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Research Article

Maize GOLDEN2-LIKE proteins enhance drought tolerance in rice by promoting stomatal closure

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Abstract

Drought has become one of the most severe abiotic stresses experienced in agricultural production across the world. Plants respond to water deficit via stomatal movements in the leaves, which are mainly regulated by abscisic acid (ABA). A previous study from our lab showed that constitutive expression of maize (*Zea mays* L.) GOLDEN2-LIKE (GLK) transcription factors in rice (*Oryza sativa* L.) can improve stomatal conductance and plant photosynthetic capacity under field conditions. In the present study, we uncovered a function of ZmGLK regulation of stomatal movement in rice during drought stress. We found that elevated drought tolerance in rice plants overexpressing *ZmGLK1* or *GOLDEN2* (*ZmG2*) was conferred by rapid ABA-mediated stomatal closure. Comparative analysis of RNA-sequencing (RNA-seq) data from the rice leaves and DNA affinity purification sequencing (DAP-seq) results obtained in vitro revealed that ZmGLKs played roles in regulating ABA-related and stress-responsive pathways. Four upregulated genes closely functioning in abiotic stress tolerance with strong binding peaks in the DAP-seq data were identified as putative target genes of ZmGLK1 and ZmG2 in rice. These results demonstrated that maize GLKs play an important role in regulating stomatal movements to coordinate photosynthesis and stress tolerance. This trait is a valuable target for breeding drought-tolerant crop plants without compromising photosynthetic capacity.

Introduction

Global crop production must be approximately doubled by 2050 to meet the demands of the increasing human population (FAO 2009; Tilman et al. 2011). However, yield improvement has stagnated in recent years and is clearly projected to fall short of the expected demand (Ray et al. 2013). Yield stagnation in major crops is caused by a combination of factors including climate change, soil erosion, and cultivar restriction (Ray et al. 2012). Drought is one of the most severe natural hazards in agricultural production; it has affected large agricultural areas and been exacerbated worldwide over the last 50 yr (FAO 2021). Rice (*Oryza sativa* L.) serves as a staple food for nearly half of the world's population, but it is a high water consuming crop and is particularly susceptible to drought. Over 50% of the world's rice production is estimated to be affected by drought stress (Ambavaram et al. 2014). Development of low-water consuming and drought-tolerant rice varieties is urgently needed to meet global food demand under changing climatic conditions (Todaka et al. 2012).

Stomata are the main channels for the gas exchange between plant and the atmosphere. When plants suffer from water

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deficit or ge exposed to other environmental stimuli e. lo light intensity, lo air humidity, high CO, levels, and p hoto ynthesis, stoma ta re rapidly closed, especially in ang iosperms Sierl et al. 2018. This dynamic movement is driven by turg or pressure ch anges in the guard cells, as a result of the activation of anion ch annels and the inhibition of inward rectifying K^+ ch annels, which encoded by *K+ CHANNEL IN ARABIDOPSIS THALIANA KAT* and *ARABIDOPSIS K+ TRANSPORTER AKT* genes. im et al. 2010. The efflux of anions and small metabolites, including Cl^-, NO_3^-, and malate, causes membrane depolarization to activate the outward rectifying K^+ channel and facilitates K^+ efflux, further reducing turg or pressure inside the guard cells and leading to the stoma tal closure Priday et al. 2007. Under ge de ficit conditions, the phytohormone abscisic acid B plays a the primary regulator of stoma tal movement to prevent ge loss, in which endogenous B levels are controlled by precise balance between biosynthesis and c atabolism, which also influenced by transport and conjugation process. ushiro et al. 200; Hsu et al. 201. B is initially synthesized from C_0 c arotenoids to form x anthophylls e. 9 *cis* violax anthin and 9 *cis* neox anthin; a C_15 intermediate, x anthoxin, is formed in the plastids via oxidative cleavage catalyzed by 9 *cis* epoxyc arotenoid dioxygenase NCED. . anthoxin is then exported to the cytosol and converted to B through a 2- step reaction via short-ch ain dehydrogenase/reductase 1 SDR1/ B2 and ar abidopsis aldehyde oxidase 3 O3 Seo and oshib 200; ion and Zhu 2003.

Transcription factors TFs are crucial regulators of many biological processes, including responses to environmental signals and hormone regulation. These regulatory functions are accomplished through binding to specific *cis* elements in the promoter regions of target genes Tod a et al. 2012. Numerous biotic stress responsive TFs have been identified in plants; for instance, WR. , M. B, and DREB/CBF TFs have all been reported as key regulators of plant stress responses M an et al. 201. GOLDEN- L. E GL TFs, generally act as transcriptional activators of chloroplast development and biogenesis Rossini et al. 2001; W ang et al. 2013 and play important roles in regulating nuclear photosynthesis related genes Chen et al. 2016. . n maize *Zea mays* L., two *GLK* genes, *ZmGLK1* and *GOLDEN2 ZmG2*, have shown differential expression patterns between mesophyll cells and the bundle sheath H all et al. 1998; Ch ang et al. 2012. Ectopic overexpression of maize *GLK* genes in rice induces chloroplast development in bundle sheath cells and activities in ar cellular plasmodesmata connections, considering the key step in forming intermediate proto ar anatomy in the transition from C_3 to C photosynthesis W ang et al. 2017. previous study from our lab showed that constitutive *ZmGLK* expression in rice leads to increased x anthophyll content and further mitigates the photoinhibition under high light conditions, resulting in enhanced photosynthetic capacity with higher stoma tal conductance and improved biomass and grain yield in the field Li et al. 2020. Moreover, GL s also function in biotic stress responses hm ad et al. 2019 and p hoto protection Murmu et al. 2014; for example, GL s affect stomatal movement in ar abidopsis *Arabidopsis thaliana* when exposed to o one N ak ashoji et al. 2016.

In this study, we uncovered the dual function of maize GL s, and that ectopic overexpression of *ZmGLK1* and *ZmG2* in rice conferred improved drought tolerance by promoting stomatal closure in response to ge de ficit, while maintaining high stomatal conductance to obtain efficient photosynthesis when sufficient ge ar available. We further showed that ar bidopsis stomatal movement is mediated by B - involved path ay under drought conditions. These results suggest that *GLK* genes may be promising candidates for breeding rice varieties with high stomatal flexibility and sustainable yield, which could strongly improve agricultural production and increase food security in the context of climate change.

Results

Z GL 1 and Z G conferred improved drought tolerance

In our previous study, ectopic ro n ur ais expression of maize lines constitutively expressing *ZmGLK1* or *ZmG2* driven by the maize *Ubiquitin ZmUBI* promoter performed improved photosynthesis rates and higher stomatal conductance Li et al. 2020. We further explored the stomatal responses of ur ais ectopic rice plants to ge de ficit with pot experiments in the greenhouse. Surprisingly, ur ais ectopic rice plants exhibited strongly enhanced drought tolerance than wild-type WT plants after recovery from a 10- drought treatment Fig. 1. Specifically, the survival rates of *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* plants were 530% to 60% after the 6-d recovery period, which were significantly higher than that of the WT 1 .3%; Fig. 1B. Moreover, the relative water content RWC in the leaves of WT and ur ais ectopic plants remained from 9 .7% to 95.3% before drought but decreased to 73.1% in the WT after ge ar withheld for 7 d. . n comparison, *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* plants maintained relatively high RWC, especially *ZmUBI*$_{pro}$*:ZmG2*, ranging from 86% to 90.9%. After 10 d of drought stress, the RWC values of WT and *ZmUBI*$_{pro}$*:ZmGLK1* plants decreased to 11.6% to 1 .9%, which were significantly lower than those of *ZmUBI*$_{pro}$*:ZmG2* plants 1 .5% to 18.6%; Fig. 1C. These results indicated ZmGL 1 and ZmG both conferred higher capacities for water conservation and thus drought tolerance.

We next tested the ir ro ot performance of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants to PEG induced osmotic stress as a drought simulation. After ir ro ots in 20% PEG 6000 for 10 d, *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants showed less wilting and chlorosis compared to the WT Supplemental Fig. S1. The maximum quantum efficiency of photosystem . PS.; F_v/F_m as measured as an important indicator of plant physiological status under stress conditions, and the

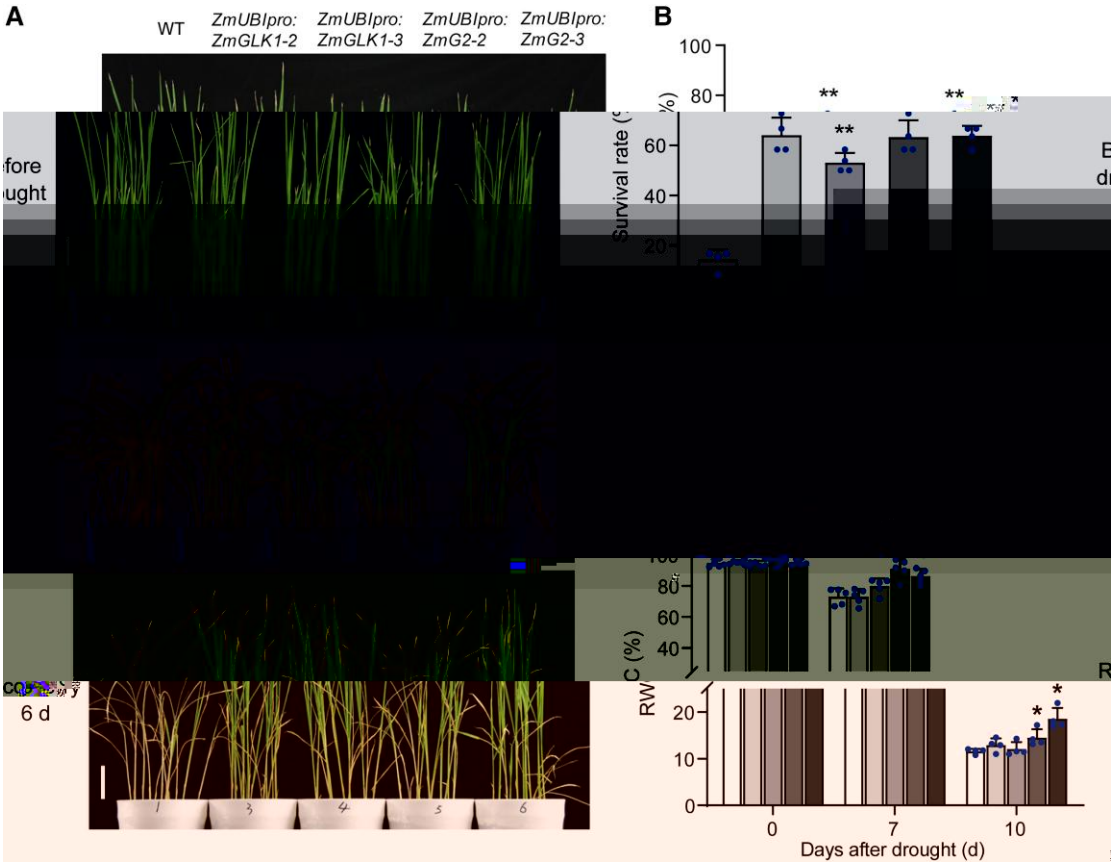


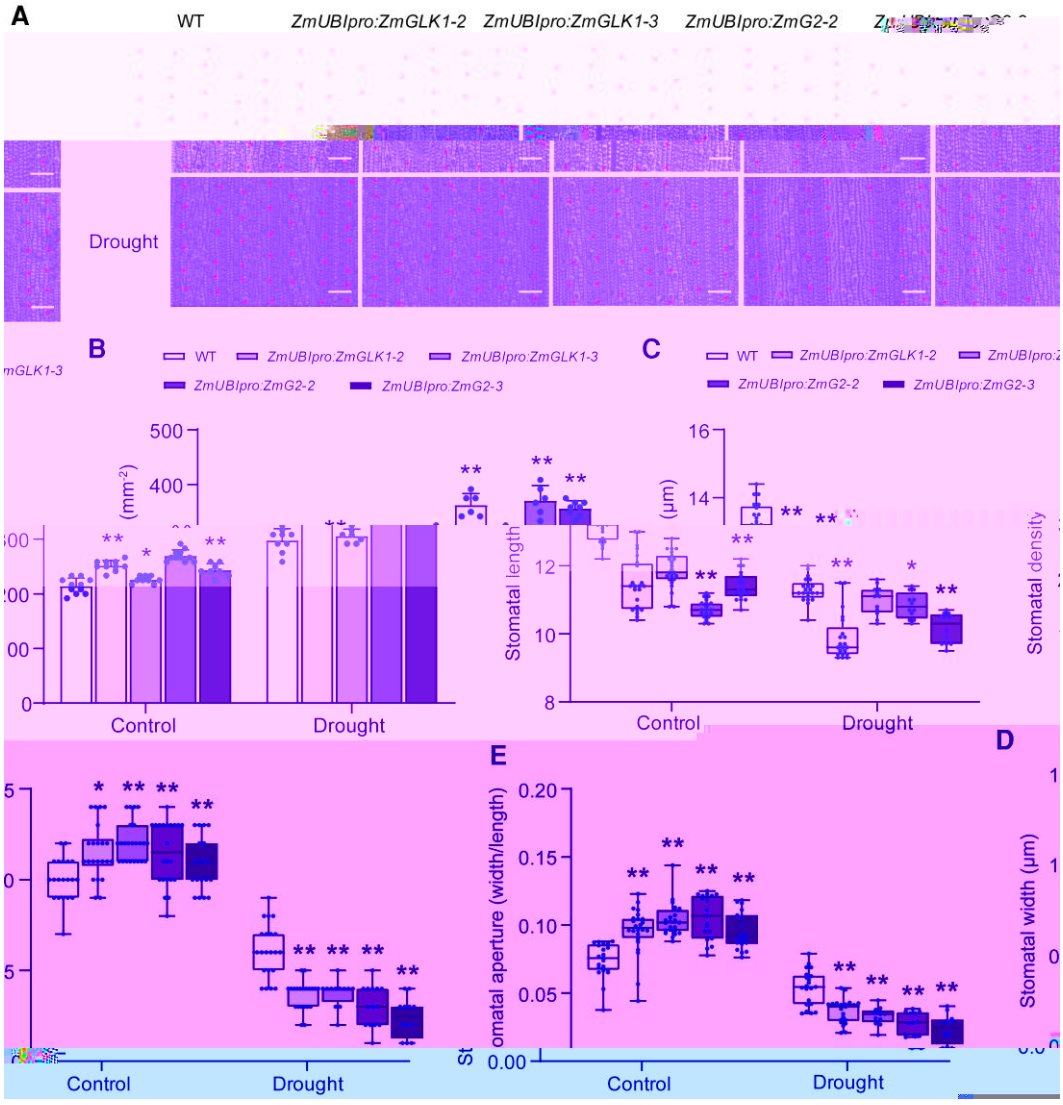
Figure 1. Overexpression of *ZmGLK1* and *ZmG2* in rice increases drought tolerance. **A)** Phenotypes of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants during drought stress. Three week old WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice seedlings grown in soil were drought stressed by withholding water for 10 d and then allowed for a 6 d recovery period. The upper, middle, and lower panels show representative plants before drought stress, after 10 d of drought stress, and after the 6 d recovery, respectively. Scale bars: 3 cm. **B)** Survival rates of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants after 10 d of drought stress followed by 6 d of recovery. Data are presented as the mean ± SD from biological replicates. **C)** The RWC of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice leaves after 0, 7, and 10 d of drought stress. Data are presented as the mean ± SD

10 d of PEG treatment (Supplemental Fig. S1B). We also monitored changes of RWC in rice seedlings during PEG treatment. The results showed that transgenic plants retained significantly higher RWC compared to the WT. Specifically, RWC values were 11.% to 12.1% and 9.5% to 9.7% higher in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants, respectively, compared with the WT (Supplemental Fig. S1C). These results together indicated that overexpression of *ZmGLK1* and *ZmG2* in rice significantly improve the tolerance to drought and osmotic stress.

ZmGLK1 and ZmG2 decrease stomatal conductance and increase water use efficiency

To further investigate the physiological mechanism underlying the elevated drought tolerance conferred by ZmGLK1 and ZmG2, we evaluated the effects of drought treatment on stomatal traits of rice seedlings grown in the pots in the greenhouse, since stomatal aperture changes are mainly responsible for gas exchange and water respiration in plants, serving as the dominant limitation to photosynthesis under drought. We therefore first measured stomatal conductance and photosynthetic related parameters under control conditions using a LI-COR 6400. Portable photosynthesis system. The results revealed significantly higher stomatal conductance in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice seedlings (0.118–0.139 and 0.126–0.131, respectively) compared with the WT (0.083) under control condition; while the transgenic plants also performed higher photosynthesis rates, intercellular CO_2 concentrations Ci, and transpiration rates (Supplemental Fig. S2A, B, C, and D). In contrast, after 7 d of drought treatment, *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants displayed sharply decrease in stomatal conductance (0.066–0.073 and 0.045–0.059, respectively), where as that of WT remained relatively stable under drought conditions (0.087; Supplemental Fig. S2B). The photosynthesis rates, Ci, and transpiration rates showed corresponding declines in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants during water deprivation (Supplemental Fig. S2A, C, and D).

We next compared the stomatal traits between WT and *ZmUBI*$_{pro}$*:ZmGLK1* or *ZmUBI*$_{pro}$*:ZmG2* rice plants under



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both control and drought conditions. Transgenic plants presented higher stomatal density in the leaves but had significantly shorter stomata compared to the WT regardless of conditions Fig. 2A, to C. Importantly, the stomata were prominently wider in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice leaves compared to the WT under control conditions Fig. 2D, where under drought stress, the stomatal widths were significantly decreased in transgenic plants to a lower level than WT, consistent with the stomatal aperture Fig. 2E.

Considering the relative low light intensity in the growth chamber could lead to the stomatal closure, we further conducted pot experiments in the greenhouse with natural light to exclude the influence of low light. As expected, the results showed consistency with the chamber experiments Fig. 1. All plants were severely impaired due to the rapid loss of water, during the 10 d drought duration Supplement Fig. S3; Fig. 3. After rewatering for 7 d, we observed the higher survival rate in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants Fig. 3B, as well as the significantly higher RWC of leaves than WT either during the drought or the recovery stage Fig. 3C. Moreover, we monitored the dynamics of photosynthesis rate and stomatal conductance throughout the duration of drought, and the *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants performed higher photosynthesis rate and stomatal conductance under sufficient water condition. Nevertheless, the photosynthesis rate and stomatal conductance of all plants were greatly declined as the

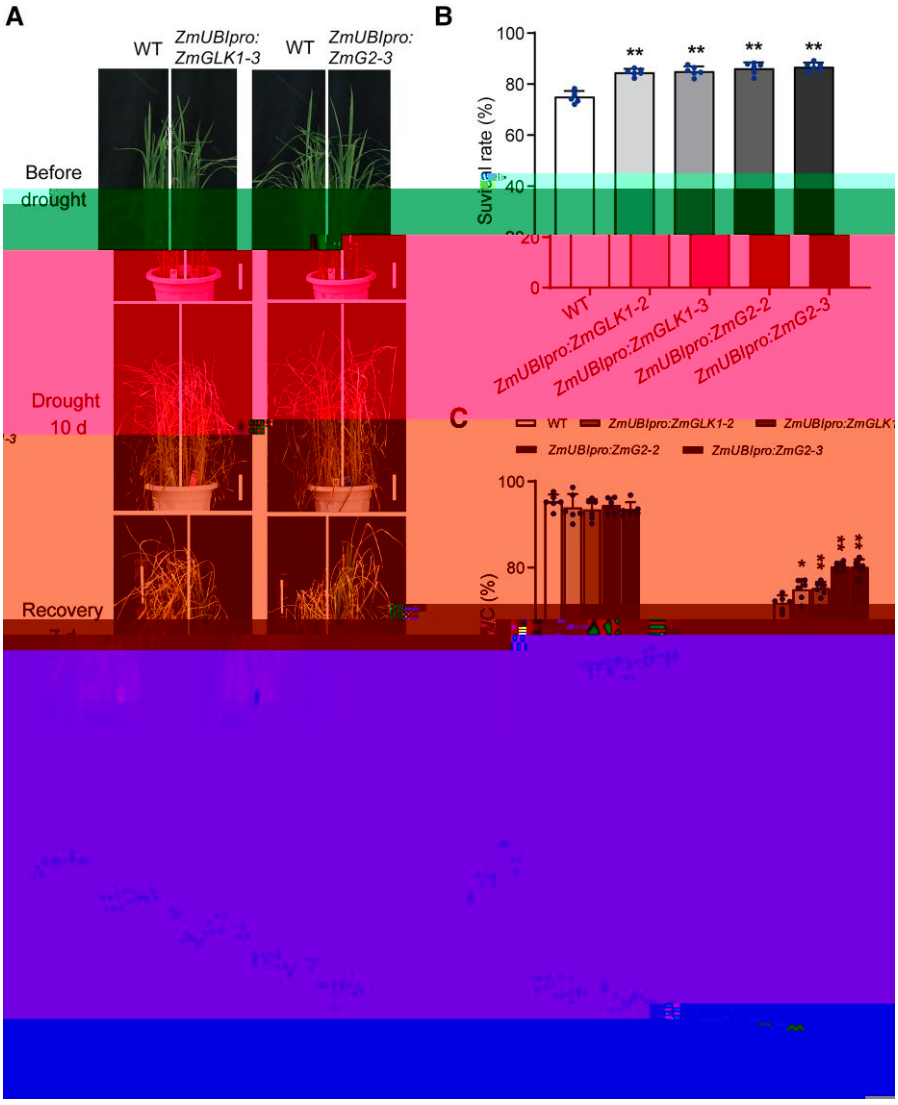

Figure 3. ZmGLK1 confers reduced stomatal closure to prevent water loss in rice during drought. **A)** Phenotypes of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants during drought stress. Sixty-day-old WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants grown in soil in the greenhouse (the natural light intensity was ~1,000 μmol m^{-2} s^{-1}) were drought stressed by withholding water for 10 d and then rewatered for a 7-d recovery period. The upper, middle, and lower panels show representative plants before drought stress, after 10 d of drought stress, and after the 7-d recovery, respectively. Scale bar = 10 cm. **B)** Survival rates of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants after 10 d of drought stress followed by 7 d of recovery. **C)** The RWC of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice leaves after 0 and 7 d of drought stress and after 7 d of recovery. **D, E)** Dynamic changes of photosynthesis rate **D)** and stomatal conductance **E)** of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants during the drought stress. Data are presented as the mean ± SD from 3 to 6 biological replicates. *$P < 0.05$, **$P < 0.01$ Student's *t* test.

drought deepened, of which *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants presented lower photosynthesis rate and the stomatal conductance compared to the WT (Fig. 3, D and E). These results together clearly indicated that the rapid stomatal closure is triggered by greater deficiency in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants, further contributing to the elevated drought tolerance.

Reduction of hydrogen peroxide and ABA enabled *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* accumulation

During the drought stress, ABA is the pivotal phytohormone that regulates stomatal movement to respond drought (Chen et al. 2020). To further dissect the underlying mechanism associated with stomatal movement induced by *ZmGLK1* and *ZmG2*, we treated rice plants with ABA to clarify whether the rapid stomatal closure is ABA induced. After 0.5 h of applying 100 μM ABA, *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants showed strongly decreased photosynthesis rates, accompanied with the reduced stomatal conductance (Fig. , and B). Accordingly, the Ci and transpiration rate were significantly lower in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* rice plants compared with the WT after ABA application (Fig. , C and D). The effects of exogenous ABA application on photosynthetic traits and stomatal conductance in

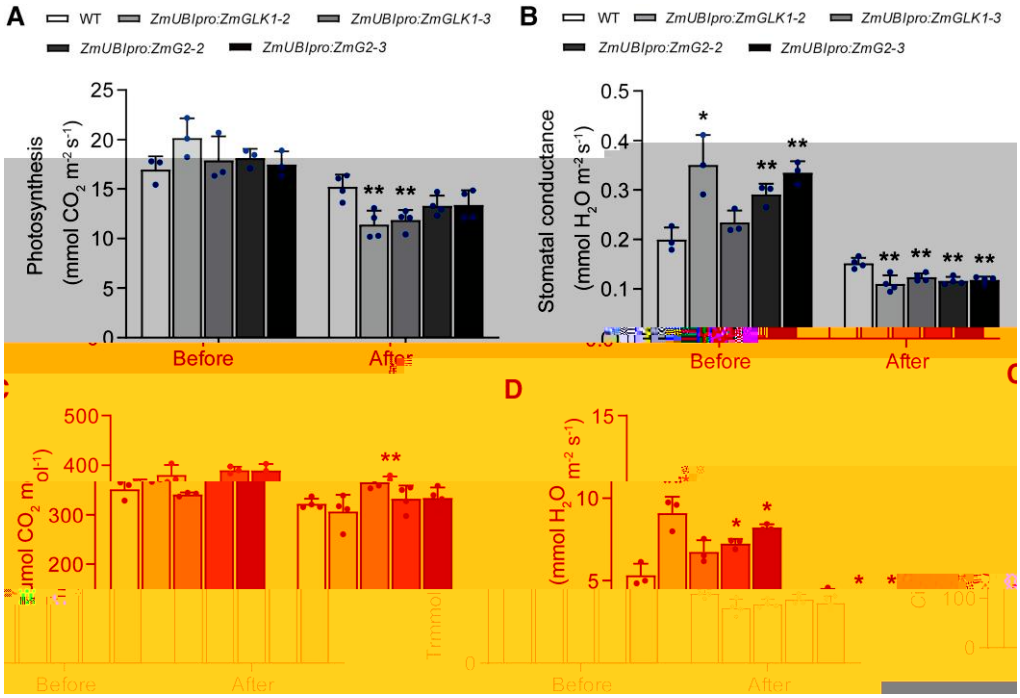

Figure 4. Exogenous B application reduced the photosynthesis rate and stomatal conductance in rice plants overexpressing *ZmGLK1* or *ZmG2* compared to the WT. **A)** Photosynthesis rates, **B)** stomatal conductance, **C)** Ci, and **D)** transpiration rates of 3 -week-old WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants grown in soil before and 5 h after B treatment. Data are shown as the mean ± SD from 3 biological replicates. *$P < 0.05$, **$P < 0.01$ Student's *t* test.

the WT and transgenic plants mimicked the results obtained from the drought stress treatments, which indicated the regulation of rapid stomatal closure in response to water deficit stress conferred by ZmGLK1 and ZmG2 as B mediated.

ZmGLK1 and ZmG2 regulate expression of genes responsible for stomatal movement

To further understand the molecular mechanisms regulated by ZmGLK1 under drought stress, we next compared the expression levels of several key genes associated with stomatal movement in WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants under control and drought stress conditions. Under control conditions, several key genes were highly expressed in the transgenic plants compared with the WT but profoundly down regulated in response to drought stress. These comprised genes encoding proteins associated with inward rectifying shaker like potassium channels 3 *OsKATs* and 1 *OsAKT1* gene, 1 H$^+$-ATPase *OsAHA7*, and several stress responsive genes including *OsbZIP23*, *OsP5CS1*, and *OsLEA3*; Fig. 5. These results demonstrated that ZmGLK1 and ZmG2 improved drought tolerance by down regulating genes involved in stomatal movement when suffering from water deficit.

Genome wide transcriptomic analysis was also conducted in WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants after 3 h after B treatment to investigate the early global effects of ZmGLK1 and ZmG2 introduced by B, especially on stomatal movement. WT plants clearly showed distinct expression patterns compared with *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* plants, as demonstrated by the clear separation with principal component analysis PC ; Fig. 6. Specifically, after B treatment, 7 and 775 genes were significantly upregulated in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* plants, respectively, compared with the WT, of which 8 genes were upregulated in both transgenic lines Fig. 6B. Gene Ontology GO term enrichment analysis revealed that the upregulated differentially expressed genes DEGs in *ZmUBI*$_{pro}$*:ZmGLK1* and *ZmUBI*$_{pro}$*:ZmG2* plants functioned in multiple biological processes but primarily in the B and water deprivation pathways Fig. 6, C and D. Next, we performed DNA affinity purification sequencing DAP seq analysis to identify genes directly regulated by the ZmGLK1 TFs. This analysis revealed 6,601 and 6,565 putative binding sites of ZmGLK1 and ZmG2 in the rice genome, respectively, with more than half of the identified sites being bound by both ZmGLK1 and ZmG2 Supplemental Fig. S . Of the 3,835 binding sites shared by ZmGLK1 and ZmG2, 17. % were localized to promoters, 8.59% to exons, and 52 6% to intergenic regions Supplemental Fig. S B. Motif analysis demonstrated that the most enriched core motifs found in the ZmGLK1 and ZmG2-binding regions were GCCTCT and G TTCT Supplemental Fig. S , C and D. Fifty nine genes identified from the DAP seq data as potential targets of ZmGLK1 and ZmG2 in rice were also identified from the RNA-sequencing RNA-seq data as differentially expressed

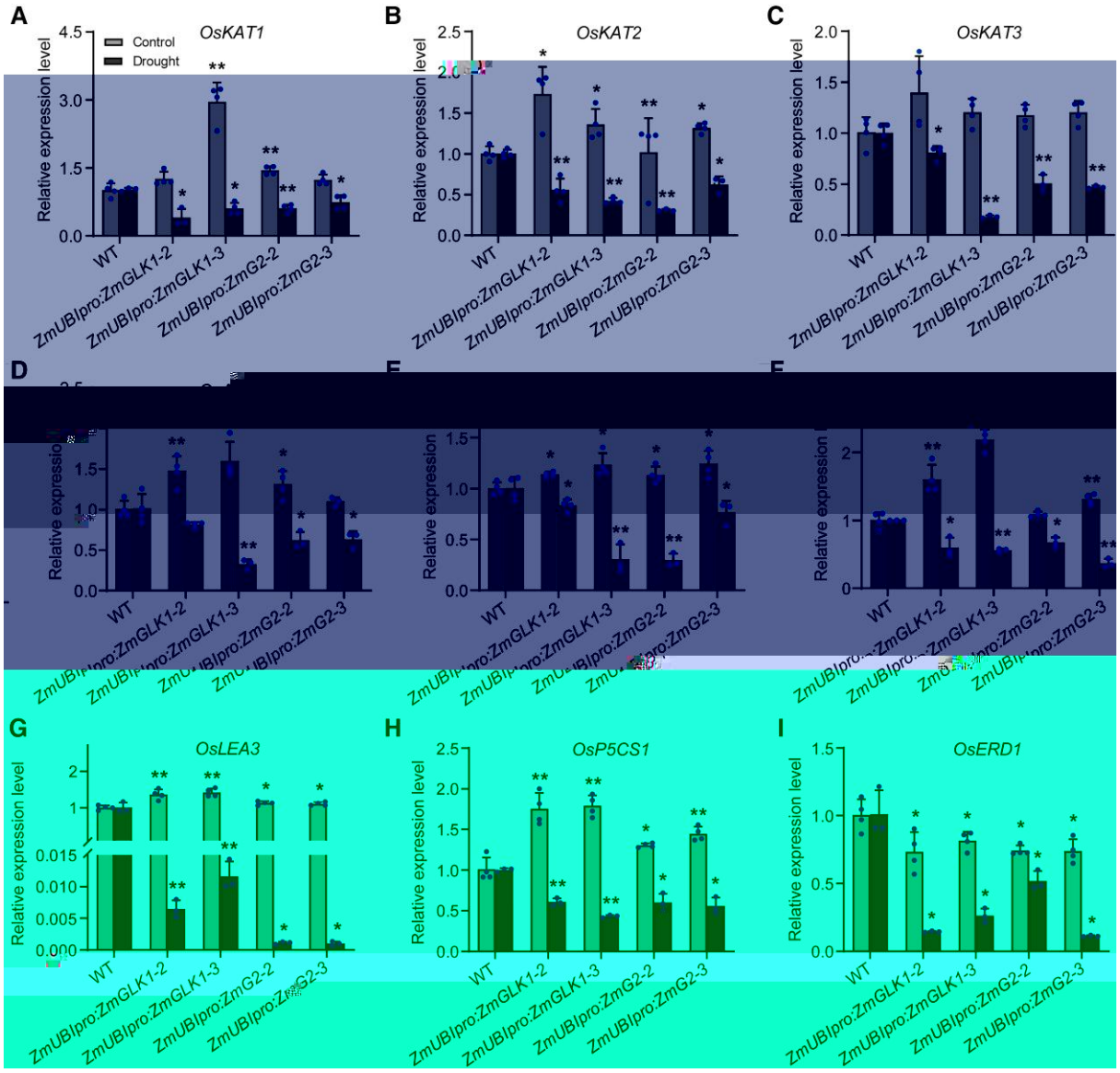

Figure 5. Relative expression levels of genes involved in stomatal movement and stomatal aperture in WT, *ZmUBI$_{pro}$:ZmGLK1*, and *ZmUBI$_{pro}$:ZmG2* rice under normal conditions and after 7 d of drought stress. Expression levels of **A)** *OsKAT1*, **B)** *OsKAT2*, **C)** *OsKAT3*, **D)** *OsAKT1*, **E)** *OsAHA7*, **F)** *OsZIP23*, **G)** *OsLEA3*, **H)** *OsP5CS1*, and **I)** *OsERD1*. Gene expression levels were measured by RT-qPCR in the leaves of 3-week-old rice plants grown in soil under normal conditions or drought stress for 7 d. Data are presented as the mean ± SD from 3 biological replicates. *$P < 0.05$, **$P < 0.01$ Student's *t* test.

in plants overexpressing *ZmGLK1* or *ZmG2* (Fig. 6B; Supplemental Table S1). We noticed that upregulated DEGs were annotated to biotic stress tolerance and showed strong binding peaks in the DAP-seq analysis simultaneously. Therefore, these genes were identified as putative target genes of ZmGLK1 and ZmG2 in rice, including rice genes *Filamentation Temperature Sensitive Protein H6 OsFtsH6*, *Cytochrome P450 Family 714 B1 OsCYP714B1*, *Red Chlorophyll Catabolite Reductase 1 OsRCCR1*, and *Subtilisin-like Protease 57 OsSub57* (Fig. 7, A to D). The gene expression from RNA-seq data of these genes is prominently higher in *ZmUBI$_{pro}$:ZmGLK1* and *ZmUBI$_{pro}$:ZmG2* rice plants (Fig. 7, E to H). Further reverse transcription quantitative PCR (RT-qPCR) analysis verified that these genes were highly induced in *ZmUBI$_{pro}$:ZmGLK1* and *ZmUBI$_{pro}$:ZmG2* rice under drought stress conditions (Fig. 7, I to L). These putative target genes may contribute to enhanced drought tolerance by enabling rapid stomatal movement when suffering from water deficit.

Discussion

GLK TFs have long been regarded as some of the most important regulators of chloroplast biogenesis and photosynthetic organelle formation; they have been identified in Arabidopsis, tomato *Solanum lycopersicum* L., and maize

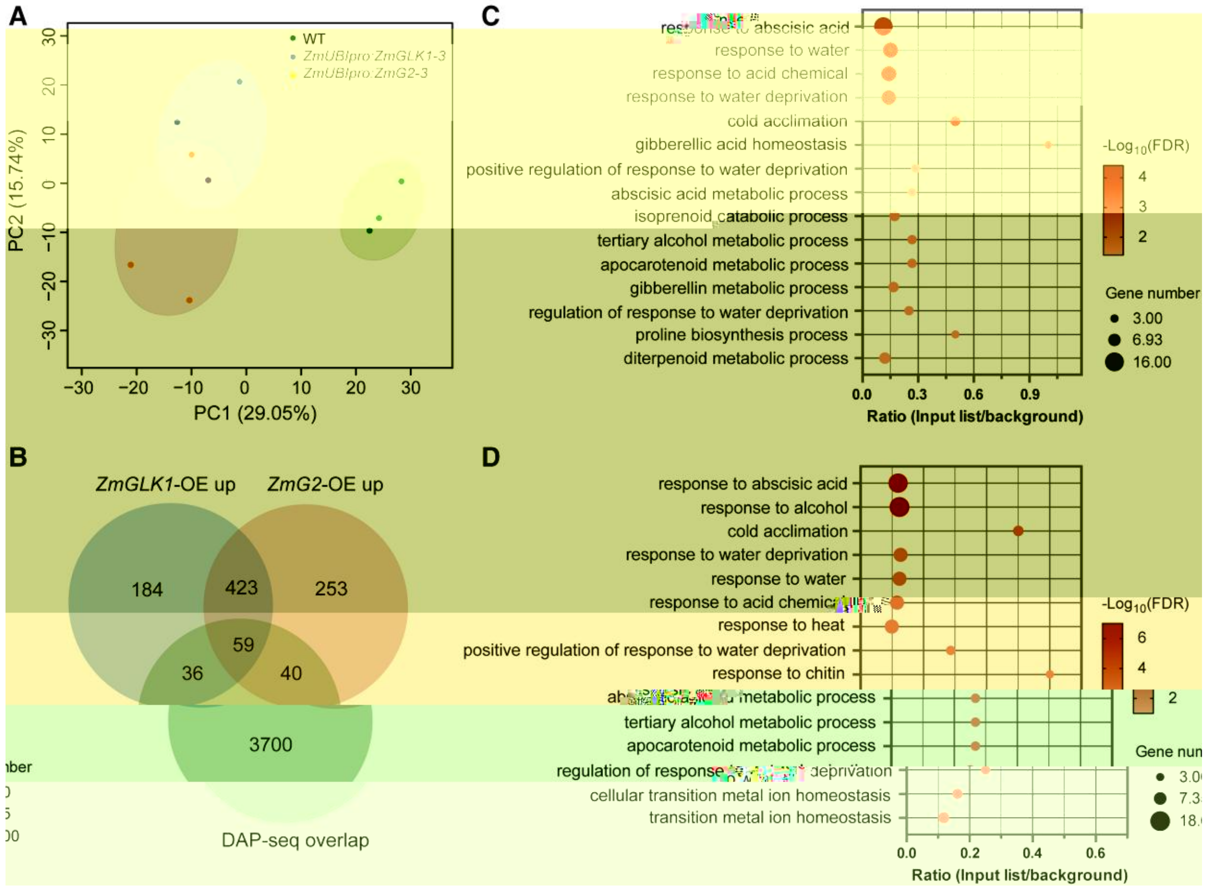

Figure 6. Transcriptomic analysis of WT, $ZmUBI_{pro}$:ZmGLK1, and $ZmUBI_{pro}$:ZmG2 rice plants at 3 h after ABA treatment. **A)** PCA of gene expression in WT, $ZmUBI_{pro}$:ZmGLK1-3, and $ZmUBI_{pro}$:ZmG2-3 rice plants based on RNA-seq data. **B)** Unique and overlapping DEGs upregulated in $ZmUBI_{pro}$:ZmGLK1 and $ZmUBI_{pro}$:ZmG2 rice plants compared to the WT and unique and overlapping ZmGLK1 and ZmG2 target genes identified from DAP-seq data. DEGs were identified based on $|\log_2$ (fold change)$| > 1$ and $P < 0.05$ by "DESeq" R package. **C, D)** GO function categories for DEGs upregulated in $ZmUBI_{pro}$:ZmGLK1 **C)** and $ZmUBI_{pro}$:ZmG2 **D)** rice plants compared to the WT. Bubble size indicates the number of DEG counts in the corresponding GO category; bubble intensity corresponds to the $-\log_{10}$ false discovery rate [FDR] value; and the *x* axis indicates the ratio of DEGs in each GO category to all genes in the category.

Rossini et al. 2001; Waters et al. 2009; Powell et al. 2022). In rice, ectopic expression of maize *GLK* genes *ZmGLK1* and *ZmG2* promotes a proto-Kranz status in the leaf anatomy, increasing chloroplast and mitochondrial development in rice vascular sheath cells (Wang et al. 2017). A previous study by our lab has revealed that rice plants overexpressing maize *GLK* genes have increased biomass and grain yield as a result of improved photosynthetic capacity and reduced photoinhibition under high and fluctuating-light conditions (Li et al. 2020).

In the present study, we uncovered that overexpression of maize *GLK* genes *ZmGLK1* and *ZmG2* in rice enhanced drought tolerance by promoting stomatal closure. Specifically, when plants were grown under standard, well-watered conditions, we observed smaller stomatal size but higher stomatal density and stomatal aperture in rice plants overexpressing *ZmGLK1* or *ZmG2* compared with WT plants (Fig. 2, B and E). These results were consistent with earlier studies showing that *ZmGLK1* and *ZmG2* overexpression led to increased stomatal conductance in field-grown rice (Li et al. 2020), greenhouse-grown rice (Yeh et al. 2022), and Arabidopsis (Nagatoshi et al. 2016). In contrast, under drought stress, the stomata of *ZmGLK1* or *ZmG2* overexpressing rice plants rapidly closed (Fig. S2B and 3E), improving drought tolerance by preventing water loss. Previous studies in rice have reported that small, high-density stomata can close quickly, thus promoting resilience against drought stress (Caine et al. 2019; Caine et al. 2023); these prior results were consistent with those of the present study. Notably, differences in stomatal status between control and drought-stressed plants as a result of *ZmGLK1* or *ZmG2* overexpression were directly caused by regulation of genes involved in stomatal movement, namely, in K^+ channels and H^+-ATPase genes, i.e. *OsKATs*, *OsAKT1*, and *OsAHK7*; Fig. 5). Upregulation of K^+ channel genes by *ZmGLK1* or *ZmG2* overexpression under normal conditions was in line with a previous study in Arabidopsis showing that GLK is a positive regulator of K^+ channel genes and stomatal movement (Nagatoshi et al. 2016); thus, this rapid

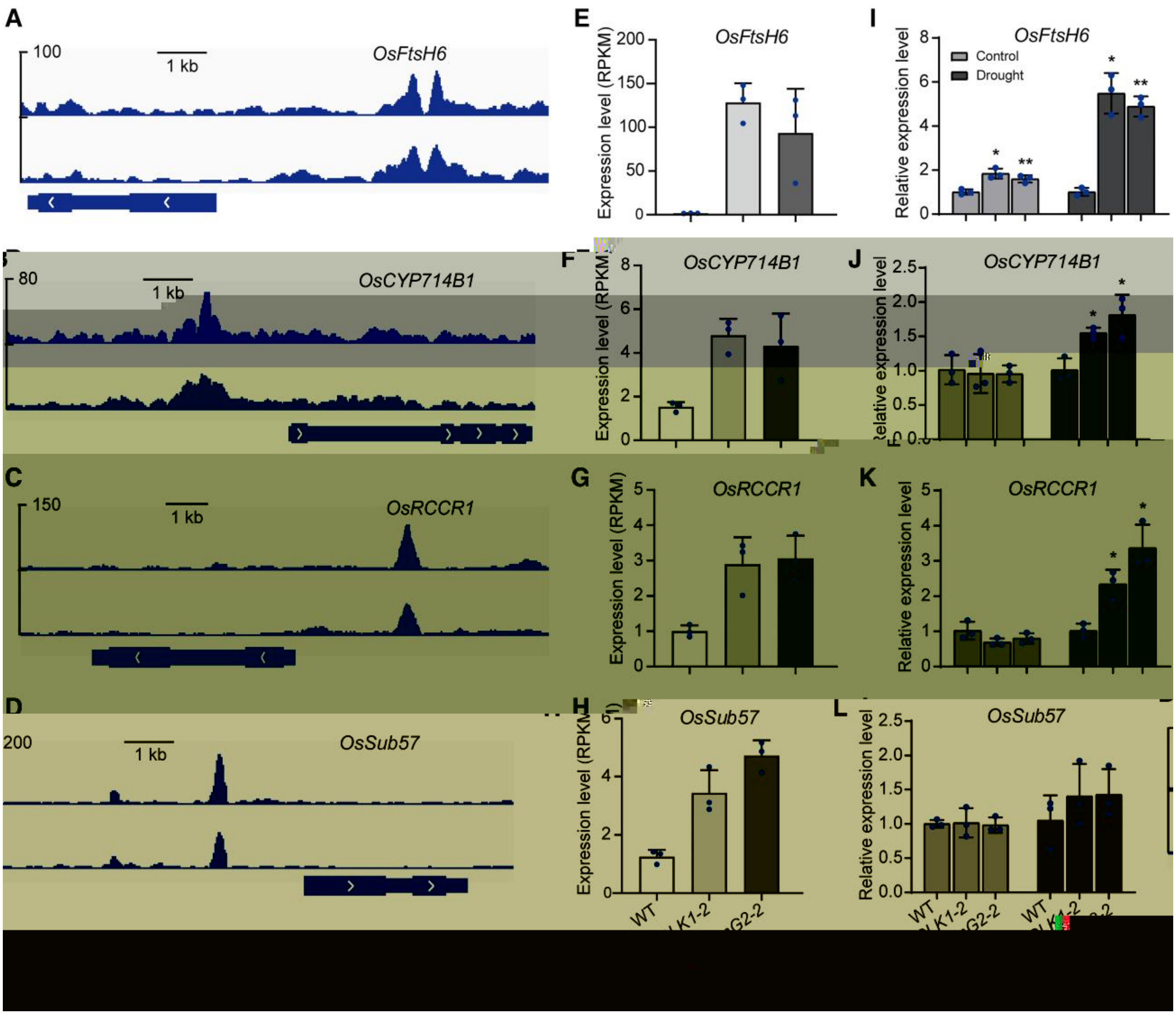

Figure 7. Putative ZmGLK1 and ZmG2 target genes in rice. **A to D)** ChIP-seq indicated that ZmGLK1 and ZmG2 preferentially bound to the promoters of *OsSub57* **A)**, *OsFtsH6* **B)**, *OsCYP714B1* **C)**, and *OsRCCR* **D)**. **E to H)** Expression levels of *OsSub57* **E)**, *OsFtsH6* **F)**, *OsCYP714B1* **G)**, and *OsRCCR* **H)** in WT rice and in rice overexpressing *ZmGLK1* or *ZmG2* as determined with RNA-seq analysis. Gene expression was calculated in RPKM. **I to L)** Relative expression levels of *OsSub57* **I)**, *OsFtsH6* **J)**, *OsCYP714B1* **K)**, and *OsRCCR* **L)** in WT, *ZmUBIpro:ZmGLK1*, and *ZmUBIpro:ZmG2* rice under control conditions and after 7 d of drought stress as determined with RT-qPCR. Data are presented as the mean ± SD from 3 biological replicates. $*P < 0.05$, $**P < 0.01$ Student's *t* test.

stomatal closure of transgenic rice plants resulted directly from a significant reduction in the expression levels of those genes under drought conditions.

Notably, we verified that the regulation of rapid stomatal closure in response to water deficit was ABA mediated, supported by the exogenous application of ABA inducing faster stomatal closure in $ZmUBI_{pro}$:*ZmGLK1* and $ZmUBI_{pro}$:*ZmG2* lines compared with the WT (Fig. [illegible]B), which mimicked the effects of drought stress. Our finding is consistent with the previous study that suggested that fast stomatal closure requires a high ABA sensitivity (Cândido-Sobrinho et al. 2022). Our results also implied that ZmGLKs may function in the ABA biosynthesis pathway, as indicated by the higher ABA accumulation (Supplemental Fig. S5) along with the abundant expression of several key genes involved in ABA biosynthesis (e.g. *OsNCED2*, *OsNCED3*, *OsAAO3*, and *OsZEP1*) in response to drought (Supplemental Fig. S6). ABA biosynthesis starts with the epoxidation of zeaxanthin, and this xanthophyll precursor therefore plays an important role in ABA biosynthesis. We previously discovered that ZmGLKs increase levels of xanthophylls, including zeaxanthin and lutein (Li et al. 2020), which may lead to the improved ABA biosynthesis in this way. Moreover, a study in Arabidopsis showed that GLKs directly activate the expression of *WRKY40*, and GLK-WRKY40 together negatively regulates ABA signaling (Ahmad et al. 2019), suggesting a possible regulatory role of ZmGLKs in the ABA signaling pathway. We also proposed that the C4-like traits conferred by ZmGLKs as mentioned above may contribute to the rapid stomatal

closure. This has been demonstrated by model simulations and experimental studies in many major C crops because of the possible more rapid stomatal closure compared to C_3 crops in response to darkness, resulting in the high water use efficiency (WUE) (Mcusker and Lynch 2016; Wang et al. 2021; Okita et al. 2022). Notably, previous studies have demonstrated that faster stomatal closure in terms is associated with reduced responsiveness to ABA and suggests compared to angiosperms (Lima et al. 2019; Cardoso-Sobrinho et al. 2022), while other plant transport proteins and osmolytes between guard cells and subsidiary cells in grass species contributes to the fast stomatal movement (Chen et al. 2017). Rice plants overexpressing *ZmGLK*s have improved chlorophyll content (Li et al. 2020), consistent with *SlGLK* gene expression in tomato plants (Powell et al. 2012; Nguyen et al. 2021); this may contribute to rapid stomatal closure at the metabolic level.

To further reveal the mechanism underlying ZmGLK-regulated stomatal movement, we conducted comparative analysis of RNA-seq and DAP-seq data. This analysis revealed several potential target genes, including *OsFtsH6*, *OsCYP714B1*, *OsRCCR1*, and *OsSub57* (Fig. 7). *OsFtsH6*, which belongs to the *OsFtsH* gene family, is involved in D1 turnover step of the PSII repair cycle. D1 turnover comprises removal of oxidized D1 proteins by FtsH protease located in the chloroplast, followed by coordinated assembly of newly synthesized D1 proteins into the thylakoid membrane (Wang et al. 2016). The high levels of D1 protein observed in *ZmGLK1* and *ZmG2* overexpressing plants in our previous study (Li et al. 2020) prompted us to hypothesize the potential regulatory function of ZmGLKs on *OsFtsH6* expression. *OsCYP714B1* encodes gibberellin (GA) 13-oxidase, which plays a critical role in GA 13-hydroxylation to regulate plant growth (Magome et al. 2013). *OsRCCR1* encodes a chlorophyll degradation enzyme; knockout mutation of *OsRCCR1* leads to chlorotic lesions in older leaves and early senescence (Tang et al. 2011). Further, *OsSub57* is annotated as encoding a subtilisin homolog that is induced by drought stress (Nadi et al. 2017; Zheng et al. 2022), but its function remains unknown. Nevertheless, it remains an open question whether the transcription regulation conferred by the heterologous gene is conserved or distinct from the native species, due to the complexity of gene regulatory system.

Stomatal closure is considered as the first reaction to drought stress in most plants, preventing water loss through transpiration. Improving the rapidity of stomatal response is a feasible and effective strategy to maximize photosynthesis and WUE simultaneously (Lawson and Vialet-Chabrand 2019). This ordinary investigations into the functions of the potential target genes and regulatory roles as well as the quantification of stomatal kinetics of *ZmGLK* overexpression plants are still needed to understand the mechanism by which ZmGLKs shape the stomatal movements, to coordinate the trade-offs between photosynthesis and drought tolerance. Further exploration will provide insights and useful targets for crop breeding, enabling creation of elite varieties with both high photosynthetic capacity and drought tolerance.

Materials and methods

Plant growth conditions

The WT rice *O. sativa* spp. *japonica* cultivar Nipponbare and homozygous lines described by Li et al. (2020) *ZmUBI*$_{pro}$:*ZmGLK1* and *ZmUBI*$_{pro}$:*ZmG2* were used in this study. For hydroponic culture, rice seedlings were grown in modified Kimura B solution (0.5 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.75 mM K_2SO_4, 1.0 mM CaCl

S[illegible] t[illegible] e s[illegible] e[illegible] W[illegible] sc[illegible] e ec[illegible] c oscopy

Ride le yes ere diet ghed from control or drou[illegible] he ere ged pl nts nd immedi gely cut into 3 × 3 mm pieces, excludi[illegible] the veins. S mples ere directly xed in .5% v/v lut[illegible] dehyde in 0.1 M phosph ge bu er pH 7 0 nd then xed id 1% osmium tetroxide. fter shi[illegible] ice id 0.1 M phosph ge bu er, s mples ere dehydr ged r gu lly in h ed nol series 30%, 50%, 60%, 70%, 80%, 90%, nd 100% or 15 min e ch, ollo ed by incub ti[illegible] in tert ry but nol or 35 min. Then, s mples ere dried usi[illegible] critic l point dryer, p ced on the s mple st e, nd then co ed id old. Stom t ere observed nd photo r phed usi[illegible] S - 8010 sc nni[illegible] electron microscope Hit chi, J p n. The si e, number, nd perture si es o stom t ere c lcu l ted usi[illegible] .m eJ so t re.

Q ntific tion of e ogenous ABA con ent

The uppermost exp nded le yes o control nd drou[illegible] he stressed rice seedli[illegible] s ere det ched nd fl sh fro en in li quid nitro en. Ground s mples 100 m e ch ere ex cted id methonitrile solution con ini[illegible] internl st nd rd °C overni[illegible] t. S mples ere centri ged, nd the resulti[illegible] super nt t ex cted . in. The combined ex cts ere puri ed on C_{18} silic column nd dried id nitro en . fter resolvi[illegible] in meth nol nd p ssi[illegible] throu[illegible] 0.22- µm lter, B s qu nti ed on HPLC–t ndem m ss spectroscopy MS/MS system s described by Liu et l. 2015 .

Exo enous ABA t e t ent

Forty d y old rice seedli[illegible] s ro n in pots ere spr yed id 100 µM B solution con ini[illegible] 0.5% [v/v] T een 20 s surf ct nt until the le yes ere mois. The volume o B solution pplied s consistent bet een seedli[illegible] s. to .5 l fter tre tme[illegible] s exch nge p r meters nd stom t l r its ere ev lu ted s described bove.

RNA ex t ction nd RT qPCR

The uppermost fully exp nded le yes ere h rvested from 3 old rice seedli[illegible] s ro n in pots under norm l conditions or drou[illegible] h stress or 7 d. S mples ere fl sh fro en in liquid nitro en nd ground to po der, nd then tot l RN s ex t cted id TR ol re ent . nvitro en . RN purity nd qu ntity ere ev lu ted usi[illegible] N noDrop2 000 spec trophotometer Thermo Fisher Scientific, S . fter DN se tre tme[illegible] cDN s synthesi ed from 1 µ o tot l RN per s mple usi[illegible] the Revert id First Str nd cDN Synthesis it Thermo Fisher Scientific, S . RT-qPCR s per formed usi[illegible] . OD S BR Green mix id RO. TO. OBO on B. Qu ntStudio 6 Flex instrument pplied Biosystems, S . Rel tive tr nscript levels ere c lcu ted id the $2^{-\Delta\Delta CT}$ method Liv k nd Schmitt en 2001 id 3 biolo ic l replic tes for e ch tre tme[illegible] usi[illegible] *OsActin* s the inter l control. Primers re listed in Supplement l T ble S .

RNA seq ys s

3 fter exo enous B tre tme[illegible] le yes ere collected from - old rice seedli[illegible] s ro n in pots. Tot l RN s ex t cted id TR ol re ent nd then RN integrity s ssessed id the ilent 2100 Bio nly er ilent Technologies, S . RN - seq libr ries ere constructed from WT, *ZmUBI$_{pro}$:ZmGLK1-3*, nd *ZmUBI$_{pro}$:ZmG2-3* rice pl nts usi[illegible] the TruSeq Str nded mRN LT S mple Prep . it .Illumin S id 3 biolo ic l replic tes per line. The resulti[illegible] 9 libr ries ere sequenced on the .Illumin HiSeq . Ten sequenci[illegible] pl orm. fter removi[illegible] the d pter se quences nd lo - qu lity re ds, cle n re ds ere m pped to the *O. sativa* cv. Nipponb re re erence enome usi[illegible] H.S T . im et l. 2015 nd Bo tie Ding me et l. 2009 . Gene expression levels ere c lcul ted in re ds per kilob se of tr nscript per million m pped re ds RP M usi[illegible] Cuffli ks. DEGs ere identified id the DESeq" R p ge e. The thresholds for cl ssific tion s DEG in the tr nsgenic lines comp red to the WT ere $P < 0.05$ nd $|\log_2$ fold ch nge$| > 1$.

DAP seq nal sis

The full len[illegible] coding sequences of *ZmGLK1* nd *ZmG2* ere mpli ed from cDN of the m ize ccession B73. E ch se quence s recombined into the p.. - H LO vector usi[illegible] LR Clon se .. nvitro en . The H LO-ZmGL 1 nd H LO-ZmG2 proteins ere ener ted usi[illegible] 500 l e ch of the p.. - H LO-ZmGL 1 nd p.. - H LO-ZmG2 pl smids

considered significant at $P < 0.05$. Figures were generated using GraphPad Prism 9.0 and Adobe Illustrator for CS3.

Accession numbers

RNA sequence data generated in this study have been deposited in the NCBI BioProject database under accession number PRJNA 718861 for RNA-seq and PRJNA 719016 for DAP-seq. The sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *ZmGLK1* GenBank: FJ318580 and *ZmG2* GenBank: FJ318579.

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Author contributions

W.Z. and J.A. conceived and designed the experiments. J.A., J.L., S.W., J.G., and R.G. performed most of the experiments. Z.L. and H.P. performed the DAP-seq experiments. P.W. critically commented and edited the manuscript. The manuscript was prepared by J.A., J.L., and W.Z. All authors discussed and commented on the manuscript.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Enhanced tolerance of *ZmUBIpro:ZmGLK1* and *ZmUBIpro:ZmG2* rice plants to drought stress induced by 20% PEG 6000.

Supplemental Figure S2. Overexpression of *ZmGLK1* or *ZmG2* in rice led to decreased stomatal conductance and photosynthetic parameters in response to drought.

Supplemental Figure S3. Dynamic changes of soil water content during the drought stress in the greenhouse experiment.

Supplemental Figure S4. Genome-wide summary of the regulatory network motif enrichment of ZmGLK1 and ZmG2 based on DAP-seq data.

Supplemental Figure S5. Changes in endogenous ABA contents in WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice leaves under normal conditions and after 7 d of drought stress.

Supplemental Figure S6. Relative expression levels of ABA biosynthesis genes in the leaves of WT, *ZmUBI*$_{pro}$*:ZmGLK1*, and *ZmUBI*$_{pro}$*:ZmG2* rice plants under normal conditions and after 7 d of drought stress.

Supplemental Table S1. Relative change of gene expression level of 59 overlapped genes from RNA-seq and DAP-seq analyses.

Supplemental Table S2. Primers used for RT-qPCR.

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Conflict of interest statement. The authors declare that they have no conflicts of interests.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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