the manuscript. FL and HY designed the experiments. YP, YC, JQ, and FS carried out the experiments and analyzed the data. LZ and QY assisted in experimental operations. XZ, YL, and FF reviewed the manuscript. WL and HY edited the manuscript. All authors read and approved the final manuscript.

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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.2022.851531/full#supplementary-material
Iniguez, I. P., Ramirez, M., Barbazuk, W. B., and Hernandez, G. (2017). Identification and analysis of alternative splicing events in Phaseolus vulgaris and Glycine max. BMC Genomics 18:630. doi: 10.1186/s12866-017-4054-2

Jang, H. Y., Rhee, J., Carlson, J. E., and Ahn, S. J. (2014). The Cmeliculain aquaporin CmPIP2;1 is regulated by phosphorylation at Ser273, but not at Ser277, of the C-terminus and is involved in salt- and drought-stress responses. J. Plant Physiol. 171, 1401–1412. doi: 10.1016/j.jplph.2014.06.009

Jiang, J., Liu, X., Liu, C., Liu, G., Li, S., and Wang, L. (2017). Integrating omics and alternative splicing reveals insights into grape to high temperature. Plant Physiol. 173, 1502–1518. doi: 10.1104/pp.16.01305

Kelemen, O., Convertini, P., Zhang, Z., Wen, Y., Shen, M., Falaleeva, M., et al. (2013). Group A PP2Cs evolved in land plants as key regulators of intrinsic desiccation tolerance. Nat. Commun. 4:2219. doi: 10.1038/ncomms3219

Li, H., Ding, Y., Shi, Y., Zhang, X., Zhang, S., Gong, Z., et al. (2017). MKP3- and MKP6-mediated IEC1 phosphorylation negatively regulates IEC1 stability and freezing tolerance in Arabidopsis. Dev. Cell. 43, 630–642. doi: 10.1016/j.devcel.2017.09.025

Li, Y., Chang, Y., Zhao, C., Yang, H., and Ren, D. (2016). Expression of the inactive Liang, B., Sun, Y., Wang, J., Zheng, Y., Zhang, W., Xu, Y., et al. (2021). Liu, Y., Wang, L., Zhang, D., and Li, D. (2013). Expression analysis of segmentally Liu, Z., Qin, J., Tian, X., Xu, S., Wang, Y., Li, H., et al. (2018). Global profiling of alternative splicing landscape responsive to drought, heat and their combination in wheat (Triticum aestivum L.). Plant Biotechnol. J. 32:e52457. doi: 10.15252/embr.202152457

Shi, Y. (2009). Serine/threonine kinases: mechanism through structure. Cell 139, 468–484. doi: 10.1016/j.cell.2009.10.006

Sidonskaya, E., Schweighofer, A., Shubchynsky, V., Kammerhofer, N., Hofmann, J., Wieczorek, K., et al. (2016). Plant resistance against the parasitic nematode Heterodera schachtii is mediated by MPK3 and MKP6 kinases, which are controlled by the MAPK phosphatase AP2C1 in Arabidopsis. J. Exp. Bot. 67, 107–118. doi: 10.1093/jxb/erv440

Singh, A., Apar, S., Kapoor, S., Tyagi, A. K., and Pandey, G. K. (2010). Protein phosphatase 2C interacts with and negatively regulates the function of CIPK9 under potassium-deficient conditions in Arabidopsis. J. Exp. Bot. 69, 4003–4015. doi: 10.1093/jxb/erq182

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85. doi: 10.1016/0003-2687(85)90442-7

Wang, J., Ding, H., Yuan, B., Wang, S., Su, C., Yao, B., et al. (2015). ABA signaling is directly dephosphorylated by OsMAPK3 and OsbHLH002 to negatively regulate the expression of OsMAPK3 and OsbHLH002 in rice. Plant Mol. Biol. 89, 55–67. doi: 10.1007/s11105-012-0489-4

Wang, J., Ding, H., Yuan, B., Wang, S., Su, C., Yao, B., et al. (2015). ABA signaling is directly dephosphorylated by OsMAPK3 and OsbHLH002 to negatively regulate the expression of OsMAPK3 and OsbHLH002 in rice. Plant Mol. Biol. 89, 55–67. doi: 10.1007/s11105-012-0489-4

Wang, Y. L., Zong, X. J., and Li, D. Q. (2010). Analysis of response to salt stress and drought in rice. J. Integr. Plant Biol. 52, 442–452. doi: 10.1111/j.1744-7909.2010.00906.x

Wang, J., Jiang, M. Y., Ma, F. F., and Ding, H. D. (2011). Expression characterization and function analysis of ZmPK7 from Zea mays seeds. J. Exp. Bot. 62:15537. doi: 10.1371/journal.pone.0015357

Wang, J., Ding, H., Zhang, A., Ma, F., Cao, J., and Jiang, M. (2010). A novel mitogen-activated protein kinase gene in maize (Zea mays), ZmMPK3, is involved in response to diverse environmental cues. J. Integr. Plant Biol. 52, 442–452. doi: 10.1111/j.1744-7909.2010.00906.x

Wang, Y. G., Fu, F. L., Yu, H. Q., Hu, T., Zhang, Y. Y., Tao, Y., et al. (2018). Interaction network of core ABA signaling components in maize. Plant Mol. Biol. 96, 245–263. doi: 10.1007/s11103-017-0692-7

Wang, Y. L., Zong, X. J., and Li, D. Q. (2010). Analysis of response to photosynthetic characteristic and antioxidant enzyme activities in transgenic tobacco transformed with zmmpk7 under salt stress. J. Nuclear Agric. Sci. 5, 1086–1092.

Wang, Z., Ji, H., Yuan, B., Wang, S., Su, C., Yao, B., et al. (2015). ABA signaling is finely tuned by antagonistic HAB1 variants. Nat. Commun. 6:8138. doi: 10.1038/ncomms9138

Xia, C., Gong, Y., Chong, K., and Xu, Y. (2021). Phosphatase OsP2C27 directly dephosphorylates OsMPK3 and OsHHL102 to negatively regulate cold tolerance in rice. Plant Cell Environ. 44, 491–505. doi: 10.1111/pce.13938

Xiang, Y., Sun, X., Gao, S., Qin, F., and Dai, M. (2017). Deletion of an endoplasmic reticulum stress response element in a ZmPP2C-A gene facilitates drought tolerance of maize seedlings. Mol. Plant 10, 456–469. doi: 10.1093/molp.10.10.005

Xue, W., Wang, D., Zhang, S., Ebright, J. N., Ji, F., Jakab, S., et al. (2008). Genome-scale expression analysis of protein phosphatase 2C in rice and Arabidopsis. BMC Genomics 9:550. doi: 10.1186/1471-2164-9-550

Yun, Z., Wang, J., Wang, F., Xie, C., Lv, B., Yu, Z., et al. (2021). MKP3-6-induced degradation of ARR10/12 promotes salt tolerance in Arabidopsis. EMBO Rep. 22:e25427. doi: 10.15252/embr.202125427
Yang, Z., Yang, J., Wang, Y., Wang, F., Mao, W., He, Q., et al. (2020). PROTEIN PHOSPHATASE95 regulates phosphate homeostasis by affecting phosphate transporter trafficking in rice. Plant Cell 32, 740–757. doi: 10.1105/tpc.19.00685

Yu, J., Miao, J., Zhang, Z., Xiong, H., Zhu, X., Sun, X., et al. (2018). Alternative splicing of OsLG3b controls grain length and yield in japonica rice. Plant Biotechnol. J. 16, 1667–1678. doi: 10.1111/pbi.12903

Zhang, J., Wan, L., Igathinathane, C., Zhang, Z., Guo, Y., Sun, D., et al. (2021). Spatiotemporal heterogeneity of chlorophyll content and fluorescence response within rice (Oryza sativa L.) canopies under different nitrogen treatments. Front. Plant Sci. 12:645977. doi: 10.3389/fpls.2021.645977

Zhou, Y., Zhou, D. M., Yu, W. W., Shi, L. L., Zhang, Y., Lai, Y. X., et al. (2021). Phosphatidic acid modulates MPK3- and MPK6-mediated hypoxia signaling in Arabidopsis. Plant Cell 34, 889–909. doi: 10.1093/plcell/koab289

Zhu, F. Y., Chen, M. X., Ye, N. H., Shi, L., Ma, K. L., Yang, J. F., et al. (2017). Proteogenomic analysis reveals alternative splicing and translation as part of the abscisic acid response in Arabidopsis seedlings. Plant J. 91, 518–533. doi: 10.1111/tpj.13571

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