

MYC2 regulates stomatal density and water use efficiency via targeting EPF2/EPFL4/EPFL9 in poplar

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Abstract: Although polyploid plants have lower stomatal density than their diploid counterparts, the molecular mechanisms underlying this difference remain elusive. Here, we constructed a network based on the triploid poplar transcriptome data and TGM algorithm and found that *PpnMYC2* was related to stomatal development-related genes *PpnEPF2*, *PpnEPFL4*, and *PpnEPFL9*. The interactions between *PpnMYC2* and *PagJAZs* were experimentally validated. *PpnMYC2*-overexpressing poplar and *Arabidopsis thaliana* had reduced stomatal density. Poplar overexpressing *PpnMYC2* had higher water use efficiency and drought resistance. RNA-sequencing data of poplars overexpressing *PpnMYC2* showed that *PpnMYC2* promotes expression of stomatal density inhibitors *PagEPF2* and *PagEPFL4* and inhibits expression of the stomatal density positive regulator *PagEPFL9*. Yeast one-hybrid system, electrophoretic mobility shift assay, ChIP-qPCR and dual-luciferase assay were employed to substantiate that *PpnMYC2* directly regulated *PagEPF2*, *PagEPFL4*, and *PagEPFL9*. *PpnMYC2*, *PpnEPF2* and *PpnEPFL4* were significantly upregulated, whereas *PpnEPFL9* was downregulated during stomatal formation in triploid poplar. Our results are of great significance for revealing the regulation mechanism of plant stomatal occurrence and polyploid stomatal density, as well as reducing stomatal density and improving plant water use efficiency by overexpressing *MYC2*.

Introduction

Stomata are channels on the surface of plant leaves (Hetherington & Woodward, 2003; Guo *et al.*, 2021), through which transpiration and carbon dioxide uptake mainly occur, play a crucial role in maintaining plant photosynthesis and water use efficiency (WUE) (Durand *et al.*, 2019; Shen *et al.*, 2021; Li *et al.*, 2021; Liu *et al.*, 2021). Multiple influencing factors regulate stomatal development, and the key transcription factors involved in the regulation of each stage of stomatal development include *bHLH*-type transcription factor members *SPEECHLESS* (*SPCH*), *MUTE*, *FAMA*, *SCREAM* (*SCRM*), and *SCRM2* (Bergmann & Sack, 2007; Kanaoka *et al.*, 2008; Wang *et al.*, 2021), as well as R2R3-type *MYB* transcription factors encoded by *FOUR LIPS* (*FLP*) and *MYB88* (Lai *et al.*, 2005). The transcription factors *SPCH*, *MUTE*, and *FAMA* of the *bHLH* family regulate stomatal formation directly (Ohashi-Ito & Bergmann, 2006; Han *et al.*, 2018a; Wang *et al.*, 2021). *SPCH* functions primarily in the early stage of stomatal formation. Its expression level is affected by an inhibitory signal transduction cascade of stomatal precursors, composed of cell surface receptor complexes of the *ERECTA* receptor-like kinase family and receptor-like protein TOO MANY MOUTHS (TMM) composition (Shpak *et al.*, 2005). The genes of the *EPF/EPFL* family indirectly affect stomatal development, for example, *EPFL9* positively regulates stomatal density, and *EPF1* and *EPF2* negatively regulate stomatal density. Studies have shown that as ligands, *EPFL9*, *EPF1* and *EPF2* can directly bind to receptors such as TMM/*ERECTA* (Hara *et al.*, 2007; Hunt & Gray, 2009; Lee *et al.*, 2015), thereby affecting the expression of downstream *SPCH* or *MUTE* (Pillitteri & Torii, 2012), and ultimately regulating stomatal development (Lee *et al.*, 2015; Xia *et al.*, 2022). A single *ERECTA* family (ERf) without a TMM recognize *EPFL4* and *EPFL6* of the *EPF/EPFL* family, and they can reduce stomatal density when combined with ERf receptors (Abrash, Davies & Bergmann, 2011; Lin *et al.*, 2017; Jiao *et al.*, 2022). However, the upstream regulatory genes of *EPF2*, *EPFL4*, and *EPFL9* remain unclear.

The basic helix-loop-helix (*bHLH*) transcription factor gene family is one of the largest gene families and is widely involved in plant growth and development and abiotic stress responses (Carretero-Paulet *et al.*, 2010). The *bHLH* transcription factor contains a *bHLH* characteristic domain, and some of its conserved amino acids determine its recognition of the so-called core E-box hexanucleotide consensus sequence -CANNTG- (Carretero-Paulet *et al.*, 2010; Feller *et al.*, 2011). *MYC2*, *MYC3*, and *MYC4* all belong to the *bHLH* family, which can bind to G-box or G-

box-like motifs in gene promoters (Shen *et al.*, 2016), and are key regulators of the jasmonic acid (JA) signaling pathway (Niu *et al.*, 2011). Double or triple mutants of *MYC2*, *MYC3*, and *MYC4* affect stomatal development in *Arabidopsis*, but single mutation experiments did not find a significant effect on stomatal development (Han *et al.*, 2018b), and whether it has a regulatory effect on the *EPF/EPFL* family has not been reported.

Generally, the stomata in polyploid plants become more prominent, but the stomatal density decreases (Xu *et al.*, 2016; Monda *et al.*, 2016; Kang & Wei, 2022). At present, the molecular mechanism underlying the regulation of decreased stomatal density in polyploid leaves remains unknown. In this study, the perennial woody triploid and diploid poplar ((*P. pseudo-simonii* × *P. nigra* 'Zheyin 3#' (2n = 2x = 38)) × 'Beijing poplar' (*P. beijingensis* (2n = 2x = 38))) were used as research materials. Based on the transcriptome data and the triple-gene mutual interaction (TGMI) algorithm, a correlation network was constructed to analyze the transcription factors associated with the expression of *PpnEPF2*, *PpnEPFL4* and *PpnEPFL9*, and the transcription factor *PpnMYC2*, whose expression was significantly upregulated in triploid poplar apical buds and young leaves, was screened out. Therefore, we aimed to investigate whether there is a regulatory relationship between *PpnMYC2* and *PpnEPF2*, *PpnEPFL4*, *PpnEPFL9*. To this end, we cloned the *PpnMYC2* gene from poplar, and proved the relationship between *PpnMYC2* and *PagEPF2*, *PagEPFL4*, *PagEPFL9* through yeast one-hybrid system, electrophoretic mobility shift assay (EMSA), ChIP-qPCR and dual-luciferase assay. *PpnMYC2* overexpression and interference lines were constructed, and the changes in stomatal density and gene expression of *PagEPF2*, *PagEPFL4*, and *PagEPFL9* in the overexpression and interference lines compared with the wild type (WT) were studied. This study revealed the molecular mechanism by which *PpnMYC2* affects plant stomatal density, provides some theoretical support for analysis of the causes of stomatal density variation in polyploid plants, and provides a meaningful target gene for the molecular-design breeding of water-saving plants.

Materials and Methods

Materials and growing conditions

The material for the gene cloning test was obtained from three all-sibling offspring populations obtained from the cross of the same parents 'Zheyin 3 poplar' (*P. pseudo-simonii* × *P. nigra* 'Zheyin 3#' (2n = 2x = 38)) × 'Beijing poplar' (*P. beijingensis* (2n = 2x = 38)), including two triploid populations and one diploid population. Two heterozygous polyploid populations were obtained by crossing 2n female gametes with male gametes produced by first-division restitution (FDR) and second-division restitution (SDR), respectively (hereafter referred to as triploid-F and triploid-S). We randomly selected 10 genotypes from these populations and propagated them asexually using cuttings for leaf stomatal phenotypic studies and gene tissue-specific expression assays (Wu *et al.*, 2021).

The transgenic vector material was derived from 84 K poplar (*Populus alba* × *Populus glandulosa*, Pag). 84 K poplar seedlings were cultured in MS medium in a chamber at 25 °C with 16h/days of light per day.

Nicotiana benthamiana plants were grown in a culture chamber at 25 °C with 16 h/days of light and filled with a 3:1 mixture of soil and perlite. They were used for subcellular localization, split luciferase complementation, and dual-luciferase reporter gene detection assays after 30 days of growth.

Arabidopsis thaliana plants were grown in a climate chamber (16 h light/8 h dark, 23 °C) and were subsequently used for gene transformation.

Network construction using TGMI algorithm

The triploid and diploid poplars obtained from the parents of 'Zheyin 3 poplar' and 'Beijing poplar' were used as materials, and triploid and diploid poplars grown in the greenhouse of Beijing Forestry University for 4 months were collected. Different tissues of poplar, including apical buds and leaves, with three biological replicates per tissue, were sequenced to obtain transcriptome datasets (available at CNCB with the accession no. PRJCA018931). RNA-seq was performed by Novogene Co. Ltd using Illumina HiSeq X Ten platform, and approximately 6 GB reads were generated from each library. The clean reads mapped to *Populus trichocarpa* genome 4.1 using TopHat2 (Kim *et al.*, 2013), and the FPKM of each gene was calculated based on the length of the gene.

Meanwhile, all the transcription factors of *Populus trichocarpa* were obtained from the Plant Transcription Factor Database (<http://planttfdb.gao-lab.org/>). The FPKM values of these transcription factors were obtained from the previously obtained triploid and diploid poplar transcriptome datasets. Subsequently, all the pathway genes

related to stomatal formation were screened out from the Gene Ontology database, and the TGMI algorithm was used to perform correlation analysis on the expression levels of these stomatal formation-related pathway genes and transcription factors (Gunasekara *et al.*, 2018), the frequencies of TFs on pathway genes were displayed in the descending order. TGMI calculates the frequency of all TFs in all significantly interacting gene blocks and ranks them, with the higher frequency TFs being usually the true pathway regulators.

Construction of plasmid vectors and gene transformation

The CDS sequence of *PpnMYC2* was amplified from the heterologous triploid cDNA and inserted into the pBI121-GFP and pROKII-RNAi vectors containing the CaMV35S promoter (35S) to generate 35S::*PpnMYC2-GFP-nos* and 35S::*PpnMYC2-Loop-PpnMYC2-nos* loop constructs. Primers are listed in Table S1.

Agrobacterium-mediated transformation of 84 K asexual lines was performed following a previously described genetic transformation method (Liu *et al.*, 2014). We identified positive transgenic lines by PCR and then obtained overexpression and silent lines with different expression levels of *PpnMYC2* by RT-qPCR screening and used them for plant and stomatal phenotypic studies, phytohormone assays, photosynthetic parameter determination, and RNA-seq experiments.

RNA-seq experiment of transgenic lines and data analysis

The apical buds of *PpnMYC2* overexpression lines were harvested. For each line, apical buds (including the first fully unfolded leaf) of five plants with similar growth were selected and mixed. Ten apical buds of WT plants were collected and divided into two groups as controls. Transcriptome sequencing of the samples was performed by Novogene Co. Ltd (Beijing, China) using the same protocols. Clean reads were mapped to the 84K polar genome using HISAT2 (Huang *et al.*, 2020). Then, the FPKM of each gene was calculated based on the length of the gene and the read counts mapped to this gene. The DESeq R package was used for the differential gene expression analysis (Wang *et al.*, 2010). Genes for which an adjusted p -value ≤ 0.05 was found by DESeq were assigned as differentially expressed.

Statistical analysis

SPSS software (IBM Corp., Armonk, NY, USA) was used to perform the statistical analyses, and the data are presented as mean $\pm$ standard deviation (SD). Student's t -test, one-way ANOVA, and two-way ANOVA were used to determine variance among the mean values (asterisks denote significant differences: * $P < 0.05$; ** $P < 0.01$) (Lu *et al.*, 2022).

All other routinely used methods including scanning electron microscopy analysis (Methods S1), tissue-specific expression of genes (Methods S2), subcellular localization analysis (Methods S3), transcriptional activation analysis in yeast cells (Methods S4), yeast two-hybrid assays (Methods S5), split luciferase complementation assays (Methods S6), GST (glutathione-S-transferase)-fusion protein pull-down assay (Methods S7), gas exchange analysis (Methods S8), gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis (Methods S9), drought tolerance assay and physiological analysis (Methods S10), yeast one-hybrid assays (Methods S11), ChIP-qPCR assays (Methods S12), electrophoretic mobility shift assays (EMSAs) (Methods S13), and dual-luciferase assays (Methods S14), used in this study are described in the Methods.

Results

Lower stomatal density and larger stomatal size in triploid poplar leaves

It has been reported that triploid poplars have significant vegetative growth and resistance advantages compared to all-sibling diploid poplars (progeny of the same parents of triploids). The most significant advantages are greater height, larger diameter at the base, and stronger drought resistance (Liao *et al.*, 2016; Liao *et al.*, 2018). We found that triploid poplars had greater plant height and leaf size than diploid poplars (Fig. 1a, b). The leaf area of the triploid-FDR and triploid-SDR types was 150.76 cm² and 152.63 cm², respectively, while that of the diploid was only 98.30 cm² (Fig. 1c).

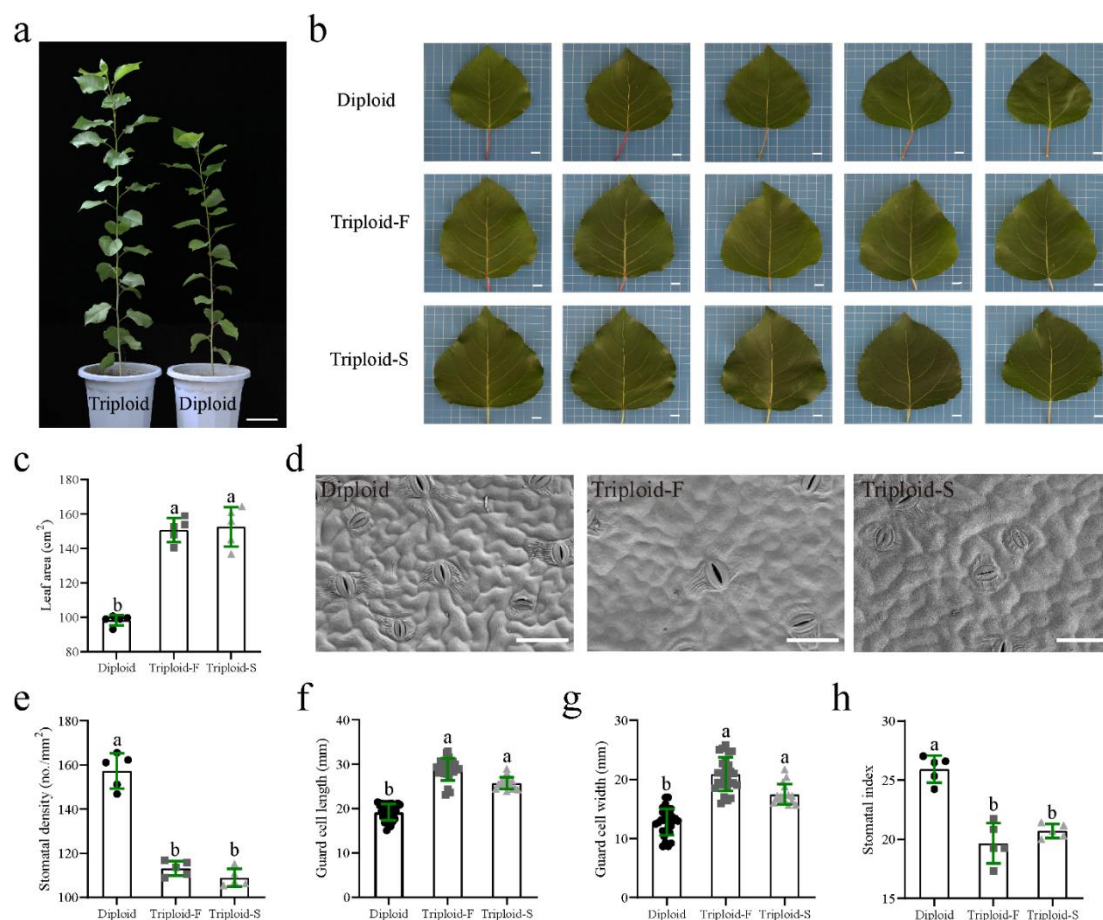


Figure 1. Phenotypes of diploid and triploid poplars. (a) Three-month-old triploid and diploid poplars, bar = 10 cm. (b) diploid and triploid poplar leaves, bars = 1 cm. (c) leaf area of diploid and triploid poplars, $n=5$. (d) Scanning electron micrographs of the stomata of diploid and triploid poplars, bars = 50 μm . (e) Stomatal density of diploid and triploid poplars, $n=5$. (f) Length of guard cells of diploid and triploid poplars, $n>5$. (g) Width of guard cells of diploid and triploid poplars, $n>5$. (h) Stomatal index of diploid and triploid poplars, $n=5$. F, first division restitution (FDR) gametes; S, second division restitution (SDR) gametes. Values represent the mean $\pm$ SD. Different letters denote statistically significant differences resulting from Duncan's test following one-way ANOVA.

A larger leaf area may lead to larger stomatal size and lower stomatal density. Stomatal density was significantly lower and stomatal size was significantly larger in triploids than in diploids (Fig. 1d). The stomatal density of FDR- and SDR-type triploids was 28.09 % and 31.32 % smaller than that of diploids, respectively (Fig. 1e), and the differences in stomatal density of leaves in different leaf positions were all significant (Fig. S1). In contrast, the guard cell lengths of FDR- and SDR-type triploid leaves were 50.43 % and 33.96 % larger than those of diploids (Fig. 1f), and the widths of guard cells of FDR- and SDR-type triploid leaves were 64.08 % and 37.20 % larger than those of diploids (Fig. 1g), respectively. Subsequently, we performed stomatal index calculations based on the number of stomata and epidermal cells and found that the stomatal index of FDR- and SDR-type triploids was also significantly lower than that of diploids (Fig. 1h).

The TGMI network clearly reflects the higher ranked transcription factors and pathway genes that may be associated with the traits we are interested in. Therefore, we screened out 17 pathway genes involved in the stomatal formation process that were related to upstream transcription factors, and the upstream transcription factors were also sorted according to the relative frequency. The highest one was *HEC2-1*, followed by *MYC2* (Fig. S2). Using network construction based on the TGMI algorithm, we found that *MYC2* was highly correlated with stomatal development-related genes (Fig. 2a). Meanwhile, we found that *MYC2*, *EPF2* and *EPFL4* were all upregulated in triploid poplars and were positively correlated in the dataset, while *EPFL9* was downregulated in triploid poplars and was negatively correlated with *MYC2* in the dataset (Fig. S3). Thus, we hypothesized that *PpnMYC2* specifically targets *PpnEPF2*, *PpnEPFL4*, and *PpnEPFL9* to regulate stomatal density and WUE in triploid poplars.

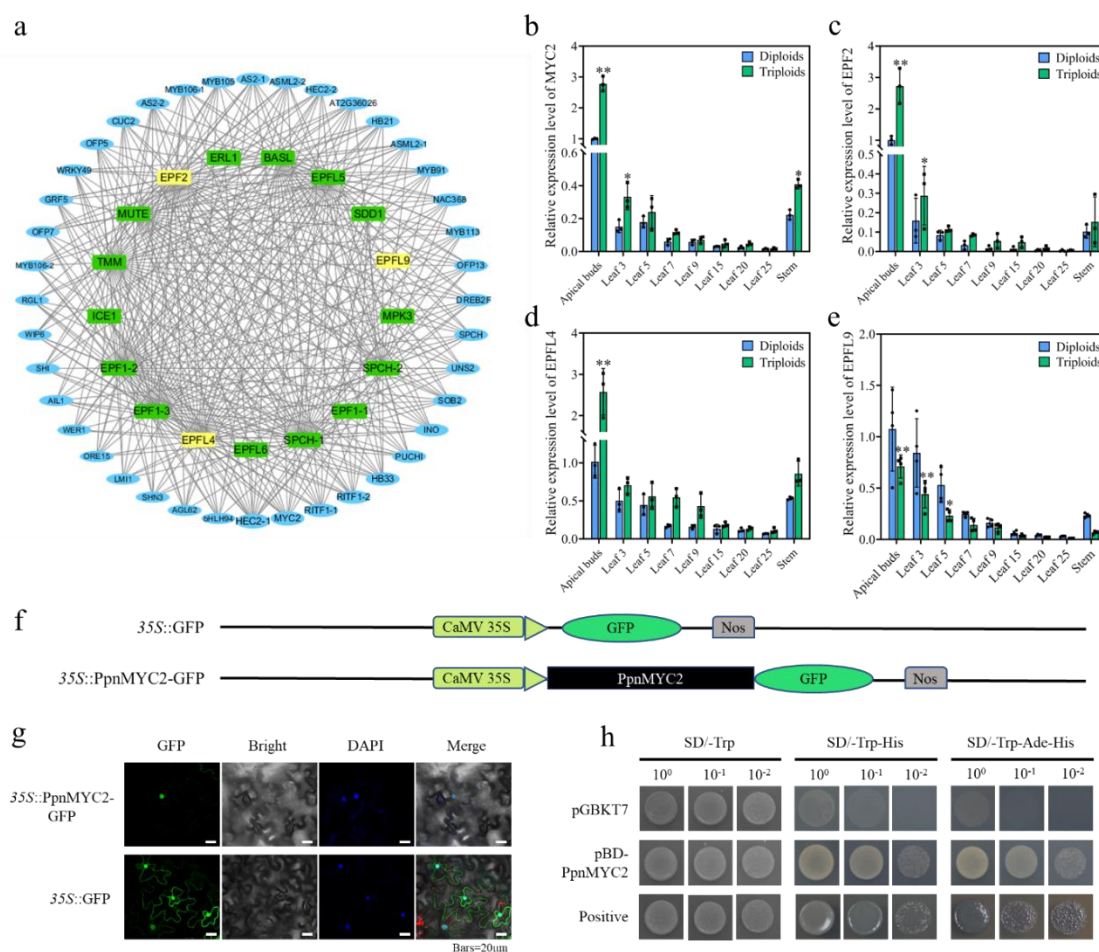


Figure 2. Expression analyses of *PpnMYC2*, *PpnEPF2*, *PpnEPFL4*, and *PpnEPFL9* in triploid poplars and subcellular localization and transcriptional activation analysis of *PpnMYC2*. (a) Regulatory network generated by the triple-gene mutual interaction (TGMI) algorithm for the stomatal development pathway. Green and yellow nodes represent stomatal development-related genes. Blue nodes are transcription factors. Tissue-specific expression of *PpnMYC2* (b), *PpnEPF2* (c), *PpnEPFL4* (d), and *PpnEPFL9* (e) in diploid and triploid poplars. Values represent the mean $\pm$ SD ($n=3$). *, $P < 0.05$; **, $P < 0.01$ (determined by Student's t -test). (f) Vector construction diagram of pBI121-*PpnMYC2*-GFP. (g) Subcellular localization of the *PpnMYC2* protein. Laser-scanning confocal microscopy images of tobacco leaves transiently expressing 35S::GFP and 35S::PpnMYC2-GFP. 4',6-diamidino-2-phenylindole (DAPI) is a nuclear dye. (h) Transactivation assay of *PpnMYC2* in yeast cells. The full length of *PpnMYC2* was fused in frame with the GAL4 DNA-binding domain in pGBKT7 and transformed into yeast strain AH109. The vector pGBKT7 was used as a negative control. The pBD-*PpnGRF5-1* strain was expressed in yeast as a positive construct as reported before.

Tissue-specific expression, subcellular localization and transcriptional activity assay of *PpnMYC2*

According to the transcriptome sequencing data, *PpnMYC2* was highly expressed in apical buds of triploid poplars (Fig. S3). To investigate the tissue-specific expression pattern of *MYC2*, we used ePlant (http://bar.utoronto.ca/eplant_poplar/) to analyze the expression of *MYC2* and found that it was primarily expressed in the young leaves of poplar (Fig. S4). Subsequently, we collected nine different tissues from 3-month-old triploids and their full-sibling diploid poplars (Fig. S5), and RT-qPCR was used to quantify *MYC2*, *EPF2*, *EPFL4*, and *EPFL9*. *PpnMYC2* had a broadly similar tissue-specific expression pattern in diploid and triploid poplars. The expression of *PpnMYC2* was higher in the apical buds than in any other tissue, and the expression level of *PpnMYC2* decreased with an increase in leaf number (Fig. 2b). Therefore, this gene may function mainly in the apical buds and young leaves (Fig. 2b). *PpnMYC2* was significantly expressed in apical buds, 3rd leaf position, and young stem segments in triploids compared to diploids (Fig. 2b). Therefore, we hypothesized that *PpnMYC2* affected stomatal development in triploids. In addition, we found that the expression trends of *PpnEPF2* and *PpnEPFL4* in diploid and triploid poplars were very similar to *PpnMYC2* and were also expressed at higher levels in young tissues (Fig. 2c, d). However, *PpnEPFL9* has the opposite expression tendency to *PpnMYC2* in diploid and triploid poplars (Fig. 2e).

To investigate whether stomatal density was associated with the high *PpnMYC2* expression in triploids, we

measured *PpnMYC2* expression in three genotypes randomly selected from three populations: an all-sibling diploid and two triploids (FDR and SDR). The expression levels of *PpnMYC2* in apical buds, the 3rd and 5th leaves of each genotype were detected by RT-qPCR (Fig. S6). The results showed that the expression of *PpnMYC2* in different diploid and triploid genotypes was negatively correlated with stomatal density (Figs. S1, S6). Stomatal density in diploids and triploids increased with increasing leaf number until the 10th leaf position, whereas the expression of *PpnMYC2* decreased (Figs. 2b, S1, S6).

To determine the subcellular localization of *PpnMYC2*, we generated the 35S::*PpnMYC2*-GFP and 35S::GFP constructs and transiently expressed them in *Nicotiana benthamiana* leaves (Fig. 2f). 4',6-diamidino-2-phenylindole (DAPI) staining confirmed that the 35S::*PpnMYC2*-GFP fusion protein was localized in the nucleus, whereas the 35S::GFP control protein was distributed in the nucleus and cytoplasm (Fig. 2g).

To determine whether *PpnMYC2* had transcriptional activity, we performed a transcriptional activity assay using *PpnMYC2*. We found that transformants carrying GALBD-*PpnMYC2* and the positive control grew well on SD/-Trp/-His and SD/-Trp/-Ade/-His media, whereas those carrying the negative control pGBKT7 vector did not grow properly (Fig. 2h), indicating that the GALBD-*PpnMYC2* fusion protein was active in yeast cells.

MYC2 transcription factor physically interacts with JAZ repressors of JA signaling

To investigate whether *PpnMYC2* interacts with *JAZs* in poplar, we cloned four *JAZ* genes, *PagJAZ10*, *PagJAZ1-2*, *PagJAZ1-5*, and *PagJAZ1-6*, from 84K poplar. We tested the physical interactions between *PpnMYC2* and each of the four *PagJAZs* using the Y2H system. The interactions between *PpnMYC2* and *PagJAZ10*, *PagJAZ1-2*, *PagJAZ1-5*, and *PagJAZ1-6* were demonstrated through a gradient dilution (Fig. 3a). However, *PpnbHLH153*, which belongs to the same family as *PpnMYC2*, did not interact with *PagJAZs* (Fig. S7a). In addition, we used a split-luciferase complementation assay to test the interaction between *PpnMYC2* and *PagJAZs*. We injected 35S::*nLUC* and 35S::*cLUC*, 35S::*PpnMYC2*-*nLUC* and 35S::*cLUC*, 35S::*nLUC* and 35S::*cLUC*-*PagJAZs*, and 35S::*PpnMYC2*-*nLUC* and 35S::*cLUC*-*PagJAZs* into the leaf epidermal cells of *Nicotiana benthamiana*. The results showed that only the co-expression of *PpnMYC2* and *PagJAZs* at the same locus caused luciferase remodeling and a more intense fluorescent signal (Fig. 3b). Bioluminescence was not observed when 35S::*PpnMYC2*-*nLUC* or 35S::*cLUC*-*PagJAZs* were co-expressed with 35S::*cLUC* or 35S::*nLUC* (Fig. 3b). At the same time, no bioluminescence was observed after co-injection of *PpnbHLH153* with *PagJAZs* into the leaf epidermal cells of *Nicotiana benthamiana* (Fig. S7b). *In vitro* pull-down assays using GST-*PpnMYC2* recombinant proteins showed direct interactions with *PagJAZ10*-His6, *PagJAZ1-2*-His6, *PagJAZ1-5*-His6, and *PagJAZ1-6*-His6 (Fig. 3c). Based on the above results, we conclude that *PpnMYC2* interacts with *PagJAZ10*, *PagJAZ1-2*, *PagJAZ1-5*, and *PagJAZ1-6*.

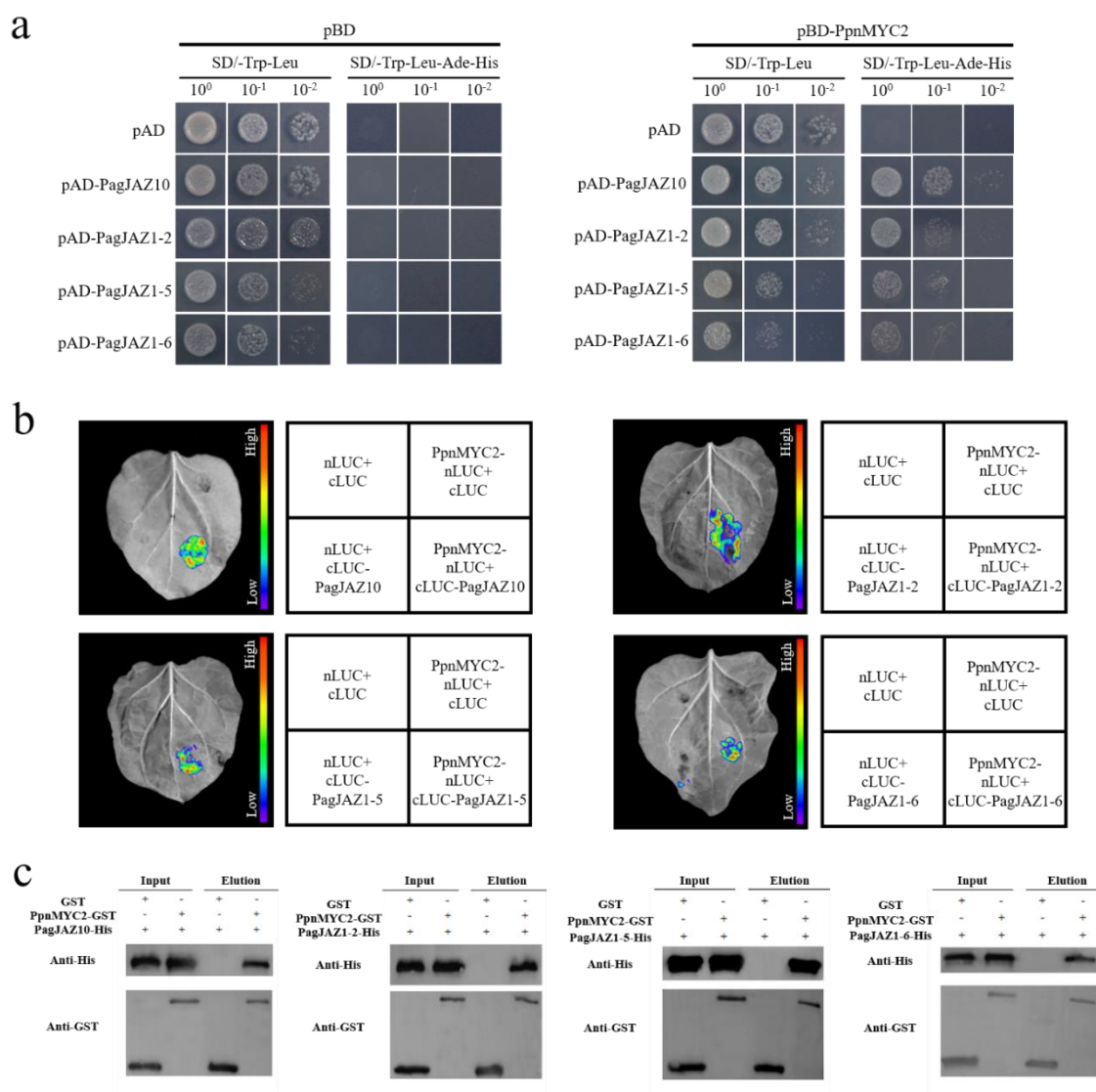


Figure 3. *PpnMYC2* interacts with *PagJAZs*. (a) Yeast cells of co-transformants of *PpnMYC2* and *PagJAZs* grown on SD/-Trp-Leu and SD/-Trp-Leu-Ade-His medium at 30 °C for 3 days. *PpnMYC2* was fused to the DNA-binding domain (BD), and *PagJAZs* were fused to the transcription activation domain (AD). pAD and pBD are negative controls. (b) Split luciferase complementation assay reveals the interaction between *PpnMYC2* and *PagJAZs*. *PpnMYC2* was fused to the N-terminal portions of LUC (nLUC), and *PagJAZs* were fused to the C-terminal portion of LUC (cLUC). *Agrobacterium* carrying different plasmids, as indicated, were co-expressed in *Nicotiana benthamiana*. Representative images of *N. benthamiana* leaves 48 h after infiltration are shown. The color scale represents LUC activity. The experiment was repeated three times with similar results. (c) Pull-down assays show the interaction between *PpnMYC2* and *PagJAZs* *in vitro*. The GST-*PpnMYC2* and *PagJAZs*-His6 recombinant proteins were mixed with a pull-down binding buffer and incubated with GST beads. The pulled-down protein was detected by immunoblotting. A mixture of GST and *PagJAZs*-His6 was used as a negative control.

PpnMYC2 regulates stomatal density in *PpnMYC2* transgenic lines

To test whether the reduced stomatal density in triploid poplar was caused by the high expression of *PpnMYC2*, we first overexpressed *PpnMYC2* in *Arabidopsis*. We compared the stomatal density and size of overexpressed *PpnMYC2* and WT *Arabidopsis*. We found that the stomatal density and stomatal index of the overexpressed *PpnMYC2* lines were significantly lower than those of WT, with 0.54, and 0.85-fold of WT, respectively (Fig. S8a-c). Statistically, we found that the length and width of guard cells of the overexpressed *PpnMYC2* lines were significantly greater than those of WT, with 1.23- and 1.27-fold of WT, respectively (Fig. S8d, e).

Afterwards, we constructed poplar *PpnMYC2* overexpression and interference transgenic lines (35S::*PpnMYC2*-GFP-*nos* and 35S::*PpnMYC2*-Loop-*PpnMYC2*-*nos* vectors). We used 84 K poplars as our gene transformation system and obtained ten independent transgenic lines overexpressing *PpnMYC2* (*PpnMYC2*-OE) and

ten independent RNA-interference transgenic lines (*PpnMYC2-RNAi*) (Fig. S9a, b). We selected *PpnMYC2-OE-8* and *PpnMYC2-OE-9* from the *PpnMYC2-OE* lines, and *PpnMYC2-RNAi-3* and *PpnMYC2-RNAi-8* from *PpnMYC2-RNAi* lines for follow-up studies (Fig. 4a).

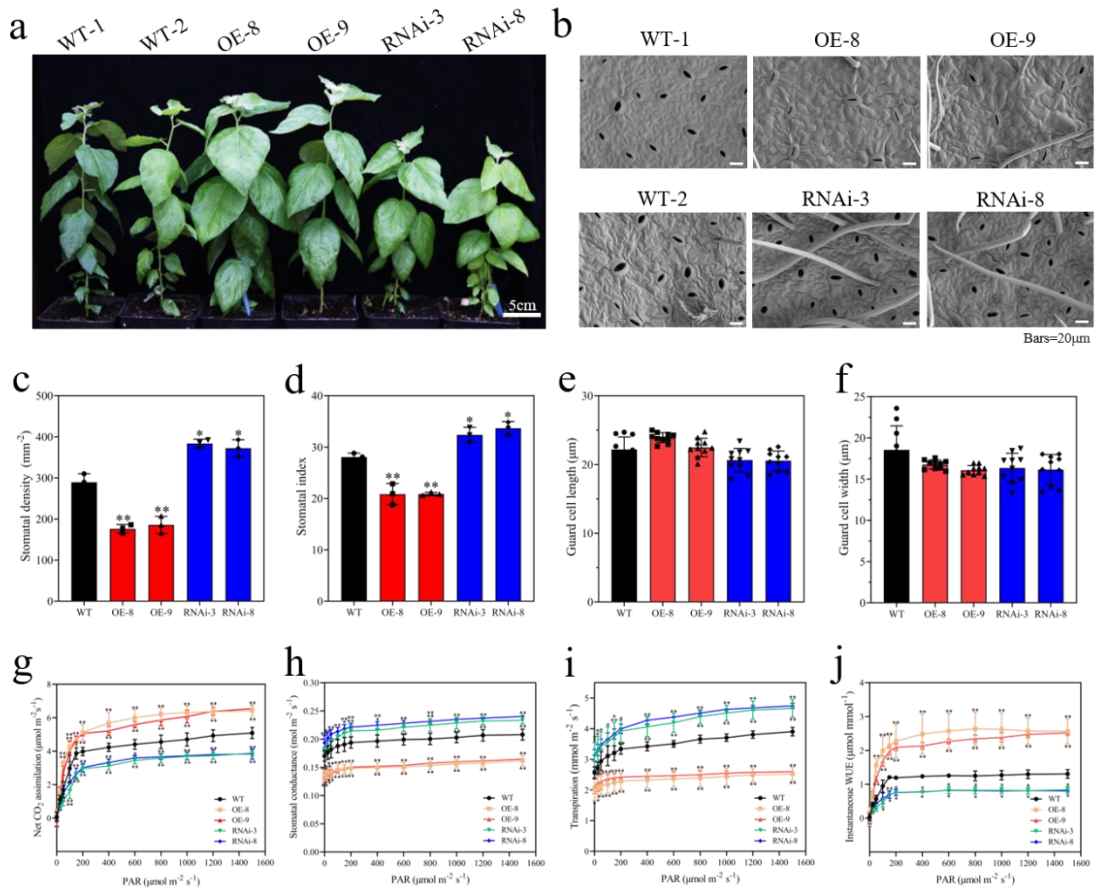


Figure 4. Effects of overexpression and interference of *PpnMYC2* on stomatal density and WUE in poplar. 5-week-old seedling phenotypes (a), stomatal phenotype (b), stomatal density (n=3) (c), stomatal index (n=3) (d), guard cell length (n=10) (e), and guard cell width (n=10) (f) of overexpressed and interfered *PpnMYC2* poplars. (g) Net CO₂ assimilation (*A*)–light curve. (h) Stomatal conductance (*G_s*)–light curve. (i) Transpiration rate (*T_r*)–light curve. (j) Instantaneous WUE–light curve, n=3. Experiments were performed three times with similar results. Values represent the mean ± SD. Asterisks denote significant differences: **: $P < 0.01$, *: $P < 0.05$ (determined by Student's *t*-test).

Based on the previously obtained gene regulatory module results, we consider that *PpnMYC2* regulates the expression of *EPF2*, *EPFL4*, and *EPFL9*, and therefore alters stomatal density in the leaves. To verify this, we observed the stomatal density of the transgenic and WT poplars. We found that the stomatal density of the 10th leaf from *PpnMYC2-OE-8* and *PpnMYC2-OE-9* transgenic lines was 0.61 and 0.64-fold that of WT, respectively (Fig. 4b, c). The stomatal density of the 10th leaf from *PpnMYC2-RNAi-3* and *PpnMYC2-RNAi-8* transgenic lines was 1.32 and 1.29-fold of WT, respectively (Fig. 4b, c). Although the area of the 10th leaf of the OE lines was significantly larger than that of the WT and that of the RNAi lines was significantly smaller than that of the WT (Fig. S10a), the total number of stomata in the entire leaf of the OE lines was still significantly smaller than that of the WT, while that of the RNAi lines was significantly larger than that of the WT. (Fig. S10b). The stomatal index showed a similar trend, with *PpnMYC2-OE* lines showing a significantly lower stomatal index than WT, whereas the stomatal index of *PpnMYC2-RNAi* lines was significantly higher than that of the WT (Fig. 4d). The differences in guard cell length and guard cell width between *PpnMYC2-OE*, *PpnMYC2-RNAi*, and WT plants were not significant (Fig. 4e, f).

Based on the above phenotypic changes, stomatal density should also affect WUE; therefore, we determined the gas exchange parameters of different lines. We found that *PpnMYC2-OE* poplars had significantly higher net CO₂ assimilation (*A*) than WT, whereas *PpnMYC2-RNAi* poplars had significantly lower *A* than WT poplars (Fig. 4g). Stomatal conductance (*G_s*) also showed a similar trend as stomatal density, and *PpnMYC2-OE* and *PpnMYC2-RNAi* lines were significantly different from the WT (Fig. 4h). However, the transpiration

ion rate (Tr) of *PpnMYC2-OE* lines was lower than that of the WT, while that of *PpnMYC2-RNAi* lines was higher than that of the WT (Fig. 4i). The instantaneous WUE of *PpnMYC2-OE* was the highest and was significantly higher than that of WT, whereas that of *PpnMYC2-RNAi* was significantly lower than that of WT (Fig. 4j). Therefore, we speculated that *PpnMYC2* might increase the WUE of poplars by affecting the expression of *EPF2/EPFL4/EPFL9* to reduce stomatal density.

Transcriptome analysis of *PpnMYC2-OE* transgenic poplars reveals that stomatal development-related genes are activated

Phenotypic changes, particularly lower stomatal density in the *PpnMYC2-OE* lines, prompted us to identify altered differential expressed genes (DEGs). To this end, we performed RNA-seq experiments on *PpnMYC2-OE* and WT 84 K poplar. Sequence reads obtained from these lines were aligned to the 84K poplar genome (Huang *et al.*, 2020). In total, 14741 DEGs (corrected $P < 0.05$) were identified by comparing transgenic and WT 84 K poplars. Among these DEGs, 7480 were upregulated and 7261 were downregulated (Fig. 5a, Table S2). GO enrichment analysis of 14741 DEGs revealed that pathways of cellular response to stress (GO:0033554, 59 genes), carbohydrate biosynthetic process (GO:0016051, 55 genes), superoxide metabolic process (GO:0006801, 11 genes), and photosystem I reaction center (GO:0009538, 12 genes) were significantly enriched (Fig. 5b, Table S3). Through KEGG enrichment analysis, it was found that carbon fixation in photosynthetic organisms, phenylalanine, tyrosine, and tryptophan biosynthesis, lysine biosynthesis, starch and sucrose metabolism, and circadian rhythm-plant were significantly enriched (Fig. 5c, Table S4).

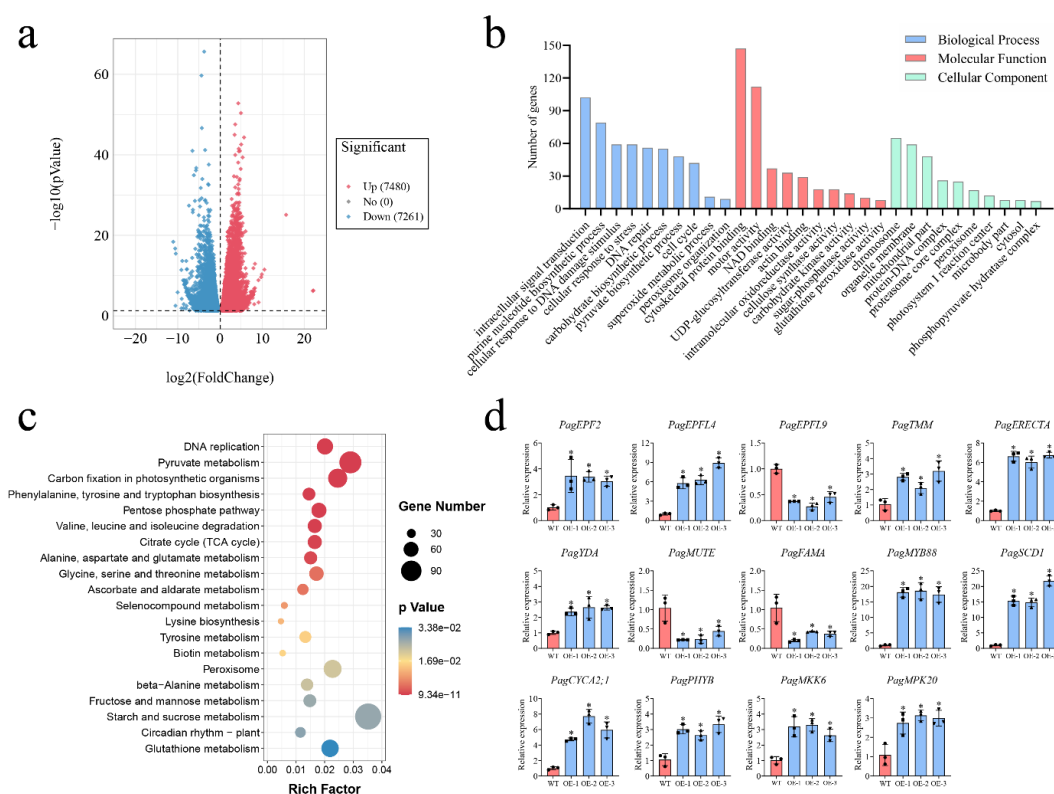


Figure 5. Transcriptome analysis of *PpnMYC2-OE* and WT poplar apical buds. (a) The identified DEGs are shown in the volcano plot. (b) GO term enrichment for DEGs in *PpnMYC2-OE* and WT plants. (c) KEGG enrichment analysis of DEGs in *PpnMYC2-OE* and WT plants. The X-axis represents the rich factor, the Y-axis represents KEGG pathways, the bubble size represents the number of genes annotated to a KEGG pathway, and the color represents the enrichment p -value. (d) RT-qPCR detection of *PagEPF2*, *PagEPFL4*, *PagEPFL9*, *PagTMM*, *PagERECTA*, *PagYDA*, *PagMUTE*, *PagFAMA*, *PagMYB88*, *PagSCD1*, *PagCYCA2.1*, *PagPHYB*, *PagMCK6*, and *PagMPK20* in WT and *PpnMYC2* overexpression plants, $n = 3$. *: $P < 0.05$ (determined by Student's t -test).

We screened the genes related to stomatal development in *PpnMYC2-OE* and WT plants and found that the genes related to stomatal density, such as *PagEPF2*, *PagEPFL4*, *PagTMM*, *PagERECTA*, and *PagYDA*, were significantly overexpressed, and *PagEPFL9* was significantly downregulated expression (Fig. S11, Tabl

e S5). However, the expression levels of genes that directly promote the stomatal formation, such as *PagSCRM*, *PagMUTE*, and *PagFAMA*, were decreased (Fig. S11, Table S5). Overexpression of *PpnMYC2* also affected the expression of stomatal differentiation-related genes *PagMYB88*, cytokinesis-related genes *PagSCD1* and *PagCYCA2;1*, MAP kinase-related genes *PagMPK6*, *PagMPK15*, *PagMPK20*, *PagMPK3*, *PagMPK4*, *PagMKK2*, *PagMKK3*, *PagMKK4*, and *PagMKK6* (Fig. S11, Table S5). The expression level of *PagPHYB* was significantly different in *PpnMYC2*-OE plants (Fig. S11, Table S5). Therefore, we detected the expression levels of these genes using RT-qPCR. The expression levels of *PagEPF2*, *PagEPFL4*, *PagTMM*, *PagERECTA*, *PagYDA*, *PagMYB88*, *PagSCD1*, *PagCYCA2;1*, *PagPHYB*, *PagMKK6*, and *PagMPK20* were significantly upregulated, and the expression levels of *PagEPFL9*, *PagMUTE* and *PagFAMA* were significantly downregulated in *PpnMYC2*-OE plants (Fig. 5d). We also found a specific correlation between stomatal development-related genes, with positive and negative correlations (Fig. S12). Therefore, we speculate that the effect of *PpnMYC2* on the stomatal density of plants is due to the influence of the above genes; however, these specific regulatory relationships require further experimental verification.

PpnMYC2* regulates stomatal density by regulating *PagEPF2*, *PagEPFL4* and *PagEPFL9

The TGMI network analysis showed that the transcript levels of *PpnEPF2*, *PpnEPFL4* and *PpnEPFL9* were correlated with *PpnMYC2* (Fig. 2a). In addition, the tissue-specific expression profiles of *PpnMYC2*, *PpnEPF2*, *PpnEPFL4*, and *PpnEPFL9* showed that the transcript levels of *PpnEPF2* and *PpnEPFL4* were positively correlated with those of *PpnMYC2*, while the transcript level of *PpnEPFL9* was negatively correlated with that of *PpnMYC2* (Fig. 2b-e). Moreover, *PagEPF2* and *PagEPFL4* were significantly upregulated and *PagEPFL9* were significantly downregulated in *PpnMYC2* overexpression plants (Fig. 5d). Since the *EPF/EPFLs* genes of the EPF family are mainly expressed in meristemoids and mesophyll cells, we observed the expression of *PpnMYC2* in poplar leaves overexpressing *PpnMYC2*, which was driven by the constitutive 35S promoter. It was found that GFP expressed by fusion of *PpnMYC2* emitted green fluorescence in mesophyll cells and lower epidermal cells of leaves, while no GFP signal was found in WT poplar (Fig. S13), indicating that *PpnMYC2* and *EPF/EPFLs* had the same expression site. Based on these findings, we hypothesized that *PpnMYC2* directly promotes the expression of *PagEPF2* and *PagEPFL4* and directly represses the expression of *PagEPFL9*. To test this, we first analyzed the cis-elements of the *PagEPF2*, *PagEPFL4*, and *PagEPFL9* promoters using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). We found a series of cis-elements involved in plant development, including light-responsive elements, such as the AE-box, AT1-motif, Box 4, G-box, and TCT-motif (Table S6). In the *PagEPFL4* promoter sequence, we also found a series of cis-elements associated in plant development, such as ACE, AT1-motif, Box 4, GATA-motif, GT1-motif, TCT-motif, and MYC-motif (Table S7). By analyzing the promoter region of *PagEPFL9*, we found that this promoter region also contains motifs that can be bound by MYC transcription factors (Table S8).

By comparing the protein sequence of *PpnMYC2* with the MYC2 of *Populus trichocarpa* and *Arabidopsis thaliana*, we found that they have the same domain in the 447-501 region, and we speculated that it had the same binding element as MYC2 in *Arabidopsis thaliana*, such as CACGTG and CATGTG (Fig. S14). After we analyzed the 2 kb proximal promoter region upstream of the *PagEPF2* translation start site in 84 K poplar, we found that the cis-element (CACGTG) of the *PpnMYC2* binding site was 785 bp upstream of the *PagEPF2* promoter. We amplified the 2 kb promoter region and approximately 150 bp flanking the motif (300 bp in total), and named them *PagEPF2p* and *PagEPF2p-1* (-785 bp site) (Fig. 6a). Additionally, we analyzed the 2 kb proximal promoter region upstream of the *PagEPFL4* translation start site, finding that the cis-element (CATGTG) of the *PpnMYC2* binding site existed at 1481 bp upstream of the *PagEPFL4* promoter. We amplified the 2 kb promoter subregions and approximately 150 bp flanking the motif (300 bp), and named them *PagEPFL4p* and *PagEPFL4p-1* (-1481 bp site) (Fig. 6a). At the same time, we analyzed the 2 kb proximal promoter region upstream of the *PagEPFL9* translation start site and found that the cis-element (CATGTG) of the *PpnMYC2* binding site existed at 1909-bp upstream of the *PagEPFL9* pro

moter. We amplified the 2 kb promoter subregions and approximately 150 bp flanking the motif (300 bp) and named these as *PagEPFL9p* and *PagEPFL9p-1* (-1909 bp site) (Fig. 6a). We then used the region from the promoter to drive the LacZ reporter gene in the yeast one-hybrid system. We found that *PpnMYC2* stably interacted with the CACGTG and CATGTG motifs in the *PagEPF2*, *PagEPFL4* and *PagEPFL9* promoter regions. Mutations of this cis-element sequence in the *PagEPF2*, *PagEPFL4* and *PagEPFL9* promoter fragments abolished *PpnMYC2* binding (Fig. 6b).

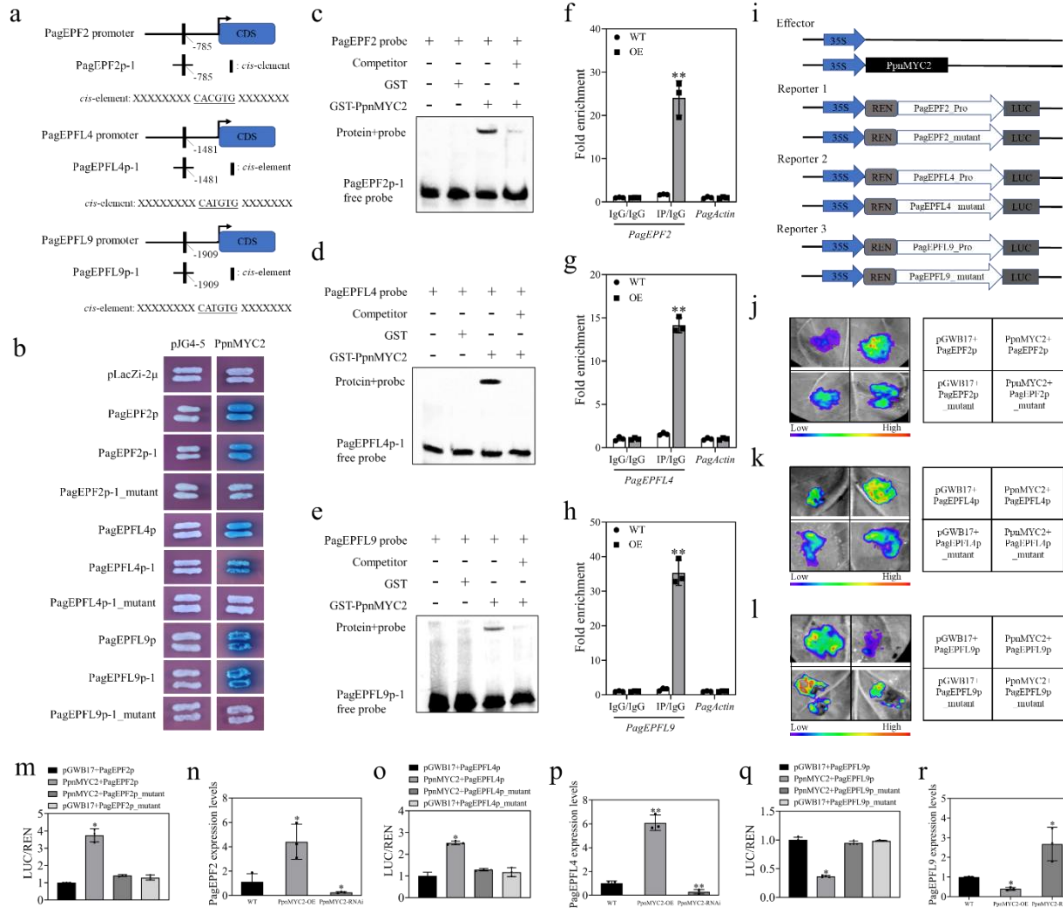


Figure 6. *PpnMYC2* binds directly to the *PagEPF2*, *PagEPFL4* and *PagEPFL9* promoters. (a) The putative *PpnMYC2* binding elements in the *PagEPF2*/*PagEPFL4*/*PagEPFL9* promoters. The absolute value of each number indicates the distance from the start codon. (b) Y1H assays showing the direct binding of *PpnMYC2* to the elements in the *PagEPF2*/*PagEPFL4*/*PagEPFL9* promoters. Blue colonies indicate a strong association of *PpnMYC2* with a specific promoter segment. (c, d, e) EMSAs showing that the GST-*PpnMYC2* recombinant protein binds to biotin-labeled probes of *PagEPF2*, *PagEPFL4*, and *PagEPFL9*. The probes were truncated from *PagEPF2*, *PagEPFL4*, and *PagEPFL9* promoters with putative binding sites (CACGTG/CATGTG). (f, g, h) ChIP-qPCR analysis. The abundance of the *PagEPF2*, *PagEPFL4*, and *PagEPFL9* promoter sequences were obtained for chromatin preparation before immunoprecipitation (Input), and was immunoprecipitated with GFP polyclonal antibody (ChIP) and IgG. *PagActin* (*Potri.019G010400*) was used as a negative control. Three independent biological replicates of the ChIP assay were performed (n=3). (i) Diagram of the carrier structure of effector and reporter. (j) Transient expression assays showing that *PpnMYC2* directly promotes the expression of *PagEPF2*. (k) Transient expression assays showing that *PpnMYC2* directly promotes the expression of *PagEPFL4*. (l) Transient expression assays showing that *PpnMYC2* directly suppresses the expression of *PagEPFL9*. Representative images of *Nicotiana benthamiana* leaves 48 h after infiltration are shown. (m) Dual-luciferase assay of *PagEPF2*:: LUC expression. The expression of REN was used as an internal control. LUC/REN ratio represents the relative activity of the *PagEPF2* promoter. (n) RT-qPCR analysis of *PagEPF2* expression. The RNA was extracted from *PpnMYC2*-OE and *PpnMYC2*-RNAi poplar leaves. (o) Dual-luciferase assay of *PagEPFL4*:: LUC expression. The expression of REN was used as an internal control. LUC/REN ratio represents the relative activity of the *PagEPFL4* promoter. (p) RT-qPCR analysis of *PagEPFL4* expression. The RNA was extracted from *PpnMYC2*-OE and *PpnMYC2*-RNAi poplar leaves. (q) Dual-luciferase assay of *PagEPFL9*:: LUC expression. The expression of REN was used as an internal control. LUC/REN ratio represents the relative activity of the *PagEPFL9* promoter. (r) RT-qPCR analysis of *PagEPFL9* expression. The RNA was extracted from *PpnMYC2*-OE and *PpnMYC2*-RNAi poplar leaves. Values shown are mean $\pm$ SD (n = 3). **: $P < 0.01$, *: $P < 0.05$ (determined by Student's *t*-test).

To verify binding further, EMSAs were performed to determine whether *PpnMYC2* could directly bind to the *PagEPF2*, *PagEPFL4*, and *PagEPFL9* promoters in vitro. As shown in Fig. 6c, d, e, *PpnMYC2* specifically bound to the promoter fragments containing normal CACGTG and CATGTG sequences. In addition, ChIP-qPCR revealed that, compared with that in the WT, the promoter fragments of *PagEPF2*, *PagEPFL4* and *PagEPFL9* in the ChIP were significantly enriched in OE leaves, and the enrichment degree was

greater than 10-fold (Fig. 6f-h). These results confirmed that *PpnMYC2* could bind to the *PagEPF2*, *PagEPFL4*, and *PagEPFL9* promoters in vivo.

Then, we examined whether *PpnMYC2* could directly regulate the transcription of *PagEPF2*, *PagEPFL4* and *PagEPFL9* using a dual-luciferase reporter assay. We constructed 35S::*PpnMYC2* effector, *PagEPF2p*::LUC, *PagEPF2p_mutant*::LUC, *PagEPFL4p*::LUC, *PagEPFL4p_mutant*::LUC, *PagEPFL9p*::LUC, *PagEPFL9p_mutant*::LUC reporter plasmids (Fig. 6i), and the *Agrobacterium* containing the above plasmids was injected into the epidermal cells of *Nicotiana benthamiana* leaves. We found that the *PagEPF2p*::LUC or *PagEPFL4p*::LUC reporter genes were activated and the *PagEPFL9p*::LUC reporter genes were inhibited in the presence of *PpnMYC2* and *PagEPF2p*, *PpnMYC2* and *PagEPFL4p*, and *PpnMYC2* and *PagEPFL9p*, but not otherwise (Fig. 6j-m, o, q). Next, we verified the correlation between the expression of *PagEPF2*, *PagEPFL4*, *PagEPFL9*, and *PpnMYC2* in transgenic lines. RT-qPCR analysis showed that the expression of *PagEPF2* and *PagEPFL4* was promoted in the leaves of *PpnMYC2-OE* but suppressed in the leaves of *PpnMYC2-RNAi* (Fig. 6n, p) and the expression of *PagEPFL9* was promoted in the leaves of *PpnMYC2-RNAi* but suppressed in the leaves of *PpnMYC2-OE* (Fig. 6r). Therefore, we demonstrated that *PpnMYC2* directly binds to the promoters of *PagEPF2*, *PagEPFL4* and *PagEPFL9*, promoting *PagEPF2* and *PagEPFL4* expression and suppressing *PagEPFL9* expression.

***PpnMYC2* enhances drought tolerance of poplars under drought stress**

To explore the role of *PpnMYC2* in drought stress response, WT, *PpnMYC2-OE* and *PpnMYC2-RNAi* poplars were placed under drought stress. On the 12th day, the leaves of *PpnMYC2-RNAi* plants were severely withered, and the leaves of WT plants also began to wilt, while the leaves of *PpnMYC2-OE* plants were still plump (Fig. 7a). After 2 days of resuming watering, the phenotypes of WT and *PpnMYC2-OE* plants recovered, while the phenotypes of *PpnMYC2-RNAi* plants did not change significantly (Fig. 7a).

There were no significant differences in the physiological indexes of WT, *PpnMYC2-OE* and *PpnMYC2-RNAi* poplars under the control condition (Fig. 7b-g). Under drought stress, the catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and ascorbate peroxidase (APX) enzyme activities of *PpnMYC2-OE* poplar were significantly higher than those of WT poplar, while the CAT, POD, SOD and APX enzyme activities of *PpnMYC2-RNAi* poplar were significantly lower than those of WT poplar (Fig. 7b-e). Meanwhile, the MDA content and H_2O_2 content of *PpnMYC2-OE* poplar were significantly lower than those of WT poplar, while these contents of *PpnMYC2-RNAi* poplar were significantly higher than those of WT poplar (Fig. 7f, g), indicating that the cell membrane of *PpnMYC2-RNAi* poplar was most seriously damaged. The data showed that under natural dehydration conditions, the survival rate of *PpnMYC2-OE* poplar was the highest, which was significantly higher than that of WT, and the survival rate of *PpnMYC2-RNAi* poplar was the lowest (Fig. 7h). And the water loss rate of *PpnMYC2-OE* poplar leaves in vitro was slower than that of WT and *PpnMYC2-RNAi* poplar leaves (Fig. 7i). Overall, under drought stress, *PpnMYC2* enhances drought resistance by reducing stomatal density.

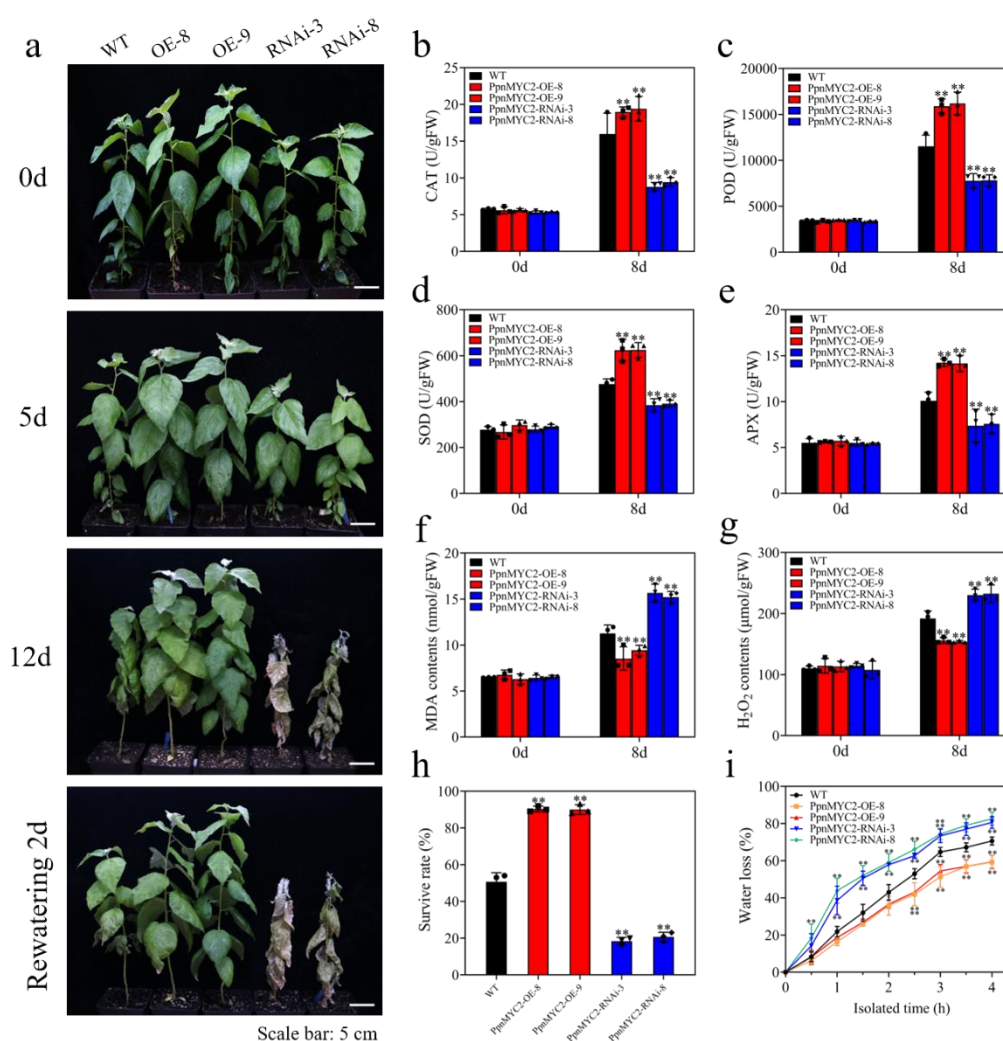


Figure 7. *PpnMYC2* enhances drought tolerance in poplars. (a) Morphological differences in drought assays. CAT (b), POD (c), SOD (d) and APX (e) enzyme activity in the leaves of WT, *PpnMYC2*-OE and *PpnMYC2*-RNAi plants under normal and drought stress conditions. MDA content (f), H₂O₂ content (g), survive rate (h) and water loss (i) in the leaves of WT, *PpnMYC2*-OE and *PpnMYC2*-RNAi plants under normal and drought stress conditions. Values are means $\pm$ SD. Asterisks denote significant differences: **, $P < 0.01$ (Student's *t*-test, WT vs transgenic lines within the same time point, $n=3$).

Discussion

Polyploid plants generally exhibit characteristics such as reduced stomatal density (Monda *et al.*, 2016; Wei *et al.*, 2019). Stomata are essential for the photosynthesis and transpiration of plants (Wang, Chen & Xi ang, 2007b; Lin *et al.*, 2022). Stomatal density affects plant growth, WUE, and drought tolerance (Tanaka *et al.*, 2013; Guo *et al.*, 2019). Studies have shown that stomatal density is affected by multiple genes of the *EPF/EPFL* family, such as *EPF2* (Hunt & Gray, 2009), *EPFL9* (Sugano *et al.*, 2010; Xia *et al.*, 2022), and *EPFL4* (Lin *et al.*, 2017). So far, their role in polyploid poplar remains unknown, and the reason for the lower stomatal density in polyploid plant leaves compared to that in diploid plant leaves remains unclear.

MYC2 interacts with JAZ and regulates plant stomatal density suppressors *EPF2*, *EPFL4* and promoter *EPFL9*

The reduction of stomatal density in polyploid leaves leads us to the following question: can we screen the upstream regulatory factors of *EPF2*, *EPFL4* and *EPFL9* through gene expression analysis during polyploid plant leaf formation? To this end, we constructed a gene network based on the TGMI algorithm using transcriptome sequencing data of polyploid poplar and found that *PpnMYC2* and *PpnEPF2/PpnEPFL4/PpnEPFL9* were highly correlated (Fig. 2a). *MYC2* belongs to the *bHLH* family and can bind to the G-box or G-box-like motifs in gene promoters (Shen *et al.*, 2016), and can interact with the JA pathway inhibitor JAZs

(Niu *et al.*, 2011). The interaction experiment between MYC2 and JAZ10, JAZ1-2, JAZ1-5, JAZ1-6 proves that there is indeed an interaction between them, which may affect the combination of MYC2 to downstream genes. The promoter regions of *PagEPF2*, *PagEPFL4*, and *PagEPFL9* were analyzed, and G-box cis-acting elements of *PpnMYC2* were found in their promoter regions (Tables S6-S8). The yeast one-hybrid, EMSAs, ChIP-qPCR and dual-luciferase assays were used to verify whether *PpnMYC2* regulates *PagEPF2*, *PagEPFL4* and *PagEPFL9*, and we found that *PpnMYC2* can directly bind to the CACGTG and CATGTG cis-acting elements in the promoter regions of *PagEPF2*, *PagEPFL4* and *PagEPFL9* and affect their expression (Fig. 6). The *MYC2* gene was able to activate the expression of its downstream target genes due to its transcriptional activation activity (Shen *et al.*, 2016; Liu *et al.* 2019; You *et al.*, 2019). Meanwhile, studies have reported that it also binds to the promoters of genes and inhibits their expression (Song *et al.*, 2014). Therefore, it was reasonable that *PpnMYC2* inhibited the expression of *PpnEPF2* and *PpnEPFL4* while increasing the expression of *PpnEPFL9* in our study. Based on these results, we generated transgenic poplar lines with *PpnMYC2* overexpression and interference. Our findings indicated a significant reduction in stomatal density in the *PpnMYC2*-OE line, whereas the *PpnMYC2*-RNAi line exhibited a significant increase in stomatal density (Fig. 4b, c). The stomatal density in *Arabidopsis* leaves overexpressing *PpnMYC2* was also significantly reduced (Fig. S8), suggesting that this regulatory factor may have general significance in plants. And the expressions of *PagEPF2* and *PagEPFL4* were significantly upregulated and the expression of *PagEPFL9* was significantly downregulated in *PpnMYC2*-OE poplar (Fig. 5d). In summary, we demonstrated that *MYC2* is a transcription factor that regulates the stomatal density suppressors *EPF2* and *EPFL4* and the stomatal density promoting factor *EPFL9*, and can interact with JAZ10, JAZ1-2, JAZ1-5, JAZ1-6 (Fig. 8a).

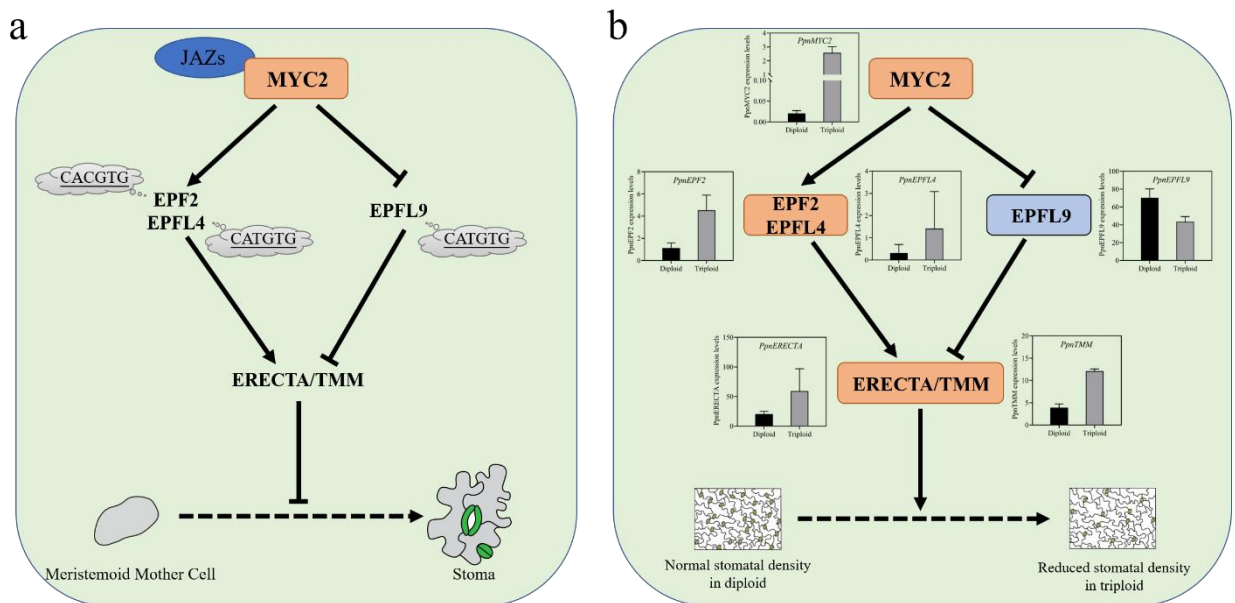


Figure 8. MYC2 regulates stomatal density patterns in plants. (a) MYC2 interacts with JAZ protein and promotes the expression of *EPF2* or *EPFL4* and suppresses the expression of *EPFL9*. *EPF2*, *EPFL4* and *EPFL9* can bind to the receptors TMM or ERECTA and promote or suppress their expression, ultimately reducing stomatal density. (b) A regulatory model for decreased stomatal density in polyploid plant leaves. Orange indicates genes that are upregulated during stomatal formation in polyploid plants, and blue indicates genes that are downregulated during stomatal formation in polyploid plants. The histogram next to the gene indicates the FPKM value of the gene in diploid and triploid poplars. Solid arrows represent facilitation, solid lines without arrows represent inhibition.

Studies have shown that the pathway of *EPF2* regulating stomatal density includes receptor kinases ERECTA and TMM (Hunt & Gray, 2009), and *EPF2* is sensed by a receptor complex composed of receptor-like proteins TMM and ERECTA, ERL1, and ERL2. These receptors activate mitogen-activated protein (MAP) kinase modules, including MAPKKK YODA (YDA), which is required for the regulation of stomatal development (Bergmann, Lukowitz & Somerville, 2004; Wang *et al.*, 2007a). MAP kinase phosphorylates and disrupts the expression of the transcription factor *SPCH*, affecting stomatal density during leaf development (Hunt & Gray, 2009; Jewaria *et al.*, 2013). *EPFL9* competes with *EPF2* for binding to ERECTA/TMM receptor

s, inhibiting receptor function, and promoting stomatal development (Hunt, Bailey & Gray, 2010; Pillitteri & Torii, 2012). *EPFL4* and *EPFL6* can bind to *ERECTA*, affecting downstream MAP kinase phosphorylation and stomatal density (Abrash *et al.*, 2011; Lin *et al.*, 2017; Jiao *et al.*, 2022). Through RNA-sequencing and RT-qPCR detection of *PpnMYC2*-OE and WT plants, we found that *PagTMM*, *PagERECTA*, and other genes were significantly upregulated (Fig. 5d); thus, *PpnMYC2* may promote the expression of the stomatal development-related genes *PagTMM* and *PagERECTA* by promoting the expression of *PagEPF2* and *PagEPFL4* and suppressing the expression of *PagEPFL9*, thereby affecting stomatal density in plants (Fig. 8a).

Transcriptional regulation mechanism of decreased stomatal density in plant polyploid leaves

Numerous studies have shown that polyploid leaves have larger stomata and a significantly lower stomatal density (Hetherington & Woodward, 2003; Oliveira *et al.*, 2004; Padoan *et al.*, 2013; Monda *et al.*, 2016). For example, the stomatal density of tetraploid *Arabidopsis* and rice is significantly lower than that of diploid plants (Monda *et al.*, 2016; Xiong *et al.*, 2022). The stomatal density of tetraploid poplar, eucalyptus, and triploid eucalyptus was also significantly lower than that of diploids (Lu *et al.*, 2014; Yang *et al.*, 2018; Ren, Jing & Kang, 2021; Liu *et al.*, 2022). By observing and comparing the stomata of triploid and diploid poplar leaves, we found that the stomata of triploid poplar enlarged and the stomatal density decreased (Fig. 1d-g). Since we have shown that *MYC2* is a regulator of *EPF2*, *EPFL4* and *EPFL9*, it is suggested that the expression changes of related genes may be a fundamental reason for the occurrence and density changes of stomata in triploid poplars. In *Arabidopsis*, *MYC2*, *MYC3*, and *MYC4* genes have functional redundancy (Han *et al.*, 2018b), but we found that *MYC3* and *MYC4* do not exist in poplar, which may be the difference between *Arabidopsis* and poplar. Differential analysis of gene expression in apical buds and young leaves of triploid poplars revealed that the expression levels of *PpnMYC2*, *PpnEPF2*, and *PpnEPFL4* were significantly higher than those in diploid poplars, while expression of *PpnEPFL9* was significantly lower than that in diploid poplars (Figs. S3, 2b-e). Meanwhile, the expression levels of *PpnTMM* and *PpnERECTA* in triploid poplars were higher than those in diploid poplars (Fig. 8b), and both were expressed in young tissues. The significantly upregulated expression of *MYC2* and *EPF2/EPFL4* and the downregulated expression of *EPFL9* during stomatal formation are some of the reasons for the decreased stomatal density in triploid poplars (Fig. 8b).

***MYC2* affects WUE by regulating plant leaf stomatal density**

WUE is a quantitative measure of the biomass produced per volume of water evaporated by plants, which is an essential physiological characteristic for plants to effectively use water resources to fix carbon dioxide to maintain growth and development (Romero-Romero *et al.*, 2018). Plant WUE is determined by photosynthesis and transpiration (Wang, Chen & Xiang, 2007b). Stomatal density affects plant transpiration and WUE (Wang, Chen & Xiang, 2007b; Bertolino, Caine & Gray, 2019), and stomatal density regulation genes can regulate plant WUE. For example, *Arabidopsis thaliana* *GTL1* regulates stomatal density by inhibiting *SD* *DI*, affecting WUE (Yoo *et al.*, 2010). The transcription factor *ZmNAC49* represses the expression of *ZmMUTE*, reduces stomatal density and transpiration rate, and improves WUE in maize (Xiang *et al.*, 2021). *SITLF* *P8* regulates stomatal density to reduce water loss and improve WUE (Li *et al.*, 2020). Overexpression of *EPF1* in both poplar and rice produces plants with significantly reduced stomatal density, leading to improved WUE (Wang *et al.*, 2016; Caine *et al.*, 2019). Triploid poplars have a higher WUE because of their lower stomatal density and higher photosynthetic capacity, the WUE of triploid poplar is 10%-15% higher than that of diploid (Liao *et al.*, 2018). In this study, the photosynthetic parameters of transgenic and WT plants were measured, and it was found that *PpnMYC2*-OE lines showed significantly lower stomatal conductance and transpiration, whereas assimilation was significantly higher. This may be related to the fact that it has higher rubisco enzyme activity and chlorophyll content. We repeated the assay three times in the same environment and obtained similar results (Fig. 4). And the water loss rate of *PpnMYC2*-RNAi plants was significantly higher than that of WT and *PpnMYC2*-OE plants (Fig. 7i), which coincided with the changes in WUE. Meanwhile, we found that the drought resistance of *PpnMYC2*-OE plants after drought stress treatment were

significantly higher than those of WT, while that of *PpnMYC2-RNAi* lines was significantly lower than that of WT (Fig. 7). This indicates that *MYC2* likely contributes to WUE by regulating stomatal density, which may be one of the reasons for the increase of WUE in triploid poplar.

It has been reported that the interaction of JAZ and *MYC2* may promote its response to drought stress (Song et al., 2022). On the one hand, the reason was related to the increase of certain compounds, such as POD, SOD and CAT, thereby improving the drought resistance of plants. On the other hand, studies have shown that *MYC2* silencing expression could inhibit the closing of stomata and reduce drought tolerance (Chen et al., 2020; Xu et al., 2022). Therefore, in poplar, overexpression of *MYC2* may not only reduce stomatal density, but may also promote stomatal closure. Meanwhile, *MYC2* was constitutively expressed driven by the 35S promoter in our study, so we hypothesized that this gene might affect WUE and drought tolerance of plants not only by affecting the stomatal density of leaves, but also by influencing phenotypic changes in other tissues of the plant (e.g., roots). In this study, through correlation analysis and expression detection of *MYC2*, *EPF2*, *EPFL4* and *EPFL9*, and further verification of the regulatory relationship between them, it was proven that *MYC2* could directly promote the expressions of *EPF2* and *EPFL4* and suppress the expression of *EPFL9*, and affect the stomatal density and WUE of plants in combination with TMM or ERECTA receptors. Our study demonstrates another new function of *MYC2* as a regulator of stomatal density, revealing the reason for the decrease in stomatal density in polyploid plant leaves. This study proposes a new direction for studying the function of *MYC2*, provides another target gene for plant molecular design breeding, and highlights new strategies for water-saving plant breeding.

Acknowledgements

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Competing interests

The authors declare no conflict of interest.

Author contributions

YX performed most experiments and data analysis, and XK guided the overall research. SJ and WW helped with the transgenic experiment and protein–DNA interaction experiments. KD generated the triploid poplars and performed RNA-seq on triploids. YX and XK wrote the manuscript.

Data availability

All data are presented in the manuscript. Raw RNA-sequencing data generated in this study have been submitted to the CNCB (<https://ngdc.cnbc.ac.cn/gsa/>) with the accession no. PRJCA018931.

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