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RESEARCH PAPER

ZCN8 encodes a potential orthologue of *Arabidopsis* FT florigen that integrates both endogenous and photoperiod flowering signals in maize

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Abstract

Higher plants use multiple perceptive measures to coordinate flowering time with environmental and endogenous cues. Physiological studies show that florigen is a mobile factor that transmits floral inductive signals from the leaf to the shoot apex. *Arabidopsis* FT protein is widely regarded as the archetype florigen found in diverse plant species, particularly in plants that use inductive photoperiods to flower. Recently, a large family of *FT* homologues in maize, the *Zea CENTRORADIALIS* (*ZCN*) genes, was described, suggesting that maize also contains FT-related proteins that act as a florigen. The product of one member of this large family, *ZCN8*, has several attributes that make it a good candidate as a maize florigen. Mechanisms underlying the floral transition in maize are less well understood than those of other species, partly because flowering in temperate maize is dependent largely on endogenous signals. The maize *indeterminate1* (*id1*) gene is an important regulator of maize autonomous flowering that acts in leaves to mediate the transmission or production of florigenic signals. This study finds that *id1* acts upstream of *ZCN8* to control its expression, suggesting a possible new link to flowering in day-neutral maize. Moreover, in teosinte, a tropical progenitor of maize that requires short-day photoperiods to induce flowering, *ZCN8* is highly up-regulated in leaves under inductive photoperiods. Finally, vascular-specific expression of *ZCN8* in *Arabidopsis* complements the *ft-1* mutation, demonstrating that leaf-specific expression of *ZCN8* can induce flowering. These results suggest that *ZCN8* may encode a florigen that integrates both endogenous and environmental signals in maize.

Key words: florigen, *FT* orthologue, long-distance signalling, maize, photoperiod induction, teosinte, transcription factor.

Introduction

Timing of the transition from vegetative to reproductive growth in higher plants is a finely orchestrated process that is controlled through the integration of environmental cues and developmental signals. Genetic and molecular studies have defined key components that cause flowering to occur for optimal reproductive success (reviewed in Amasino and Michaels, 2010). More recently, a leaf-derived protein, identified as the product of the *Arabidopsis Flowering Locus T* gene (*FT*), has been identified and is now widely accepted as the long sought mobile florigen inferred from early physiological studies (Corbesier *et al.*, 2007; Giakountis and Coupland, 2008). The FT protein, which encodes a phosphatidylethanolamine-binding (PEBP)-related kinase, interacts with Flowering Locus D (FD), a bZIP protein, at the vegetative shoot apex (Abe *et al.*, 2005; Wigge *et al.*, 2005). The FT–FD complex subsequently activates transcription of floral meristem genes that start the flowering process, such as *APETALA1* (*AP1*).

Activation of *FT* in response to inductive environmental cues, such as photoperiod, and subsequent movement of FT protein to the shoot apex, satisfies one of the original criteria for a florigenic substance (i.e. that it is a phloem mobile signal that passes through living tissue to its target). Another tenet of the florigen hypothesis is that the mobile inductive signal is universal and should be found in all flowering plants (Zeevaart, 1976). Although there is no

a priori reason why all plants should have similar versions of FT protein that signal time to flowering, FT orthologues are being discovered in many plant species (e.g. Bohlenius *et al.*, 2006; Gyllenstrand *et al.*, 2007; Hayama *et al.*, 2007; Hou and Yang, 2009; Blackman *et al.*, 2010; Kong *et al.*, 2010), including the grasses (Yan *et al.*, 2006; Faure *et al.*, 2007; Danilevskaya *et al.*, 2008; Komiya *et al.*, 2008; Kikuchi *et al.*, 2009). Therefore, the role of FT-like proteins in transmitting inductive signals in diverse plants suggests that it has a conserved ancestral function.

Genetic analyses of *Arabidopsis* flowering time mutants have defined a complex network of interacting flowering time genes that have orthologues in a wide variety of plants. However, given the complex nature of the floral induction process, it might be expected that other floral induction mechanisms unique to a particular subgroup, such as monocots, might have evolved that rely on pathways tailored for specific environmental conditions or developmental programmes. Indeed, many of the conserved flowering time genes first identified in *Arabidopsis* are present in the grasses, but some genes appear to be unique and are not present in other species (Colasanti and Coneva, 2009). In maize, only two genes have been shown to have a major effect on flowering time when mutated—*indeterminate1* (*id1*) and *delayed flowering1* (*dlf1*). Numerous quantitative trait loci (QTLs) associated with effects on flowering in domesticated maize have been discovered (Chardon *et al.*, 2004; Buckler *et al.*, 2009), but the identity of the genes underlying these QTLs has yet to be revealed.

Mutant *id1* plants exhibit an extreme late flowering phenotype in which they produce more than twice as many leaves as normal maize and usually fail to form axillary ear inflorescences (Singleton, 1946; Colasanti *et al.*, 1998). Loss-of-function *dlf1* mutants exhibit a less severe flowering time defect; that is, they make several more leaves and usually flower 1–2 weeks later than normal plants under field conditions (Neuffer *et al.*, 1997). The *id1* gene encodes a putative transcription factor that is expressed exclusively in the non-photosynthetic region of developing, immature leaves (Colasanti *et al.*, 1998; Wong and Colasanti, 2007), whereas DLF1 is a bZIP protein that has been proposed to regulate flowering in the shoot apical region (Muszynski *et al.*, 2006).

Functional equivalents of *id1* have been identified in rice (*OsId1*, *Ehd2*, or *RID*), and reduced expression or elimination of this gene was found to cause a severe delay in flowering (Matsubara *et al.*, 2008; Park *et al.*, 2008), or even result in rice plants that never flower (Wu *et al.*, 2008). In dicot species, no clear orthologue of maize *id1* has been identified amongst the large *id1*-related gene family (*IDD* genes) found in all higher plants (Colasanti *et al.*, 2006), although a mutation in an *Arabidopsis IDD* gene was reported recently to have a minor effect on flowering time (Seo *et al.*, 2011). Conversely, the *dlf1* gene encodes a putative transcription factor that is a likely orthologue of the *Arabidopsis FD* gene (Muszynski *et al.*, 2006). If DLF protein acts in a manner similar to FD, an equivalent to FT may be presumed to exist in maize as well. Maize contains a large family of *FT*/*TFL*-related genes, named *Zea CENTRORADIALIS* (*ZCN*) genes (Danilevskaya *et al.*, 2008). Sequence comparisons separate the 25 *ZCN* genes into both *FT* and *TFL* (*TERMINAL FLOWER*) clades. Ectopic expression of seven *ZCN* genes most similar to *TFL* in transgenic maize showed a possible role in inhibiting flowering, therefore suggesting a role similar to that of *TFL* genes found in *Arabidopsis* and other species (Danilevskaya *et al.*, 2010).

The question remains as to which, if any, of the maize *ZCN* genes have a role similar to that of *Arabidopsis FT* in encoding a florigen protein. The initial analysis of the maize *ZCN* gene family by Danilevskya *et al.* (2008) pointed to *ZCN8* as a strong candidate to encode an FT orthologue based on high sequence similarity to *FT*, a leaf-specific expression pattern, and possible interaction with DLF. To provide further evidence as to whether maize contains a putative FT-like florigen, the expression and function of the *ZCN8* gene are examined here. Transcripts of the *ZCN8* gene are localized to mature leaves of temperate maize and teosinte, a maize ancestor that requires short-day photoperiods to induce flowering. Inductive short-day photoperiods result in a dramatic increase in *ZCN8* expression in teosinte, whereas a less prominent increase was detected in temperate maize. In addition, ZCN8 expression was found to be dependent on the activity of the *id1* gene in temperate maize. Finally, evidence that ZCN8 may have the ability to act as a florigen was demonstrated by expressing it under the control of a phloem companion cell-specific promoter that completely rescued the *ft* mutant phenotype in *Arabidopsis*.

Materials and methods

Plant growth conditions and genotyping

All plants were grown in the Phytotron facility at the University of Guelph. Temperate maize (*Zea mays* ssp *mays*; B73 inbred) and tropical maize, teosinte (*Zea mays* ssp *parviglumis*) were grown in a 2:1 mixture of Sunshine Mix and Turface clay pellets. One corner of each teosinte seed was clipped (~2 mm) to aid in germination. All plants were grown in Conviron growth chambers under full spectrum mixed fluorescent and incandescent light of 500 μmol m^{-2} s^{-1}. Long-day (LD) conditions consisted of 15 h light/9 h darkness, and short-day (SD) conditions were 9 h light/15 h darkness. Day temperatures were set at 27 °C and night temperatures at 23 °C. Plants were fertilized at initial planting with 14-14-14 slow release fertilizer. Maize segregating normal (wild type; WT) plants and the *id1-m1* mutant allele were genotyped at the three visible leaf stage (V3) by PCR as described in Wong and Colasanti (2007). B73 inbred maize was used as the WT control; the *id1-m1* mutant allele used was backcrossed 10 times into the B73 background. Tissue samples were taken at various stages of growth as indicated below. For floral induction experiments, all plants were grown in LD conditions until they made four leaves (V4), at which point half of the plants were transferred to SD conditions until they made eight visible leaves.

WT *Arabidopsis thaliana* ecotype Landsberg (Ler) and *ft-1* mutant and transgenic lines were grown on Sunshine Mix soil in 3 inch pots after 4 d of seed stratification at 4 °C. Plants were grown in Conviron chambers under full spectrum light of 150 μmol m^{-2} s^{-1} at temperatures of 22 °C during the day and 20°C at night. For

LD experiments, plants were grown in light for 16 h and 8 h of darkness, while SD growth was 8 h days/16 h nights.

Recombinant plasmid construction

All constructs were made using Gateway® Cloning vectors and kits (Invitrogen). For all genes and promoters the pDONR-221 P4-P1r empty entry vector was used. The *ZCN8* gene was amplified from cDNA derived from B73 maize leaf total RNA using primers flanking the entire coding sequence (ZmZCN8F and ZmZCN8R) (Supplementary Table S2 available at *JXB* online). ENTRY vector constructs were then used for Gateway recombination into the destination vector pK2GW7 for cauliflower mosaic virus (CaMV) 35S overexpression. For construction of the SUC2::ZCN8 plasmid, separate entry clones containing ZCN8 and the SUC2 promoter were recombined into plasmid pK7m24GW3 (University of Ghent, Belgium) via multi-site Gateway recombination.

Arabidopsis *transformation with* Agrobacterium tumefaciens

Transformation of WT Ler-0 and *ft-1* mutant plants with *Agrobacterium* bacteria carrying recombinant constructs was performed using the floral dip method (Clough and Bent, 1998). For each construct, several independent lines were selected and T_3 transgenic plants were analysed for flowering time and other phenotypes. CaMV35S::ZCN8 overexpression constructs were transformed into Ler-0 and *ft-1* plants; SUC2::ZCN8 lines were generated in the *ft-1* mutant background only.

RNA isolation, PCR, and quantitative real-time PCR

Total RNA from maize and teosinte was extracted from frozen tissue using TRIZOL reagent (Invitrogen) and purified on RNeasy columns (Qiagen). *Arabidopsis* total RNA was extracted from frozen tissue as described previously (Tanimoto *et al.*, 2008). Reverse transcription reactions were performed using the Quanta cDNA Superscript system (Quanta) according to the manufacturer's instructions. Primer sets used for quantitative real-time PCR (qRT-PCR) are listed in Supplementary Table S2 at *JXB* online. qRT-PCR was performed with 3–6 independent biological replicates and three technical replicates for each sample. Data were analysed using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). Expression levels of specific genes were normalized to maize *β-tubulin* or *Arabidopsis* glyceraldehyde 3-phosphate dehydrogenase (Supplementary Table S2). Statistical significance between any pair of relative expression means was assessed with a *t*-test.

Results

Selection of the maize ZCN8 *gene for functional testing*

The maize genome contains at least 25 members of the PEBP gene family, encompassing both *FT*- and *TFL*-related genes (Danilevskaya *et al.*, 2008). Several lines of evidence suggest that *ZCN8* could be the functional equivalent of *FT* in maize. Although sequence comparisons of *ZCN* genes with *FT* and *FT*-related genes make it difficult to identify any particular ZCN as the clear orthologue of FT, as shown in Fig. 1A, ZCN8 is highly similar to FT and the rice orthologue of FT, Hd3a (74% amino acid identity to both). Moreover, the gene structure of *ZCN8* is similar to that of *FT* and *Hd3a* (Fig. 1B). A preliminary semi-quantitative PCR analysis by Danilevskya *et al.* (2008) and qRT-PCR analysis in this study (data not shown) suggests that *ZCN8* is one of only two *ZCN* genes (the other being *ZCN26*) that is expressed mainly in leaf tissue, the presumed location

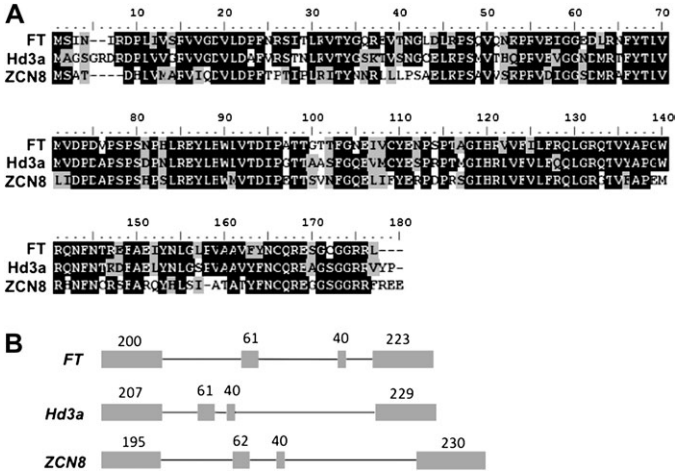

Fig. 1. Comparison of maize ZCN8, rice Hd3a, and *Arabidopsis* FT. (A) Sequence alignment of amino acid sequences. Identical regions are shaded in black. (B) Gene structures of *ZCN8*, *Hd3a*, and *FT*. Boxes represent exons and thin lines are introns. Exons size is indicated above each box.

where a florigen-encoding gene would be expressed. Therefore, the *ZCN8* gene was selected for further analysis.

ZCN8 *expression is highly up-regulated upon floral induction in mature leaves of teosinte*

Teosinte (*Z. mays* ssp *parviglumis*) is an obligate SD plant that has an absolute requirement for SD photoperiods to induce flowering (Emerson, 1924). In contrast, in temperate maize, typified by inbred line B73, the transition to flowering occurs after a particular number of leaves are produced. B73 plants with 7–8 visible leaves are at the floral transition stage; that is, the vegetative shoot apex has initiated all leaves, but conversion into an inflorescence meristem has yet to commence (McSteen *et al.*, 2000). For this study, expression of *ZCN8* in B73 was analysed at the 8-leaf floral transition stage after growth under SD conditions, the same exposure given to teosinte for photoinduction (Fig. 2; Supplementary Fig. S1 at *JXB* online). Growth of teosinte under SD conditions for ⩾7 d results in 100% conversion of plants to reproductive growth (data not shown).

For both maize and teosinte, the blade region of leaf 4 and leaf 7 was isolated from each plant to assess expression levels of *ZCN8* by qRT-PCR. Only leaf tissue was analysed because *ZCN8* transcripts were not detected in immature leaf tissue or shoot apical regions (data not shown). In addition, samples were taken at several time points during the course of a day (2, 6, 10, and 14 h after dawn) to assess the effects of light and dark exposure on *ZCN8* expression. As shown in Fig. 2A, *ZCN8* leaf transcript levels were much higher under SD conditions compared with LD-grown teosinte plants at all time points, with *ZCN8* levels ~10 times higher overall for time points 2, 6, and 10 h, and up to 60–80 times higher at the 14 h time point. Interestingly, SD-grown B73 leaves also showed an increase in *ZCN8* transcript levels, but the induction level was much lower than that observed for teosinte (Fig. 2B). Another

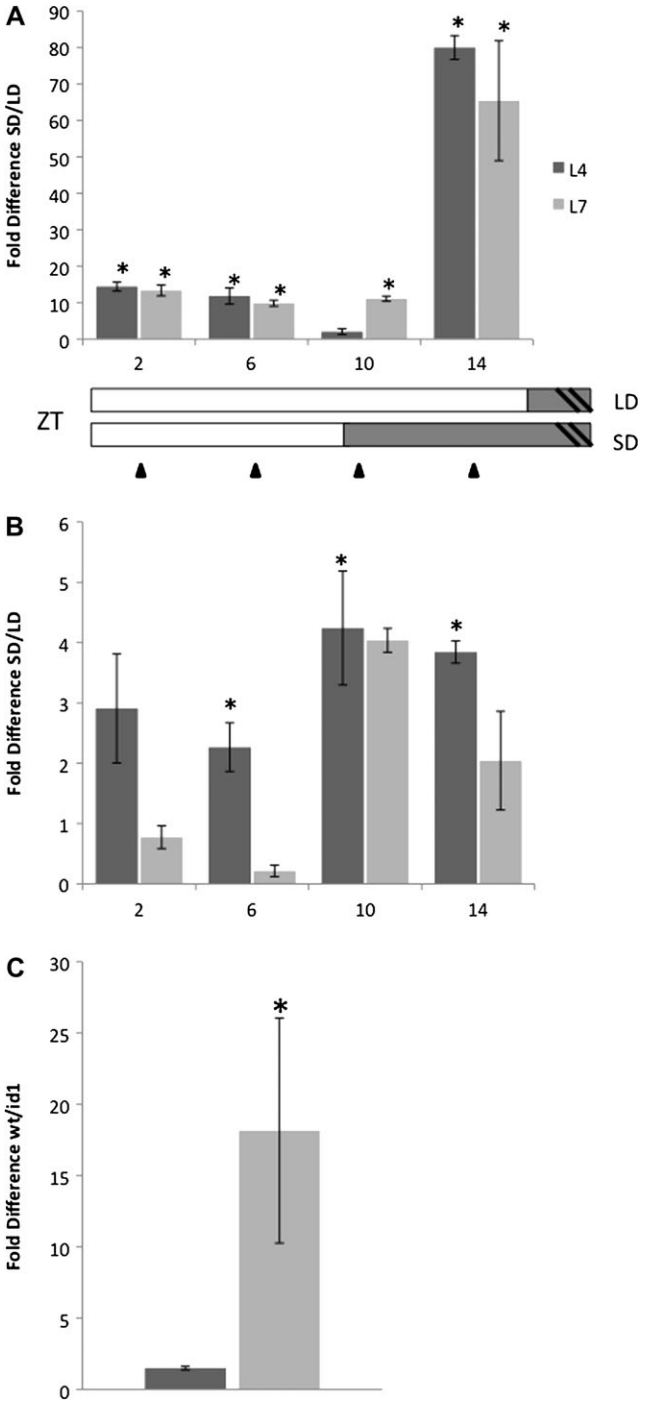

Fig. 2. Quantitative RT-PCR analysis of *ZCN8* expression in maize and teosinte leaves at the V8 stage. (A) Expression of *ZCN8* in short days (SDs) relative to long days (LDs) in teosinte for four Zeitgeber time (ZT) points, where ZT0 represents the start of light cycle. (B) Comparison of *ZCN8* expression in SD- relative to LD-grown maize at the same ZTs as in (A). (C) Fold change in *ZCN8* expression in leaves of *id1* mutants relative to WT plants. Samples were taken from leaf 4 (dark bar) and leaf 7 (light bar) at ZT6. All expression values are normalized to maize *β-tubulin*. $2^{-\Delta\Delta Ct}$ values with standard error are reported, where the average normalized expression in LDs for each ZT point is used as a calibrator. Relative quantification values obtained represent the fold change in *ZCN8* expression in SDs compared with LDs at each ZT. In (C), average normalized expression in *id1* leaves is used as a calibrator to generate fold induction in *ZCN8* expression in WT relative to *id1* plants. Statistical significance (P <0.05, denoted by an asterisk) of fold difference values is determined by a *t*-test. Horizontal bars show day/night cycles, and sampling time points are denoted by inverted triangles.

interesting observation is that *ZCN8* expression in teosinte shows greater relative induction at 14 h after dawn (Fig 2A). At this time point plants have been grown in the dark for 5 h, suggesting that *ZCN8* mRNA may accumulate in the dark. A similar effect was observed in B73 plants, but to a much lesser extent (Fig. 2B). However, the expression differences were much more variable in B73, so it is difficult to assign a pattern to the expression profile.

ZCN8 *expression is dependent on* id1 *activity*

Expression of *ZCN8* in normal ('WT') and *id1* mutant maize leaves was analysed to determine whether the absence of *id1* activity affected *ZCN8* transcript accumulation. Loss-of-function *id1* mutants are indistinguishable from normal maize until the floral transition point (Coneva *et al.*, 2007). In addition, *id1* transcript accumulation is highest at the floral transition stage; therefore, expression levels in leaves from plants at this stage of development were determined. As shown in Fig. 2C, *ZCN8* levels in leaves of *id1* mutants are severely reduced compared with WT plants at the transition stage. The difference in transcription was most dramatic in leaf 7, where WT plants had on average >15 times the level of *ZCN8* RNA. This contrasts with the SD versus LD analysis in B73 (Fig. 2B), where higher expression differences occur in leaf 4 compared with leaf 7 under SD conditions. Overall, these results show that normal *id1* function in leaves is associated with high levels of *ZCN8* expression. Analysis of expression in immature leaves and shoot apical regions of *id1* mutants showed no *ZCN8* accumulation in these tissues, demonstrating that loss of *id1* function does not alter the *ZCN8* expression pattern (data not shown).

Ectopic overexpression and phloem-specific expression of ZCN8 *in* Arabidopsis *causes early flowering and complements the* ft *mutant phenotype*

Ectopic overexpression via the 35S promoter and phloem companion cell-specific expression via the SUC2 promoter provided evidence that *ZCN8* encodes a protein that acts as a floral regulator in *Arabidopsis*. A total of five independent transgenic lines with the 35S::ZCN8 construct were selected for flowering time analysis; lines Z2 and Z5 were created by transforming the 35S::ZCN8 construct directly into *ft-1* mutant plants, and X2, Y1 and F3 lines carried the construct in Ler WT plants. For most of these lines, flowering time was shown to be significantly earlier than in WT or *ft-1* mutant plants under inductive LD conditions

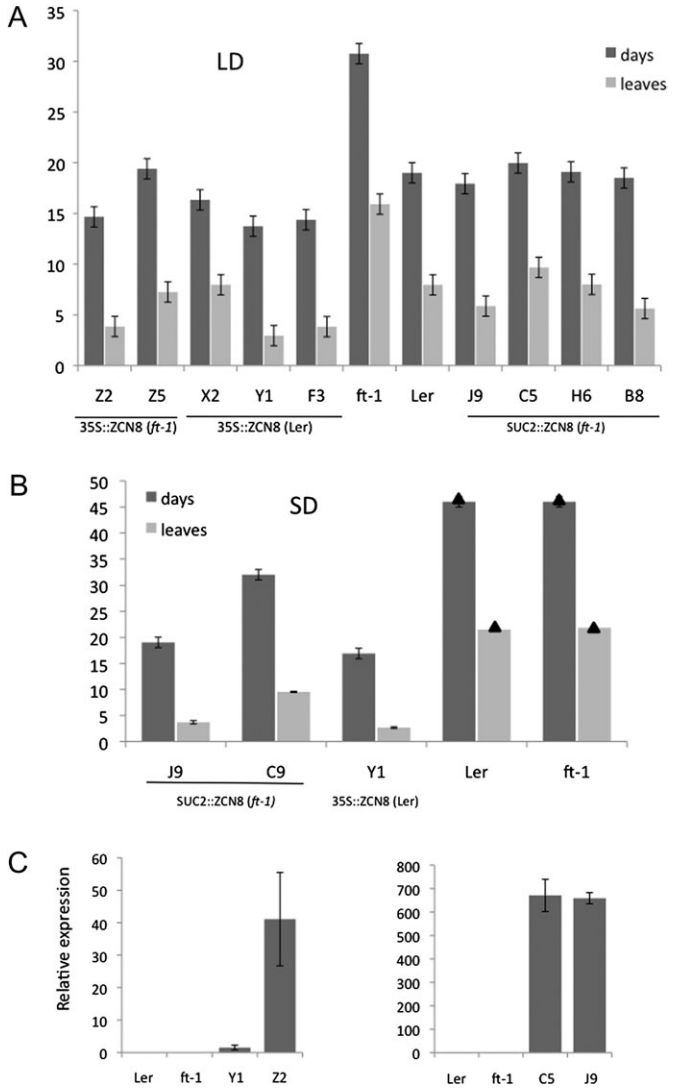

Fig. 3. Ectopic expression of maize *ZCN8* causes early flowering in *Arabidopsis*. (A) Days and leaves to bolting for several ectopic overexpression (35S) and phloem companion cell-specific expression (SUC2) *ZCN8* transgenic *Arabidopsis* lines in the Ler or *ft-1* background grown in long-day conditions. (B) Days and leaves to bolting for transgenic lines as well as Ler and *ft-1* plants grown in short days. Arrows at the tops of bars for *ft-1* and Ler indicate that plants have not flowered. (C) Expression of *ZCN8* in selected transgenic lines compared with untransformed Ler and *ft-1* plants was determined by quantitative RT-PCR.

(Fig. 3A; 4A Supplementary Table S1 at *JXB* online). Lines Y1 and F3 flowered 3–5 d earlier and made 4–5 fewer leaves than the Ler controls, whereas X2 made about the same number of leaves as Ler, even though it flowered ~2 d earlier. This discrepancy may reflect the pleiotropic effects of *ZCN8* overexpression in *Arabidopsis*. For many lines, ectopic expression caused developmental abnormalities, such as leaf curling (Fig. 4B, C) and floral defects (not shown). Terminal flower phenotypes, where the main inflorescence is converted into a single flower, were often observed as well (Fig. 4B, C). Similar abnormalities are also observed when *FT* is overexpressed by the 35S promoter (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999).

Expression of *ZCN8* in phloem companion cells also rescued the *ft-1* mutation, but did not cause developmental defects (Fig. 4A, D). Ten SUC2::ZCN8 lines in *ft-1* mutants were created (Supplementary Table S1 at *JXB* online); flowering time and leaf number for four lines under LDs are shown in Fig. 3A. Under these conditions, all lines flowered at about the same time and made as many leaves as the WT (~20 d, eight leaves) or were slightly earlier.

Flowering of the overexpressing line Y1 and the two SUC2::ZCN8 lines (C9 and J9) was compared with that of the WT and *ft* mutants under non-inductive SD conditions (Fig. 3B). As under LD conditions, Y1 flowered extremely early, making fewer than three leaves on average, and showing a terminal flower phenotype in some cases (Fig. 4C). SUC2::ZCN8 lines grown in SDs flowered at about the same time as WT plants grown under inductive LDs (Figs 3A, B, 4D). As was observed with LD growth, SUC2::ZCN8 lines showed no developmental defects. Several transgenic lines were selected for qRT-PCR analysis of expression levels in mature leaves. As can be seen in Fig. 3C, all lines showed expression of the *ZCN8* transgene. Unexpectedly, the SUC2::ZCN8 lines showed significantly higher expression than the line Y1, which had one of the strongest phenotypes and showed developments defects. It is possible that ectopic expression of *ZCN8* in the shoot apical region has a more dramatic effect than phloem companion cell expression (see Discussion).

Overall, these data show that ectopic expression of *ZCN8* in *Arabidopsis* is able to compensate for the lack of a functional *FT* gene. Similarly, overexpression under SD conditions simulates growth under inductive LDs, as would be expected for ectopic expression of *FT*.

Discussion

Long-standing physiological evidence predicted the existence of a mobile, leaf-derived flower-inducing signal, even though the identification and isolation of florigenic compounds turned out to be elusive. It is now widely accepted that *Arabidopsis* FT protein is a florigen and most, if not all, plants may have orthologous versions of FT that act as mobile flowering signals (Zeevaart, 2008). Here, the ubiquitous presence of *FT*-related genes that encode florigens is further supported by evidence that maize *ZCN8* encodes a putative FT orthologue. This supports other findings that report the presence of *FT*-like genes in grasses, such as wheat and barley (Yan *et al.*, 2006). Whether *ZCN8* encodes the sole florigen in maize is not clear, as there are at least 25 members of the *ZCN* gene family (Danilevskaya *et al.*, 2008). In rice, the *Hd3a* gene was the first *FT* orthologue identified in grasses, and subsequently was shown to migrate from leaf to shoot apex (Kojima *et al.*, 2002; Tamaki *et al.*, 2007). More recently another rice gene with similarity to *Hd3a*, *RFT1*, was found to have a role in controlling flowering (Komiya *et al.*, 2008). Therefore,

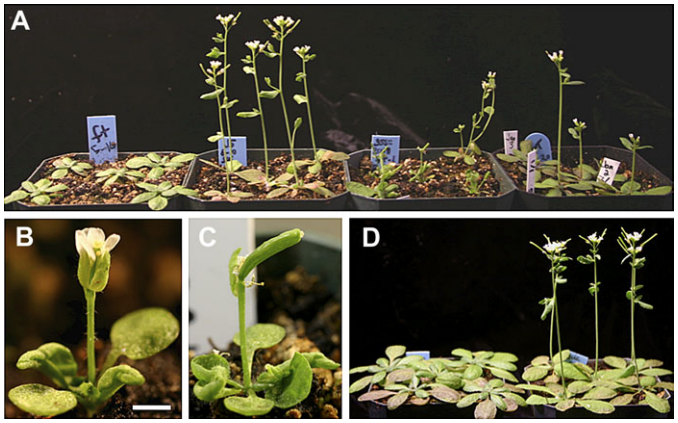

Fig. 4. Ectopic *ZCN8* expression affects flowering in transgenic *Arabidopsis*. (A) Growth under LD conditions after 24 d. Left to right: *ft-1* mutants in the Ler background; SUC2::ZCN8 in *ft-1*; 35S::ZCN8 in *ft-1*; wild-type. All plants are in thge Ler ecotype background. (B and C) Examples of terminal flower and curling leaf phenotypes typical of 35S::ZCN8 transgenic plants in the Ler and *ft-1* background, respectively. Scale bar=0.5 cm. (D) Comparison of flowering of SUC2::ZCN8 transgenic plants (right) with the *ft-1* mutant under short-day conditions after 60 d.

multiple *FT* orthologues exist in rice. Similarly, the *Arabidopsis* gene *TWIN SISTER OF FT* (*TSF*) was shown to act redundantly with *FT* in controlling flowering (Yamaguchi *et al.*, 2005; Mathieu *et al.*, 2007; Jang *et al.*, 2009). Given that maize is an allotetraploid resulting from genome duplication (Gaut and Doebley, 1997), the existence of multiple, functional florigen genes would not be unexpected. However, since ZCN8 has the high amino acid sequence similarity to FT (Fig. 1A) and is the only ZCN reported to interact strongly with DLF1 protein so far (Danilevskya *et al.*, 2008), it is a good candidate for a maize florigen. Moreover, the *ZCN8* gene maps very close to a strong maize QTL for flowering time on chromosome 8, *Vgt2* (Chardon *et al.*, 2005; Coles *et al.*, 2010). The identity of the gene underlying this QTL has not yet been revealed, but *ZCN8* would be a viable candidate. The leaf-specific expression of *ZCN8* demonstrated here is further proof that *ZCN8* encodes a mobile flowering signal.

ZCN8 *expression is highly up-regulated in leaves of SD-induced teosinte*

Teosinte, the presumed ancestor of modern maize, has an absolute requirement for SD photoperiods to induce flowering. The domestication of maize over the course of the last 9000 years involved the migration of tropical lines derived from teosinte into higher latitudes (Goodman, 1988; Matsuoka *et al.*, 2002). Adaptation to longer days of summer and colder climates at these latitudes required that temperate maize become less sensitive to photoperiod and more dependent on endogenous signals so that floral transition occurs at the appropriate time for reproductive success (Gouesnard *et al.*, 2002). This study finds that *ZCN8* transcript levels are greatly up-regulated in leaves of teosinte plants that are exposed to SD treatment. Further, expression levels were higher relative to non-inductive conditions at several different time points throughout the day (Fig. 2B; Supplementary Fig. S1 at *JXB* online). Whether *ZCN8* fluctuates in a diurnal or circadian pattern, as is found for *FT* orthologues in other species such as rice and *Arabidopsis*, cannot be concluded from these data. However, the high levels of induction clearly show that *ZCN8* levels are associated with teosinte leaves that are synthesizing florigen. Interestingly, *ZCN8* transcription also increased in B73 plants that do not require SD treatment to flower, although relative induction levels are much lower than those observed in teosinte and the expression levels of different times of day are not consistent (Fig. 2B). This suggests that a residual photoperiod pathway may still function in B73, although at a much more muted level since the autonomous pathway has superseded photoperiod-induced flowering in temperate maize. Careful analysis of flowering time under LD and SD photoperiods showed that B73 plants flower at the same time; however, they make ~2 fewer leaves under SD conditions than under LD conditions, suggesting that the photoperiod pathway retains a minor role in controlling flowering in temperate maize (Coles *et al.*, 2010).

ZCN8 *expression in the leaf is controlled by endogenous flowering signals*

Although a florigen was first proposed to mediate flowering in response to photoperiod induction (such as LD-grown *Arabidopsis* and SD-grown rice), grafting experiments demonstrated that it also has a role in transmitting leaf-derived signals in day-neutral plants (Lang, 1977). Temperate maize exemplified by the B73 inbred used in this study relies almost exclusively on an autonomous mechanism that signals time to flowering after the shoot apex has produced a particular number of leaves. The *id1* gene is expressed exclusively in developing leaves; moreover, *id1* mRNA and ID1 protein levels do not fluctuate in a diurnal pattern. Therefore, *id1* has been proposed to be a regulator of a leaf-derived florigenic signal of the autonomous pathway in maize (Colasanti *et al.*, 1998; Wong and Colasanti, 2007). Transcript levels of *ZCN8* are greatly reduced in the leaves of *id1* mutants (Fig. 2C), suggesting that ZCN8 relays the flowering signal from an upstream endogenous signal in maize. Analysis of flowering in day-neutral tomato and tobacco species similarly shows that FT orthologues acts as a florigen in these species (Lifschitz and Eshed, 2006; Lifschitz *et al.*, 2006). The results presented here extend the role of *FT*-like florigen genes to a day-neutral monocot species and provide further evidence that FT orthologues act as universal mobile stimuli in all flowering plants.

The finding that *id1* acts upstream of *ZCN8* supports studies in rice that show that the rice *id1* orthologue (*RID1*, *OsId1*, or *Ehd2*) acts upstream of the *Hd3a* gene (Matsubara *et al.*, 2008; Park *et al.*, 2008; Wu *et al.*, 2008). Therefore, the leaf-specific *id1-ZCN8* hierarchy appears to be conserved in maize and rice, which are both grasses of

tropical origin. However, other elements are not conserved, such as the rice *Early heading day 1* (*Ehd1*) gene, a B-type response regulator that also acts upstream of *Hd3a* and is a component of the SD photoperiod pathway (Doi *et al.*, 2004). No *Ehd1* orthologue has been reported in maize; therefore, *Ehd1* may represent an activator of *Hd3a* expression that is unique to rice. It has been proposed that the rice *id1* orthologue is a master regulator of both photoperiod and autonomous pathways (Wu *et al.*, 2008). Whether maize *id1* controls both photoperiod and autonomous pathways seems unlikely since studies show no diurnal fluctuation of *id1* expression and no induction of *id1* in photoperiod-induced teosinte (Coneva *et al.*, 2007; Wong and Colasanti, 2007).

ZCN8 *integrates both endogenous and photoperiod signals*

Overall the findings presented here suggest that *ZCN8* integrates signals from both the photoperiod and autonomous pathways in maize. A simple model is shown in Fig. 5A, where floral-inductive, leaf-derived signals impinge on *ZCN8* from an autonomous pathway, mediated by *id1* activity, and from signals activated by the inductive SD photoperiod. In this proposed model, ZCN8 is a checkpoint that can assimilate different inductive signals. Whether *id1* has a role in mediating the floral transition in teosinte or other tropical maize that requires SD photoperiods to induce flowering is unknown. Nevertheless, this study suggests that the evolution of day-neutral maize from a tropical ancestor retained the function of ZCN8 as an integrator of inductive signals, whether they are environmental or autonomous.

Phloem-specific expression and overexpression of ZCN8 *rescues the* Arabidopsis ft-1 *mutant and causes extreme early flowering*

Ectopic expression of *ZCN8* in transgenic *Arabidopsis* plants provides further proof that ZCN8 has the properties of a universal, leaf-derived florigen. Initial studies in *Arabidopsis* found that overexpression of *FT* driven by the 35S promoter rescued the *ft* mutation and also caused extremely early flowering (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999), as did overexpression of the *TSF* gene (Yamaguchi *et al.*, 2005). In addition to early flowering, these studies report developmental anomalies in 35S::FT plants similar to what is found in the current study (i.e. terminal flower formation and curling of the first few leaves). Therefore, *ZCN8* overexpression has the same effect as overexpression of the *FT* gene in *Arabidopsis*; but most importantly has the ability to complement the *ft* mutation. Later experiments showed that *FT* expression under the control of the phloem companion cell-specific SUC2 promoter could also rescue the *ft* mutation (Jaeger and Wigge, 2007; Mathieu *et al.*, 2007). In this study, the finding that the SUC2::ZCN8 transgene complements *ft* in *Arabidopsis* (Figs. 3A, 4) demonstrates an important point; namely, that maize ZCN8 synthesized in phloem cells is able to activate floral transition at the shoot apex. Similar to what is observed for *Arabidopsis* FT and rice Hd3a proteins, it is likely that ZCN8 acts as a florigen and moves to the shoot apex to mediate the transition. However, it is possible that ZCN8 causes the production of a secondary florigenic signal in the leaf and that this component migrates to the apex. Further studies would be required to show definitively that ZCN8 is a mobile signal. Nevertheless, the data presented here provide strong evidence that ZCN8 has properties of a florigen. The observation that most SUC2::ZCN8 transgenic lines flower at the same time as or only slightly earlier than WT plants suggests that phloem-specific expression does not cause developmental abnormalities associated with ectopic overexpression. Interestingly, this was supported by the finding that expression levels of ZCN8 in leaves were often found to be higher that in the 35S lines (Fig.3C). This may suggest that phloem-specific expression has an intrinsic 'gating' mechanism that regulates the level of ZCN8 (or FT) protein that migrates to the shoot apex.

Does id1 *act as an autonomous activator of* ZCN8 *expression in temperate maize?*

The nature of the upstream mechanism that controls *ZCN8* expression in the leaf remains an open question. In *Arabidopsis*, the role of the *CONSTANS* gene (*CO*) as a sensor of photoperiod signals via the circadian clock and the direct activation of *FT* expression in leaves is well established (reviewed in Turck *et al.*, 2008). In maize, the *conz1* gene, a homologue of *Arabidopsis CO*, was shown to exhibit a diurnal expression pattern, suggesting a possible role in photoperiod control of flowering (Miller *et al.*, 2008). However the role of *conz1* in flowering time control has not been reported, and whether CONZ is an activator of ZCN8 needs to be investigated. It will be interesting to ascertain whether teosinte and tropical maize, which depend on SD photoperiods to flower, contain a functional CO orthologue that activates *ZCN8* expression. In maize, even less is known about the mechanisms underlying endogenous flowering signals. The finding that absence of *id1* activity reduces *ZCN8* expression suggests that an ID1–ZCN8 regulatory module may exist in day-neutral maize leaves, similar to the CO–FT module in *Arabidopsis* and the Hd1–Hd3a module in rice. It is of interest to note that the temporal and spatial expression patterns of *id1* and *ZCN8* do not overlap; that is, *id1* activity is confined to the immature, non-photosynthetic part of developing leaves (Colasanti *et al.*, 1998; Wong and Colasanti, 2007), whereas *ZCN8* expression is localized to mature leaves (Fig. 5B). Therefore, it is unlikely that *ZCN8* is a direct target of the ID1 transcriptional regulator in the way that *FT* is a direct target of CO (Samach *et al.*, 2000). One possibility is that ID1 acts in the basal portion of the leaf to regulate the production of an unidentified mobile signal that moves to the mature, green portion of the leaf to activate *ZCN8* expression. Given that activation is indirect in this scenario, numerous intermediary factors may exist to control *ZCN8* expression (Fig. 5A). An alternative possibility

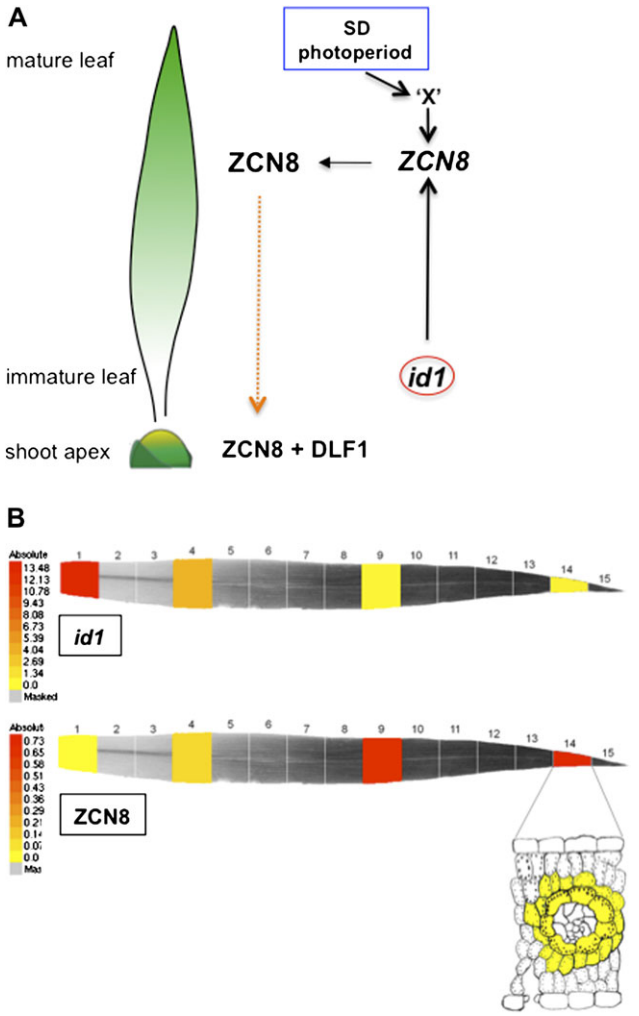

Fig. 5. Model of *id1* and *ZCN8* interaction in controlling flowering in maize. (A) *ZCN8* expression is dependent on *id1* in temperate maize and on SD induction in teosinte and tropical maize. *id1* activity in immature leaves of temperate maize induces downstream expression of *ZCN8* in mature leaves. In photoperiod-dependent maize, such as teosinte, SD conditions activate *ZCN8* expression by an unknown intermediate (x). ZCN8 protein is translated in vascular cells of mature leaves and migrates via phloem sieve elements to the shoot apex (orange dotted line) where it interacts with the DLF1 protein to activate floral meristem identity genes. (B) Electronic fluorescent pictograph (eFP) heat map images show absolute expression levels of *ZCN8* and *id1* in leaf 3 of B73 maize plants (Li *et al.*, 2010), illustrating that transcript accumulation patterns of *id1* and *ZCN8* do not overlap. The expanded cross-section for the lower image shows no detectable *ZCN8* transcript in bundle sheath and mesophyll cells. [eFP browser image from BAR (Botanical Array Resource), University of Toronto.]

is that ID1 functions in early stages of leaf development to generate a mature leaf that is competent to produce florigenic signals, including expression of *ZCN8*. This latter scenario is intriguing in that it suggests an epigenetic role for *id1* in patterning the mature leaf expression profile. Increasing evidence from studies of vernalization and autonomous pathways in *Arabidopsis* suggest that epigenetic mechanisms, such as RNA-mediated DNA methylation and establishment of chromatin states, are central components of flowering control (reviewed in He, 2009; Amasino, 2010). Further, recent studies show that *FT* and *TSF* expression is subject to epigenetic control through specific chromatin modifications (Adrian *et al.*, 2010; Yang *et al.*, 2010). Preliminary evidence that ID1 protein interacts with proteins that have putative roles in mediating RNA-directed DNA methylation and chromatin modification support this idea (M. Tanimoto, R. Hilborn, A. Kozaki and J. Colasanti, unpublished). It will be of interest to determine whether epigenetic programming during leaf development is the basis of autonomous regulation of leaf competence to produce florigen.

Supplementary data

Supplementary data are avaialbe at *JXB* online.

Figure S1. Quantitative RT-PCR analysis of *ZCN8* expression in leaf 4 and leaf 7 of teosinte and B73 maize plants at the V8 stage.

Table S1. Days to flowering and total leaf number at flowering for all 35S::ZCN8 and SUC2::ZCN8 transgenic constructs in *Arabidopsis* under long-day and short-day growth conditions.

Table S2. Primers used for qRT-PCR and gene cloning.

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