

ZmCCT regulates photoperiod-dependent flowering and response to stresses in maize

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Abstract

Background: Appropriate flowering time is very important to the success of modern agriculture. Maize (Zea mays L.) is a major cereal crop, originated in tropical areas, with photoperiod sensitivity. Which is an important obstacle to the utilization of tropical/subtropical germplasm resources in temperate regions. However, the study on the regulation mechanism of photoperiod sensitivity of maize is still in the early stage. Although it has been previously reported that ZmCCT is involved in the photoperiod response and delays maize flowering time under long-day conditions, the underlying mechanism remains unclear.

Results: Here, we showed that ZmCCT overexpression delays flowering time and confers maize drought tolerance under LD conditions. Implementing the Gal4-LexA/UAS system identified that ZmCCT has a transcriptional inhibitory activity, while the yeast system showed that ZmCCT has a transcriptional activation activity. DAP-Seq analysis and EMSA indicated that ZmCCT mainly binds to promoters containing the novel motifs CAA AAA TC and AAA TGG TC. DAP-Seq and RNA-Seq analysis showed that ZmCCT could directly repress the expression of ZmPRR5 and ZmCOL9, and promote the expression of ZmRVE6 to delay flowering under long-day conditions. Moreover, we also demonstrated that ZmCCT directly binds to the promoters of ZmHY5, ZmMPK3, ZmVOZ1 and ZmARR16 and promotes the expression of ZmHY5 and ZmMPK3, but represses ZmVOZ1 and ZmARR16 to enhance stress resistance. Additionally, ZmCCT regulates a set of genes associated with plant development.

Conclusions: ZmCCT has dual functions in regulating maize flowering time and stress response under LD conditions. ZmCCT negatively regulates flowering time and enhances maize drought tolerance under LD conditions. ZmCCT represses most flowering time genes to delay flowering while promotes most stress response genes to enhance stress tolerance. Our data contribute to a comprehensive understanding of the regulatory mechanism of ZmCCT in controlling maize flowering time and stress response.

Keywords: DAP-Seq, Maize, Flowering time, Circadian period, Stress response, ZmCCT

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functionally characterized including the CCT domain-containing genes \( ZmCCT \), \( ZmCCT9 \), and the maize \( CONSTANS LIKE 3 \) (\( ZmCOL3 \)), Zea mays MADS-box genes \( ZMM4 \) and \( ZmMADS69 \), the circadian clock component genes \( ZmCCA1 \) and \( ZmCCA1a \), Zea mays \( CENTRORADIALIS 8 \) (\( ZCN8 \)), and Zea mays NF-Y transcriptional factor gene \( ZmNF-YA3 \). CCT domain genes play an important role in flowering time regulation in maize [4]. \( ZmCOL3 \) appears to affect flowering time primarily under long days (LD) conditions. \( ZmCOL3 \) represses flowering time by activating the expression of \( ZmCCT \) [4], which functions as a flowering repressor in maize under LD conditions [5, 6]. A Harbinger-like transposable element that tropical SD maize germplasms do not have, acts in \( cis \) to promote flowering under LD conditions by repressing the expression of \( ZmCCT9 \) [7]. Under LD conditions, \( ZmCCT9 \) delays flowering time by negative regulation of the florigen gene \( ZCN8 \) [7]. \( ZCN8 \) underlies the major flowering time quantitative trait locus \( qDTA8 \) that is involved in photoperiod sensitivity [8, 9]. The MADS-box transcription factors play important roles in flower development and plant inflorescence [10]. \( ZmMADS69 \) suppresses the expression of the flowering repressor \( ZmRap2.7 \), thereby promotes the expression of \( ZCN8 \) and causing early flowering [10]. \( ZMM4 \) promotes floral transition, and the maize transgenic plants over-express \( ZMM4 \) flowered earlier than the wild type [11]. Plants adjust daily changes and seasonal changes by the circadian clock, an endogenous mechanism that controls a wide range of biological processes [12, 13]. \( ZmCCA1 \), an ortholog of \( AtCCA1 \), is expressed in a rhythmic pattern, and the overexpression of \( ZmCCA1 \) in \( A. thaliana \) delayed flowering time [14]. Shi et al. [15] showed that \( ZmCCA1a \) is likely to be an important component of the circadian clock in maize, and flowering time was delayed in the \( ZmCCA1a \)-overexpressing \( A. thaliana \) lines under LD conditions. NF-Ys are widespread in eukaryotes. Several studies showed that NF-Ys play an important role in flowering time regulation [16–19]. In \( Arabidopsis \), the \( nf-yc3/yc4/yc9 \) triple mutant produced almost twice as many total leaves before flowering compared to the wild type under LD but not SD conditions, indicating that \( NF-YS \), \( NF-YS \) and \( NF-YS \) genes are involved in photoperiod-dependent flowering regulation [16]. The overexpression of the \( OsNF-YC2 \) could rescue the late-flowering phenotype of the \( Arabidopsis \) \( nffc3/yc4/yc9 \) triple mutant [18]. \( ZmNF-YA3 \) encodes an NF-YA subunit in maize, and the \( zmnf-ya3 \) mutant showed delayed flowering under LD conditions, whereas there was no significant difference in flowering time compared to the WT under SD conditions [19].

A growing number of evidence showed that photoperiod responsive genes play important roles in plant response to biotic and abiotic stresses [20–23]. Tian et al. [20] showed that transgenic \( Arabidopsis \) plants overexpressing \( ZmCCA1.1 \) exhibited higher tolerance to drought stress. \( AtPpPR7 \), a central component of the \( Arabidopsis \) clock, negatively regulates stress responses via direct regulation of drought- and abscisic acid-responsive genes [21]. Wang et al. [22] identified new genes responsible for \( R \)-gene-mediated resistance to downy mildew in \( Arabidopsis \), and they are controlled by the circadian rhythm regulator \( CCA1 \). Abscisic acid, osmotic and salt stresses could induce the expression of \( AtCOL4 \) [23], which is an essential regulator of abiotic stress tolerance in \( Arabidopsis \). Su et al. [19] showed that \( ZmNF-YA3 \) enhances stress resistance in maize under LD conditions rather than positive regulation of flowering time. The relative water contents (RWC) of \( zmnf-ya3 \) mutant was significantly lower than WT plants after 1 and 4 days of drought and heat stress induction under LD conditions, respectively. \( ZmNF-YA3 \) improves plant tolerance to drought and heat stresses via direct regulation of the expression of the \( bHLH92 \), \( PAM \) and \( JA \) activator gene \( MYC4 \) [19].

A previous study revealed that \( ZmCCT \) plays a negative role in regulating flowering time in maize [5, 6]. Hung et al. [5] showed that the expression of \( ZmCCT \) alleles from diverse teosintes is higher than that from temperate maize and confer delayed flowering phenotype under LD conditions. In early-flowering maize, Yang et al. [6] detected a CACTA-like transposable element within the \( ZmCCT \) promoter that markedly reduces flowering time. The CACTA-like transposable element represses the expression of \( ZmCCT \) to reduce photoperiod sensitivity. Consequently, maize could flower early under LD conditions. \( ZmCCT \) is a homolog of the rice photoperiod response regulator gene \( Ghd7 \). \( Ghd7 \) is diurnally expressed, and the expression of \( Ghd7 \) was much higher under LD compared to SD conditions. \( Ghd7 \) is expressed in young tissues, such as developing leaves, apical meristem and leaf sheaths [24]. The teosinte \( ZmCCT \) allele showed a diurnal expression pattern under LD conditions, with higher transcription levels observed in the light [6]. \( ZmCCT \) is expressed in the leaves and shoot apical meristem at 3–7 leaf stages, plant ovule and pollen [25–27]. Enhanced expression of \( Ghd7 \) under LD conditions delays heading date in rice [24]. The expressions of \( Hd3a \) and \( Ehd1 \) were suppressed by \( Ghd7 \) under LD conditions [24]. Ku et al. [25] showed that the RWC of the \( HZ4-NIL \) containing \( ZmCCT \)-associated QTL was remarkably higher than that of \( HZ4 \) after drought and heat stress induction. The co-expression analysis and the diurnal rhythms of stress response-related genes suggest \( ZmCCT \) as a crucial functional crosslink linking photoperiod with stress tolerance responses under LD conditions.
conditions [25]. However, the molecular mechanisms of ZmCCT in photoperiod-dependent flowering time regulation and response to biotic/abiotic stresses in maize are still ambiguous. Here we showed that overexpression of ZmCCT delays flowering time and confers drought tolerance in maize under LD conditions. Implementing the Gal4-LexA/UAS system revealed that ZmCCT has a transcriptional inhibitory activity, while the yeast system showed that ZmCCT has a transcriptional activation activity. The DAP-Seq assay and EMSA showed that ZmCCT binds to the promoters containing the motifs CAAAATTC and AAATGGTC. Moreover, DAP-Seq and RNA-Seq analyses showed that ZmCCT regulates genes implicated in photoperiod-dependent flowering time regulation, stress response and plant development. This work contributes to a comprehensive understanding of the molecular mechanism of photoperiod affecting maize flowering time and stress response.

**Results**

**Phenotypic variations in flowering time and drought stress tolerance under LD conditions**

Seven T0 transgenic plants were obtained from three independent transgenic events. Compared to the WT plants, T2 families of transgenic plants exhibited 5.9 days delayed anthesis under LD conditions (Fig. 1a, b). Under SD conditions, no significant differences were observed between OE-ZmCCT and the WT plants in the number of days to anthesis (Fig. 1a, b). The total leaf number (TLN) of the OE-ZmCCT was higher than WT plants under LD conditions (Fig. 1c). These results suggest that overexpression of ZmCCT delays maize flowering under LD conditions. Moreover, the OE-ZmCCT and WT plants exhibited significant differences in response to drought stress under LD conditions (Fig. 1d). The relative water content (RWC) was estimated to investigate the response of the OE-ZmCCT and WT plants to

![Fig. 1](image-url)
drought stress at the physiological level. The RWC of OE-ZmCCT plants was significantly higher than that of the WT plants after drought stress induction (Fig. 1e). These results indicate that ZmCCT has dual functions in regulating maize flowering time and stress response under LD conditions.

**RNA-Seq identification of genes affected by ZmCCT**

To understand the regulatory network of ZmCCT in response to photoperiod-mediated flowering, RNA-Seq analysis was conducted using total RNA extracted from leaves of the wild type (WT) and ZmCCT over-expressing plants (OE-ZmCCT) at V6 growth stage under both SD and LD conditions. On average, 21.4–27.3 million 150-nt clean reads were generated for each cDNA library (12 cDNA libraries in total), and ~83% of the reads were uniquely mapped to the maize reference genome V4 (Additional file 1). To dissect the changes in the gene expression in response to LD conditions, differentially expressed genes (DEGs) between the WT and OE-ZmCCT plants under SD and LD conditions were investigated. Genes showed significant changes in expression (> two-fold, i.e., log2 foldchange > 1 or log2 foldchange < −1, padj < 0.05) were selected for further analysis. Accordingly, 91 and 746 genes DEGs were identified under SD and LD conditions, respectively (Fig. 2a). To validate the differential expression observed from RNA-Seq analysis between the WT and OE-ZmCCT under SD and LD conditions, RT-qPCR on 20 DEGs under LD conditions was performed (Additional file 2). RT-qPCR of those 20 DEGs showed similar levels of differential expression patterns observed from the RNA-Seq analysis, indicating

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**Fig. 2** Transcriptomic analysis of ZmCCT. **a)** Venn diagram shows DEGs identified between the WT and OE-ZmCCT plant under SD and LD conditions. **b)** GO annotation of DEGs between the WT and OE-ZmCCT plant under LD conditions. **c)** Heat map shows differential expression of genes related to flowering time and stress response. The FPKM was employed used to make heat map.
the reliability of RNA-Seq analysis in the identification of DEGs (Additional file 3, Fig. S1). The agriGO v2.0 analysis toolkit was employed to perform Gene Ontology (GO) enrichment analysis [28]. Out of the 746 DEGs identified between the WT and OE-ZmCCT under LD conditions, 599 DEGs were functionally annotated (Fig. 2a, b). The GO term enrichment analysis of the 599 DEGs revealed that these genes are mainly involved in cellular process (GO:0050794, $p$ value $= 5.2 \times 10^{-4}$), response to stimulus (GO:0050896, $p$ value $= 8.00 \times 10^{-10}$), metabolic process (GO:0005007, $p$ value $= 1.2 \times 10^{-6}$), biological regulation (GO:00065007, $p$ value $= 3.4 \times 10^{-5}$), developmental process (GO:0044767, $p$ value $= 0.001$), flower development (GO:0009908, $p$ value $= 4.1 \times 10^{-4}$). The most significant subcategory was “response to abiotic stimulus” (GO:0009628, $p$ value $= 2.00 \times 10^{-21}$). The other interesting significant subcategories are “response to stimulus” and “flower development”. Heatmap showed some differentially expressed genes between the WT and OE-ZmCCT under LD conditions (Fig. 2c, Additional file 4).

**ZmCCT has both transcriptional activation and inhibitory activities**

To investigate whether ZmCCT can act as a gene expression promotor or repressor, transactivation analysis of ZmCCT in yeast was performed. ZmCCT exhibits obvious transcriptional activation activity (Fig. 3a). However, ZmCCT encodes a CCT-domain protein (Fig. 3b) that has been reported to have a transcriptional inhibitory activity [21, 29]. To confirm that, the Gal4-LexA/UAS system that analyzes the proteins for positive or negative transcriptional potential was implemented. ZmCCT significantly decreased the expression of the reporter gene (Fig. 3c), indicating that ZmCCT has a transcriptional inhibitory activity. Altogether, ZmCCT is likely to have both transcriptional activation and inhibitory activities.

**DAP-Seq identifies ZmCCT target genes**

DAP-Seq assay was employed to identify the potential target genes directly regulated by ZmCCT. Using the Illumina platform (PE150 sequencing strategy), the DAP-Seq assay produced about 30 million reads of two biological
replicates. Out of those reads, about 21 million reads were uniquely mapped to the maize_V4 reference genome. The effective mapping ratio was about 72%. The MACS2 algorithm version 2.2.7.1 was employed to identify ZmCCT binding sites. A total of 15,862 peaks across the entire maize V4 genome in the two biological replicates are significantly associated with two motifs, i.e., AAATGGTC and CAAAAATTC (Fig. 4a) (p-value < 0.05). From all detected peaks, about 21% (3133 peaks) were located to genomic regions including the promoter (−3kb to TSS), 5’UTR, 3’UTR, intron and exon (TTS to 3kb) (Fig. 4b). The 3133 peaks correspond to 2041 genes. GO enrichment analysis revealed that these genes are significantly enriched in the response to stimulus and flower development subcategories (Fig. 4c). Out of 2041 genes, ZmCCT is likely to bind to the promoter region of 1602 genes, and these genes are mainly involved in the cellular process (GO:0009908, p-value = 5.1 × 10⁻⁴), organelle organization (GO:0006996, p-value = 4.5 × 10⁻⁴), flower development (GO:0009908, p-value = 6.2 × 10⁻⁴), transcription factor activity (GO:0000995, p-value = 4.5 × 10⁻⁴) and response to stimulus subcategories (GO:0050896, p-value = 2.1 × 10⁻⁴).

To further validate the expression level of those genes as putative targets directly regulated by ZmCCT, we analyzed the overlapping genes between DEGs generated from the WT and OE-ZmCCT under LD conditions and putative target genes directly regulated by ZmCCT in the upstream regions. Twenty-one DEGs that are likely to be implicated in flowering time regulation, stress response and flower development were identified (Additional file 5). Out of those 21 genes, 11 genes were upregulated in the OE-ZmCCT plants, whereas the remaining 10 genes were downregulated.

**Binding motif analysis revealed novel ZmCCT cis-elements**

The two novel DNA motifs AAATGGTC and CAAAAATTC were selected as candidate binding sites by MEME-ChIP software (Fig. 4a). To confirm whether ZmCCT could bind to the AAATGGTC and CAAAAATTC motifs, the electrophoretic mobility shift assay (EMSA) was performed using a purified recombinant ZmCCT protein and labeled DNA probes containing the ZmCCT binding sites, i.e., AAA TGGTC and CAAAAATTC. As shown in Fig. 4d, ZmCCT protein could bind to the AAATGGTC and CAAAAATTC motifs. The addition of 100× unlabeled competitors reduced the detected binding sites of ZmCCT, and it could not bind to the mutated probes (ACGCTAGA and TCC GCGCT). In the absence of the ZmCCT protein, except for the free probe, no binding band was observed. These results confirmed the specific binding of the ZmCCT protein to the AAATGGTC and CAAAAATTC motifs.

**ZmCCT directly regulates genes related to photoperiod-dependent flowering time**

The DAP-Seq and RNA-Seq analyses identified 10 potential target genes of ZmCCT related to flowering regulation (Additional file 6), from which two PSEUDO RESPONSE REGULATOR (ZmPRR5), a REVEILLE 6 (ZmRVE6) and a CONSTANS-LIKE (ZmCOL9). To investigate whether ZmCCT could regulate the expression of these potential target genes, a dual-luciferase transient transcriptional activity assay (referred to as Dual-LUC hereafter) in N. benthamiana leaves was carried out. The 35S::ZmCCT served as an effector and LUC (the firefly luciferase-coding gene) driven by different promoter regions (−3000 to −100bp) of the potential target genes as a reporter (Fig. 5a). The results indicated that ZmCCT protein promotes the expression of three of the ten target genes, while represses the expression of the remaining seven genes (Fig. 5b). In particular, ZmCCT binds to the promoters of ZmPRR5 (−1567bp), ZmRVE6 (−420bp) and ZmCOL9 (−1315bp) to promote the expression of ZmRVE6, and represses the expression of ZmPRR5 and ZmCOL9 (Fig. 5b, Additional file 3). Three of those nine genes, i.e., ZmVOZ1, ZmARR16, ZmHY5, and ZmMPK3, are known to be involved in drought stress response [30–33]. The results further showed that ZmCCT binds to the promoters of ZmVOZ1 (−927bp) and ZmARR16 (−2438bp) to repress their expression, and to the promoters of ZmHY5 (−2940bp) and ZmMPK3 (−2841bp) to promote their expression (Fig. 5b, 6a, Additional file 7). Three of those nine genes, i.e., ZmVOZ1, ZmARR16, ZmHY5, and ZmMPK3, are known to be involved in drought stress response [30–33]. The results further showed that ZmCCT binds to the promoters of ZmVOZ1 (−927bp) and ZmARR16 (−2438bp) to repress their expression, and to the promoters of ZmHY5 (−2940bp) and ZmMPK3 (−2841bp) to promote their expression (Fig. 5b, 6a, Additional file 7). To further confirm that, we measured the mRNA levels of these nine genes by RT-qPCR in the WT and OE-ZmCCT plants between the well-irrigated and drought-stressed treatments under LD conditions. RT-qPCR results showed that the expression of ZmHY5 and ZmMPK3 was upregulated in the OE-ZmCCT and WT plants in response to drought stress, with the expression levels were much higher than those in the WT (Fig. 6b).
Fig. 4 (See legend on previous page.)
**Fig. 5** *ZmCCT* regulates a set of genes involved in maize photoperiod-dependent flowering time. **a)** The reporter construct was transiently expressed in *N. benthamiana* leaves together with the control vector or 35S::*ZmCCT* effector. Pro genes represent the promoters of the target genes. **b)** The LUC/REN ratio representing the relative activity of the gene promoters (*n* = 3, *p* < 0.05; **p** < 0.01)

**Fig. 6** *ZmCCT* regulates a set of genes involved in stress response. **a)** The LUC/REN ratio representing the relative activity of the gene promoters. **b)** Expression analysis of the targeted genes in the WT and OE-*ZmCCT* plants at the V6 growth stage under drought and LD conditions by RT-qPCR (*n* = 3, *p* < 0.05; **p** < 0.01)
higher in the OE-ZmCCT compared to the WT plants (Fig. 6b). Meanwhile, ZmVOZ1 and ZmARR16 were downregulated in the OE-ZmCCT and WT plants after drought stress induction, and the expression levels were much lower in the OE-ZmCCT compared to the WT plants (Fig. 6b). The expression levels of the remaining 5 genes did not reveal any significant difference between the well-irrigated and the drought-stressed treatments (Fig. 6b). These results indicate that ZmCCT responds to drought stress primarily by direct regulation of the expression of those four drought-stress-related genes (ZmHY5, ZmMPK3, ZmVOZ1 and ZmARR16).

**ZmCCT directly regulates several development-related genes**

Six DEGs related to the developmental process were identified as direct targets of ZmCCT (Additional file 5). Four genes were upregulated and two genes were downregulated in the OE-ZmCCT plants. DAP-Seq results showed that ZmCCT has binding sites to the promoters of Zm00001d018977 (ZmMET1, −1223 bp), Zm00001d037982 (FAC, −2850 bp), Zm00001d034929 (ZmLUG3, −2995 bp), Zm00001d044815 (BOB1, −972 bp), Zm00001d008882 (ZmM2, −2958 bp), and ZmGB1 (−1587 bp) (Additional file 5). These genes are involved in regulating plant growth and development.

**Discussion**

Flowering time is an important agronomic trait that determines plant adaptation and distribution. The CCT domain-containing gene ZmCCT has been previously reported to play a negative role in regulating flowering time in maize [5, 6]. ZmCCT is a homolog to the rice photoperiod response regulator gene Ghd7. Enhanced expression levels of Ghd7 under LD conditions delay heading date in rice [24]. Here we have employed several molecular techniques including DAP-Seq and RNA-Seq analyses to elaborate the molecular mechanisms of ZmCCT in photoperiod-dependent flowering time regulation and response to biotic/abiotic stresses in maize. Our data showed that ZmCCT negatively regulates maize flowering time and confers maize drought tolerance under LD conditions. The observed transcriptional activation and inhibitory activities displayed by ZmCCT when employing either the yeast and Gal4-LexA/UAS transcriptional systems might be due to the interaction with different transcription factors as the Yes-associated protein (YAP) and PDZ-binding motif (TAZ) do, which are known as oncogenic transcriptional co-activators and key regulators of stem cell function [34, 35]. Kim et al. [36] showed that YAP and TAZ could also function as transcriptional co-repressors when interacting with the TEA domain (TEAD) transcription factor. However, further studies are needed to explore the molecular mechanism of ZmCCT.

**ZmCCT delays flowering by regulating photoperiod-dependent flowering genes**

The observed delayed flowering time in response to the overexpression of ZmCCT under LD conditions is consistent with previous studies [5, 6] where ZmCCT, a homolog of the rice photoperiod response regulator Ghd7, was consistently expressed at higher levels and confer delayed flowering in the teosintes under LD conditions. The DAP-Seq and RNA-Seq analyses results showed that ZmCCT directly promotes the expression of ZmRVE6, while represses the expression of ZmPRR5 and ZmCOL9, resulted in a delayed flowering phenotype in maize. These findings suggest that ZmCCT delays flowering by upregulating the expression of a rhythmic gene ZmRVE6 and downregulating the expression of the circadian oscillator gene ZmPRR5 and a photoperiod-sensitivity gene ZmCOL9. These three genes (ZmRVE6, ZmPRR5 and ZmCOL9) are involved in the photoperiod-dependent flowering time regulation and circadian rhythm in maize [37–40]. ZmPRR5, which is related to the domestication of maize, reduces the expression levels in the late-flowering phenotype Ify1 mutant compared to the WT [37, 38]. ZmRVE6 is a homolog of the Arabidopsis REVEILLE 8 (AtRVE8) that is involved in the photoperiodic flowering of Arabidopsis and shows a rhythmic expression in maize [39, 40]. Moreover, RVE8 induced several evening-phased oscillator genes in Arabidopsis, including PRR5 [41]. ZmCOL9 belongs to C2C2-CO-like-transcription factor 5 and participates in the photoperiod-dependent teosinte flowering pathway [42, 43]. ZmCOL9 is a homolog of the negative regulator of flowering in the photoperiod pathway in Arabidopsis AtCOL9 [44], and DTH2 that acts as an activator of rice heading under long-day conditions [45].

Moreover, RNA-Seq analysis revealed 10 DEGs between the WT and OE-ZmCCT plants under LD conditions. These genes belong to the circadian rhythm, photoperiodism regulation and flower development (Additional file 8), and comprise 3 CCT-domain-containing protein, 3 MADS transcription factor, Adagio1, PHOT2, phyB2 and GRASS3. The expression levels of seven out of those ten genes are downregulated in the OE-ZmCCT plants. The circadian clock is an endogenous mechanism for keeping time, which allows organisms to coordinate biological processes and provide an adaptive advantage [46, 47]. Our data showed that ZmCCT regulates the expression of ZmHY5, ZmARR16, ZmCML36 and ZmLIP, which are related to the maintenance of the
circadian period. More specifically, ZmCCT promotes the expression of ZmHY5 but represses the expression of ZmARR16, ZmCML36 and ZmLIP under LD conditions, which may prolong the circadian period thus delay flowering time. Under the blue light, a mutation in the HY5 in Arabidopsis remarkably shortens the circadian period [48]. AtHY5 affects the clock via the transcriptional repression of PRR5 [48]. Homologs of the ZmARR16 in Arabidopsis, i.e., ARR3 and ARR4, are key genes for a proper circadian period and define an additional level of the circadian clock regulation [49]. ARR3 or ARR4 mutant could prolong the period of the clock regardless of the presence or absence of light [49]. In Arabidopsis, the cytosolic-free Ca\(^{2+}\) ([Ca\(^{2+}\)\_cyt]) circadian oscillations could influence the function of the circadian clock via the Ca\(^{2+}\)-dependent action of CALMODULIN-LIKE24 (CML24) [50]. The cml23-2cml24–4 double mutant prolongs the circadian period [50]. The Arabidopsis homolog of the ZmLIP (AtLIP1) acts as a unique negative regulator in controlling the light input of the circadian clock, which is necessary for precise clock entrainment in plants. The lip1–1 mutant shortens the circadian period by 1.5–2h than the wild-type plants under continuous red light in Arabidopsis [51].

Taken together, our results suggest that ZmCCT could directly or indirectly regulate the expression of several photoperiod-dependent and circadian clock maintenance flowering time genes to affect maize flowering time.

**ZmCCT improves stress tolerance by regulating a set of stress response genes**

Among the target genes of ZmCCT, several genes were shown to be associated with plant response to various biotic and abiotic stresses, which is consistent with a previous study where ZmCCT has been reported to be implicated not only in regulating flowering time but also in the stress response [25]. In particular, the overexpression of ZmCCT confers maize drought tolerance under LD conditions by regulating the expression of drought-responsive genes. After drought stress induction, ZmHY5 and ZmMPK3 expression in transgenic plants overexpressing the ZmCCT transgene was strongly elevated compared to the WT, suggesting that the effect of ZmHY5 and ZmMPK3 on drought stress is mediated through the promotion of ZmCCT. These results are consistent with previous studies where drought stress promotes the expression of ZmHY5 and ZmMPK3 [30, 31]. Similarly, the strong downregulation of the drought-responsive genes ZmVOZ1 and ZmARR16 in the OE-ZmCCT plants compared to the WT after drought induction suggests that ZmCCT mediates drought tolerance by suppressing the expression of ZmVOZ1 and ZmARR16. A previous study showed that ZmARR16 was downregulated in the ear, leaf and kernel of maize under drought stress [32]. The voz1voz2 double mutant enhanced drought-stress tolerance in Arabidopsis [33]. Furthermore, ZmCCT may have a role in response to biotic stresses by regulating target genes. For instance, ZmVOZ1 was downregulated in response to the infection with the rice black-streaked dwarf virus (RBSDV) [52]. Moreover, the expression of ZmMPK3 was elevated in response to the infection with Setosphaeria turcica and necrotrophic fungi Cercospora zeae-maydis and Cercospora Zeina [53, 54]. Besides, the strong promoted expression of the defense and immune responsive genes ZmSYD, ZmGB1 and ZmRPS2 [55–59] in the transgenic plants carrying the ZmCCT transgene compared with WT suggests that ZmCCT plays a crucial role in maize response to biotic stresses.

**ZmCCT might be involved in regulating plant development**

The identification of several ZmCCT target genes implicated in regulating plant growth and development, such as ZmGB1, ZmLUG3 and ZmM2 suggests that ZmCCT plays indirect roles in regulating plant development. ZmGB1 encodes a heterotrimeric G protein β subunit and regulates shoot meristem development in maize [60]. ZmLUG3 protein belongs to the Gro/Tup1 family, which acts as negative transcriptional regulators and play important roles in the growth and developmental processes of many organisms [61]. ZmM2 is involved in maize ear development and is regulated by the BRANCHED SILKLESS 1 (BD1) [62]. The mRNA of ZmM2 was not detected during the development of bd1 ears [62]. Besides, ZmCCT regulates the expression of the Arabidopsis and rice homologs ZmFAC, and ZmMET1 which are known to be involved in plant development [63–65]. ZmCCT may be involved in plant growth and development by regulating those development related genes.

Accordingly, we proposed a regulatory model for the role of ZmCCT in regulating flowering time and stress response in maize (Fig. 7). Previous studies on the model plant Arabidopsis revealed that ZmPRR5 and ZmRVE6 may form a negative feedback loop in maize [40, 41]. Interestingly, three genes, i.e., ZmHY5, ZmVOZ1 and ZmARR16, might have dual functions in regulating flowering time and stress response [30, 32, 33, 48, 49, 66]. Our data showed that ZmCCT represses the expression of most flowering time genes, whereas promotes the expression of most stress-responsive genes. Two lines of evidence suggest that ZmCCT delays flowering by repressing flowering time-related genes and enhances stress tolerance by promoting the stress-responsive genes. Firstly, the RNA-Seq results showed a strong downregulation of seven of the ten DEGs related to flowering time in the transgenic plants overexpressing the ZmCCT transgene.
compared to the WT. Secondly, out of the identified 48 DEGs related to stress response, 40 genes were strongly upregulated in the transgenic plants overexpressing the ZmCCT compared to the WT.

**Conclusions**

ZmCCT has dual functions in regulating maize flowering time and stress response. ZmCCT negatively regulates flowering time and enhances maize drought tolerance under LD conditions. ZmCCT delays flowering by repressing flowering time-related genes and enhances stress tolerance by promoting the stress-responsive genes. Based on the results of this study, a model for the regulatory role of ZmCCT in flowering time and stress response in maize was proposed. Our results contribute to a comprehensive understanding of the regulatory mechanisms of ZmCCT in regulating flowering time and stress response in maize.

**Methods**

**Plant materials and growth conditions**

In this study, the full-length coding sequence of the ZmCCT was inserted into pCAMBIA1300-35S binary vector to overexpress ZmCCT. The wild-type (WT) B104 and ZmCCT overexpression transgenic (OE-ZmCCT) plants with B104 background were used. The B104 and Maize genetic transformation were performed by Beijing bomeixingao Technology Company. Seeds of the WT and OE-ZmCCT plants provided by Beijing bomeixin-gao Technology Company. The OE-ZmCCT and WT plants were grown in growth chambers (GR64, Conviron, Canada) under either long days (LD) and short days (SD) conditions. For LD conditions 15 h light/9 h dark, day temperature 28 °C, night temperature 22 °C, with a 40% relative humidity, a light intensity of 105 μmol m⁻² s⁻¹ in Zhengzhou, China, in the spring of 2018 were implemented. Meanwhile, under SD conditions, plants were subjected to 9 h light/15 h dark, other parameters were the same as for LD conditions. In this study, leaf blade tissue was collected from the leaves of the OE-ZmCCT or WT plants grown either under LD or SD conditions at the V6 growth stage. Each sample was collected from four different randomly selected plants. Three biological replicates were collected at the same time.

**RNA extraction and RNA sequencing (RNA-seq)**

For each sample, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), and then purified by magnetic stand (Invitrogen). The cDNA libraries were prepared using 5 μg of the total RNA following the Illumina standard protocol (TruSeq Standard RNA LT Guide). Twelve separate cDNA libraries were constructed. An Agilent 2100 Bioanalyzer system was employed to perform the
quality control checks of all libraries. Qualified cDNA libraries were sequenced using the Illumina HiSeq 4000 system, and 150 bp paired-reads were generated.

RNA-Seq data analysis
The RNA-Seq data analysis was performed according to the method described by Cao et al. [67]. To validate the differences in the expression levels observed by RNA-Seq between the WT and OE-ZmCCT plants under LD and SD conditions, we performed RT-qPCR on 20 differentially expressed genes. The primer sequences used in the RT-qPCR assay are listed in Additional file 2. All analyses were conducted with three technical and biological replicates.

Transcriptional activation assay in yeast
To analyze the transcriptional activity of ZmCCT, the yeast strain AH109 (Stratagene, USA) that contains the lacZ and HIS3 reporter genes was used. The coding sequence (CDS) of ZmCCT cloned from the maize inbred line CML288 was inserted into the pBD-GAL4 vector via BamHI and NdeI restriction enzymes to produce ZmCCT-GAL4 fusion protein. The negative control pBD-GAL4, positive control pGAL4 and pBD-ZmCCT plasmids were transfected into the AH109 cells. The transfected yeast cells were transferred to YPDA or SD/−Trp/−His medium and cultured at 30°C for 3–5 days. The β-galactosidase filter assay was performed to determine the β-galactosidase activity of the transfected yeast cells (PT3024–1).

Gal4/UAS system assay
The reporter (UAS-GUS) and effector (VP16 and Gal4) constructs were previously described by Tiwari et al. [68]. The ZmCCT-GAL4 effector construct contains the full-length coding sequence of ZmCCT fused into the N-terminus of the Gal4 DNA-binding domain under the control of CaMV-35S promoter. To normalize the expression of the GUS reporter gene, the 35S-LUC construct was co-transformed as an internal control. GUS and firefly luciferase (LUC) enzymatic assays were performed to determine the β-galactosidase activity of the transfected yeast cells (PT3024–1).

DNA affinity purification sequencing (DAP-Seq) experiments
DAP-Seq assay was carried out as described by O’Malley et al. [69]. The NEB Next® DNA Library Prep Master Mix set for Illumina kit (NEB #E6040S) was implemented to prepare the DAP-Seq gDNA library. The pFN19K Vector (cat#G184A, Promega) was employed to fuse the ZmCCT into the HaloTag. The TNT SP6 High-Yield Wheat Germ Protein Expression System (L3260, Promega) was then implemented to express the ZmCCT-HaloTag fusion protein. Magen HaloTag Beads (G7281, Promega) was used to purify the fusion protein. The ZmCCT-HaloTag fusion protein and 500 ng of library DNA were co-incubated in 40 μl PBS buffer with a slow shaking for 1.5 h in a cold room. The beads were five-times washed in 200 μl PBS + NP40 (0.005%). The supernatant was discarded and an aliquot of 25 μl of elution buffer was added. Finally, beads were incubated at 98°C for 10 min to elute DNA fragments. According to the fragment size of the library, the DAP-Seq library concentration for a given read count was measured. The mock DAP-Seq libraries used as a negative control were prepared as previously described except for adding protein to the beads.

DAP-Seq data analysis
The clean reads were aligned to the maize_V4 reference genome using the software Bowtie 2 version 2.3.4.3 at the default parameters [70]. The MACS2 algorithm (Model-Based Analysis of ChiP-Seq) version 2.2.7.1 was implemented to identify peaks with BAMPE mode [71]. Genes that contain peaks located within 3 kb upstream the TSS (transcription start site) or downstream TTS (transcription termination site) were defined as target genes of the ZmCCT.

Gene ontology (GO) enrichment analysis
GO enrichment analysis was performed using the AgriGO analysis toolkit version 2.0 [28]. According to maize GAMER, the GO annotations of maize B73_V4 protein-coding genes were used as a reference [72]. The type of GO in agriGO v2.0 was set as Plant GO slim. Fisher’s exact test was employed to determine the significance levels. The BY procedure in the agriGO v2.0 toolkit was implemented for multiple test corrections.
Electrophoretic mobility shift assay (EMSA)
The probes used for EMSA were labeled and annealed under the guidance of DIG Gel Shift Kit standard procedure (Roche). All binding reaction components were thawed on ice. For a binding reaction of 20 μl, 4 μl of binding buffer, 1 μl of poly [d (I-C)] (1 μg/μl), 1 μl of poly L-lysine (0.1 μg/μl), 2 μl labeled probe (0.4 ng/μl), 1 μl purified ZmCCT fusion protein (25–75 ng/μl) and double distilled water were added. After mixing the mixture carefully, it was incubated for 15 min at room temperature. A native polyacrylamide gel of 6–8% acrylamide in 0.5× TBE buffer was prepared, and the binding reaction was loaded onto the gel and run in 10 cm × 10 cm × 0.1 cm PAGE at 80 V. After electrophoresis, an equilibrated nylon membrane was carefully placed onto the gel for 30 min at 400 mA to transfer the binding reaction. The membrane was then placed on a development folder or hybridization bag and 1 ml CSPD working solution was applied. After incubation at 37°C for 10 min to enhance the luminescent reaction, images were taken.

Supplementary Information
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Availability of data and materials
The raw data can be accessed from the NCBI Sequence Read Archive (SRA) platform under the accession number PRJNA727729 (http://www.ncbi.nlm.nih.gov/bioproject/727729).

Declarations

Ethics approval and consent to participate
The research on plants, including the collection of plant material complies with relevant institutional, national, and international guidelines and legislation. Experimental research and field studies on plants were carried out by Huihui Su, Jiachen Liang and Haiyang Cheng. The inbred of maize B104 and ZmCCT overexpression transgenic (OE-ZmCCT) plants with B104 background were used in our experiment which were collected from Beijing bomeixingao Technology Company.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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