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Full Length Research Paper

Effects of night break on accumulation of *HD6* mRNA in tropical photoperiod-sensitive maize

Xiaomin Wei[1,2], lixia Ku[1], Siyuan Li[1], Shaofang Zhang[1], Xintao Wang[1] and Yanhui Chen[1]*

[1]College of Agronomy, Henan Agricultural University, Zhengzhou, Henan 450002, China.
[2]Department of Pharmacy Engineering, Zhengzhou Animal Husbandry Engineering College, Zhengzhou, Henan 450011, China.

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To increase the genetic diversity of maize grown in temperate zones, it is essential that tropical germplasm be integrated, but a major limiting factor is photoperiod sensitivity. To address the molecular basis of this sensitivity, a tropical maize inbred line CML288 was grown under inductive short-day (SD) and non-inductive night break (NB) conditions in which the inductive dark period was broken by a brief illumination. Homologous amplification and rapid amplification of cDNA ends (RACE) technology were used to clone the maize *HD6* gene, an ortholog of rice *Hd6* that is involved in the photoperiodic flowering pathway, and functions as a critical component of the circadian clock. Detailed expression analysis of this gene were conducted using real-time fluorescence quantitative PCR in the stem apexes and leaves at the 5th-leaf stage, the developmental stage most sensitive to NB. The results demonstrated that diurnal expression rhythms of *HD6* were altered in both the stem apexes and the leaf by NB treatment, and its mRNA accumulation gradually declined with the increase of NB treatment days. Additionally, the degree of delay in day to tassel (DTT) and day to pollen (DTP) was also proportional to the number of NB treatment days. The consistent effect of NB on *HD6* accumulation, together with the flowering delay suggests that the *HD6* gene may participate in the photoperiodic floral induction.

Key words: Maize (*Zea mays* L.), night break, *HD6* gene, expression analysis, photoperiod sensitivity.

INTRODUCTION

Light is one of the major environmental stimuli controlling flowering. In addition to the intensity and quality of light, the duration of light per day, or photoperiod, is a major determinant of the timing of flowering (Thomas and Vince-Prue, 1997; Samach and Coupland, 2000; Thomas, 2006; Jaeger et al., 2006). Based on responsiveness to daylength, flowering plants can be classified into 3 groups: long-day plants (LDPs) that flower when daylength exceeds a critical duration, short-day plants (SDPs) that flower faster under short day (SD) conditions, and day-neutral plants that flower independently of daylength (Ishikawa et al., 2005).

Maize (*Zea mays* L.) is domesticated from tropical Latin America teosinte, a plant requiring short day photoperiods to flower (Miller et al., 2008; Coles et al., 2010). Although the majority of maize varieties are generally characterized as day-neutral plants, achieved through centuries of domestication and artificial selection, numerous tropical landraces of maize remain sensitive to photoperiod (Miller et al., 2008). As a result, tropical maize exhibits delayed flowering time, increased plant height, and increased number of leaves when grown in temperate long-day photoperiods. Moreover, some tropical varieties do not flower under these conditions (Giauffret et al., 2000; Gouesnard et al., 2002; Wang et al., 2008; Coles et al., 2010). These phenomena undoubtedly raise barriers to the use of diverse tropical germplasm resources for breeding programs of temperate maize. Therefore, it is vital for maize breeders to understand the genetic basis of photoperiod sensitivity (PS).

Previous research of the response to photoperiod were conducted using a constant photoperiod, in which plants were planted in both long- and short-day environments as well as conducting reciprocal transfer experiments in

*Corresponding author. E-mail: chy9890@163.com. Tel: 86-371-63558032. Fax: 86-371-63558032.

which plants were transferred from long to short photoperiods and vice versa (Wu et al., 2007; Wang et al., 2008; Miller et al., 2008; Coles et al., 2010). Sequential planting in which plants were sown on different dates sequentially and grown under natural and extended photoperiod (Birch et al., 1998a, b; Tan et al., 2000) is also used. However, maize is originally an SDP, and the molecular basis of its PS has never been analysed by night break (NB). NB has been used extensively as an experimental tool to study the photoperiodic control of flowering for many years (Moore et al., 1967; Lumsden et al., 1982; O'Neill et al., 1994; Sage-Ono et al., 1998; Ishikawa et al., 2005). Its effect on flowering is most evident in SDPs, where it inhibits flowering after a very short exposure to light during the night (Hamner and Bonner, 1938; Thomas and Vince-Prue, 1997). Further investigation of NB treatments in various SDPs demonstrated that the circadian clock is involved in the photoperiodic regulation of flowering, and NB disrupts a circadian clock-regulated process required for flowering (Ishikawa et al., 2005; Hayama et al., 2007). Thus, NB treatment provides a rigorous negative control for experimental treatments that induce flowering.

The circadian clock, as an endogenous time-keeping mechanism, enables organisms to coordinate their activities with external light/dark cycles by anticipating the coming of dawn or dusk (Searle and Coupland, 2004). Circadian clocks are divided conceptually into three parts: the input signaling pathways, the central oscillator that is itself composed of a transcription-translation negative feedback loop to generate rhythm of approximately 24 h, and the output signaling pathways from the oscillator to various clock-controlled processes (Sugano et al., 1999; Yang et al., 2003; Daniel et al., 2004; Portolés and Más, 2007; Smith et al., 2008). In addition to feedback regulation, circadian clock function also relies on accurate post-transcriptional regulation of clock components (Sugano et al., 1999; Yang et al., 2003; Bae and Edery, 2006; Portolés and Más , 2007). In this sense, casein kinase 2 (CK2) is a critical component of the circadian clock systems (Allada and Meissner, 2005; Mizoguchi et al., 2006; Portoles and Mas, 2007), and has been shown to phosphorylate clock proteins in several organisms including *Arabidopsis thaliana, Neurospor acrassa* and *Drosophila melanogaster*, affording them proper clock function to bring forth optimal phase relationships between the endogenous rhythms and the external day-night cycles (Yang et al., 2002; Daniel et al., 2004; Allada and Meissner, 2005; Smith et al., 2008). In *A. thaliana*, Circadian clock-associated 1 (CCA1), its partially redundant homolog late elongated hypocotyl (LHY), and timing of CAB expression 1 (TOC1), make up the central oscillator of the circadian clock. The central oscillator plays an important role in photoperiodic flowering by regulating the rhythmic expression of the flowering-related genes including Gigantea (GI), Constans (CO), and Flowering Locus T (FT) (Daniel et al., 2004; Mizoguchi et al., 2005; Portolés and Más, 2007; Ding et al., 2007). CK2 can phosphorylate CCA1 to confer normal function to the central oscillator to regulate the activity of diurnal rhythms of the output genes that set the light sensitive phase for triggering the floral transition (Sugano et al., 1998, 1999; Daniel et al., 2004). In *Oryza sativa*, the *Hd6* gene encodes an α-subunit of CK2 that delays flowering time under long-day conditions (Takahashi et al., 2001), although this process is independent of the circadian clock mechanism in which the *OsLHY*, as a core component of the circadian clock of rice and the sole ortholog of *Arabidopsis CCA1/LHY*, was also phosphorylated by Hd6 (Ogiso et al., 2010). To date, a few of the reports involving maize CK2 have focused on its subcellular localization, the pattern of expression of its α/β subunits, and its interaction with substrate Rab17 in growth-related processes under stress conditions (Peracchia et al., 1999; Riera et al., 2001; Riera et al., 2004). However, there have been few reports on its role in the photoperiodic floral process in maize.

To dissect the genetic basis of maize PS, a tropical maize inbred line CML288 was grown under SD and NB conditions. We cloned *HD6*, an ortholog of rice *Hd6*, from CML288 using homologous amplification and 3′ RACE, and then analyzed NB effects on *HD6* mRNA accumulation and flowering time. Photoperiodic flowering occurs when a time-measuring process initially sensed in the leaf, leads to the synthesis of a flower-promoting stimulus that is translocated to the shoot apex, resulting in flower bud differentiation (Corbesier et al., 2007). Therefore, HD6 mRNA in leaves and shoot apexes (SA) were estimated in the presence or absence of NB. In the present study, the main objectives were to (1) determine whether NB affects maize flowering time and which developmental stage is the most sensitive to NB (2) clone HD6 gene in maize and analyze its expression in leaves and shoot apexes (SA) of different developmental stages under various photoperiodic regimes. The results showed that the suppression of accumulation of *HD6* mRNA by NB treatment days was consistent with the delay degree of day to tassel (DTT) and day to pollen (DTP) by NB treatment days, demonstrating that *HD6* might be involved in photoperiodic floral induction of maize.

MATERIALS AND METHODS

Plant materials and photoperiodic treatments

The tropical maize inbred line CML288 was obtained from the Mexico International Maize and Wheat Improvement Center (CIMMYT). This species is normally viable when grown under tropical short-day conditions (Sanya, China, 18°45′N, 109°30′E), but tassels very late, and does not silk in temperate long-day environments (Zhengzhou, China, 34°43′N, 113°43′E) (Wu et al., 2007; Wang et al., 2008).

Photoperiodic treatment was carried out in the trial area of Zhengzhou, Henan Province, China. Seeds were planted in plastic pots (35 cm diameter h 28 cm depth) in summer in 2008 and 2009. Germinated seedlings were grown in one canopy with SD conditions consisting of 9 h light/15 h dark (9:00 to 18:00 o'clock). For NB treatment, plants at the 4th, 5th, 6th, 7th, and 8th new full-

expansion leaf stage were transferred to another canopy to receive 30 min of illumination from 2 cool-white fluorescent tubes (100 µmol $m^{-2}s^{-1}$) in the middle of the 15 h night for 1, 3, 5, 7, 10, or 15 days, and then transferred back to SD conditions. In contrast, control plants were placed under constant SD conditions. The temperature in the canopy during the night was controlled to approximate the outside temperature. To determine the effect of NB on flowering, about 10 plants in each treatment grew under SD conditions until flowering to calculate DTT and DTP. The remaining plants were prepared for gene expression analysis. At the 5th stage, the 5th leaves and stem apexes (SAs) were excised every 4h of one photoperiod, starting at 0:00 o′clock just before NB treatment. From the 4th to 8th new full-expansion leaf stage, leaves and SAs were harvested at midnight. All samples were frozen immediately with liquid nitrogen and stored at -80℃ prior to RNA extraction. Each tissue sample was collected from three different, randomly selected plants. Three biological replicates were collected at the same time.

Nucleic acid isolation and cDNA synthesis

Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA synthesis was performed using Oligo$(dT)_{15}$ and reverse transcriptase M-MLV (RNase H^-) (TaKaRa). All protocols were performed according to the manufacturer’s instructions. cDNA was stored at -20℃ for gene clone and expression analysis.

Isolation of *HD6* homologs from maize

Partial clones of the *HD6* gene were amplified with gene-specific primers (F1:5′-TCCATCGTTCGTCGGCTCG-3′ and F2:5′-TAACAAGTTGATCATGGTTG-3′). These primers were designed based on the *Oryza sativa Hd6* (ABB17669) from the NCBI database (http://www.ncbi.nlm.nih.gov). A primer (5′-CTCCAGGTACTTCAAGGGT-3′) based on the partial clone was used to obtain the 3′ sequence with the 3′-Full RACE Core Set kit (TaKaRa). The full-length cDNA of the *HD6 gene* was obtained with primers whose design was based on the spliced sequence of the partial clone and the 3′ sequence.

Gene expression analysis

Stored cDNA was used as template for gene expression analysis. Real-time PCR was performed on the ABI 7500 Real time PCR system using SYBR Premix Ex Taq II (TakaRa) for analyzing gene expression. The amplification reactions were performed under the following PCR conditions: one cycle at 94℃ for 180 s, followed by 35 cycles of 94℃ for 10 s, 48℃ for 30 s and 72℃ for 30 s. Each reaction was performed in triplicate. The primers across an intron for *HD6 gene* were HQ_1 (5′-CCCGACACTAACAGATTA-3′) and HQ_2 (5′-AGCCGGAGTTTCCGAA-3′). The primers for a reference gene 18S were S_1 (5′-CCTGCGGCTTAATTGACTC-3′) and S_2 (5′-GTTAGCAGGCTGAGGTCTCG-3′). The reaction volume was 25 µl, consisting of 10 × PCR buffer (Mg^{2+}), 4 µl; 10 mmol·L^{-1} dNTP, 0.5 µl; 20× SYBR dye, 1 µl; Plus-Taq, 0.3 µl; forward and reverse primers, 0.6 µl; water, 16 µl; and cDNA template, 2 µl. The housekeeper gene 18S was used as endogenous control to normalize expression in maize tissues.

RESULTS

The effects of NB on flowering in maize

To determine whether NB treatment affected maize flowering time, one 30 min NB treatment was performed at the 4th, 5th, 6th, 7th, and 8th leaf developmental stage. The results showed that NB caused detectable flowering delay even at the earliest stage examined and that the extent of delay in DTT was consistent with that in DTP (Figure 1A). Moreover, when treated at the 5th-leaf stage, DTT and DTP were increased by 5.2 and 5.5 d respectively, which were the greatest changes induced of all of the various NB treatments, indicating that the 5th-leaf stage is the most sensitive to NB.

Therefore, the 5th-leaf stage was targeted to determine the effect of time on NB treatment. Each NB was scheduled to last 1, 3, 5, 7, 10, or 15 days. The results clearly demonstrated that the length of NB treatment was positively correlated to DTT and DTP (Figure 1B). When NB treatment was applied for 15 days, DTT and DTP were increased 20 and 17 d, respectively compared with control plants, but complete inhibition of flowering did not occur. These results indicate that the tropical maize inbred line CML288 is an ideal PS material and that NB treatment provides an appropriate experimental tool for investigating the molecular basis of the photoperiodic control of the flowering of maize.

Isolation of *HD6* from maize and function analyses

Full-length cDNA of the *HD6 gene* (GenBank Accession Number ABO09630) were amplified using primers based on the spliced sequence of the partial clone and 3′RACE clone. It contained an ORF of 999 bp encoding 332 amino acids. A homology tree created using DNAMAN 6.0 software showed that the deduced polypeptide sequence of *HD6* had high homology with CK2α derived from other plants. It was found that *HD6* has 99% identity with ZmCK2*α*-3, 95% with OsHd6 and TaCK2*α*, 92% with AtCK2Aα-2 and CK2α-1 (Figure 2).

Multiple alignments of the predicted complete amino acid sequences of HD6 with CK2α from several plant species indicated that HD6 of maize was more similar to ZmCK2α-3 than to other CK2α. Moreover, CK2 domain I, CK2 domain II, the ATP binding site and NLS, sites essential to CK2α activities conserved in other plant species were also found in ZmHD6. All of these sites were located at the same positions as those of CK2α from other plants. This stringent conservation among evolutionary diverse plant species indicate that *HD6* may function as a CK2α (Figure 3).

NB altered diurnal oscillations of *HD6* mRNA levels

Under SD conditions, low levels of *HD6* mRNA were observed both in leaves and SAs during the day, then its expression started increasing toward dusk, and peaked at 21:00 and 5:00 o′clock of darkness respectively (Figure 4A and B).

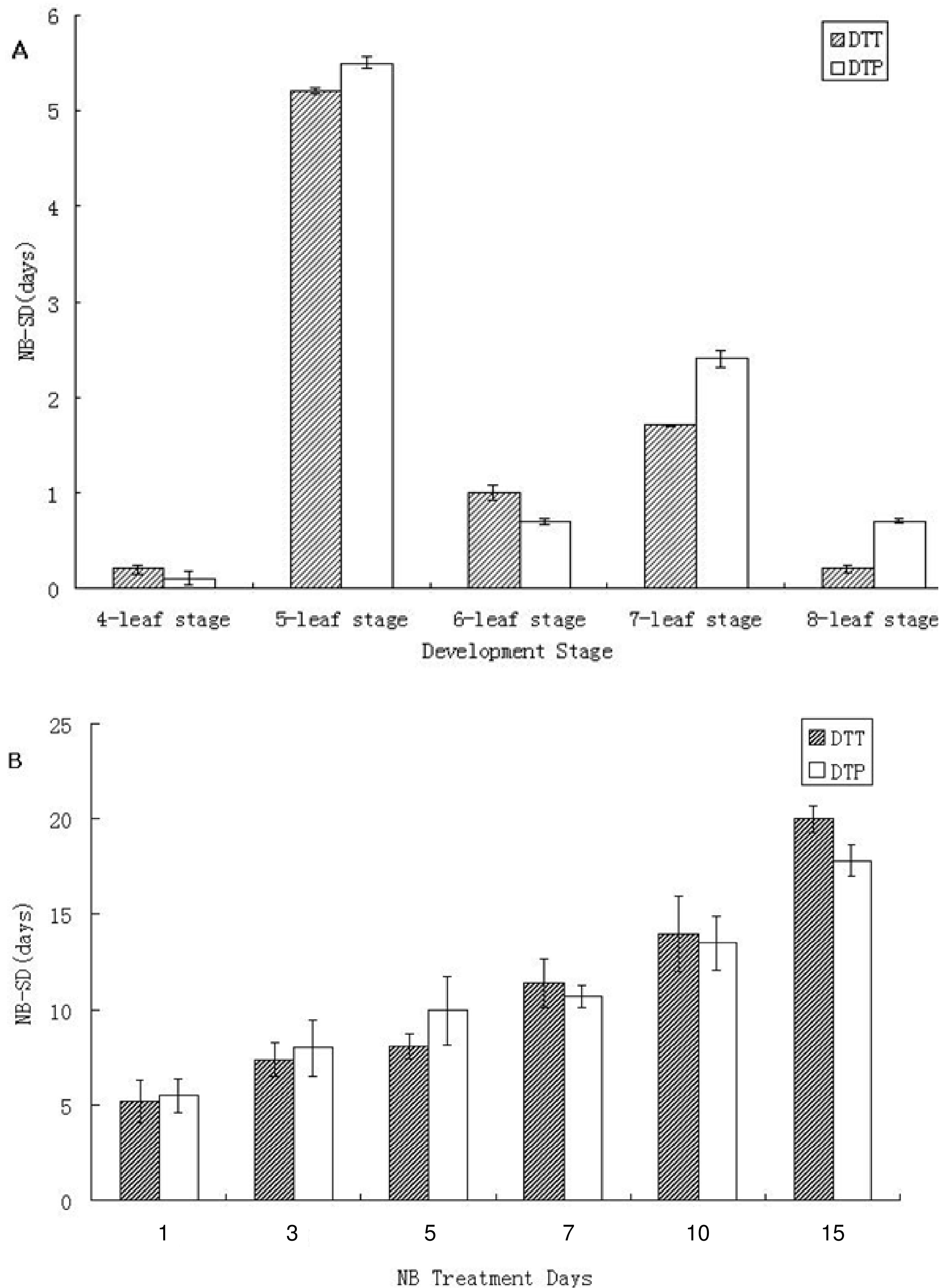

Figure 1. Effect of NB on the flowering time of maize. (A) The flowering times of plants treated with one NB at different developmental stages. Day to tassel (DTT) and day to pollen (DTP) were the dates of tassel or pollen emergence of each plant. Each figures in graphs illustrated the mean of five plants by subtracting values of control plants (SD). (B) The flowering times of plants treated with NB for different lengths of time at the 5th-leaf stage.

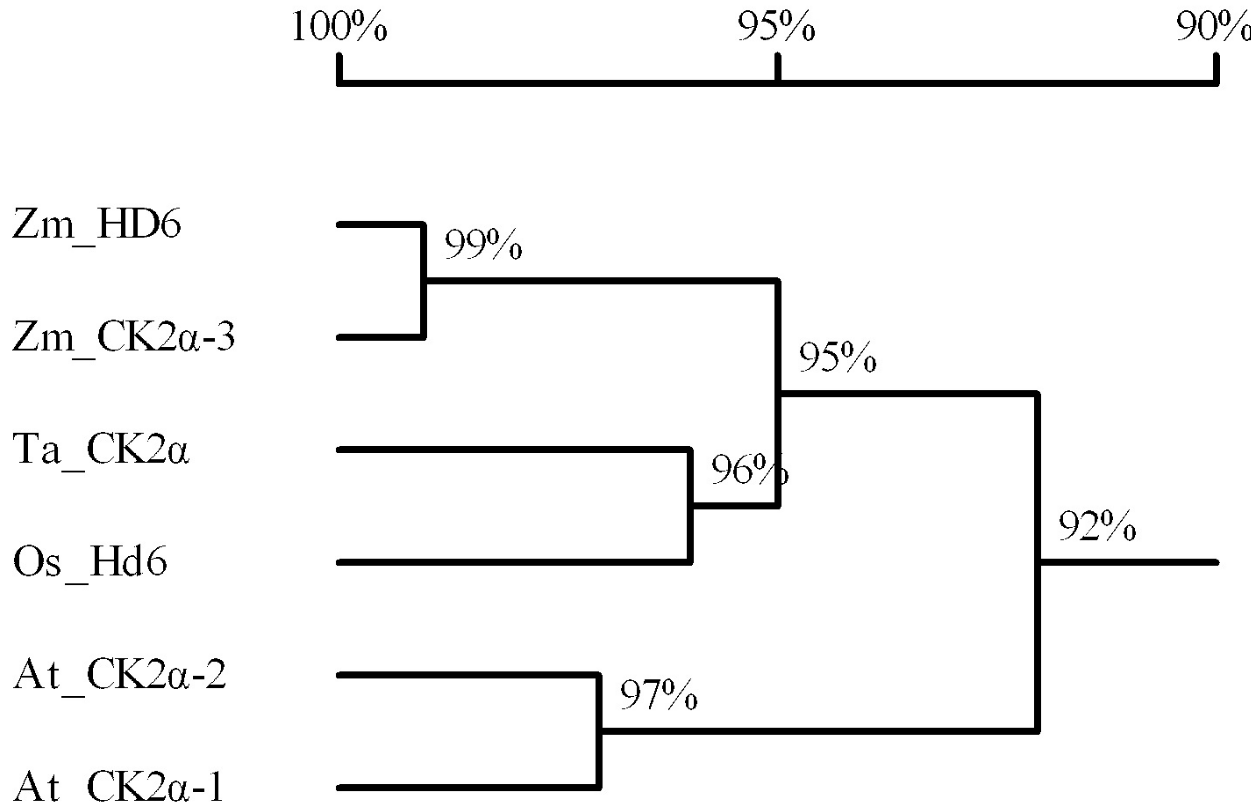

Figure 2. A phylogenetic tree of maize HD6. The predicted sequences of HD6 proteins were aligned using DNAMAN software. Protein sequences taken from GenBank are the following: *Zea mays CK2α-3* (AF239819), *Oryza sativa Hd6* (ABB17669), *Triticum aestivum L. CK2α* (AB052133), *Arabidopsis CK2A2* and *CK2A1* (Q08466, Q08467).

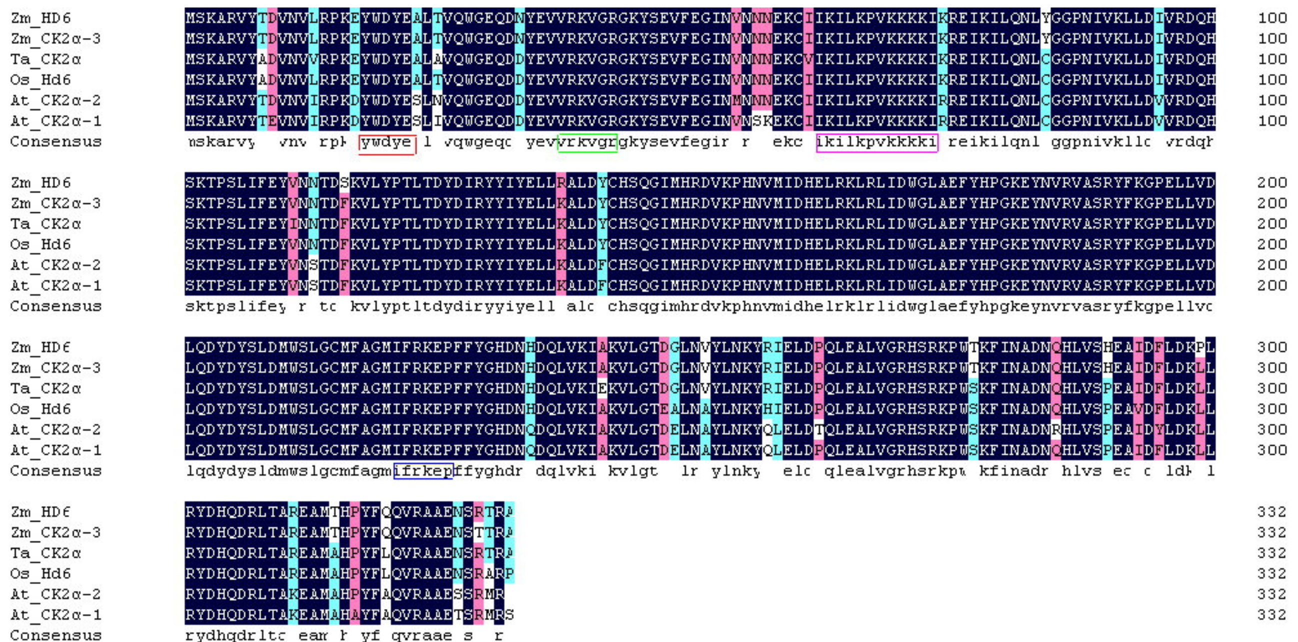

Figure 3. Multi-alignment of *HD6* with several *CK2α* from other flowering plants. Blue shadows indicate invariant residues in all aligned kinases. Colorful frames below the alignment indicate functionally important conserved domains. The red frame denotes CK2 domain I, the green frame denotes the residues of the ATP binding site, the pink frame denotes the nuclear localization signal (NLS), and the purple frame denotes CK2 domain II.

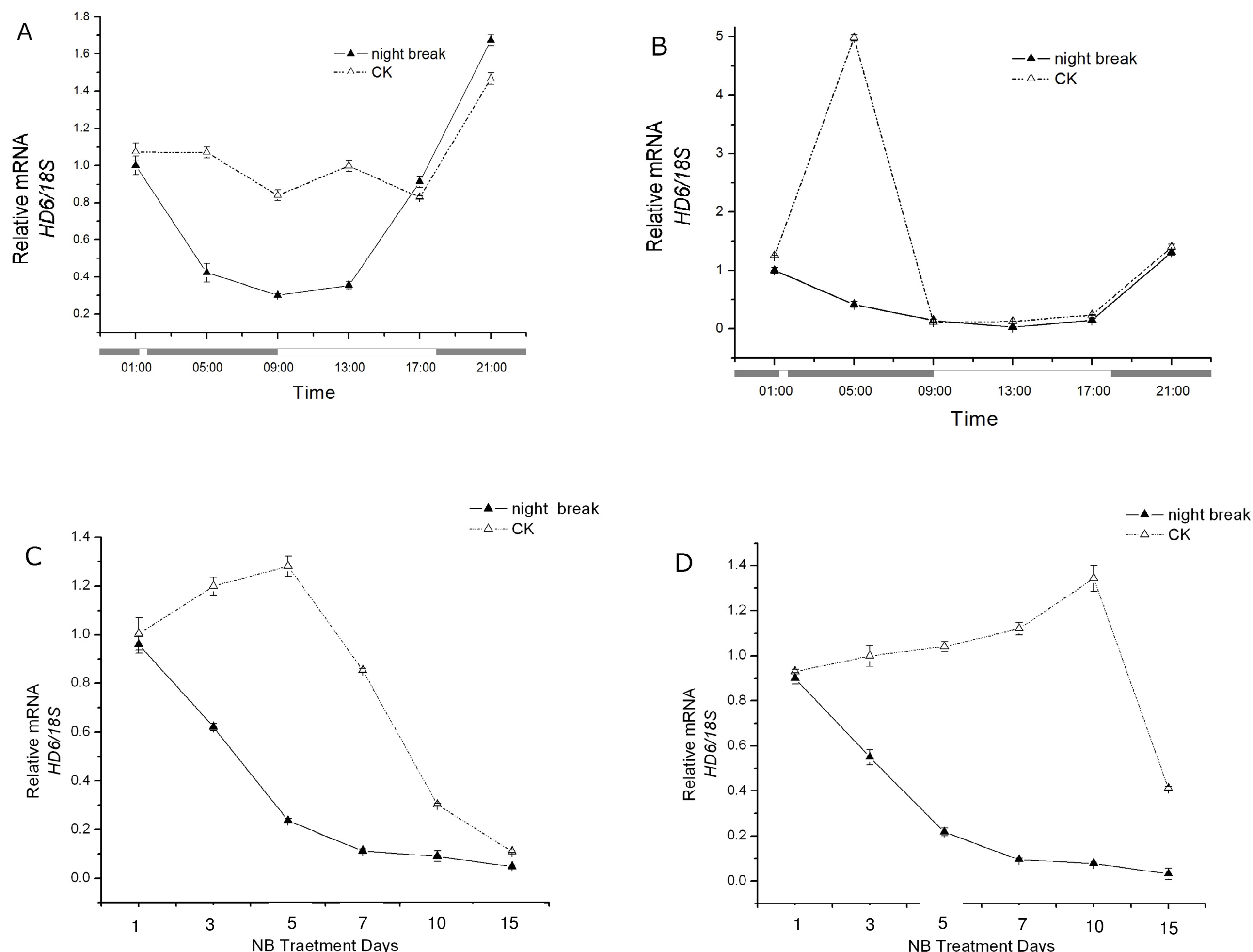

Figure 4. Real-time PCR was performed to quantify Zm *HD6*. Each mRNA was normalized relative to the *18S* mRNA level. Error bars show SE of 3 PCR experiments. A biological replicate provided a similar result. (A) and (B) A single NB treatment altered the rhythmic expression pattern of *HD6* in both leaves and SAs. The white and black bars at the bottom represent the light and dark period respectively. (C) and (D) The suppression of *HD6* mRNA in leaves and SAs were proportional to the number of NB treatment days.

In contrast, a 30 min NB treatment applied at 1:15 o′clock dramatically reduced *HD6* mRNA levels in leaves and SAs during the night. Its transcript abundance in leaves started to increase at dawn and reached approximately the same levels found in controls around 17:00 o′clock. In SAs, *HD6* mRNA reverted to the same levels as controls after dawn. Thus, exposure to night break altered the rhythmic expression pattern of *HD6* both in leaves and SAs, though the circadian patterns between them differ (Figure 4A and B).

The NB effect on the suppression of *HD6* mRNA was consistent with its effect on flowering

Because a single NB treatment altered the rhythmic expression pattern of *HD6*, we investigated the effect of multiple days of NB treatment on *HD6* expression. *HD6* expression was examined on the 1st, 3rd, 5th, 7th, 10th, and 15th day after the 5th-leaf stage at the peak of its expression. In the control plants that were not exposed to night breaks, *HD6* expression in leaves and SAs peaked at the fifth and tenth day after the 5th-leaf stage. In the presence of NB, no peak of *HD6* expression in leaves and SAs was observed. On the contrary, the abundance of the mRNAs in leaves was 2% (Figure 4C) and 6% (Figure 4D) of peaks in control plants. Furthermore, NB inhibited *HD6* expression both in leaves and in SAs, and the suppression of *HD6* mRNA was proportional to the number of NB treatment days. This curve of *HD6* mRNA suppression was very similar to a curve produced in a previous study of the NB effects on flowering. Thus, the effect of NB on the suppression of *HD6* mRNA was consistent with its effect on flowering delay. Taken together, these results strongly suggested that *HD6* might be involved in the photoperiodic floral inductive process of maize.

DISCUSSION

Since the 1930s, NB discovered by Hamner and Bonner (1938), has become a research tool for the response of plants to photoperiod. One or more brief irradiations with light during the inductive night serve as an effective NB treatment. NB effect has been used in both physiological and molecular research to suppress and unravel the complicated process of photoperiodic floral induction, and is the "litmus test" for whether an event is associated specifically with floral induction. Although there are many NB experiments on SDPs that have an obligate, qualitative sensitivity to photoperiod (Murakami et al., 2007; Ishikawa et al., 2005; O'Neill et al., 1998; Cháb et al., 2008), there is no similar study on maize.

Maize was domesticated in Southern Mexico and its presumed ancestor, teosinte *(Z. mays L.* ssp. *parviglumis*), likely evolved photoperiod sensitivity to synchronize its reproductive phases to the short-day season (Campos et al., 2006; Miller et al., 2008). Although photoperiod sensitivity in temperate maize was substantially reduced during the adaptation process by post-domestication evolution, it is very common in maize lines adapted to the tropics (Gouesnard et al., 2002). In this study, the tropical maize line CML288 was tested and found to be very sensitive to NB, so it can be classified as a qualitative SDP. In contrast, 5 and 7 days NB treatment at the 5-leaf stage were required for temperate maize lines B73 and Huangzao4 to produce a visible delay in DTT and DTP, reflecting that tropical and temperate germplasm produce different responses to NB.

In this study, the NB effect on the flowering delay was detectable at all stages examined. Furthermore, when treated with a single NB treatment at different developmental stages, the DTT and DTP of the 5th-leaf plants were the longest, and delayed about 5 d compared to control plants, indicating that the 5th-leaf stage is the most sensitive to NB. These results coincide with the study by Cole et al. that showed the photoperiod-sensitive phase of maize occurs early in development, often before plants have reached the 8-leaf stage (Coles et al., 2010). While Birch et al. research demonstrated that the inductive stage in maize commences and ends around the 8-leaf stage or a bit later (Birch et al., 1998a, b).

NB inhibited a number of SDPs flowering, such as rice, Pharbitis and Chenopodium, and it inhibited the expression of *FT* orthologs in these species, while mRNA accumulation of *CO* orthologs were not affected (Liu et al., 2001; Ishikawa et al., 2005; Hayama et al., 2007; Cháb et al., 2008). The functions of key regulators *FT* and CO in the photoperiodic flowering pathway are well characterized in *Arabidopsis* and other plant species. However, there are a few genes, studied using the NB tool, whose role in the photoperiodic floral induction process remain obscure (Sage-Ono et al., 1998; O'Neill et al., 1994, 1998; Kim et al., 2003). Our data demonstrated that accumulation of *HD6* mRNA in maize was inhibited by NB and that the curve of this inhibition by NB treatment days was consistent with that of flowering delay. Additionally, rhythmic expression of *HD6* was upregulated during the night of a 24 h circadian period in both leaves and SAs. These results are congruent with O'Neill criteria that state that mRNAs functionally associated with floral induction should (a) be regulated by darkness, (b) demonstrate a circadian rhythm in their regulation of abundance, and (c) be affected by NB treatment (O'Neill et al., 1994). Therefore, we speculate that *HD6* may be involved in the regulation of photoperiodically floral induction of maize.

Although a clear involvement with this process cannot be established from our findings, we also found that *HD6* of maize showed high homology with the *HD6* gene of rice and the *CK2α* gene of *Arabidopsis* and shared the same functional domains. The study in rice revealed that *Hd6* encodes a CK2α subunit and that it delays flowering under LD conditions by phosphorylation of an unknown protein that could work together with Hd1 to repress expression of *FT-Like* genes, such as *Hd3a* and *RFT1*, but this process is disconnected from circadian clock activity (Ogiso et al., 2010). Inconsistent with this, photoperiodic flowering-responses in *Arabidopsis* is influenced by circadian rhythms. The effects of CK2 on the circadian clock were due to its phosphorylation of a central clock component CCA1, which is important for the normal functioning of the central oscillator. Overexpression of one of its regulatory subunits, CKB3, increased CK2 activity and shorted the period of the circadian clock, resulting in flowering earlier in both long-day and short-day photoperiods (Sugano et al., 1999; Daniel et al., 2004). Therefore, the next step will be to identify the target protein that could be phosphorylated by *HD6* of maize to evaluate whether the role of *HD6* in flowering-time regulation is similar to rice or *Arabidopsis*. The possibility that *HD6* may have a unique regulatory function not seen in rice and *Arabidopsis* cannot be excluded. To understand fully the mechanisms underlying the functions of *HD6*, overexpression of this gene in transgenic plants may be particularly useful in this regard.

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