In search for maize florigen

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In search for maize florigen

by

Xin Meng

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Erik W Vollbrecht, Co-major Professor
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Iowa State University
Ames, Iowa

2011

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# TABLE OF CONTENTS

**ABSTRACT** ...................................................................................................................... iv

**CHAPTER ONE: GENERAL INTRODUCTION** .................................................................1

<table>
<thead>
<tr>
<th>Dissertation organization</th>
<th>General introduction of Arabidopsis Flowering Locus T (FT) and its orthologs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

**CHAPTER TWO: MOLECULAR CHARACTERIZATION OF MAIZE FT-LIKE ZCN GENES** ...............9

<table>
<thead>
<tr>
<th>Abstract</th>
<th>Introduction</th>
<th>Materials and methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plant materials and growth conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diurnal experiment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Short day inductive experiment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNA isolation, RT-PCR and quantitative RT-PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue imaging</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arabidopsis T-DNA constructs and transformation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Statistical analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Accession number</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survey of FT-like ZCN gene transcription in leaves of temperate and tropical lines under SD and LD photoperiods</td>
</tr>
<tr>
<td>Responses of FT-like ZCN genes to a floral inductive SD treatment</td>
</tr>
<tr>
<td>Diurnal expression of three FT-like ZCN genes under SD and LD photoperiods</td>
</tr>
<tr>
<td>Overexpression of FT-like ZCN genes in Arabidopsis ft mutants</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Discussion</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT-like ZCN genes display distinct expression patterns in the leaf of maize lines with different photoperiod sensitivities</td>
</tr>
<tr>
<td>ZCN8 transcription is diurnally regulated in photoperiod-sensitive tropical lines but not in day-neutral lines</td>
</tr>
<tr>
<td>Arabidopsis complementation experiment suggests that ZCN8, ZCN14 and ZCN15 are putative maize floral activators in which ZCN8 is the most promising one</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acknowledgments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>27</td>
</tr>
</tbody>
</table>

**CHAPTER THREE: THE FT-LIKE ZCN8 GENE FUNCTIONS AS A MAIZE FLORAL ACTIVATOR IN THE id1-dlf1 GENETIC PATHWAY ** ..............................52

<table>
<thead>
<tr>
<th>Abstract</th>
<th>Introduction</th>
<th>Materials and methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plant materials and growth conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNA isolation, RT-PCR and quantitative RT-PCR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis T-DNA constructs and transformation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Discussion</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
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<th>Acknowledgments</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>27</td>
</tr>
</tbody>
</table>
The mobile floral-promoting signal, florigen, is thought to consist of, in part, the FT protein named after the *Arabidopsis thaliana* gene *Flowering locus T*. FT is transcribed and translated in leaves and its protein moves via the phloem to the shoot apical meristem where it promotes the transition from vegetative to reproductive development. In search for a maize FT-like floral activator(s), seven *Zea mays* CENTRORADIALIS (ZCN) genes encoding FT homologous proteins were studied. ZCN8 stood out as the only ZCN having the requisite characteristics for possessing florigenic activity. In photoperiod sensitive tropical lines, ZCN8 transcripts were strongly up-regulated in a diurnal manner under floral-inductive short days. In day-neutral temperate lines, ZCN8 mRNA level was independent of day length and displayed only a weak cycling pattern. ZCN8 is normally expressed in leaf phloem but ectopic expression of ZCN8 in vegetative stage shoot apices induced early flowering in transgenic plants. Silencing of ZCN8 by artificial microRNA resulted in late flowering. ZCN8 was placed downstream of *indeterminate 1* (*id1*) and upstream of *delayed flowering 1* (*dlf1*), two other floral activator genes. A flowering model was proposed to link photoperiod sensitivity of tropical maize to diurnal regulation of ZCN8.
CHAPTER ONE: GENERAL INTRODUCTION

Dissertation organization

The dissertation is composed of four chapters. The first chapter is a general review of research progress of the floral activator Flowering Locus T (FT) and its orthologs in plants. The second and third chapters are modified from the paper published in the Plant Cell (Meng et al., 2011) and describe the search for maize floral activator(s) among FT-like ZCN genes and the proof of its function. In the last chapter of the general conclusion, a model of maize flowering time is proposed and future work is discussed. As to contributions of the authors listed in the paper, Olga Danilevskaya initiated the project and proposed the hypothesis; Xin Meng is the primary researcher and designed and performed all experiments, analyzed the experimental data and wrote the draft; Olga Danilevskaya and Michael Muszynski edited and finalized the draft. The research was performed based on the background from our four papers (Danilevskaya et al., 2008a; Danilevskaya et al., 2008b; Danilevskaya et al., 2010; Hayes et al., 2010).

The work is fulfilled under supervision of Dr. Olga Danilevskaya, who is a research scientist at Pioneer Hi-Bred and a collaborating professor in the Department of Genetics, Development and Cell Biology (GDCB) in Iowa State University. Dr. Erik W Vollbrecht, my co-major advisor and an associate professor in the GDCB department, gave me a lot of advice in pursuit of my Ph.D degree.

General introduction of Arabidopsis Flowering Locus T (FT) and its orthologs

Flowering time is an important process of a plant’s life cycle and is regulated by environmental and developmental cues to assure flowering under favorable conditions. During plants’ development and growth, flowering time is determined by the floral transition, a critical switch from vegetative to reproductive stages. Crop flowering at appropriate time ensures its maturity and yield. Moreover, success of adaptation of new crop varieties to local environments is affected by their timing of flowering. Thus, appropriate genetic modification of crop flowering time would make great contributions to agriculture. The
importance of flowering time to crop production results in tremendous efforts made to reveal mechanisms which underlie flowering time.

Studies of plant flowering time can be tracked back to almost two hundred years ago. In his review, Zeevaart described that as early as the nineteenth century Julius Sachs noticed that one certain substance produced in the leaf under light mediated the formation of flowers of shoots in darkness. The hypothesis of an unknown substance in the leaf under flowering inductive condition inducing flowering of shoots under flowering non-inductive condition was proposed and supported by classical grafting experiments performed in plant species sensitive to photoperiod in which a donor leaf treated under inductive short days (SDs) induced flowering of a receptor under long days (LDs) (Zeevaart, 2006). This special substance was named “florigen” by Chailakhyan (Chailakhyan, 1937). Since successful flowering induction of one species by another species in grafting experiments, it was proposed that florigen has a universal function in plants (Zeevaart, 1976). These findings implied that florigen may act as a mobile long-distance signal that moves from the leaf to shoot apices. However, no compounds were found to be florigen by physiological-biochemical approaches until the emergence of molecular genetics. Grafting experiments and transgenic approaches in Arabidopsis (Corbesier et al., 2007; Jaeger and Wigge, 2007; Notaguchi et al., 2008) revealed that the protein encoded by the Flowering locus T (FT) gene had florigenic activity. The FT protein shares sequence similarity with mammalian phosphatidylethanolamine-binding protein (PEBP) (Kardailsky et al., 1999b) which has been annotated as a kinase regulator (Banfield and Brady, 2000). Arabidopsis FT is shown to behave as a “flower-forming” signal, because it is transcribed and translated in leaves but its protein subsequently moves through the phloem to the shoot apical meristem where it induces the floral transition upon reaching a critical concentration (Tsuji et al., 2008; Turck et al., 2008; Zeevaart, 2008).

In Arabidopsis, several genetic pathways regulate flowering time such as photoperiod, vernalization, autonomous and gibberellins (GA) pathways (Mouradov et al., 2002). In each pathway, several key members connect upstream and downstream components to allow perceived signals to converge on them. These key members are named integrators. With the discovery of more and more components in the pathways controlling flowering, almost all of
them were identified to regulate $FT$ activity, demonstrating that $FT$ is an essential integrator of Arabidopsis flowering time pathways (Mouradov et al., 2002).

In the photoperiod pathway, $PHYTOCHROME B$ ($PHYB$), $CRYTOCHROME 2$ ($CRY2$), $GIGANTEA$ ($GI$), $CONSTANS$ ($CO$) and $FT$ are important genes to regulate Arabidopsis flowering under LDs (Turck et al., 2008). In the vernalization pathway two critical genes, $FRIGIDA$ ($FRI$) and $FLOWERING LOCUS C$ ($FLC$), function as negative regulators (Kim et al., 2009). Without cold temperature treatment, high expression level of $FLC$ suppresses the expression of $FT$ and prevents plants from flowering. The autonomous pathway is composed of endogenous factors regulating flowering independent of environmental cues, including genes determining the competency of adult and juvenile phases. Progress has recently been made on how RNA metabolism regulates Arabidopsis phase and floral transitions. Two microRNAs, miR156 and miR172, were identified to mediate the transition from juvenile to adult phase in Arabidopsis with sequential action (Wu et al., 2009). By targeting the $SQUAMOSA PROMOTER BINDING PROTEIN LIKE$ ($SPL$) gene family, miR156 expression maintains the juvenile phase and prevents precocious flowering. Along with development of plants, expression of miR156 decreases and miR172 is highly activated, which inhibit expression of $APETALA2-LIKE$ ($AP2-like$) and remove repression of $FT$ expression to promote flowering (Aukerman and Sakai, 2003; Mathieu et al., 2009).

$FT$ function as a floral activator is widely conserved in angiosperms, although $FT$ is regulated differently depending on the photoperiod sensitivity of the species (Turck et al., 2008). $FT$ orthologs have been identified as floral activators in many species including rice ($Oryza sativa$ ssp. $japonica$) $Heading Date 3a$ ($Hd3a$) and $Rice Flowering Locus T1$ ($RFT1$) (Kojima et al., 2002; Komiya et al., 2008), tomato ($Solanum lycopersicum$) $Single Flower Truss$ ($SFT$) (Lifschitz and Eshed, 2006; Lifschitz et al., 2006), wheat ($Triticum aestivum$) $TaFT$ (Yan et al., 2006), barley ($Hordeum vulgare$) $HvFT$ (Faure et al., 2007), morning glory ($Pharbitis nil$) $PnFT1/2$ (Hayama et al., 2007), sugar beet ($Beta vulgaris$) $BvFT2$ (Pin et al., 2010) and sunflower ($Helianthus annuus$) $HaFT1$ and $HaFT4$ genes (Blackman et al., 2010).
Compared with the extensive genetic and molecular analyses of Arabidopsis flowering time, studies in maize were lagged behind due to the scarcity of flowering time mutants. However, the complexity of the genetic architecture of flowering time in maize was revealed through natural variation in quantitative trait loci (QTL) controlling this trait. Maize was domesticated in Mexico from its wild ancestor, teosinte (Zea mays ssp. parviglumis) which requires SDs to flower (Matsuoka et al., 2002). This ancestral photoperiod sensitivity is most evident in tropical varieties of maize which flower normally under SDs but have a greatly delayed transition when grown under LDs (Colasanti and Muszynski, 2009). In contrast, most maize lines seem to have lost much of their photoperiod sensitivity as they are grown in temperate environments where they flower, set seed and mature under LDs. To search for QTLs controlling maize flowering time, either large mapping populations created from parents with different flowering time were exploited or association tests in representative inbred lines were performed. Chardon et al., used a meta-analysis approach to analyze twenty-two populations and found 62 consensus QTLs in which six QTLs were located on Chromosomes 1, 6, 8 and 9 had major effects in flowering time (Chardon et al., 2004). Later, one flowering time QTL on Chr8, Vegetative to generative transition 1 (Vgt1), was cloned by positional cloning and confined to a 2 kb non-coding region thought to regulate a flowering time gene ZmRap2.7 located downstream (Salvi et al., 2007). Recently, Buckler et al., used maize nested association mapping (NAM) populations to identify many flowering time QTLs, including the six major QTLs, and found they only had relatively small effects compared with the flowering time QTLs in Arabidopsis, rice, barley and sorghum (Buckler et al., 2009). Additionally, two other research groups developed mapping populations created from temperate and tropical lines and did fine mapping of maize flowering time. Their results showed that a major QTL on chromosome 10 is highly correlated with maize photoperiod sensitivity (Ducrocq et al., 2009; Coles et al., 2010).

In addition to QTL studies of maize flowering time, a few maize genes promoting or repressing flowering time were cloned and characterized including indeterminate1 (idl), delayed flowering1 (dlf1), ZmRap2.7 and Zea mays MADS 4 (ZMM4) (Colasanti et al., 1998; Muszynski et al., 2006; Salvi et al., 2007; Danilevskaya et al., 2008b). The idl and dlf1 genes were isolated from loss-of-function late flowering mutants. idl is specific to monocots
and encodes a zinc finger transcription factor (Colasanti et al., 1998). \textit{dlf1}, the ortholog of Arabidopsis \textit{FD}, encodes a basic leucine zipper (bZIP) transcription factor and its mRNA is highly expressed in the shoot apex (Muszynski et al., 2006). These two genes are the key components of the maize autonomous pathway regulating flowering time (Colasanti and Muszynski, 2009). \textit{ZmRap2.7}, an \textit{APETALA2 (AP2)}-like transcription factor, is thought to be a repressor of flowering time in maize (Salvi et al., 2007). \textit{ZMM4} is a member of the maize MADS-Box gene family and an ortholog of \textit{Arabidopsis APETALA1 (AP1)/FRIUTFUL (FUL)} and regulates floral induction and inflorescence development (Danilevskaya et al, 2008).

Despite some progress understanding the molecular genetic mechanisms for maize flowering, the nature of maize \textit{FT} orthologs are still unknown. Because of high conservation of function of \textit{FT} and its orthologs in other species, I hypothesized that there are \textit{FT}-like genes functioning as floral activators in maize. In my research, I took advantage of molecular expression analysis, genetics and transgenic approaches to identify maize floral activator(s) among maize twenty-five maize PEBP homologs named \textit{Zea mays CENTRORADIALIS (ZCN)} genes after the first cloned PEBP plant gene from Antirrhinum (Danilevskaya et al., 2008a). There were several specific objectives I wanted to achieve:

1. **Objective one**: Test the correlation between transcription of \textit{FT}-like \textit{ZCN} genes and the floral transition in maize lines of different flowering time.
2. **Objective two**: Examine \textit{FT}-like \textit{ZCN} gene function as putative floral activator(s) by complementing Arabidopsis \textit{ft} late flowering mutants
3. **Objective three**: Test genetic interactions between \textit{FT}-like \textit{ZCN} genes and \textit{id1} and \textit{dlf1} network.
4. **Objective four**: Test \textit{ZCN} gene function as floral activator(s) by their ectopic or reduced expression in maize.

The details of methods, materials and experimental results will be discussed in following chapters.
References


structure analysis of a major flowering time quantitative trait locus on maize chromosome 10. Genetics 183, 1555-1563.


CHAPTER TWO: MOLECULAR CHARACTERIZATION OF MAIZE FT-LIKE ZCN GENES

Modified from a paper published in the Plant Cell (Meng et al., 2011)

Abstract

Arabidopsis thaliana *Flowering Locus T (FT)* is a leaf-produced floral activator and its orthologous function is highly conserved in angiosperms. *FT* encodes a protein homologous to mammalian phosphatidylethanolamine-binding proteins (PEBPs). In search for a maize FT-like floral activator(s) among maize PEBP genes, seven *Zea mays* CENTRORADIALIS (ZCN) genes encoding *FT* homologous proteins were studied. Four maize lines with different photoperiod sensitivities were chosen to analyze correlations between the timing of ZCN gene expression and the timing of the floral transition. Of the seven ZCN genes, transcript accumulation of ZCN8, ZCN18 and ZCN26 correlates to the floral transition in photoperiod-insensitive temperate and photoperiod-sensitive tropical lines at different stages. Expression analysis of these three ZCN genes under long days (LDs) and short days (SDs) demonstrated that only ZCN8 mRNA expression displays distinct diurnal patterns in temperate and tropical lines responding to different day lengths. In day-neutral temperate lines ZCN8 mRNA level was independent of day length and displayed a weak cycling pattern. In contrast, in photoperiod sensitive tropical lines, ZCN8 transcripts were strongly up-regulated in a diurnal manner under floral-inductive short days. Although ectopic expression of ZCN8, ZCN14 and ZCN15 complemented the Arabidopsis late flowering *ft* mutant, the combination of results obtained indicates that ZCN8 is the most promising candidate of the maize floral activator. However, additional functional evidence is required to confirm that ZCN8 is a maize floral activator, which is presented in chapter three.

Introduction

Several genetic pathways regulate the floral transition in Arabidopsis, including the autonomous, gibberellin, photoperiod and vernalization pathways (Mouradov et al., 2002). *FT* is a key integrator because almost all pathways converge on it and *FT* transmits the floral inductive signal to downstream floral identity genes (Mouradov et al., 2002). Therefore, the
temporal and spatial expression of \( FT \) function is essential for proper flowering time regulation. Arabidopsis is sensitive to photoperiod with long days (LDs) promoting flowering. In the photoperiod pathway, \( FT \) transcription in the vasculature of the leaf is regulated by \( CONSTANS (CO) \) (Samach et al., 2000; An et al., 2004; Turck et al., 2008). \( CO \) encodes a transcription factor containing two B-box zinc finger domains and a CCT (CO, CO-Like, TOC1) domain near the carboxy terminus (Putterill et al., 1995; Robson et al., 2001). \( CO \) transcripts predominantly accumulate in the shoot and leaves under LDs (Putterill et al., 1995) and are diurnally regulated with a peak at dusk under both LDs and short days (SDs) (Suarez-Lopez et al., 2001). \( CO \) protein accumulation is stabilized toward the end of the day only under LDs, whereas SD condition triggers \( CO \) degradation (Valverde et al., 2004). Subsequently, the accumulation of \( CO \) protein drives accumulation of \( FT \) mRNA at the end of the day under LDs but not under SDs and \( FT \) transcripts induced in the leaf display diurnal expression pattern with a peak at dusk (Kardailsky et al., 1999b; Suarez-Lopez et al., 2001). \( FT \) mRNA levels stay low under SDs but can be induced to floral-promoting levels by a minimum three day exposure to LDs (Corbesier et al., 2007). Thus, transient \( FT \) mRNA accumulation caused by a short inductive photoperiod is sufficient to promote flowering (Corbesier et al., 2007).

The function of \( FT \) as a floral activator is widely conserved in plants, although \( FT \) is regulated differently depending on the photoperiod sensitivity of the species (Turck et al., 2008). In contrast to Arabidopsis, which is a LD plant, most rice cultivars flower earlier under SDs due to their tropical origin. \textit{Heading Date 3a} (\( Hd3a \)), a rice ortholog of \( FT \), was identified as a floral activator under SDs (Kojima et al., 2002). Later, \textit{Rice flowering locus T1 (RFT1)} , the \( Hd3a \) paralog located within 6 kb of \( Hd3a \) on chromosome 6, was identified as a major floral activator under LD conditions (Komiya et al., 2008; Komiya et al., 2009). Both genes show a diurnal expression pattern with transcript abundance peaking at dawn (Kojima et al., 2002; Komiya et al., 2009). Both genes encode PEBP–like proteins that were shown to move from the leaf to shoot apex (Tamaki et al., 2007; Komiya et al., 2009). Rice is extremely sensitive to day-length variation and flowering can be modulated by photoperiod differences as short as 30 minutes. \( Hd3a \) is highly expressed in photoperiods \(<13~\text{hrs} \) but its expression decreases sharply in photoperiods \(>13.5~\text{hrs} \) and its expression is not detectable
under longer photoperiods. This level of precision is achieved by the control of expression of two floral regulators of $Hd3a$: a floral activator, *Early heading date1* (*Ehd1*) and a floral repressor, *Grain number, plant height and heading date7* (*Ghd7*) (Itoh et al., 2010). *Ehd1* encodes a protein with sequence similarity to B-type response regulators and *Ghd7* encodes a CCT domain protein (Itoh et al., 2010).

*FT* and its orthologs encode a protein homologous to mammalian phosphatidylethanolamine-binding protein (PEBP). In plants, the first cloned PEBP gene was *CENTRORADIALIS (CEN)* which controls determinacy of the *Antirrhinum* inflorescence (Bradley et al., 1996). All PEBP proteins share a highly conserved putative ligand-binding pocket and an external loop at their C-terminal (Banfield et al., 1998; Banfield and Brady, 2000). In Arabidopsis, the PEBP gene family consists of six gene members including *FT*, *TWIN SISTER OF FT (TSF)*, *TERMINAL FLOWER 1 (TFL1)*, *BROTHER OF FT and TFL1 (BFT)*, *ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUE (ATC)*, and *MOTHER OF FT AND TFL1 (MFT)*. The functions of *FT* and *TFL1* have been extensively studied. They antagonistically determine Arabidopsis flowering time and inflorescence architecture (Kobayashi et al., 1999). Similar to *FT*, *TSF* is a floral activator in Arabidopsis but promotes flowering mainly under SDs (Yamaguchi et al., 2005). *ATC* protein sequences have the highest similarity to those of *Antirrhinum CEN*. *ATC*, *BFT* and *MFT* are found to have *TFL1*-like function and play redundant roles with *TFL1* to regulate the floral transition and inflorescence organ development (Mimida et al., 2001; Yoo et al., 2004; Yoo et al., 2010). Recently, *MFT* expression in embryo was found to be affected by ABA, GA and brassinosteroid signaling pathways to modulate seed germination (Xi et al., 2010; Xi and Yu, 2010).

The key amino acids defining *FT/TFL1* antagonistic function have been identified. Hanzawa et al., and Ahn et al., analyzed flowering time of transgenic Arabidopsis plants overexpressing chimeric proteins and confirmed that the amino acids in the ligand-binding pocket and the external loop of *FT* and *TFL1*, Tyr85 (FT)/His 88(TFL1) and Gln 140(FT)/Asp144(TFL1) and the segment 128-141 (FT)/131-145 (TFL1), are crucial for their functions as either a floral activator or repressor (Hanzawa et al., 2005; Ahn et al., 2006). More evidence confirmed the importance of the segment in the fourth exon to *FT/TFL1*
antagonistic activity. In sugar beet, the conversion of $BvFT1/2$ function from activator to repressor of flowering was shown by swapping the segment 134-141($BvFT2$)/138-144 ($BvFT1$) in their fourth exon, which further supports the idea that the external loop is critical for $FT/TFL1$ antagonistic function (Pin et al., 2010).

The research objective of this dissertation is to search for $FT$-like genes promoting flowering in maize. However this task is not trivial due to the expansion the PEBP gene family in maize (Danilevskaya et al., 2008a). There are twenty-five PEBP homologs which were divided into four clades including $TFL1$-like, $MFT$-like, $FT$-like I and $FT$-like II. Each of the 15 FT-like genes displays a unique spatial-temporal expression pattern and only six, $ZCN7$, $ZCN8$, $ZCN12$, $ZCN14$, $ZCN18$ and $ZCN26$, are expressed in leaves (Danilevskaya et al., 2008a). These six ZCN genes are potential floral activator candidates. It is important to point out that $ZCN15$, which maps to a region syntenic to the rice chromosome 6 region where $Hd3a/b$ resides, is expressed predominantly in kernels and not in leaves, suggesting it may be not involved in control of flowering (Danilevskaya 2008a). Therefore functional analysis and experimental evidence are necessary to determine which of the ZCN genes possesses florigenic activity.

To identify maize $FT$ orthologs, I took advantage of the tremendous natural diversity in photoperiod response and flowering time in maize. Maize was domesticated in Mexico from its wild ancestor, teosinte ($Zea mays$ ssp. $parviglumis$); a species which requires SDs to flower (Matsuoka et al., 2002). As a result of artificial selection by humans, maize is adapted to growth in an extraordinary large geographical area, from latitude 58º north to 35º south (Kuleshov, 1933). Most maize lines grown in temperate environments seem to have lost much of their photoperiod sensitivity as they complete the life cycle under LDs, the same as in SDs.

The experimental approaches I chose to determine which of the seven ZCN genes might have florigenic function included analyses of the dynamics of transcript accumulation in four maize lines with different flowering times and photoperiod sensitivities. I expect that the transcription level of maize $FT$-like gene that might possess florigenic activity would be elevated during the floral transition, similar to accumulation of $FT$ transcript in Arabidopsis and rice (Kardailsky et al., 1999b; Kojima et al., 2002; Komiya et al., 2008). Expression of
seven maize FT-like ZCN genes was examined in the extremely early flowering line Gaspé Flint (Brawn, 1968), the temperate inbred B73 (Troyer, 2000) and two photoperiod sensitive tropical lines CML311 and CML436 (Russel and Stuber, 1983). I found that ZCN8, ZCN18 and ZCN26 mRNA accumulation in leaves always preceded the floral transition of the shoot apical meristem in all four maize lines tested, regardless of photoperiod. Nevertheless, only ZCN8 transcription is diurnally regulated in photoperiod sensitive tropical lines but showed an attenuated cycling pattern in day-neutral temperate lines. In addition, only ZCN8 transcript is induced after a seven day floral-inducing SD exposure in tropical lines, suggesting ZCN8 is involved in photoperiod sensitivity. A potential function as a floral activator was further tested by complementation of the Arabidopsis ft mutant. Over expression of three genes, ZCN8, ZCN14 and ZCN15 in the Arabidopsis ft mutant promotes flowering, suggesting that functional analysis in maize is required to identify maize floral activator(s).

**Materials and Methods**

**Plant Materials and Growth Conditions**

Gaspé Flint, B73, CML311 and CML436 maize lines were chosen for this research due to their distinct photoperiod sensitivities and differences in flowering time. Gaspé Flint is an extremely early temperate variety which flowers after producing 8-9 leaves. B73 is a temperate inbred line which flowers after producing 19-20 leaves. Gaspé Flint and B73 are considered day-length insensitive. CML311 and CML436 are two tropical inbred lines. CML311 is a moderately photoperiod sensitive line producing 19-20 leaves under SDs and 23-24 leaves under LDs. CML436 is a late highly photoperiod sensitive line producing 19-20 leaves under SDs and 27-30 leaves under LDs. Seeds were germinated directly in soil and seedlings grown in growth chambers. LDs were set up for 16 h light and 8 h dark and SDs were 8 h light and 16 h dark. The temperature varied from 25-27°C during the day and 23-24°C during the night.

Seeds of two Arabidopsis late flowering mutant allele, ft-2 and ft-3, were ordered from Arabidopsis Biological Resource Center (ABRC). Wild type ecotype is Landsberg erecta (Ler). Detailed information for the mutant has been described (Koornneef et al.,
All plants were grown in growth chambers under LDs with 16 h light at 25°C during days and 23°C during nights.

**Tissue Collection**

Maize tissues were collected at different vegetative growth stages, mainly before and after the floral transition. Vegetative growth stages (V stages) were defined according to the full extension of the leaf collar of the uppermost leaf (Ritchie et al., 1997). Stage VE is defined as the time when the first true leaf emerges from the coleoptile. The floral transition was detected when the SAM was more elongated compared to its width. Collection of Gaspé Flint tissue began after five days of growth under SDs or LDs. Collection of B73, CML311 and CML436 tissue began after two weeks of growth under SDs or LDs. B73 tissue collection stopped after elongation of the SAM was observed under SDs and after the apex of the inflorescence finished spikelet initiation under LDs. Collection of CML311 and CML436 tissue was stopped after spikelet initiation was observed on the apical inflorescence under both SDs and LDs. All tissue sampling was performed in the morning and tissue from at least 3 plants was pooled together.

Arabidopsis leaf tissue was collected before bolting and saved for genotyping and transgene expression analysis.

**Diurnal experiment**

For the diurnal experiment under SDs, tissue from Gaspé Flint, B73 and CML436 was collected near the time of the floral transition as determined by examination of the SAM of representative plants. CML436 was planted first, B73 two weeks later and Gaspé Flint last, such that all three lines would transition near the same time under SDs. After one more week of growth, tissue from all genotypes were harvested every 4 h over a 68 h period. Tissues sampled at each time point included leaf blades from the uppermost leaf, immature leaves and shoot apices of two randomly selected plants. Three biological replicates were collected at the same time.

For the diurnal experiment under LDs, tissue of Gaspé Flint and B73 were collected around the floral transition. CML436 was harvested at V5 when CML436 was still at a
vegetative growth stage and at V9 when CML436 started the floral transition. All other procedures were the same as those under SDs.

**Short Day Inductive Experiment**

The two tropical varieties were grown under long days until stage V4-V5, were exposed to seven short days and then returned to long days. Control plants were kept continuously in LDs. Before the SD treatment, blades of the uppermost leaf of 3 plants were harvested. During the SD treatment and next seven days after returning to LD conditions, blades of the uppermost leaf of 5 plants were harvested every other day. After that, leaf blades of 5 plants were harvested according to their V stages. At the time, leaves were harvested and plants were dissected to check development of their SAM. All tissue collections were performed in the morning.

**RNA Isolation, RT-PCR and Quantitative RT-PCR**

Total RNA was isolated with TRIzol Reagent (Roche Applied Science). The Turbo DNA-free kit (Applied Biosystems) was used to remove DNA from the RNA samples. cDNA synthesis was performed with Superscript® III First-Strand Synthesis system (Invitrogen). All processes were done following the manufacturers’ instructions. PCR amplifications were performed using Expand Long Template DNA polymerase with 35 cycles for all transcripts (Roche Applied Science). PCR products were detected by Ethidium Bromide on Agarose gels. Quantitative RT-PCR amplifications were performed using the TaqMan® probe based detection system (Applied Biosystems). Primers and probes were designed according to nucleotide sequences of *FT*-like genes published in GenBank. All primers and probes are shown in Table 1. In qRT-PCR reactions, three biological replicates were used and each PCR was performed in triplicate. The relative expression level of genes was calculated by their quantification normalized to *ubiquitin 5*. The processes were performed following the User Bulletin #2 from Applied Biosystems at [http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf](http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf)
**Tissue Imaging**

Shoot apices and inflorescence meristems were dissected and fixed in 50% acetic acid. Images were taken with a Nikon SMZ1500 microscope and attached Nikon digital camera DXM1200.

**Arabidopsis T-DNA Constructs and Transformation**

GATEWAY® TECHNOLOGY (Invitrogen, CA) was used for vector construction. The promoters, the cDNA sequences and PINII terminator were put into pENTR™/D-TOPO vector (Invitrogen, CA) through ligation. Then LR recombination reaction was performed between the pENTR constructs and destination vectors having ATTR recombination sites (Invitrogen, CA) to generate binary expression vectors. The expression vectors were introduced into Agrobacterium strain GV3101 (pMP90RK). The floral dip method was used to transform Arabidopsis plants (Clough and Bent, 1998). Plants used for transformation were Arabidopsis ft homozygous mutant. In T1 plants, homozygous mutants were confirmed by PCR and transgene expression was examined by RT-PCR. In the T2 generation, two independent transgenic lines with 10 individuals were chosen to collect leaf number data.

**Statistical Analysis**

Mean values and standard deviations were calculated by linear regression using the Minitab 15® Statistical Software (Minitab Inc., PA). The difference in leaf number was tested by a one-way ANOVA taking the phenotype and the presence or absence of the transgene as the sources of variation. Tukey’s family error rate was chosen for one-way multiple comparisons with a p-value level of significance (α-level) equal to 0.05 and 0.1.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers:

EU241923 (ZCN7), EU241924 (ZCN8), EU241928 (ZCN12), EU241929 (ZCN14), EU241930 (ZCN15), EU241933 (ZCN18), EU241937 (ZCN26).
Results

Survey of *FT*-like *ZCN* gene transcription in leaves of temperate and tropical lines under SD and LD photoperiods

Four lines which vary significantly in their time to flower and sensitivity to photoperiod were used to assess transcript accumulation of seven *FT*-like *ZCN* genes in their leaves before and after the floral transition under SDs and LDs (Figure 1). To monitor the floral transition, shoot apices were dissected and images taken documenting the morphology of the shoot apical meristem (SAM) at each sampling time point. The transition from vegetative to flowering was judged by the extent of elongation of the apex and the appearance of branch meristems on the flanks of the SAM marking an early reproductive stage (Irish and Nelson 1991).

Survey of *ZCN* gene transcript accumulation is presented in Figure 2. Because the floral transition in the early-flowering, temperate Gaspé Flint line takes place between the fifth and sixth days after sowing when plants are still at the VE stage (emergence), leaves wrapped in the coleoptile were collected (Figure 2A). Under SDs, only *ZCN14* was expressed in leaves at the second and third days after sowing. When the leaves emerged from the soil on the fifth day after sowing and became photosynthetically competent, *ZCN8* and *ZCN18* mRNA were detected. Transcripts from both genes accumulated to moderately high levels after the floral transition at stages V1 and V2. *ZCN26* transcript was only weakly detected in green leaves after the floral transition. Under LDs, the floral transition occurred at the same stage as in SDs and the overall transcription patterns for the *ZCN* genes surveyed were similar to those under SDs. A low level of *ZCN8* mRNA was detected earlier than in SDs in the coleoptile-wrapped leaf at the second day after sowing. After leaves fully emerged from the coleoptile and became green, *ZCN8* expression was high at all stages. No transcripts of *ZCN7*, *ZCN12* and *ZCN15* were detected in leaves under either SDs or LDs (Figure 2A).

The floral transition in the temperate mid-maturity B73 line occurs at stages V4 –V5 under both SDs and LDs (Figure 2B). *ZCN8*, *ZCN18* and *ZCN26* transcripts were detected before and after the floral transition in fully mature green leaves under both photoperiods. *ZCN8* mRNA accumulation was low at early time points, increased moderately prior to the
floral transition and was abundant and stable thereafter. \(ZCN7\) transcripts were not detected under SDs but its unspliced form was found under LDs. \(ZCN12\) was not expressed at stages V2-V5 but mRNA was detected later at stages V6-V10 that is consistent with our previous results (Danilevskaya et al., 2008a). No \(ZCN14\) and \(ZCN15\) mRNAs were detected at vegetative stages but a low level of transcript could be seen after the floral transition. \(ZCN18\) and \(ZCN26\) were constitutively expressed in leaves across all stages under both SDs and LDs (Figure 2B).

Under SDs the floral transition in the tropical lines CML311 and CML436 takes place around stages V4-V5 which is similar to the temperate B73 line (Figure 2C and 2D). Under SDs, the expression patterns of the \(ZCN\) genes were nearly identical in both lines and very similar to their patterns in B73. In the tropical line CML436 unspliced \(ZCN8\) RNA was detected at early vegetative stages but fully spliced mRNA was detected prior to and after the floral transition.

Under LDs CML311 and CML436 transitioned at stages V7-V8 and V10-V11, respectively. The expression patterns of \(ZCN8\), \(ZCN12\), \(ZCN15\), \(ZCN18\) and \(ZCN26\) were similar to their patterns under SD conditions. Unspliced \(ZCN7\) mRNA was present only in CML436 grown under LDs. However, under LDs a novel \(ZCN14\) mRNA pattern was observed in both tropical lines. The abundance of \(ZCN14\) mRNA gradually increased before the floral transition preceding the onset of the \(ZCN8\) expression suggesting a possible role of \(ZCN14\) in promoting flowering under LDs.

Overall, the transcriptional analyses revealed that three genes, \(ZCN8\), \(ZCN18\) and \(ZCN26\), displayed expression patterns in the mature leaf before and after the floral transition which are consistent with a hypothetical function as a floral activator. However, the expression pattern of \(ZCN14\) suggests it may also play a role in the floral transition under LDs in some tropical lines.

**Responses of \(FT\)-like \(ZCN\) genes to a floral inductive SD treatment**

Arabidopsis plants grown under non-permissive SDs and then exposed to three inductive LDs will express \(FT\) and flower (Corbesier et al., 2007). Conversely, exposure of the obligate SD plant goosefoot (\(Chenopodium rubrum\)) to LDs will induce \(FT\)-like gene
expression resulted in early flowering (Chab et al., 2008). Tropical maize varieties are either moderately or highly sensitive to photoperiod and will flower earlier under SDs than under LDs. A SD inductive experiment was conducted to assess the response of two tropical lines to a limited exposure of SD photoperiod.

Pilot experiments indicated that a SD treatment of either three or five days was not long enough to induce an earlier floral transition in the tropical lines CML311 and CML436 grown under continuous LDs (Figure 3). However, after a treatment of seven SDs, the SAM started transitioning in both CML311 and CML436 at growth stage V5, which is earlier than controls grown under continuous LDs (Figure 4). The inflorescence meristem continued to develop into tassel primordia indicating the transition to reproductive growth was complete and irreversible. To quantify the effect of the inductive SD treatment on flowering time, total leaf number was counted for plants grown under LDs and treated for seven SDs (Table 2). CML311 and CML436 plants produced an average of 21 and 23 leaves, respectively, when grown under continuous LDs. Plants of both lines given the seven day SD treatment produced 19 leaves on average which was significantly fewer than those of controls. Thus, the SD treatment induced an earlier floral transition.

The expression patterns of the \( FT \)-like \( ZCN \) genes were determined in leaf blade tissue and shoot apices dissected from plants grown under continuous LDs but treated with seven SDs (Figure 4). Out of seven \( ZCN \) genes assayed, only \( ZCN8 \) and \( ZCN12 \) mRNAs were induced in leaves of plants induced by a transient SD treatment (Figure 4A and B). \( ZCN8 \) mRNA was detectable after one (CML436) or three (CML311) short day treatment and its abundance sharply increased during the rest of the treatment time period. The induction of \( ZCN12 \) mRNA was observed 2-3 days later than \( ZCN8 \). After a return to LDs, \( ZCN8 \) mRNA level dropped significantly but was still detectable, while \( ZCN12 \) expression was not detectable.

Expression of \( ZMM4 \), a floral transition MADS-box gene (Danilevskaya et al., 2008b), is a marker for the completing the floral transition. Its transcript accumulation was assayed in shoot apices sampled from the same plants given the SD inductive photoperiod treatment (Figure 4C and D). \( ZMM4 \) transcript was detected in shoot apices during and after the SD treatment. Unspliced \( ZCN7 \) and \( ZCN8 \) transcripts were always present in shoot
apices but mature ZCN8 mRNA was only detected in leaves. This observation hints that ZCN8 expression might be also regulated via pre-mRNA splicing. ZCN15, ZCN18 and ZCN26 transcripts were not detected in shoot apices. Notably, ZCN14 was the only FT-like ZCN gene whose fully spliced transcript was detected in shoot apices. Mature ZCN14 mRNA was detected in shoot apices after the floral transition and this expression pattern placed this gene apart from the other FT-like ZCNs, as none produced fully spliced mRNA in the shoot apex. Therefore, the inductive SD experiment narrowed down the list of candidate floral activators to ZCN8 as the only FT-like gene whose expression pattern was the most consistent as functioning as a floral activator under SDs.

**Diurnal expression of three FT-like ZCN genes under SD and LD photoperiods**

A hallmark of Arabidopsis FT gene expression, which directly contributes to its function as a floral activator, is its diurnal, circadian pattern with transcript abundance peaking at dusk under permissive LDs in the leaf (Turck et al., 2008). Rice Hd3a mRNA accumulation is also diurnally regulated under SDs with a peak of expression just before dawn (Kojima et al., 2002). Thus, I expect any ZCN gene with FT-like function should display a diurnal expression patterns in the leaf of photoperiod-sensitive lines responding to photoperiod. ZCN8, ZCN18 and ZCN26 transcripts accumulate in leaves, before and after the floral transition, from plants grown under either SD or LD photoperiods. Whether their mRNA accumulation showed a diurnal expression pattern in leaf blades of Gaspé Flint, B73 and CML436 was investigated by sampling plants every 4 hours during three days of vegetative growth for a set of plants grown under LDs and a set of plants grown under SDs (Figure 5).

In Arabidopsis, the diurnal expression of GI and CO activates FT expression under LDs (Turck et al., 2008). Expression of the maize homologs of GIGANTEA (Gigz1) and CO (Conz1) was also tested in the diurnal experiment (Miller et al., 2008). In the experiments these genes showed a diurnal pattern of expression as was described previously (Miller et al., 2008) (Figure 6). Their diurnal expression patterns were similar in the lines under either LDs or SDs with a peak in the end of day.
In the extremely early flowering day-neutral genotype Gaspé Flint, ZCN8 mRNA accumulation did not display a consistent diurnal oscillation under SDs or LDs, although accumulation tended to be higher at night (Figure 5). In the day-neutral line B73, ZCN8 mRNA expression had a weak diurnal expression pattern with low amplitude under both photoperiods but with expression peaking just before dawn. In B73, the oscillation of ZCN8 under SDs was more evident than that under LDs, however, the lowest level of ZCN8 transcript accumulation never reached zero.

In the tropically adapted, photoperiod-sensitive CML436 line, expression of ZCN8 under SDs was strikingly distinct (Figure 5). ZCN8 transcript abundance under SDs showed a clear diurnal pattern peaking at dawn. During the day ZCN8 transcript accumulation sharply declined and 8 hours after dawn ZCN8 transcript was too low to be detected. Under LDs at stage V5, when the line is in a vegetative growth stage, ZCN8 transcript accumulation was very low but was still diurnally expressed if graphed at an expanded scale (Figure 7A). However, when leaves were sampled during the floral transition under the LDs, the amplitude of the diurnal level of ZCN8 mRNA was comparable to amplitude under SDs. In addition, the peak of transcript accumulation shifted to 4 hours after dawn even though transcript accumulation at dusk remained undetectable (Figure 7B).

The expression of ZCN18 did not exhibit any consistent diurnal patterns in any inbred lines under either photoperiod (Figure 5). Notably, its expression level was very high in all lines except B73. Under SDs, ZCN26 displayed a diurnal expression pattern in B73 and CML436 with expression peaking at dawn. This was in contrast to expression in Gaspé Flint where ZCN26 mRNA was barely detectable. ZCN26 mRNA did not show a diurnal expression pattern under LDs in any of the three lines tested. Thus, only ZCN8 mRNA displayed an obvious and consistent diurnal expression pattern in the tropical line CML436 under both short and long day photoperiods.

**Overexpression of FT-like ZCN genes in Arabidopsis ft mutant**

In Arabidopsis, several FT loss-of-functions alleles were generated by ethylmethane sulfonate (EMS) or T-DNA insertion in ecotypes of *Columbia* and Landsberg erecta (Koornneef et al., 1991). Because of availability of the ft mutant to the public and the simple
plant transformation process, these ft alleles were widely used in complementation experiments to determine function of FT orthologs from other species. Two Arabidopsis EMS-induced ft alleles, ft-2 with a premature stop codon in the fourth exon and ft-3 with Arginine mutated to Histidine in the fourth exon, were selected for a complementation experiment. Several constructs constitutively expressing the ZCN genes under control of the Cauliflower mosaic virus (CaMV) 35S promoter (ProCaMV35S: ZCNs) were transformed to the Arabidopsis ft2 and ft3 alleles to test their complementation. ZCN8, ZCN14, ZCN15 and ZCN26 were chosen because ZCN8 and ZCN26 mRNA expression in leaf blades showed a high correlation with the floral transition and ZCN14 and ZCN15 exhibited highest protein sequence conservation with FT in the ligand-binding pocket and the external loop regions. T1 transgenic lines were genotyped using PCR and transgene expression was examined by RT-PCR. At least 2 independent transgenic lines in homozygous background were saved for further T2 generation analysis. Flowering time of 10 individuals of each line in the T2 generation grown under LDs was examined. The transgenic lines ectopically expressing ZCN8, ZCN14 and ZCN15 flowered significantly earlier than the ft mutant and wild type (Table 3). Especially the transgenic lines of ProCaMV35S: ZCN8 ft-2/ft-3 developed into reproductive stages with 5-6 rosette leaves and 0-1 cauline leaf (Figure 8). The transgenic plants of ProCaMV35S: ZCN14 ft-2/ft-3 and ProCaMV35S: ZCN15 ft-2/ft-3 had 5-6 rosette leaves and 2-3 cauline leaves. The transgenic line of ProCaMV35S: ZCN26 ft-2/ft-3 produced the same leaf number as those of the mutant plants. These results suggested that ZCN8, ZCN14 and ZCN15 but not ZCN26 had the ability to complement the Arabidopsis ft late flowering mutant. These three ZCN genes might be considered as putative candidates for maize floral activators.

Discussion

FT-like ZCN genes display distinct expression patterns in the leaf of maize lines with different photoperiod sensitivities

In identifying a maize florigen, expression analysis of seven FT-like ZCN genes in the leaf of four lines under either LDs or SDs and transient SD inductive conditions was conducted. Six of them, ZCN7, ZCN8, ZCN12, ZCN14, ZCN18 and ZCN26, were chosen, due to their expression in leaves in at least one developmental stage near the time of the
floral transition (Danilevskaya et al., 2008a). In addition, the ZCN15 gene was included in this study as it is syntenic to the rice flowering QTL Hd3 (Heading date3), which harbors the rice FT-like genes Hd3a and Hd3b (also known as RFT1) although it is mainly expressed in kernels not leaves and unlikely to function as florigen (Tsuji et al., 2008; Komiya et al., 2009). Each of the seven ZCN genes displays a unique combination of spatial, temporal and photoperiod-specific expression which implies functional diversification. A summary of our functional analyses of the seven FT-like ZCN genes are shown in Table 4.

ZCN8 transcript gradually accumulates in leaves at vegetative stages and reaches its highest expression level at or near the floral transition in all genotypes tested. ZCN8 mRNA accumulates rapidly in response to a floral-inductive SD treatment in photoperiod-sensitive tropical lines, correlating with an earlier floral transition. These data suggested ZCN8 expression is highly correlated to the floral transition. Its duplicate, the ZCN7 gene, appears to be non-functional, as fully spliced ZCN7 mRNA was not detected by RT-PCR from any of the tissues we tested under normal growth conditions.

Consistent with our previous data, no ZCN15 mRNA was detected in leaves from four diverse maize genotypes, as its expression is rather restricted to early stages of kernel development (Danilevskaya et al., 2008a). When during the evolution of the Poaceae ZCN15 might have acquired a function in developing kernels is an intriguing question to study. Identification of functional FT equivalents in teosinte and related species like sorghum might help to answer this question. The second closest rice FT homolog, ZCN14, is expressed predominantly in the shoot apex after the floral transition in temperate corn lines and in tropical lines grown under SDs. However in tropical lines grown under LDs, its mRNA was detected in leaves prior to the floral transition and in the apex after the transition. This observation suggests a putative function for ZCN14 in LD photoperiod response in tropical lines, but this hypothesis requires more rigorous study.

ZCN12 mRNA is detected in leaves at reproductive stages in temperate and tropical lines under SDs. Its expression is induced by the SD treatment in tropical lines, indicating that ZCN12 expression is regulated by SD photoperiods in these genotypes.

Both ZCN18 and ZCN26 appear to be constitutively expressed in leaves in all growth stages and genotypes studied. Although ZCN18 transcript accumulated to a higher level than
the other leaf-expressed ZCNs, its diurnal regulation was inconsistent in different photoperiod sensitive lines. Thus, ZCN18 function appears to be independent of the flowering time network with an unknown function. ZCN26 expression in temperate lines with normal flowering time and tropical lines under SDs exhibited a distinct diurnal pattern suggesting that ZCN26 may be connected to a clock-regulated pathway.

ZCN8 transcription is diurnally regulated in photoperiod-sensitive tropical lines but not in day-neutral lines

The diurnal expression of FT genes plays a key role in flowering of photoperiod-sensitive species. In Arabidopsis, a long day plant, FT is regulated by the circadian clock through the GI–CO photoperiodic pathway (Turck et al., 2008). In rice, a short day plant, there are two FT-like genes that produce a mobile florigen signal, Hd3a and RFT1 (also known as Hd3b). Under short days, rice flowering is regulated by a SD activation pathway, predominantly via Hd3a. Under long days, flowering is regulated by a LD suppression and activation pathway, via RFT1 (Komiya et al., 2008; Komiya et al., 2009). Accurate day length regulation of both FT-like genes is critical for the fine tuning of flowering in rice to achieve the most optimal reproductive outcome (Itoh et al., 2010).

Widely grown throughout the temperate regions of the globe, modern corn varieties have been adapted to be day-neutral although maize was originally domesticated from the obligate short-day plant teosinte (Colasanti and Muszynski, 2009). Temperate maize is considered as day insensitive even though it will produce 1-2 fewer leaves when grown under short-day compared to long-day photoperiods (Russell and Stuber, 1983). Maize genotypes adapted to growth in tropical environments retain more photoperiod sensitivity and the most sensitive lines produce up to 30 leaves under LDs compared to 19-20 leaves under SDs (Russell and Stuber, 1983).

ZCN8 diurnal expression patterns during vegetative growth were compared in the temperate early flowering cultivar Gaspé Flint, the mid-maturity temperate inbred B73, and the photoperiod sensitive tropical inbred CML436 under both short and long day photoperiods. In Gaspé Flint, ZCN8 shows no diurnal oscillation under either day length. In B73, ZCN8 mRNA has a weak diurnal expression pattern under SDs but not under LDs.
Thus, diurnal oscillations of *ZCN8* transcript levels may not be required in these genotypes to induce the floral transition. In contrast, in CML436, under inductive SDs, *ZCN8* transcript exhibits a strong diurnal expression pattern with the maximum amplitude of expression occurring at the end of the dark period, just before dawn. After its peak in expression, *ZCN8* transcript accumulation plummets to zero and stays undetectable during the rest of the light period (Figure 5A). In CML436 grown in LDs, the *ZCN8* mRNA level is very low, but it still retains a diurnal rhythm with a peak of expression around dawn. During the floral transition under LDs, the amplitude of *ZCN8* expression increased 50-100 fold compared with expression at vegetative stages under LDs. These results indicate that *ZCN8* expression in photoperiod-sensitive tropical maize is tightly regulated by photoperiod. Under less inductive long days, *ZCN8* transcription is suppressed suggesting the existence of a LD suppression pathway.

The diurnal expression of the maize homologs of *CO* (*conz1*) and *GI* (*Gigz1A and 1B*), which are upstream of the *FT*-like genes in the photoperiodic pathway, was also investigated (Miller et al., 2008). All three genes are diurnally regulated in temperate and tropical lines suggesting that the photoperiodic pathway upstream of *ZCN8* is intact in temperate and tropical genotypes. The circadian clock is also intact and functional in temperate maize as was recently published for the inbred B73 (Hayes et al., 2010). This finding suggests that temperate maize may have lost photoperiod sensitivity downstream of the circadian clock but upstream of the regulation of *ZCN8*, which might explain why the floral transition in temperate lines are less sensitive to differences in day length.

**Arabidopsis complementation experiment suggests that *ZCN8*, *ZCN14* and *ZCN15* are putative maize floral activators in which *ZCN8* is the most promising one**

A direct approach to confirm the function of *ZCNs* as floral activator(s) would be to use reverse genetics strategy to generate *ZCN* genes mutants. However, mutants of *ZCN* genes are not available in mutagenized maize populations. Due to the ease and high efficiency of Arabidopsis transformation, complementation of the Arabidopsis *ft* mutant is an alternative way to test a gene function and identify potential floral activators. Crops, vegetables and other plant species which have difficulties in generating transgenic plants
usually are studied by this approach. For example, transgenic Arabidopsis plants overexpressing the rice *Hd3a* gene had earlier flowering time and produced four leaves compared with ten leaves in Columbia wild type (Kojima et al., 2002). Similar results were obtained in rice (Kojima et al., 2002). The consistency of the results between different systems suggests that testing gene function from other plant species in Arabidopsis could provide useful information about their endogenous function.

In the complementation experiment, ectopic expression of *ZCN8, ZCN14* and *ZCN15* in Arabidopsis *ft* mutants induced early flowering in transgenic plants, indicating that they could be candidates of a maize floral activator. *ZCN26* does not rescue the Arabidopsis *ft* mutant. However, only *ZCN8* has other characteristics more consistent with the molecular requirements for FT function. Neither *ZCN14* nor *ZCN15* are expressed in the leaf, but they are expressed in tissues not related to transition from vegetative to reproductive development. One of reasons why they complemented the *ft* mutant is that their protein sequences have the residues crucial for the floral activator such as Tyr (85) and Gln (140) of FT and Tyr(134), Gly(137) and Trp(138) of BvFT2 (Hanzawa et al., 2005; Pin et al., 2010). Surprisingly, *ZCN8* has different residues, Phe(F)132, Glu(E)135 and Met(M)136, at the corresponding positions, suggesting that these positions have evolved enough flexibility for FT proteins function as a repressor or an activator of flowering (Figure 9). The transgenic lines of *ProCaMV35S: ZCN8 ft-2/ft-3* with over-expression of *ZCN8* produced terminal flowers immediately after germination and their whole life cycle is significantly shorter than that of *ProCaMV35S: ZCN14/15 ft-2/ft-3*, also indicating that *ZCN8* could bypass limiting factors such as the antagonistic activity of TFL1 to induce flowering in Arabidopsis.

Although one out of seven *FT*-like *ZCN* genes can be considered as a highly possible floral activator, its functional analysis in maize is necessary to validate this speculation. In chapter three, experiments of determining which *ZCN* genes function as maize floral activators will be described.

**Acknowledgments**

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References


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<td>ZCN8-Probe</td>
<td>CAGAACACTAGTGTCGCGCC</td>
<td>qRT-PCR</td>
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<td>ZCN18-F</td>
<td>GGCCTAGGTGGATGAGGCGAGTCTC</td>
<td>qRT-PCR</td>
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<td>ZCN18-R</td>
<td>GTCTGCGATCGGTCGAATCA</td>
<td>qRT-PCR</td>
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<tr>
<td>ZCN18-Probe</td>
<td>CGTGCTGCTGACTGCACC</td>
<td>qRT-PCR</td>
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<td>TTGCGATTGGTGAGGCTGACATC</td>
<td>qRT-PCR</td>
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<tr>
<td>ZCN26-R</td>
<td>CTTCTATGCTGGATGAGGCTGAGGCA</td>
<td>qRT-PCR</td>
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<tr>
<td>ZCN26-Probe</td>
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<td>qRT-PCR</td>
<td></td>
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<tr>
<td>gigz1-F</td>
<td>GCAGAAAGGTACATCAGGCTACCA</td>
<td>qRT-PCR</td>
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<td>qRT-PCR</td>
<td></td>
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<tr>
<td>gigz1-Probe</td>
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<td>qRT-PCR</td>
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<td>GGCAGGTGCTGTAACGAGGAGG</td>
<td>qRT-PCR</td>
<td></td>
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<tr>
<td>conz1-R</td>
<td>CCACCTCCATGACAGTGAG</td>
<td>qRT-PCR</td>
<td></td>
</tr>
<tr>
<td>conz1-Probe</td>
<td>AGCAACAGCATCCTCT</td>
<td>qRT-PCR</td>
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Table 2. Leaf number of CML311 and CML436 grown under an inductive seven SD treatment compared to continuous LDs

<table>
<thead>
<tr>
<th>Tropical lines</th>
<th>Leaf No.</th>
<th>No. of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML311- inductive SDs</td>
<td>19 ± 0.48</td>
<td>10</td>
</tr>
<tr>
<td>CML311- continuous LDs</td>
<td>21 ± 0.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>CML436- inductive SDs</td>
<td>19 ± 0.67</td>
<td>10</td>
</tr>
<tr>
<td>CML436- continuous LDs</td>
<td>23 ± 2.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
</tbody>
</table>

Leaf numbers were collected from at least 10 individual plants. Mean values and standard deviations were calculated by ANOVA analyses from Minitab Statistical Program (Minitab Inc.). <sup>a</sup> - means are statistically significant with p<0.05
Table 3. Leaf number of transgenic constructs in Arabidopsis ft mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of T1 transgenic lines</th>
<th>Total rosette leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Ler, Landsberg er)</td>
<td>6</td>
<td>9.5 ± 1.0</td>
</tr>
<tr>
<td>ft-2</td>
<td>6</td>
<td>14 ± 1.0</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN8} \text{ft-2})</td>
<td>3</td>
<td>3.5 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN14} \text{ft-2})</td>
<td>8</td>
<td>6 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN15} \text{ft-2})</td>
<td>1</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN26} \text{ft-2})</td>
<td>15</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>ft-3</td>
<td>6</td>
<td>14 ± 1.2</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN8} \text{ft-3})</td>
<td>3</td>
<td>4 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN14} \text{ft-3})</td>
<td>3</td>
<td>6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN15} \text{ft-3})</td>
<td>4</td>
<td>6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(\text{ProCaMV35S:ZCN26} \text{ft-3})</td>
<td>15</td>
<td>12 ± 0.7</td>
</tr>
</tbody>
</table>

Leaf numbers were collected from at least 10 individual plants. Mean values and standard deviations were calculated by ANOVA analyses from Minitab Statistical Program (Minitab Inc.). <sup>a</sup> - means are statistically significant with p<0.05.
Table 4. Summary of molecular characteristics of seven *FT*-like *ZCN* genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue</th>
<th>mRNA detected</th>
<th>Timing</th>
<th>Diurnal pattern</th>
<th>SD induction</th>
<th>Complementation to <em>ft</em> mutant</th>
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<tbody>
<tr>
<td>ZCN7</td>
<td>Unspliced</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>ZCN8</td>
<td>Leaf</td>
<td>Prior FT</td>
<td>SD; LD</td>
<td>induced</td>
<td>Yes</td>
<td></td>
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<tr>
<td>ZCN14</td>
<td>SAM</td>
<td>After FT</td>
<td>n/a</td>
<td>induced</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>ZCN15</td>
<td>Kernel</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>ZCN12</td>
<td>Leaf</td>
<td>After FT</td>
<td>n/a</td>
<td>Induced</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>ZCN18</td>
<td>Leaf</td>
<td>All times</td>
<td>LD</td>
<td>NO</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>ZCN26</td>
<td>leaf</td>
<td>All times</td>
<td>SD</td>
<td>NO</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

n/a-not applicable; LD-Long Day; SD-Short Day; FT-floral transition.
Figure 1. Whole plant images of the four varieties used in this study.

(A) Extreme early flowering temperate line Gaspé Flint produces 6-8 leaves under SDs or LDs. (B) Temperate B73 inbred line produces 18-19 leaves under SDs or LDs. (C) Photoperiod-sensitive tropical line CML311 produces 23-24 leaves under LDs and 18-20 leaves under SDs. (D) Photoperiod-sensitive tropical line CML436 produces 26-30 leaves under LDs and 18-20 leaves under SDs. The plants for images were grown in the summer in Iowa, USA (latitude 42°N).
Figure 2. Expression patterns of seven FT-like ZCN genes in leaves of four maize lines under SDs and LDs.

(A) RT-PCR of FT-like ZCN genes in the early flowering Gaspé Flint line. Due to transition to reproductive development at the VE stage, leaves were sampled by days after seed sowing (DAS) starting with day 3. The tissues collected at the third and fourth DAS were leaves wrapped in the coleptile. After that, the tissues collected were fully emerged leaf blades. Leaves at the fifth day after sowing were collected in the morning and the afternoon separately. (B) RT-PCR of FT-like ZCN genes in the temperate line B73. (C) RT-PCR of FT-like ZCN genes in the tropical line CML311. (D) RT-PCR of FT-like ZCN genes in the tropical line CML436. On the top of each panel the images represent the shoot apices at stages when leaf blades were collected. Black arrows and thin vertical lines demarcate the timing of the floral transition. Vegetative growth stages (V stages) were defined according to the full extension of the leaf collar of the uppermost leaf where the first V stage is designated as VE (emergence). The superscript numbers 1, 2, 3 and 4 mark early and later stages of the same V stage, as judged by different internode lengths between the uppermost leaf and the leaf below it. RT-PCR products were detected on ethidium bromide stained agarose gels. The first and last lanes of each gel image are a DNA marker and genomic PCR product, respectively. Asterisks (*) mark genes with unspliced transcripts. Bar = 100 μm.
Figure 3. The development of the shoot apical meristem of two tropical lines after three or five SDs treatment.

Plants were grown under continuous LDs until V5, transferred to SDs for 3 or 5 days and then returned to LDs. Under continuous SD treatment, CML311 and CML436 transition around stage V4-V5. Under continuous LD treatment, CML311 starts to transition around V7 and CML436 around V10. Arrows indicate the growth stage of the floral transition of the tropical lines under continuous LDs. Long days were 16 h of light and 8 h of dark. Short days were 8 h of light and 16 h of dark.
Figure 4. Effect of an inductive short day treatment on gene expression in tropical lines. (A) and (B) RT-PCR of FT-like ZCN genes in leaf blade of CML311 and CML436 plants grown for four weeks under LDs, followed by seven SDs and then transfer back to LDs for one month. The images on the top of each panel represent development of shoot apices at specific growth stages when leaf blades were collected. The images marked by the superscript letter “a” indicate leaves collected every other day. The superscript numbers 1, 2 and 3 mark early and later growth stages of the same V stage, as judged by internode lengths between the uppermost leaf and the leaf below it. The bracket and the solid lines indicate the growth stages that underwent the SD treatment. RT-PCR products were detected on ethidium bromide stained agarose gels. The first and last lanes of each gel image are marker DNA and genomic PCR, respectively. (C) and (D) RT-PCR of FT-like ZCN genes and the flowering MADS-box gene ZMM4 in shoot apices of the same plants as described above. Bar = 100 μm.
Figure 5. Diurnal expression patterns of three $FT$-like $ZCN$ genes in leaf blades of three inbred lines under SDs or LDs during vegetative growth.

Relative expression levels were determined by quantitative RT-PCR normalized to $ubiquitin5$ (the Y axis). The X axis represents time point (hours). Data points represent an average of three biological replicates with three technical replicates. Error bars represent standard deviation. The shaded bars over each chart represent dark periods.
Figure 6. Diurnal expression of Gigz1 and Conz1 in leaf blades of plants grown under either SDs or LDs.

(A) Diurnal expression of Gigz1 and Conz1 during the floral transition in all three lines under SDs and in Gaspé and B73 under LDs and during vegetative stage growth of CML436 under LDs. (B) Expression patterns of Gigz1 and Conz1 in the tropical line CML436 during the floral transition under LDs. Relative expression levels were determined by quantitative RT-PCR normalized to ubiquitin5 (the Y axis). The X axis represents time point (hours). Data points represent an average of three biological replicates with three technical replicates. Error bars represent standard deviation. The shaded bars over each chart represent dark periods.
A. Gigz1

B. Conz1

Gaspé

B73

CML436

B. CML436

LD
Figure 7. ZCN8 diurnal expression in the tropical line CML436 under LDs.

(A) ZCN8 diurnal expression in CML436 mature leaf at vegetative growth stages under LDs at an expanded scale from Figure 5. (B) ZCN8 diurnal expression in the CML436 mature leaf during the floral transition under natural LD conditions. Relative expression levels were determined by quantitative RT-PCR normalized to ubiquitin5 (the Y axis). The X axis represents time point (hours). Data points represent an average of three biological replicates with three technical replicates. Error bars represent standard deviation. The shaded bars over each chart represent dark periods.
Figure 8. Images of wild type, $ft$ mutants and transgenic plants.

The plants were thirty days old in $Ler$ background and grew under LDs.
Figure 9. The alignment of the amino acids sequences (encoding an external loop of PEBP protein) of Arabidopsis, sugar beet and maize FT-like proteins.

Three amino acids, Asn (N)134, Gln (Q)137 and Gln(Q)138 that are the major cause of BvFT1 repressive function are in bold. Maize floral activator ZCN8 carries in these position Phe(F) 132, Glu(E)135 and Met (M)136. The external loop is boxed.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession</th>
<th>Sequence</th>
</tr>
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<td>AtFT</td>
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CHAPTER THREE: THE FT-LIKE ZCN8 GENE FUNCTIONS AS A MAIZE FLORAL ACTIVATOR IN THE id1-dlf1 GENETIC PATHWAY

Modified from a paper published in the Plant Cell (Meng et al., 2011)

Abstract

In the previous chapter, three FT-like ZCN genes, ZCN8, ZCN14 and ZCN15, were identified as putative maize floral activators. Further functional analyses of FT-like ZCN genes were performed including the genetic relationship with indeterminate 1(id1) and delayed flowering 1(dlf1), protein interaction with DLF1, and transgenic manipulation of gene expression. ZCN8 stood out as a maize floral activator. Genetically, only ZCN8 was positioned downstream of id1 and in parallel to or upstream of dlf1. ZCN8 and ZCN26 mRNAs were spatially expressed in vasculature tissues of the leaf including phloem, xylem parenchyma and epidermal cells. According to functional analysis of the genes, only overexpression of ZCN8 in shoot apices induced early flowering and reduced mRNA expression resulted in late flowering. The cumulative results are consistent with ZCN8 functioning as a maize floral activator with potential florigenic activity.

Introduction

Early grafting experiments in plants suggested that the transition from vegetative to reproductive development is triggered by the movement of a hypothetical flowering hormone, named florigen (‘flower-former’), from leaves to the shoot apical meristem (Chailakhyan, 1937). Until recently, the biochemical nature of florigen remained elusive until breakthrough experiments in tomato (Lifschitz and Eshed, 2006; Lifschitz et al., 2006), Arabidopsis (Corbesier et al., 2007; Jaeger and Wigge, 2007; Notaguchi et al., 2008) and rice (Tamaki et al., 2007) revealed that the protein encoded by the Flowering locus T (FT) gene and its orthologs had florigenic activity.

Arabidopsis is photoperiod sensitive and flowers earlier under LDs than SDs. As early as 1991, Koornneef et al., isolated and described quite a few Arabidopsis late-flowering mutants from ethyl methanesulphonate (EMS) treated populations in Ler including Gigantea (GI), Constans (CO), and a number of mutants named alphabetically, for example the 4th (D)
locus obtained controlling flowering time ($FD$) and so on (Koornneef et al., 1991). $FT$ was among them. The flowering time mutants flower late only under LDs and produce more rosette and cauline leaves. Activation tagging or constitutive expression of the genes in Arabidopsis plants results in precocious flowering with much fewer leaves independent of photoperiods (Kardailsky et al., 1999a). The proportional relationship between the days to flower and leaf number illustrates the correlation between the two parameters of flowering time (Koornneef et al., 1991). Genetic analysis of double/triple mutants among the late-flowering loci proves they have epistatic interactions and are in the same genetic pathway called the photoperiod pathway (Koornneef et al., 1998). In the photoperiod pathway, GI-$CO-FT$ is the core module to regulate the floral transition. GI perceives messages from the circadian clock genes in the leaf. CO protein activates $FT$ mRNA expression in the leaf vascular tissue. Using transgenic plants ectopically expressing fluorescent-fusion or the Myc-tagged FT protein in the phloem or grafting experiments, it was shown that the FT protein moved via the phloem to the shoot apical meristem (Jaeger and Wigge, 2007; Mathieu et al., 2007; Notaguchi et al., 2008). There, the FT protein interacts with $FD$, a bZIP transcription factor, to induce expression of floral identity genes (Abe et al., 2005; Wigge et al., 2005). Hence the FT protein has all the required features expected for the hypothetical flowering hormone, florigen (Tsuji et al., 2008; Turck et al., 2008; Zeevaart, 2008).

Furthermore, progeny of the cross between transgenic plants overexpressing $FT$ and mutant plants without $FD$ expression always produces more leaves than those of the transgenic plants alone under LDs or SDs, suggesting the activation of floral identity genes by $FT$ in the shoot apical meristem requires the $FD$ activity (Abe et al., 2005; Wigge et al., 2005).

The FT protein and its orthologs are conserved as universal florigen in plants. In rice, experiments expressing Hd3a-fluorescent fusion protein in the vascular tissue of leaf blade, demonstrated the movement of Hd3a into the shoot apices (Tamaki et al., 2007). Heterografting performed in Arabidopsis, the cucurbits and tomato provided definitive evidence that FT protein, not $FT$ mRNA, has long-distance movement from the leaf to the shoot apical meristem (Lifschitz and Eshed, 2006; Lifschitz et al., 2006; Lin et al., 2007; Notaguchi et al., 2008; Notaguchi et al., 2009). Subsequently, Lifschitz et al reported that there are some limitations on FT florigen function after analyzing transgenic plants.
overexpressing fusions of SFT-GFP under the viral 35S promoter or different tissue specific promoters (Shalit et al., 2009). One limitation is the size of the protein. Transgenic plants with these fusions can promote earlier flowering. However, conflicting with results from Arabidopsis and rice, fluorescent signals were not observed in the shoot apical meristem and graft-transmissible stimulators from transgenic plants were not detected either in grafting receptors. Secondly, the selection of tissue specific promoters to ectopically expression of SFT is crucial for its forigen effect. In contrast to the phloem-specific SUC2 promoter, none of the tomato leaf-specific promoters successfully activated transgenic FT function as a long-distance moving signal in tomato (Shalit et al., 2009).

In my second chapter, it was shown that ZCN8 is the most promising candidate for the maize floral activator. ZCN8 mRNA accumulation in leaves always preceded the floral transition of the shoot apical meristem in the four maize lines tested regardless of photoperiod. ZCN8 transcription was diurnally regulated in photoperiod sensitive tropical lines but showed an attenuated cycling pattern in day-neutral temperate lines. Moreover, constitutive expression of ZCN8 rescued late flowering of Arabidopsis ft mutants.

This chapter reports functional analysis of candidate genes using a transgenic approach. In my transgenic approach, the genes were ectopically expressed certain tissues under control of tissue specific promoters or repressed by transgene-mediated gene silencing. In Arabidopsis and rice, ectopic expression of FT or Hd3a using a shoot apical meristem specific promoter such as FD promoter or a phloem specific promoter, such as Arabidopsis Sucrose-Proton Symporter 2 (AtSUC2), or Rice Phloem Protein 16 (RPP16) promoter, induces early flowering (Tamaki et al., 2007; Turck et al., 2008). In addition to transposon-inserted and chemical-induced mutants, transgene-mediated gene silencing is a powerful approach to test gene function. RNA interference (RNAi) and artificial miRNAs (amiRNAs) are major tools in the transgene-mediated gene silencing approach and amiRNAs has higher specificity to repress gene expression (Chuang and Meyerowitz, 2000; Schwab et al., 2006). There were two amiRNAs generated in Arabidopsis to silence endogenous FT expression. Both of them efficiently decreased FT transcript and resulted in late flowering comparable to ft null mutants (Schwab et al., 2006).
In this chapter genetic interaction between late flowering id1 and dlf1 mutants and FT-like ZCN genes were studied. ZCN – DLF1 protein interaction and in situ hybridization further refined functionality of FT-like ZCN genes. Over-expression of genes under control of tissue specific promoter or down-regulation by amiRNA was used to examine FT-like ZCN gene function in maize. Only ZCN8 mRNA in id1 and dlf1 mutants exhibited distinct patterns in which its transcript was not present in id1 until late inflorescence stage and not affected in dlf1. Additionally, id1 and ZCN8 were coexpressed in the transition section of a leaf and ZCN8 strongly interacted with DLF1. ZCN8 mRNA was spatially expressed in the phloem of the leaf of maize lines with different photoperiod sensitivities. Ectopic expression of ZCN8 in the shoot apex induced early flowering time and transgenic plants with repressed ZCN8 mRNA expression had late flowering time. All experimental evidence demonstrates that ZCN8 is a maize floral activator and a possible mobile flowering signal. In id1-dlf genetic flowering pathway, ZCN8 is put downstream of id1 and upstream of or in parallel to dlf1.

Materials and Methods

Plant Materials and Growth Conditions

Gaspé Flint, B73, and CML436 maize lines were chosen for this research due to their distinct photoperiod sensitivities and differences in flowering times. Seeds were germinated directly in soil and seedlings grown in growth chambers. LDs were set up for 16 h light and 8 h dark and SDs were 8 h light and 16 h dark. The temperature varied from 25-27°C during the day and 23-24°C during the night.

Both dlf1-N2461A and id1-m1 alleles were obtained from the Maize Genetic Cooperation Stock Center (http://www.maizegdb.org/cgi-bin/stockcatalog.cgi). All plants were grown in the field under LDs and genotyping was done around 14 days after planting. Tissues of wild type and homozygous were collected according to days after sowing and included leaf blades, immature leaf and shoot apices.
Tissue Collection

Tissues of B73 were collected at stages around the floral transition. When plants were at V5 stages, three individual plants were pooled together and their parts above ground were divided into four major sections for sampling including shoot apices with 1-2 immature leaves around them, the sixth and seventh leaves, the fifth leaf and the fourth leaf. Every section had three replicates.

RNA Isolation, RT-PCR and Quantitative RT-PCR

Please refer to the section of “Material and Method” in chapter two.

RNA in situ hybridization

Tissues were collected at different vegetative growth stages, mainly before and after the floral transition. The floral transition was detected when the SAM was elongated compared to its width. Stage VE is defined as the time when the first true leaf emerges from the coleoptile. Collection of Gaspé Flint leaf blade began after five days of growth under LDs at stage VE. Collection of B73 leaf blade began before the floral transition and continued to V6 under LDs. Collection of CML436 leaf blade started when plants reached V5 under SDs. All tissue sampling was performed in the morning. The procedures of tissue fixation, probe preparation and in situ hybridization were performed following the protocols described previously with minor modifications (Kidner and Timmermans, 2006). The templates for in vitro RNA syntheses were generated by PCR using primers listed on Supplemental Table 1. Antisense and sense probes were labeled with digoxigenin. Sections were viewed under Leica DMRXA research microscope and images were taken with a Leica digital camera DC500.

Yeast Two-Hybrid and GST Pull-Down Assay

Yeast two-hybrid analysis was performed using the ProQuest™ Two-Hybrid System (Invitrogen) following the manufacturer’s instructions. cDNAs of genes were cloned into both pDEST™ 32 (the Bait vector) and pDEST™ 22 (the Prey actor) to test for reciprocal interactions. All plasmids were transformed into the MaV203 yeast strain. The yeast cells
were plated on dropout media SD-/Leu/-Trp. Individual clones were suspended in sterile saline and diluted 10 fold. 2 µl of diluted clones were spotted onto plates of SD-/Leu/-Trp and SD-/Leu/-Trp/-His+3AT.

*ZCNs* and *DLF1* ORFs were firstly cloned into pENTR/SD/D-TOPO (Invitrogen). The plasmids expressing GST-gene fusions were obtained by recombination between the pENTR and pDEST™15 vectors (Invitrogen). The control plasmid only expressing GST was constructed by deleting *ZCN8* ORF in pDEST™15 vector. The plasmids were transformed into the *E.coli* BL21-AI™ (Invitrogen). The *DLF1* ORF was cloned into pET-22b (+) vector (Novagen) to get 6 X His tags fused to N-terminal of DLF1. The plasmid was transformed into BL21 (DE3) (Agilent) to express His tag-DLF1. The GST pull-down assay was performed according to the manufacturer’s protocol (Thermo Scientific). Samples were run on 10% SDS-PAGE (Bio-Rad) and analyzed by western blotting using GST and His-tag antibodies (GenScript).

**Tissue Imaging**

Please refer to the section of “Material and Method” in chapter two.

**Maize T-DNA Constructs and Transformation**

GATEWAY® TECHNOLOGY (Invitrogen, CA) was used for vector construction in maize. The promoters, the cDNA sequences and PINII terminator were put into pENTRTM/D-TOPO vector (Invitrogen, CA) through ligation. Then LR recombination reaction was performed between the pENTR constructs and destination vectors containing ATTR recombination sites (Invitrogen, CA) to generate the expression vectors. The expression vectors were introduced into Agrobacterium strain LBA4404 and used to transform plants as described by Unger et al., 2001; Cigan et al., 2005. Transgenic plants were identified by RT-PCR testing of expression of the transgene at the T0 or T1 generation. In the T1 generation, two independent events with 15 individuals for each construct were chosen for leaf number data collection. The fifth and tenth leaves were marked during the growth of plants to count total leaf number. The 21 mer (amiRNA) 5’-TCTCATAAAATATTAGCTCTT was designed for *ZCN8* artificial microRNA was
designed according the rules for artificial microRNA (Schwab et al., 2006). The 21 amiRNA was incorporated into the zma-miR396h backbone (Zhang et al., 2009) and then was cloned into the pENTR vector for engineering the co-integration vectors.

Statistical Analysis

Please refer to the section of “Material and Method” in chapter two.

Accession Numbers

Please refer to the section of “Material and Method” in chapter two.

Results

Expression of FT-like ZCN genes in dlf1 and id1 late flowering mutants

Expression of seven FT-like ZCN genes was examined in both id1-m1 and dlf1-N2461A late flowering mutants to establish their genetic interactions. Homozygous mutants introgressed into the B73 inbred background were grown and tissues sampled as described previously (Danilevskaya et al., 2008b). The morphological development of the SAM of wild type B73 (WT), dlf1 and id1 at different stages are shown in Figure 1A. The floral transition occurs two weeks later in dlf1 and one month later in id1 compared to WT. RT-PCR was performed using leaf blade tissue (Figure 1B). ZCN7, ZCN14 and ZCN15 mRNA were not detectable in any genotype. ZCN26 transcript was detected in all genotypes with a similar accumulation pattern, indicating its expression is independent of the id1-dlf1 pathway. ZCN18 showed an inconsistent pattern of expression that was difficult to interpret. Interestingly ZCN12 was ectopically expressed in the dlf1 mutant, pointing to the possibility of regulation by dlf1.

Of the ZCN genes assessed, only ZCN8 displayed a clear and distinct pattern of expression in the two late flowering mutants. In B73 plants, transcript accumulation in leaves was first detected on the 17th day after sowing and accumulation increased during development. In the dlf1 mutant, ZCN8 was expressed in leaves at all time points tested. In the id1 mutant, ZCN8 mRNA was not detected until the last stage that was well after the
floral transition. This result suggested that ZCN8 is downstream of id1 and upstream or in parallel to dlf1.

Given the placement of ZCN8 downstream of id1, a question was asked if ZCN8 or any of the other FT-like ZCNs surveyed were co-expressed with id1 in immature leaves, the tissue where id1 mRNA is predominantly produced (Colasanti 1998). For this reason, expression of the seven FT-like ZCN genes was tested in immature leaves. None of the seven ZCN genes tested was expressed in immature leaves (Figure 2A). Because ZCN14 mRNA was expressed in the shoot apex, its expression was examined in WT and id1 mutant apices. ZCN14 transcript was abundant in both WT and mutant shoot apices after the floral transition indicating its expression is independent of the id1-dlf1 pathway (Figure 2B).

id1 is expressed in the immature leaf but ZCN8 transcripts are mainly detected in the mature leaf. To determine if expression of both genes overlaps at some position along the gradient of the developing leaf, leaves from B73 plants at stage V5 were dissected into precise sections. From stage V5 plants, leaf 4 and leaf 5, the youngest, uppermost leaf with an exposed auricle/ligule (collar) region, were dissected into basal leaf sheath tissue and 12 cm sections of mature, distal leaf blade tissue (Figure 1C, sections 9-17). The next youngest leaf, leaf 6, with a photosynthetically competent distal blade tip but still immature basal region, was dissected into 4 cm sections of basal immature leaf tissue, a 20 cm section of transitional leaf tissue and a 20 cm section of mature leaf blade tissue (sections 3-8). The youngest leaf primordia enclosing the SAM and a section with the adjacent 1-2 more developed leaves above the apex comprise the last two samples (sections 1 -2). The abundance of ZCN8 and id1 transcript in each section was analyzed by quantitative RT-PCR (Figure 1C). Transcripts for both genes were not detected in the shoot apex and next adjacent section of immature leaf (sections 1 and 2). In the sixth leaf, id1 was expressed in the immature sections (sections 3-6) and the transitioning section (section 7) with expression barely detectable in the green, mature leaf blade (section 8). In the more mature fourth and fifth leaves, no id1 mRNA was detected in either the leaf sheath or leaf blade (sections 9-17). Overall, id1 expresion was highest in immature leaf sections 4-8 cm above the shoot apical meristem with expression decreasing in more mature sections of the leaf.
ZCN8 expression, on the other hand, displayed an expression pattern complimentary to that of id1. No expression was detected in the immature leaf sections up to 4cm above the shoot apex (sections 1-3). Rather, transcripts were first detected in immature leaf sections 4, 5 and 6. Transcripts were also detected in transitioning leaf tissue (section 7) with the highest accumulation in the mature leaf tissue of section 8. ZCN8 mRNA was consistently detected in the mature leaf blade sections (10–13 and 15–17) of leaf 4 and 5 but not the leaf sheath. Overall, throughout the basal to distal developmental gradient of the leaf, id1 expression is highest in basal immature leaf sections and diminishes in more distal mature blade, while ZCN8 expression is low in basal sections and increases in more mature distal tissue. Hence during leaf development, expression of id1 precedes expression of ZCN8.

**Interaction of FT-like ZCN proteins with the DLF1 protein**

In Arabidopsis, the FT protein interacts in the shoot apical meristem with the bZIP transcription factor FD to activate flower morphogenesis (Abe et al., 2005; Philip A. Wigge, 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007). Previously we have shown that the ZCN8 protein interacts with the DLF1 protein which is the presumed maize FD ortholog (Danilevskaya et al., 2008a). Yeast two-hybrid (Y2H) and GST pull down analyses were used to test the seven candidate FT-like ZCN proteins for interactions with DLF1 (Figure 3). In Y2H analysis, DLF1 showed self-interaction which is typical for bZIP transcription factors which normally function as dimers. None of the ZCN proteins showed self interactions (Figure 3A). ZCN7, ZCN8 and ZCN26 showed interactions with DLF1 in reciprocal Y2H reactions, whereas, ZCN14, ZCN12, ZCN15 and ZCN18 only interacted with DLF1 in one bait-prey combination, indicating that these ZCNs might interact weakly with DLF1 (Figure 3B). In vitro, ZCN8, ZCN14 and ZCN26 had strongest interactions with DLF1 (Figure 3C).

**RNA in situ hybridization of select FT-like ZCN genes in the leaf**

The transcripts of Arabidopsis FT and rice Hd3a and RFT1 are mainly detected in the phloem of the leaf (An et al., 2004; Tamaki et al., 2007; Komiya et al., 2009). Based on the analyses conducted, ZCN8 was the most favorable candidate for a floral activator in maize.
The spatial expression pattern of ZCN8 was determined using in situ hybridization analysis. Because ZCN7 and ZCN26 also interact with the DLF1 protein in the yeast two hybrid analyses, they were also included in the in situ hybridization experiment. ZCN7 and ZCN8 are duplicated genes sharing 94% identity at the nucleotide level (Danilevskaya et al., 2008a). Because mature, spliced ZCN7 mRNA was never detected in leaf tissues, sequences from the third intron of ZCN7 were used as a RNA probe for in situ hybridization to distinguish between these two paralogs. No signal was detected in any leaf tissues using the ZCN7 intron probe (Figure 4). Because ZCN8 transcript is abundant in the tip of mature leaf blades, this tissue was used for in situ hybridization. The tip of leaf blades from the temperate lines Gaspé Flint and B73 were harvested before and after the floral transition from plants grown under LDs. Similar tissues were harvested from the tropical line CML436 before the floral transition from plants grown under SDs. Hybridization with the ZCN8 RNA antisense probe revealed signal over vascular bundles within transverse leaf sections in all genotypes tested (Figure 5A, C and E). At higher magnification, signal was detected primarily in phloem, xylem parenchyma and epidermal cells (Figure 5B, D and F). Stages before (V4) and after (V6) the floral transition in B73 and inflorescence stages in Gaspé Flint were also examined (Figure 6). Before the floral transition, the ZCN8 signal was detected in the phloem (Figure 6A, B and C). At later stages, after the floral transition, signal was detected in additional cells, including the xylem parenchyma and sclerenchyma cells on the adaxial and abaxial sides of the vascular bundles (Figure 6D, E and F). In Gaspé Flint, when the SAM had already developed into tassel primordia, long after the floral transition, ZCN8 signal was detected only in sclerenchyma cells on the both sides of the vascular bundles (Figure 6G and H).

ZCN26 displayed spatial expression patterns similar to ZCN8. Hybridization signal was detected over the vascular bundles (Figure 5G, I, and K). Detailed images at a higher magnification showed signal over phloem, xylem parenchyma and sclerenchyma cells (Figure 5H, J, L and M).
Flowering phenotype of maize transgenic plants ectopically expressing ZCN genes

As a functional test of the candidate ZCN genes, their ectopic expression in transgenic plants was exploited. If the candidate gene functions as a floral activator and is ectopically expressed earlier in development in the shoot apex, an early flowering phenotype should be observed. The attempt was made to over express most of the FT-like ZCN genes using the ubiquitin (Ubi) promoter which is a strong constitutive promoter routinely used for transgenic studies in maize (McElroy and Brettell, 1994). Surprisingly, only ProUBi:ZCN18 and ProUBi:ZCN26 transgenic plants were obtained as plants presumably expressing the other ZCN constructs failed to regenerate plants from callus (Danilevskaya et al., 2010) (Figure 7). A weaker, constitutive promoter, ZM-GOS2 was tried to ectopically express the ZCN genes that failed to regenerate plants using the Ubi promoter (Barbour et al., 2003). However, transgenic plants were only generated with this weaker promoter driving expression of the genomic sequence of ZCN7 (ZCN7g). Even though endogenous ZCN7 does not produce fully spliced mRNA in any of the tissues tested, ProZM-GOS:ZCN7g transgenic plants did unexpectedly produce three independent events with spliced mature mRNAs (Figure 9 A). Transgenic plants bearing ProUBi:ZCN18, ProUBi:ZCN26 or ProZM-GOS:ZCN7g constructs produced the same number of leaves as their non-transgenic siblings. Hence overexpression of these three ZCN genes did not alter the floral transition and appear to play no apparent role in flowering time regulation.

In order to recover transgenic plants ectopically expressing a few of the ZCN genes, two tissue-specific promoters, ProZMM4 and ProZM-ADF4 (Zea mays Actin Depolymerizing Factor 4), were tried next (Bate and Reimann, 2009). ProZMM4 will condition expression in the shoot apex, including the SAM and nascent leaf primordia near the time of the floral transition (Danilevskaya et al., 2008b) (Figure 8 A). Using a ProZM-ADF4:GUS construct, it was found that this promoter drives strong expression of the reporter GUS (β-Glucuronidase) in the shoot apex as early as stage V3 and expression continued in these tissues at later stages (Figure 8 B). Both promoters were elected to drive expression of the most favorable candidate ZCN8. ProZMM4:ZCN8 and ProZM-ADF4:ZCN8 constructs were made, transgenic T0 plants regenerated and single transgene copy events were outcrossed to produce segregating T1 progeny, which were evaluated for flowering time phenotypes. Expression of the
transgene was confirmed by RT-PCR in all events selected for phenotyping (Figure 9 D and E). Leaf numbers were counted as a measure of the timing of the floral transition. Transgenic plants with either construct transitioned earlier, resulting in plants with fewer leaves (Table 2). The average leaf number of ProZMM4:ZCN8 plants was eighteen, one leaf fewer than their non-transgenic siblings, while the average leaf number of ProZM-ADF4:ZCN8 plants was seventeen, two leaves fewer than their non-transgenic siblings. These differences were statistically significant. The earlier flowering phenotype of the ProZM-ADF4:ZCN8 plants are consistent with a relatively higher and earlier ectopic ZCN8 expression in the shoot apex compared to the ProZMM4:ZCN8 plants (Figure 8 and 9). A representative image of ProZM-ADF4:ZCN8 transgenic plant is shown in Figure 11 A. These results demonstrate that ectopic expression of ZCN8 in shoot apices prior the floral transition promotes early flowering.

Flowering phenotype of maize transgenic plants repressing ZCN8 mRNA expression

Ectopic expression of ZCN8 in the shoot apical meristem promoting flowering in maize implies that ZCN8 is a maize floral activator. To further confirm this observation, we designed an artificial microRNA (amiRNA) to down regulate ZCN8 mRNA expression level. Due to the importance of the fourth exon of ZCN8 in its functioning as the floral activator, this region was searched for amiRNAs (Ahn et al., 2006). Based on general parameters described for the design of amiRNAs in Schwab et al., 2006, the precursor of miRNA396H is the backbone for amiRNA constructs but the original miRNA sequences of the precursor were replaced by those of amiR-ZCN8 using overlapping PCR. The amiR-ZCN8 expression was under control of the maize ubiquitin promoter. The construct was transformed into Gaspé Flint, an early flowering temperate maize line. T1 transgenic plants showed the late flowering phenotype and produced 3-4 more leaves relative to a non-transgenic control (Table 2 and Figure 10 C). Development of the shoot apical meristem of transgenic and non-transgenic plants at different stages was also examined. At the VE (emergence) stage, the shoot apical meristems of both were at the vegetative stages. At the V2 stage, the shoot apical meristems of the non-transgenic plants had already initiated inflorescence primordia. In contrast, the shoot apical meristem of the transgenic plants remained vegetative stages, suggesting that repression of ZCN8 transcripts delays the floral transition (Figure 10 B).
qRT-PCR were used to examine accumulations of the amiRNA and its effect on \textit{ZCN8} mRNA expression. In eight transgenic events tested, seven events had amiRNAs with high expression and endogenous \textit{ZCN8} mRNA expression in the corresponding events was decreased significantly compared with that in non-transgenic plants (Figure 9D). For example, the mRNA level of \textit{ZCN8} in the event with the most leaves number is reduced 10 fold in transgenic plants (Figure 9D). In addition to more leaves produced, the transgenic plants had larger leaf area and branchy tassel which are typical phenotype of plants with late flowering. Thus, these results strongly support \textit{ZCN8} function as a maize floral activator.

**Discussion**

**Genetic position of \textit{ZCN8} in the maize flowering pathway**

In maize, the genes underlying the two late flowering mutants, \textit{id1} and \textit{dlf1}, have been cloned and their relationship determined. \textit{id1} was placed upstream of \textit{dlf1} in the genetic pathway for flowering (Muszynski et al., 2006). \textit{ZCN8} transcript accumulation was characterized in the leaf blade of both these mutants. No \textit{ZCN8} transcript was detected in \textit{id1} mutant leaves, but in the \textit{dlf1} mutant \textit{ZCN8} expression was similar to wild type. Therefore, \textit{ZCN8} functions downstream of \textit{id1} and upstream or in parallel to \textit{dlf1} in the maize flowering pathway. However, \textit{id1} is exclusively transcribed in immature leaves and was shown to function non-cell-autonomously to regulate the floral transition (Colasanti et al., 1998). The ID1 protein appears to not be mobile; it stays in immature leaves (Wong and Colasanti, 2007). \textit{id1} was proposed to control the production of a mobile florigenic (F) signal that moves to the shoot apex (Wong and Colasanti, 2007; Colasanti and Muszynski, 2009). The results show that \textit{ZCN8} is likely the mobile signal (F) that functions downstream of \textit{id1}. The genetic interaction of \textit{id1} and \textit{ZCN8} suggests that \textit{id1} activity is required for \textit{ZCN8} transcription.

How \textit{id1} regulates transcription of \textit{ZCN8} is not clear. It is unlikely that \textit{ZCN8} is a direct target of \textit{id1} as there are no obvious ID1 binding sites near the \textit{ZCN8} gene sequence (Kozaki et al., 2004). In addition, the two genes are most highly expressed in non-overlapping domains of developing leaves; although both genes are moderately expressed in the transition zone of the leaf where \textit{id1} expression diminishes and \textit{ZCN8} expression initiates (Figure 4C). It is more probable that \textit{id1} regulates expression of, at least one other gene that
is required for transcription of ZCN8. ID1 may turn on expression of another transcription factor that, in turn, activates transcription of ZCN8 in the transition zone and more distal parts of the emerging leaf. Alternatively, ID1 or another input pathway may control expression of an epigenetic regulatory factor that affects the chromatin state of cis-regulatory sequences near ZCN8 and therefore affects its transcriptional competence. This may explain how ZCN8 expression is maintained in the vascular cells of the mature leaf blade, which are many mitotic generations removed from when id1 was active. Recently, several studies have shown FT is subject to epigenetic regulation as one of several inputs required for its expression (Jiang et al., 2008; Jeong et al., 2009; Adrian et al., 2010; Yang et al., 2010). But this speculation remains to be studied in maize.

**ZCN8 mRNA is expressed in the leaf phloem**

One essential functional feature of FT genes is their expression in the phloem of the leaf vasculature and transport of the translated protein to the shoot apex. Due to low FT expression in Arabidopsis and rice, indirect techniques such as endogenous promoter-GUS/GFP fusions were employed to visualize cell-specific FT expression (Corbesier et al., 2007; Tamaki et al., 2007; Komiya et al., 2009). In contrast, ZCN8 expression in the leaf was able to be detected directly by RNA in situ hybridization. An obvious ZCN8 signal was reproducibly observed in the major and minor veins of leaves sampled from different genotypes at or near the floral transition. At a higher magnification, signal was evident in the phloem, xylem parenchyma and sclerenchyma cells of the vascular bundles. The dynamics of ZCN8 spatial expression were investigated in the vasculature at several developmental stages in B73. ZCN8 signal appeared first in the phloem at vegetative stage V4. Later, during the time of the floral transition, ZCN8 signal was not only seen in the phloem but was also detected in the xylem parenchyma and the sclerenchyma cells. At an even later reproductive stage (in the early temperate line Gaspé Flint), ZCN8 signals persisted only in the sclerenchyma fiber cells. I propose that ZCN8 expression in the phloem is required prior to and during the floral transition but at later reproductive stages, ZCN8 is expressed in the sclerenchyma cells where its function, if any, is not clear.
**ZCN8 induces early flowering when ectopically expressed in the shoot apex and delays flowering when constitutively repressed**

Transgenic ectopic or decreased expression of genes is one approach to analyze gene function. Ectopic expression of both *FT* and *Hd3a* driven by phloem-specific promoters was used to induce early flowering in transgenic Arabidopsis and rice, respectively (An et al., 2004; Tamaki et al., 2007; Turck et al., 2008). Unfortunately phloem-specific promoters are not available in maize. For this reason, two well-characterized promoters, *ProZMM4* and *ProZM-ADF4*, were used to drive expression in the shoot apex. Ectopic expression of ZCN8 in the shoot apex at vegetative stages before the floral transition using the *ProZM-ADF4:ZCN8* construct promotes early flowering. Although ZCN8 expression was very high in the shoot apex with this construct (Figure 8), it did not produce an extremely early flowering phenotype similar to Gaspé Flint. To induce the floral transition, ZCN8 must interact with its partner, the DLF1 protein, in the shoot apex. The amount of DLF1 in the apex may be limiting in these early vegetative stages, as we know *dlf1* transcripts accumulate to high levels in stages just preceding the floral transition (Muszynski et al., 2006). The early flowering phenotype of the *ProZMM4:ZCN8* transgenic plants were more moderate, likely due to a weaker and later activity of this promoter in the shoot apex.

Conversely the amiRNA down regulation of ZCN8 mRNA resulted in a late flowering phenotype. These findings are direct evidence that ZCN8 has floral-inductive activity in maize. It was found that transgenic plants with the lowest endogenous ZCN8 mRNA level produced the maximum leaf number however they eventually flowered. This suggests that changes in ZCN8 expression level directly affect flowering time and that low abundant ZCN8 transcript could eventually accumulate to a sufficient threshold at later stages required to induce the floral transition. This also suggests that additional maize floral activators might exist and regulate maize flowering time.

**ZCN8 is a maize floral activator with potential florigenic function**

The flowering hormone florigen appears to be universal in flowering plants (Shalit et al., 2009) and its genetic regulation and a mode of action have been deciphered in a number of species including Arabidopsis, rice and tomato. According to the current model
established in these species, florigen is a mobile PEBP protein encoded by one (or more) $FT$-like gene which is transcribed and translated in the leaf vasculature and then moves via the phloem to the shoot apical meristem where it interacts with an FD-like bZIP transcription factor to induce flower development (Abe et al., 2005; Wigge et al., 2005; Turck et al., 2008). However, it is still unclear which $FT$ orthologous gene(s) in maize function as the mobile flowering signal. This knowledge is essential for understating the genetic regulation of flowering time in this important agronomic and model crop species. Here we have provided evidence that at least one maize gene, $ZCN8$, possesses nearly all the characteristics consistent with florigenic function.

Assuming the mechanism of florigen activity and movement is evolutionarily conserved, a number of criteria were defined for the functional analysis of a number of $FT$-like $ZCN$ genes in maize. A $ZCN$ gene with florigenic activity should (1) be expressed in leaves before the floral transition, (2) be transcriptionally responsive to inductive photoperiods in sensitive genotypes, (3) complement late flowering of Arabidopsis $ft$ mutants when ectopically expressed, (4) encode a protein that interacts with the DLF1 protein (the maize FD ortholog), (5) function genetically within a defined floral inductive network, (6) induce early flowering when ectopically expressed in the shoot apex of transgenic plants or delay flowering when its native expression is repressed and (7) encode a protein that moves through the phloem to the shoot apex. Except for the last criterion, all other criteria were applied to the analysis of seven maize $FT$-like $ZCN$ genes. A summary of the functional analyses of the seven $FT$-like $ZCN$ genes is shown in Table 3.

Out of seven $ZCN$ genes selected for functional analyses, only $ZCN8$ satisfied all six of our criteria for possessing florigenic activity. $ZCN8$ transcript gradually accumulates in leaves at vegetative stages and reaches its highest expression level at or near the floral transition in all genotypes tested. $ZCN8$ mRNA accumulates rapidly in response to a floral-inductive SD treatment in photoperiod-sensitive tropical lines, correlating with an earlier floral transition. The ZCN8 protein interacts with the DLF1 protein in the yeast two hybrid and GST pull-down assays and functions genetically downstream of the $id1$ floral activator in the $id1$-$dlf1$ flowering network. Furthermore, ectopic expression of $ZCN8$ in the shoot apex during vegetative growth stages induced early flowering in transgenic plants and decreased
mRNA expression of ZCN8 in leaf tissue delayed flowering. Collectively these data are consistent with ZCN8 functioning as a floral activator. Consistent with the florigen model, ZCN8 transcript is not detected in the shoot apex at any stage of development but ZCN8 mRNA accumulates in the leaf phloem at or near the floral transition. However, a critical but more difficult experiment is required to prove the ZCN8 protein does indeed move from the leaf to the shoot apex and thus functions as florigen in maize.

Notably, the duplicate of ZCN8, the ZCN7 gene, appears to be non-functional, as fully spliced ZCN7 mRNA was not detected by RT-PCR from any of the tissues we tested under normal growth conditions. Nevertheless, in transgenic plants over-expressing a genomic ZCN7 sequence, properly spliced mRNA was produced in leaves. This finding points toward the possibility that under some specific conditions, ZCN7 might play a functional role and its function might be regulated by RNA splicing. However, properly spliced ZCN7 mRNA in transgenic plants did not have an impact on flowering time, suggesting this gene appears to not play a role in the floral transition. Because overexpression of ZCN15 and ZCN14 in Arabidopsis ft mutant complemented the loss-of-function of FT, they were considered as putative floral activators. The evidence acquired from the genetic analysis in two late flowering mutants indicates that ZCN14 and ZCN15 do not interact with the id1-dlf1 genetic pathway. Unfortunately, transgenic plants ectopically expressing ZCN14 and ZCN15 could not be generated. Thus, without this test of their function in maize, I could not validate the hypothesis that they can promote earlier flowering in maize.

Other FT-like ZCN genes show some interesting characteristics that suggest their functional diversification. ZCN12 mRNA accumulates in leaves in the dlf1 mutant suggesting a genetic interaction between ZCN12 and dlf1 where dlf1 may be a repressor of ZCN12 expression. Moreover a weak interaction was observed between the ZCN12 and DLF1 proteins using yeast two-hybrid assay. This finding suggests ZCN12 might have a role in reproductive development and is therefore an unlikely candidate for functioning as a floral activator. Both ZCN18 and ZCN26 appear to be constitutively expressed in leaves in all growth stages and genotypes studied. ZCN18 did not interact genetically within the id1-dlf1 network and its protein did not interact with the DLF1 protein. Thus, ZCN18 function appears to be independent of the flowering time network. Although ectopic expression of
ZCN26 did not affect flowering time, endogenous expression showed a distinct diurnal pattern under SDs and the ZCN26 protein interacts strongly with DLF1. ZCN26 transcripts accumulate in leaf phloem cells but are not regulated by either idl or dlf1. Hence, ZCN26 is an intriguing candidate for further functional studies.

Acknowledgments

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References


Table 1. The sequences of primers and probes

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<td>AGCCTGCTCACGCAC</td>
<td>qRT-PCR</td>
</tr>
</tbody>
</table>
Table 2. Leaf number of transgenic maize plants

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Background</th>
<th>No. of plants</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG siblings</td>
<td>Temperate</td>
<td>32</td>
<td>19 ± 0.6</td>
</tr>
<tr>
<td>TG <em>ProZMM4</em>: ZCN8</td>
<td>Temperate</td>
<td>34</td>
<td>18 ± 0.6*a</td>
</tr>
<tr>
<td>NTG siblings</td>
<td>Temperate</td>
<td>12</td>
<td>19 ± 0.6</td>
</tr>
<tr>
<td>TG <em>ProZM-ADF4</em>: ZCN8</td>
<td>Temperate</td>
<td>13</td>
<td>17 ± 0.6*b</td>
</tr>
<tr>
<td>NTG siblings</td>
<td>Temperate</td>
<td>30</td>
<td>19 ± 0.4</td>
</tr>
<tr>
<td>TG <em>ProGOS2</em>: ZCN7g</td>
<td>Temperate</td>
<td>30</td>
<td>19 ± 0.6</td>
</tr>
<tr>
<td>NTG siblings</td>
<td>Early temperate</td>
<td>28</td>
<td>10 ± 0.8</td>
</tr>
<tr>
<td>TG <em>ProUBI</em>: ZCN18</td>
<td>Early temperate</td>
<td>20</td>
<td>10 ± 0.8</td>
</tr>
<tr>
<td>NTG siblings</td>
<td>Temperate</td>
<td>32</td>
<td>19 ± 0.5</td>
</tr>
<tr>
<td>TG <em>ProUBI</em>: ZCN26</td>
<td>Temperate</td>
<td>36</td>
<td>19 ± 0.6</td>
</tr>
<tr>
<td>NTG siblings</td>
<td>Early temperate</td>
<td>30</td>
<td>9 ± 0.6</td>
</tr>
<tr>
<td>TG <em>ProUBI</em>: amiR-ZCN8</td>
<td>Early temperate</td>
<td>27</td>
<td>13 ± 0.6*b</td>
</tr>
</tbody>
</table>

NTG – non-transgenic sibling plants. TG – transgenic plants. All constructs were expressing cDNA with exception of ZCN7g, which was a genomic fragment including exons and introns. Leaf numbers were collected from at least 10 individual plants from two independent events. Mean values and standard deviations were calculated by the ANOVA analyses from Minitab Statistical Program (Minitab Inc.).

*a* - means are statistically significant with p<0.1

*b* - means are statistically significant with p<0.05
Table 3. Summary of the functional analysis of seven FT-like ZCN genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue</th>
<th>Timing</th>
<th>Diurnal pattern</th>
<th>SD</th>
<th>Complement to ft mRNA detected</th>
<th>mRNA in id1 mutant</th>
<th>mRNA in dlf1 mutant</th>
<th>DLF1 interaction</th>
<th>TG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCN7</td>
<td>unspliced</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>weak</td>
<td>neutral</td>
<td>n/a</td>
</tr>
<tr>
<td>ZCN8</td>
<td>leaf</td>
<td>prior FT</td>
<td>SD; LD</td>
<td>Yes</td>
<td>Yes</td>
<td>NO</td>
<td>Yes</td>
<td>strong</td>
<td>early</td>
<td>late</td>
</tr>
<tr>
<td>ZCN14</td>
<td>SAM</td>
<td>after FT</td>
<td>n/a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>weak</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>ZCN15</td>
<td>kernel</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>n/a</td>
<td>No</td>
<td>n/a</td>
<td>neutral</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>ZCN12</td>
<td>leaf</td>
<td>after FT</td>
<td>n/a</td>
<td>Yes</td>
<td>n/a</td>
<td>Yes</td>
<td>weak</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>ZCN18</td>
<td>leaf</td>
<td>all times</td>
<td>LD</td>
<td>NO</td>
<td>n/a</td>
<td>Yes</td>
<td>Yes</td>
<td>NO</td>
<td>neutral</td>
<td>n/a</td>
</tr>
<tr>
<td>ZCN26</td>
<td>leaf</td>
<td>all times</td>
<td>SD</td>
<td>NO</td>
<td>no</td>
<td>Yes</td>
<td>Yes</td>
<td>strong</td>
<td>neutral</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a – not applicable. TG<sup>a</sup> - flowering phenotype of transgenic plants which ectopically expressed ZCN genes. TG<sup>b</sup> - flowering phenotype of transgenic plants which repressed ZCN8 genes; SAM: the shoot apical meristem; FT: floral transition.
Figure 1. *FT*-like *ZCN* gene expression in leaves of wild type and late flowering mutants.

(A) Images of shoot apices at growth stages (days after sowing, DAS) when leaves were collected. Red arrows mark the floral transition. (B) Expression of *FT*-like *ZCN* genes in leaf blades from wild type B73, *dlf1-N2461A* and *id1-m1*. The first and last lanes of each gel image are marker DNA and genomic template DNA, respectively. (C) RT-PCR of *ZCN8* and *id1* in dissected sections of B73 plants at V5. The images on the top show sections numbered from 1 to 17. Section 1 represents the shoot apex with the youngest enclosing leaf primordia. Section 2 is 1cm of immature leaves around the shoot apex. Sections 3-6 are immature leaves, each of 4 cm length where 3 is the closest one to shoot apex. Section 7 is the transitional part of the sixth and seventh leaves. Section 8 is mature, photosynthetically competent blade of 6th leaf. Section 9 is the sheath of the 5th leaf. Sections 10-12 are parts of the mature leaf blade of the fifth leaf, each 12 cm in length. Section 13 is the tip of the mature blade of the fifth leaf. Section 14 is the sheath of the 4th leaf. Sections 15-16 are sections of the mature leaf blade of the 4th leaf, each 12cm in length. Section 17 is the tip of the 4th leaf blade. On the X axis the numbers are marking the dissected tissue sections. The Y axis represents the relative expression level normalized to ubiquitin5. Error bars represent standard deviation. Bar = 18 cm.
Figure 2. Expression of $FT$-like $ZCN$ genes in the immature leaf or shoot apices of WT and late flowering mutants.

(A) Expression patterns of $FT$-like $ZCN$ genes in the immature leaf of WT and late flowering mutants. The numbers above the gel indicate the days after sowing (DAS). (B) $ZCN14$ expression patterns in shoot apices of WT and late flowering mutants. WT, $dlf1$ and $id1$ are in the B73 background. The WT floral transition occurred around V4, $dlf1$ around V6 and $id1$ around V11. The red arrow shows the stage of the floral transition in the wild type and two late flowering mutants.
Figure 3. Yeast two-hybrid interactions between FT-like ZCN proteins and the DLF1 protein. 
(A) FT-like ZCN and DLF1 self-interaction in yeast. (B) FT-like ZCN and DLF1 reciprocal interactions in yeast. BD: Binding Domain; AD: Activation Domain; SD: Synthetic Dextrose Minimal Medium. (C) GST pull-down assay. Arrows indicate GST and GST-ZCNs protein. (More details are in MATERIALS AND METHODS)
Figure 4. Negative controls for RNA *in situ* hybridization.

**(A)** RNA *in situ* hybridization of *ZCN7* intron probe to transverse sections of the leaf of B73. **(B)** Sense controls of *ZCN8* and *ZCN26*. Bars= 100 µm.
A. In Situ Hybridization of ZCN7 intron probe to transverse sections of mature B73 leaf.

Antisense

Sense

B. Sense controls of ZCN8 and ZCN26

ZCN8

ZCN26
Figure 5. RNA in situ hybridization of ZCN8 and ZCN26 in leaf tissue from Gaspé Flint, B73 and CML436 during the floral transition.

(A), (C) and (E) Hybridization signal of ZCN8 DIG-labeled antisense probe in transverse sections of the leaf blade tip from (A) Gaspé Flint at 5 days after sowing, (C) B73 at stage V4 and (E) CML436 at stage V5 under SDs. (B), (D) and (F) 40X magnification of the individual vascular bundle from near the midrib (marked by circles in A, C and E) showing signal in the phloem, xylem parenchyma cells and in the sclerenchyma fibers (B and D). (G), (I) and (K) Hybridization signal of the ZCN26 DIG-labeled RNA antisense probes in transverse sections of the leaf blade tip described above. (H), (J), (L) and (M) 40X magnification of an individual vascular bundle near the midrib or major vein (marked by circles in G, I and K) showing signal in the phloem, the xylem parenchyma cells and the sclerenchyma fibers (H and J). bs - bundle sheath; m - metaxylem vessels; pxv - protoxylem vessels; p- phloem; sf - sclerenchyma fibers; xp - xylem parenchyma; LD: Long day; SD: Short Day.

Bars = 200 μm in E and K; 100 μm in A, C, G and I; 50 μm in B, D, F, H, J and M.
Figure 6. RNA in situ hybridization of ZCN8 to the leaf tip of B73 at stages V4 and V6 under LDs (A), (B), (C), (D), (E) and (F) and Gaspé Flint at stage V2 after the floral transition under LDs (G) and (H).

(A), (B) and (C) Hybridization signals of ZCN8 antisense DIG-labeled probe to transverse sections of B73 mature leaf blades at stage V4, before the floral transition.  (D), (E) and (F) Hybridization signals of ZCN8 antisense DIG-labeled probe to transverse sections of B73 mature leaf blades at stage V6, after the floral transition.  In (A) and (D) transverse sections of B73 mature leaf tip showing hybridization signal mainly in vascular tissues.  In (B), (C), (E) and (F) 40X magnification of individual vascular bundles showing signal in phloem, xylem parenchyma and sclerenchyma fiber cells.  (G) 10X magnification of transverse sections of the Gaspé Flint leaf near the midrib at stage V2.  (H) 20X magnification of vascular bundles (vb) near the midribs. ZCN8 RNA antisense probe signal appeared only in sclerenchyma fiber cells (sf) of the large vascular bundles. m - metaxylem vessels; pxv - protoxylem vessels; p- phloem; sf - sclerenchyma fibers; xp - xylem parenchyma

Bars= 200 µm in (A) and (D); 100 µm in (G); 50 µm in (B), (C), (E), (F) and (H).
Figure 7. Regeneration of transgenic calli of the construct ProUBI::ZCN8.

(A) and (B) Induction and growth of calli, shoots and roots of transgenic events from transgenic embryos. No calli were regenerated on the plates and shoots and roots stopped development. (C) A transgenic event with ZCN8 transgene expression but without shoot and leaf induction. (D) A transgenic event without ZCN8 transgene expression but with shoots and leaves regeneration. (E) A negative control plant. (F) RT-PCR of ZCN8 transgene in transgenic plants or negative controls shown in (C), (D) and (E). Lane #1: 1kb plus marker; Lanes #2-3: expression of transgene ZCN8 in transgenic calli with shoot initiation arrested; Lane #4-5: expression of transgene ZCN8 in transgenic calli with shoots and leaves regeneration; Lane #6-7: Expression of transgene ZCN8 in negative control plants.
Figure 8. GUS staining of shoot apices or immature tassels of ProZMM4: GUS (A) and ProZM-ADF4:GUS (B) transgenic plants at stages V3-V6.

(A) GUS staining of shoot apices or immature tassel of ProZMM4: GUS transgenic plants at stages V3-V6. (B) GUS staining of shoot apices or immature tassel of ProZM-ADF4: GUS transgenic plants at stages V3-V6. SAMs or immature tassels were dissected and incubated at 37°C overnight in the dark in GUS staining solution. After incubation, the tissues were destained several times with 70% ethanol at room temperature and stored at 4°C in 70% ethanol prior to imaging.

ZMM4: Zea may MADS-box 4; ZM-ADF4: Zea may Actin Depolymerizing Factor; Bar=100 µm
Figure 9. Expression analysis of transgene and native gene expression in transgenic plants.

(A) RT-PCR analysis of ProZM-GOS2:ZCN7g and ProUBi:ZCN26 transgene (TG) and native gene (NG) expression in leaf tissue in independent T0 transgenic events. Two events highlighted by the white box were advanced to the T1 generation for analysis in the field. The expression of native ZCN7g includes native gene and transgene. The white arrow marked spliced ZCN7 mRNAs. (B) RT-PCR analysis of ProUBi:ZCN18 transgene (TG) and native gene (NG) expression in leaf tissue in T1 transgenic events. (C) The relative expression of ProZMM4:ZCN8 and ProZM-ADF4:ZCN8 transgene (TG) and native gene (NG) were detected by qRT-PCR in leaf tissues. Note, due to the high level of transgene mRNA in ProZM-ADF4:ZCN8 events, expression of the native ZCN8 mRNA cannot be seen at this scale. Relative expression numbers are shown over the bars. (D) qRT-PCR analysis of the relative expression of ProUBi:amiR-ZCN8 transgene and native gene in leaf tissues. In (C) and (D), transgene and native gene expression was normalized to Ubiquitin5.
Figure 10. Images of non-transgenic and transgenic plants of $Pro_{ZM-ADF4}:ZCN8$ and $Pro_{UBI}:amiR-ZCN8$.

(A) Images of wild type, non-transgenic sibling and transgenic plant from the construct of $Pro_{ZM-ADF4}:ZCN8$. (B) Images of the shoot apical meristem of non-transgenic sibling and transgenic plants of $Pro_{UBI}:amiR-ZCN8$ at stages of VE and V2. VE: at the stage of emergence. (C) Images of wild type, non-transgenic sibling and transgenic plant from the construct of $Pro_{UBI}:amiR-ZCN8$. The plants of $Pro_{ZM-ADF4}:ZCN8$ were grown in the field under LDs and the plants of $Pro_{UBI}:amiR-ZCN8$ were grown in the greenhouse under LDs.
CHAPTER FOUR: GENERAL CONCLUSIONS

A model integrating the autonomous and photoperiod pathways for flowering in maize

Knowledge of the maize flowering network is relatively rudimentary due to a limited number of flowering mutants (Colasanti and Muszynski, 2009). Our findings allow for the further elaboration of the maize flowering network (Figure 1). In this model, id1 represents the autonomous inductive pathway in maize as neither id1 transcript nor ID1 protein are diurnally regulated (Wong and Colasanti, 2007). id1 indirectly activates expression of ZCN8 in the transition zone of the immature leaf. Hence ZCN8 is regulated by the autonomous pathway in developing leaves.

In temperate, day-neutral maize lines, ZCN8 transcript accumulates at early stages of development and does not exhibit a diurnal expression pattern, which is especially evident in the extremely early flowering cultivar Gaspé Flint. This finding suggests that in temperate maize genotypes, ZCN8 transcription is disconnected from the circadian clock and, in particular, is not responsive to negative LD regulation, even though the circadian machinery appears to be intact (Hayes et al., 2010). On the other hand, in tropical genotypes, ZCN8 is regulated by signals from the photoperiod pathway. Under floral-inductive short days, ZCN8 transcript accumulation is upregulated at early vegetative stages to ensure an earlier floral transition and earlier flowering under the relatively unfluctuating tropical day lengths. But when these genotypes are grown in long-days, the amplitude of ZCN8 expression is suppressed, possibly by a putative LD suppression pathway. After an extended period of vegetative growth, the amplitude of ZCN8 expression gradually increases, allowing plants to transition to reproductive development and flower.

According to the proposed model, during selection for adaptation to growth at higher latitudes, several genetic events could have occurred, including a relaxation of the diurnal regulation of ZCN8 expression under long days and its disconnection from the circadian machinery. This might involve mutations in the ZCN8 gene per se or in the upstream regulators connecting ZCN8 expression to the circadian clock. How the regulation and/or activity of ZCN8 have been altered between photoperiod sensitive and insensitive lines is of interest to both evolutionary biology and plant breeding. Our findings reveal a putative
target gene for maize adaption outside of the tropical regions of the globe and provides guidance in the study of photoperiod sensitivity in maize.

**Future work**

**Mobility of the ZCN8 protein**

Direct experimental results showing that ZCN8 is capable of moving from leaf to shoot apices via the phloem are absent although ZCN8 has been confirmed to be a maize floral activator in our study. Such evidence is critical to conclude ZCN8 is a component of florigen. Therefore, a priority for future work on ZCN8 is to provide data showing the ZCN8 protein, similar to FT and Hd3a, is phloem-mobile. In Arabidopsis and rice, due to the low abundance of FT and Hd3a mRNA in leaf, transgenic plants overexpressing the fusion protein of FT/Hd3a with a fluorescent protein under the control of the phloem specific promoter was used to determine their movement (Corbesier et al., 2007; Tamaki et al., 2007). In contrast, ZCN8 mRNA expression in maize leaf is sufficiently abundant so that it can be detected by RNA in situ hybridization. Thus, methods directly measuring protein quantity in the leaf and the shoot apex such as mass spectrometry could be considered. *Cucurbita moschata* FT-like (*Cm-FTL*) was successfully identified to be a mobile florigenic signal in the Cucurbits by this technique (Lin et al., 2007). However, this technique mainly analyzes smaller peptides digested from proteins and might not accurately reflect whether the intact protein travels to shoot apices. Therefore, a similar transgenic approach using fusion of the target gene to a fluorescent protein, as used in Arabidopsis and rice, is also recommended.

**Identification of additional FT-like floral activators**

Additional future work I suggest, is to verify more floral activators from maize ZCN genes and establish their genetic relationship regulating the floral transition of maize lines with different photoperiod sensitivities. In Arabidopsis and rice, several PEBP genes were confirmed to be floral activators promoting flowering under different day lengths, for instance Arabidopsis *FT* and *TSF* and rice *Hd3a* and *RFT1* (Yamaguchi et al., 2005; Komiya et al., 2008). Maize has an expanded PEBP gene family, suggesting possible redundant functions of different ZCN genes. Our results showed that ZCN14 and ZCN15 complement Arabidopsis late flowering *ft* mutant and they have higher homology with monocot floral
activators and especially ZCN14 mRNA is present in leaf tissue of the tropical lines under LDs, suggesting that ZCN14 may be required for the tropical lines to flower under LDs. Because amiRNA-ZCN8 transgenic plants eventually flower although ZCN8 expression is constitutively reduced, different tissues of these transgenic plants could be used to analyze how expression of other six FT-like ZCN genes are affected. Furthermore, transgenic plants ectopically expressing ZCN14 driven by ZM-ADF4 promoter or with decreased ZCN14 mRNA level by amiRNA could be used to test whether or not ZCN14 is a floral activator.

References


Figure 1. A model integrating the autonomous and photoperiod pathways in tropical and temperate maize.

In the autonomous pathway, *id1* indirectly controls *ZCN8* transcription in the immature region of leaves which are hidden in the whorl (marked as a grey area on the leaf image). *ZCN8* but not *id1* is transcribed in the green, photosynthetically competent parts of the leaf blade. In short-day tropical maize, leaf-expressed *ZCN8* is diurnally regulated under SD and LD. But under non-permissive LDs, the amplitude of *ZCN8* expression is severely attenuated, resulting in later flowering. This suggests the possible existence of a “LD suppression pathway” in maize similar to that in rice (Komiya et al., 2009). In day-neutral temperate maize, regulation of *ZCN8* transcription is disconnected from the circadian clock and from the hypothetical “LD suppression pathway” (marked by X). Disconnection from the circadian clock results in *ZCN8* transcription displaying no diurnal regulation, even though the upstream genes, *gigz1A, 1B* (maize homolog of *GIGANTEA*) show diurnal transcript regulation. In both tropical and temperate maize, *ZCN8* transcripts are localized in the phloem of the vascular bundles (fine red lines over the leaf image) which allows the ZCN8 protein to move to the shoot apex (blue line and blue arrow), although this movement has not yet been directly demonstrated. In the shoot apex, the ZCN8 protein interacts with the DLF1 bZIP transcription factor to activate expression of floral identity genes like *ZMM4*. The onset of *ZMM4* expression marks the floral transition and initiation of reproductive development (inflorescence development) in maize.
Photoperiod-sensitive tropical maize

Photoperiod-neutral temperate maize

ZCN8 + DLF1

Inflorescence development
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