

ABSTRACT

STOKES, RUTHIE LUCILLE. Flowering Time Regulation via ZCN8 and Lipid Interaction. (Under the direction of Dr. Rubén Rellán Álvarez).

Maize was originally domesticated from its wild relative teosinte *parviglumis* in southwestern Mexico around 9,000 years ago. After domestication, maize expanded into higher latitudes in North America, where it adapted maize had to adapt to longer days. Many genes were involved in this photoperiod sensitivity adaptation, including *Zea Centroradialis 8* (ZCN8), which is a mobile florigen gene. ZCN8 underlies a flowering time QTL in American maize populations. Variants in the promoter of ZCN8, selected in high-latitude maize, lead to higher expression of ZCN8 and are associated with shorter flowering time¹. ZCN8 is a homolog of the Flowering Time (FT) protein in *Arabidopsis*. In *Arabidopsis*, FT can bind to phosphatidylcholine (PC) and phosphatidylglycerol (PG)² and modulate flowering time. Using heterologous expression, we recently showed that ZCN8 co-purifies with certain PC species, particularly abundant in high-elevation Mexican maize varieties that carry a non-functional allele of High Phosphatidylcholine 1 (HPC1) allele³. We also showed that this HPC1 allele is associated with shorter flowering times and higher fitness in highland conditions. Here, we seek to understand the mechanisms by which phospholipid interaction with ZCN8 may regulate flowering time. We show that ZCN8 produced in *Saccharomyces* is bound to PC 34:2 and generates maize protoplasts to explore lipid-protein interactions in native membrane environments. Through comparative and predictive modeling, we identify the potential lipid binding sites in ZCN8. In the future, this work can be used to crystallize ZCN8 and use the structure to identify lipid binding sites and study enzymatic activity formally. This will expound upon our knowledge of how phospholipid-protein interactions can mediate flowering time.

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Flowering Time Regulation via ZCN8 and Lipid Interaction

by
Ruthie Lucille Stokes

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APPROVED BY:

Rubén Rellán Álvarez
Committee Chair

Josh Strable

Peter Thompspon

Nora Haenn

DEDICATION

This thesis is dedicated in remembrance of my father, Donald Ray Stokes Sr., my grandparents, Freddie and Lucile Stokes, and Will and Ruth Scriven. I wish you were all here to experience this; thank you for instilling in me at an early age, the ability for me to believe in myself. I dedicate all my work towards this thesis, to my mother, Dr. Marjorie Scriven, thank you for your constant love and support, the sacrifices you have made for me, and for always pushing me to pursue my dreams. I also dedicate thesis to my wonderful older brother, Donald Ray Stokes Jr. I wouldn't be who I am today without his unconditional love. I would also like to my wonderful family, lab members, and friends for your support throughout this process.

BIOGRAPHY

Ruthie Lucille Stokes, daughter of Donald Ray Stokes Sr. and Marjorie Stokes, was born on December 1st, 1999 in Fayetteville, North Carolina. She was raised on a fourth-generation family farm in Sampson County, North Carolina, where I developed a love for finding solutions and wanting to give back to my community. In the spring of 2017, she graduated from Sampson Early College High School with both a high school diploma and two associate degree in science and arts. Following high school graduation, she attended North Carolina Central University, where she graduated in the Spring of 2021 with two Bachelor of Science degrees in Biomedical Science and Chemistry with a minor in Biochemistry and Animal Science. In the fall of 2021, she started to pursue a Master of Science degree in Biochemistry under Dr. Rubén Rellán Alvarez. She plans to join the workforce after graduation.

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TABLE OF CONTENTS

Chapter 1: Literature Review: Modern Maize Introdcion.....	1
Chapter 2: Adaptive Introgression from Teosinte Mexicana Alters Phosphatidylcholine Levels and Influences Maize Flowering Time	
Major Findings.....	20
Matierials and Methods.....	25
Discussion	28
References.....	32

LIST OF TABLES

Table 1.1	Hit the Tab key after typing Table titles to maintain separation of title and caption text	5
Table 1.2	If your caption takes up multiple lines, like this one, any line after the first should be aligned with the start of the first line. To do this, you will have to manually indent any lines after the first. There is already a Tab rule set for this on the ruler bar	7
Table 2.1	Caption text should not overlap with page numbers	10

LIST OF FIGURES

Figure 1	Map Showing the Spread of Maize from Mexico	2
Figure 2	Synthesis of Phosphatidylcholine: Kennedy Pathway	
10		
Figure 3	Predicted binding sites of ZCN8 align with binding sites of Arabidopsis FT	23
Figure 4	ZCN8 binds to Phosphatidylcholine	28

CHAPTER 1: Modern Maize Domestication

Introduction

Maize (*Zea mays*) remains a cornerstone of global agriculture, revered for its versatility and role as a staple food across diverse cultures. Originating from the domestication of teosinte in Mesoamerica around 9,000 years ago, maize has undergone significant evolutionary changes, allowing it to thrive in a wide array of climates and soil conditions. The domestication process of maize, characterized by a complex network of molecular adaptations, has resulted in the extensive genetic diversity observed in modern maize varieties (Hufford et al., 2019).

As shown in figure 1, after its initial domestication, maize began to spread across the Americas through trade routes and the migration of people. Genetic evidence shows that maize had reached regions as far south as Peru around 5,500 years ago, long before it had fully diversified within Mexico. This early spread did not involve the highland maize varieties that later developed significant genetic diversity due to admixture with *Z. mays* ssp. *mexicana* around 6,000 years ago. This admixture event, occurring in the Mexican highlands, introduced genetic variations that allowed maize to adapt to different climates and altitudes, further facilitating its spread across diverse ecological regions (Lyu 2023).

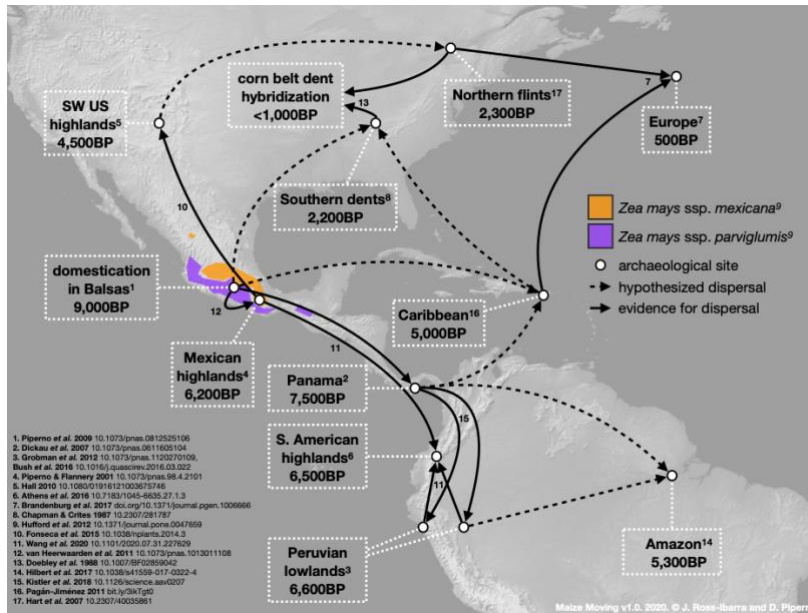


Figure 1: Map illustrating the spread of maize from its origin in central Mexico throughout the Americas, highlighting the routes of agricultural expansion and the cultivation of this vital crop across diverse regions

The spread of maize outside of Mexico also involved significant human selection and adaptation to local environments, which led to the development of various landraces with distinct characteristics suited to different climates. As maize moved northward into the present-day United States and southward into Central and South America, it underwent further diversification.

Ancient agricultural practices focused on selecting traits such as larger kernels, enhanced yield, and adaptability to various climates, showcasing the ingenuity of early cultivators. These practices have led to the development of diverse maize varieties, each with unique chemical compositions that cater to the specific culinary preferences of

different cultures across the Americas and beyond. The genetic diversity of maize today is, therefore, not just a product of natural adaptation but also of human influence (Choquette et al., 2023).

The genetic modifications that arose during maize domestication are deeply embedded in its genome, offering valuable insights into the relationships between human cultivation practices and plant evolution. Recent studies have highlighted the importance of these genetic adaptations in improving our understanding of how crops can be further optimized for agricultural efficiency and resilience (Chen et al., 2020).

One of the critical environmental factors that crops, including maize, must adapt to when introduced to new latitudes is photoperiod or day length. The duration of daylight a plant experiences directly influences its flowering time and, consequently, its overall fitness. Regions close to the equator experience relatively stable day lengths throughout the year, whereas those nearer the poles see significant seasonal variations in day length. The ability of maize to adapt to different photoperiods has been pivotal in its successful cultivation across diverse geographical locations (Kutschera et al. 2018).

Plants are broadly classified into three categories based on their sensitivity to photoperiod: short-day, long-day, and day-neutral plants. Short-day plants typically initiate flowering when the day length is shorter than 12 hours, while long-day plants require longer days to trigger flowering. Day-neutral plants, however, do not rely on day

length for flowering, making them adaptable to various photoperiod conditions. The sensitivity of plants to photoperiod is a critical aspect of their internal biological clock, significantly influencing planting schedules, cultivation practices, and overall crop yield (Jung & Müller, 2020).

Maize, which was domesticated in southwest Mexico, originally thrived under near-neutral day conditions, with day lengths ranging from 11 to 13 hours throughout the growing season. This regionally adapted maize, often referred to as tropical corn, is highly sensitive to photoperiod. When grown in long-day conditions, such as those found in the Midwest United States during summer, tropical photoperiod-sensitive maize tends to flower much later in the season. In extreme cases, it may not flower at all, which can significantly affect crop productivity (Meng., et al. 2011).

The spread of maize to northern latitudes after domestication led to the selection of genetic variants that confer photoperiod insensitivity. This adaptation was crucial for maize cultivation in regions with longer summer days, such as most of the United States. The development of photoperiod-insensitive maize, commonly referred to as temperate corn, has enabled the crop to thrive across a wide range of latitudes, from tropical to temperate regions (Choquette et al., 2023).

The regulation of flowering time in maize involves complex genetic pathways. Central to this process are genes that encode proteins responsible for the perception and transduction of photoperiod signals. Variations in these genes play a pivotal role in

determining the photoperiod sensitivity of maize varieties. In regions with long summer days, such as the U.S., photoperiod-insensitive genotypes are predominant, facilitating the widespread cultivation of maize across diverse climatic conditions (Jaillais & Parcy, 2019).

In northern latitudes, where the growing season is constrained by frost, early flowering is a critical trait for ensuring successful reproduction and optimal yield in maize (*Zea mays*). The colder temperatures characteristic of these regions present significant challenges to corn cultivation, necessitating adaptations that enhance cold tolerance. One key adaptive mechanism is the alteration of lipid composition in response to cold stress, which plays a vital role in maintaining membrane fluidity and integrity. Lipid metabolism in plants encompasses the synthesis, modification, and degradation of lipids, which are essential components of cellular membranes, energy storage, and signaling molecules. Key processes include fatty acid biosynthesis, triacylglycerol (TAG) accumulation, and phospholipid turnover. Lipids also play a crucial role in response to abiotic stresses, such as drought and temperature extremes, by maintaining membrane integrity and facilitating signaling pathways (Li-Beisson et al., 2013). This adaptation mitigates cold-induced cellular damage by ensuring that membranes remain functional under stress conditions. The synthesis of unsaturated fatty acids, catalyzed by desaturase enzymes, is particularly important as these fatty acids confer fluidity to cellular membranes, which is crucial for cold adaptation (Lavel & Benning, 2019)..

Beyond photoperiod sensitivity and cold adaptation, lipid metabolism is intricately linked with various other environmental stresses commonly encountered by maize in northern latitudes, such as drought, salinity, and nutrient deficiencies. Changes in lipid composition and metabolism in response to these stresses significantly impact the plant's overall stress tolerance and fitness. Lipid-derived signaling molecules, including phosphoinositides and sphingolipids, are central to these processes, participating in stress signaling pathways that regulate stress-responsive gene expression and physiological responses (Huang et al., 2021).

Maize's adaptability to different latitudes is shaped by a complex interplay of temperature tolerance, day length, and photoperiod sensitivity. Varieties cultivated in higher latitudes have evolved to flower more gradually, an adaptation that ensures that critical developmental stages such as flowering and kernel development occur in alignment with the specific environmental conditions of each latitude (Chen et al., 2020). The primary mechanism underlying this adaptation is maize's sensitivity to photoperiod, which refers to the duration of daylight that the plant experiences. Farmers strategically select maize varieties based on their photoperiod sensitivity, synchronizing planting schedules with regional day length patterns to optimize yields (Meng., 2011).

Maize varieties adapted to these regions often exhibit delayed flowering in response to longer days, a trait that is tightly regulated by environmental cues and genetic factors. The timing of flowering is a critical phase in the maize life cycle, with its successful

completion being essential for reproductive success and overall crop yield (Castelletti et al., 2020).

Florigen, a crucial regulator of flowering time in plants, has long captivated researchers due to its unique ability to move from the leaves to the shoot apical meristem (SAM), where it induces the transition from vegetative to reproductive growth. Florigen serves as a systemic signaling molecule that coordinates flowering in response to environmental cues such as photoperiod and temperature. In maize, the florigen is encoded by the ZCN8 gene, while in Arabidopsis, it is encoded by the FLOWERING LOCUS T (FT) gene.

Recent studies have deepened our understanding of the biochemical mechanisms underlying the mobility of florigen and its interaction with phospholipids (Wang et al., 2019; Yan et al., 2020).

Florigen is synthesized in the leaves under inductive conditions and then transported to the SAM, where it triggers the expression of floral meristem identity genes, thereby initiating the flowering process. The movement of florigen is facilitated by its interaction with cellular membranes, particularly phospholipids, which play a critical role in its transport to the SAM (Susila et al., 2021). The function of florigen in regulating flowering time is especially prominent in plants that exhibit photoperiodic flowering responses, such as Arabidopsis and various crop species, including rice, wheat, and soybean. In these plants, day length serves as a key environmental cue for determining the timing of flowering, with long days promoting flowering in long-day plants and short days promoting it in short-day plants (Jung et al., 2021).

In maize, ZCN8 is a member of the CCT (CONSTANS, CONSTANS-like, and TOC1) family, characterized by the presence of a CCT domain that facilitates protein-protein interactions and DNA binding. The primary function of ZCN8 is to regulate the transition from vegetative to reproductive growth by integrating environmental signals such as photoperiod and temperature. It operates downstream of the photoperiod pathway and modulates the expression of FT-like genes, which are essential for initiating flowering (Su et al., 2021).

Recent research has begun to explore the intricate interactions between ZCN8 and lipid metabolism, uncovering a complex network of regulatory mechanisms. These interactions can be categorized into direct and indirect effects. Directly, ZCN8 may bind to the promoters of genes involved in lipid metabolism, thereby influencing their expression. Indirectly, ZCN8 may influence lipid metabolism through hormonal pathways. Gibberellins (GAs) and abscisic acid (ABA), which are hormones known to modulate lipid metabolism, are also regulated by flowering-time genes. ZCN8 may alter the levels of these hormones, thereby indirectly affecting lipid biosynthesis and degradation (Castroverde and Dina, 2021; de Abreu. et al. 2018).

The transition to flowering involves extensive metabolic reprogramming, including shifts in lipid metabolism. The induction of flowering by ZCN8 can redirect resources from vegetative growth to the development of reproductive structures, impacting lipid composition and storage. This metabolic reprogramming is essential for providing the energy and building blocks necessary for flower and seed development. The molecular

mechanisms by which ZCN8 influences lipid metabolism involve multiple layers of regulation, including transcriptional control, protein-protein interactions, and epigenetic modifications (Nakamura, 2017).

In maize, the Kennedy pathway predominantly synthesizes PC from DAG, as seen in figure 2.



Figure 2: The biosynthesis of PC 34:2 can occur through several pathways, but the two primary routes are the Kennedy pathway (also known as the CDP-choline pathway) and the remodeling pathway.

The biosynthesis of phosphatidylcholine (PC), a key component of cell membranes, is a complex process that involves the acylation of diacylglycerol (DAG) by the enzyme phosphatidylcholine synthase. The acyl chains incorporated into PC molecules are crucial in determining the composition and properties of these phospholipids. Specifically, PC 34:2 refers to phosphatidylcholine molecules with two acyl chains, each containing 16 carbon atoms and two unsaturated bonds. The transcriptional regulation of the enzymes involved in PC synthesis exhibits diurnal oscillations, with peak expression levels occurring at specific times of the day (Nakamura et al., 2014).

Research has demonstrated that the expression of phospholipid biosynthetic genes follows a circadian rhythm, with maximal activity coinciding with the organism's active phase. This rhythmic regulation of PC synthesis is influenced by various factors,

including developmental stage and environmental cues. For instance, the cellular demand for phospholipids fluctuates throughout the day due to changes in metabolic processes and cellular activities. During periods of increased energy expenditure, such as daytime activity, there is a heightened need for membrane remodeling and lipid synthesis to support cell growth and division. This increased demand drives the upregulation of biosynthetic pathways, leading to the accumulation of phospholipids during the daytime (Nakamura et al., 2014).

In a 2014 study, Nakamura and colleagues discovered that the Arabidopsis FT protein, a key regulator of flowering time, binds specifically to diurnally oscillating phospholipids, particularly phosphatidylcholine and phosphatidylserine, in a time-of-day-dependent manner. These phospholipids exhibit rhythmic changes in abundance throughout the day, peaking during the daytime and declining at night. The binding of FT to these diurnally oscillating phospholipids enhances its stability and activity, which accelerates flowering under long-day conditions (Nakamura et al., 2014).

The interaction between FT and diurnally oscillating phospholipids represents a novel mechanism by which plants integrate environmental cues and internal rhythms to regulate flowering time. By sensing changes in the abundance of phospholipids throughout the day, FT modulates its activity in response to the circadian clock and environmental signals, ensuring that flowering occurs at the optimal time for reproductive success. Moreover, this interaction provides insights into the role of membrane dynamics in flowering time regulation. By interacting with phospholipids, FT may influence

membrane dynamics and signaling pathways that modulate flowering time, further highlighting the interconnectedness of cellular processes in plant development (Nakamura et al., 2022).

The discovery of the interaction between FT and diurnally oscillating phospholipids opens new avenues for understanding the molecular mechanisms underlying flowering time regulation, with potential implications for related pathways in maize, such as those involving the ZCN8 homolog. Future research may focus on elucidating the signaling pathways downstream of the FT-phospholipid interaction and investigating the physiological significance of this interaction in plant growth, development, and environmental adaptation.

The complex interplay between environmental cues, circadian rhythms, and lipid metabolism plays a crucial role in regulating cellular responses to diurnal variations. A particular focus has been placed on phosphatidylcholine (PC) 34:2, a specific phospholipid species that is vital for maintaining cellular structure, signaling, and membrane dynamics. The accumulation of PC 34:2 is subject to intriguing variations influenced by daytime length, highlighting the complex biochemistry underlying lipid metabolism in plants.

PC 34:2 biosynthesis primarily occurs in the endoplasmic reticulum (ER) through de novo synthesis pathways. This process is tightly regulated by the circadian clock, which contributes to the temporal dynamics of PC 34:2 accumulation. The circadian clock

integrates light-dark cycles to synchronize physiological processes with external environmental conditions, such as day length. Research indicates that changes in day length, including variations in photoperiod, can disrupt the synchrony between the circadian clock and lipid metabolism, resulting in alterations in PC 34:2 dynamics (Nakamura et al., 2014).

Experimental studies have shown that modifications in day length significantly impact the phase and amplitude of diurnal rhythms in PC 34:2 biosynthesis. These changes, in turn, lead to variations in lipid composition and abundance within maize tissues. The rhythmic regulation of phospholipid biosynthesis and turnover is essential for maintaining membrane integrity and ensuring cellular function across different metabolic demands and day lengths (Ali et al., 2022).

During periods of increased metabolic activity, such as the daytime, there is a heightened demand for PC 34:2. This demand drives the upregulation of biosynthetic pathways, leading to increased accumulation of PC 34:2. Conversely, during nighttime, when metabolic activities are reduced, the biosynthesis of PC 34:2 is attenuated, resulting in lower levels of accumulation. Day length serves as a critical environmental cue that shapes PC 34:2 dynamics by modulating the phase and amplitude of circadian oscillations. Consequently, variations in day length can disrupt the synchrony between the circadian clock and metabolic pathways, leading to significant changes in PC 34:2 levels (Nakamura et al., 2014; Yang & Guo, 2018).

Phosphatidylglycerol (PG) 36:2 is a significant phospholipid within the membranes of maize cells, playing a crucial role in maintaining membrane structure and function. The synthesis of PG 36:2 in maize involves a series of enzymatic reactions primarily occurring in the chloroplasts and the ER, similar to the synthesis pathway of phosphatidylcholine (PC) 34:2. The biosynthesis begins with the conversion of glycerol-3-phosphate (G3P) and acyl-CoA molecules into lysophosphatidic acid (LPA) through the action of glycerol-3-phosphate acyltransferase (GPAT). LPA is then converted into phosphatidic acid (PA) by acyltransferases. In the chloroplasts, PA undergoes dephosphorylation to yield diacylglycerol (DAG), a central precursor in lipid metabolism (Nakamura et al., 2019).

DAG serves as a substrate for the synthesis of various phospholipids, including PG. The acylation of DAG with glycerol-3-phosphate acyltransferase enzymes within the chloroplast membrane leads to the formation of phosphatidylglycerol phosphate (PGP). PGP is subsequently dephosphorylated to produce PG, a key constituent of thylakoid membranes involved in photosynthesis. PG 36:2 specifically refers to phosphatidylglycerol molecules with two acyl chains, each containing 18 carbon atoms (C18) and two unsaturated bonds (denoted as 2). The composition and abundance of PG 36:2 can vary depending on environmental conditions and developmental stages, reflecting the dynamic nature of lipid metabolism and cellular physiology (Kobayashi, et al., 2017).

The regulation of flowering time in maize is a complex process influenced by genetic, hormonal, and environmental factors. Recent research has implicated PG metabolism in the control of flowering time, suggesting a potential link between lipid composition and the timing of flowering. As a crucial component of thylakoid membranes, PG plays an essential role in photosynthesis and chloroplast function, processes that are vital for plant growth and development (Kobayashi et al., 2015). Emerging evidence indicates that changes in PG composition and abundance can impact flowering time in maize and other plant species. PG-derived lipid messengers are involved in signaling cascades that regulate gene expression and developmental transitions associated with flowering (Ali et al., 2022).

Furthermore, components of the PG synthesis pathway, including GPAT enzymes, have been identified as potential regulators of flowering time through their effects on lipid metabolism and associated signaling pathways. The synthesis of phosphatidylethanolamine (PE), another important phospholipid, involves a series of biochemical reactions orchestrated by enzymes localized in the ER and mitochondria, underscoring the compartmentalization and complexity of lipid metabolism within eukaryotic cells (Nakamura et al., 2014).

The Kennedy pathway is a major route for phospholipid biosynthesis and serves as the primary pathway for PE synthesis. In this pathway, PE is synthesized through the sequential acylation of G3P and DAG molecules. The acylation of G3P by GPAT generates LPA, which is subsequently acylated by lysophosphatidic acid acyltransferase

(LPAAT) to form PA. PA then undergoes dephosphorylation by phosphatidate phosphatase (PAP) to yield DAG, which serves as a precursor for PE synthesis. The synthesis of PE is critical for plant growth and development, including the regulation of flowering time (Jung et al., 2021).

Phosphatidylethanolamine (PE) metabolism has emerged as a critical factor in the regulation of flowering time in Arabidopsis, with genetic studies underscoring its importance. Mutations in genes encoding key enzymes involved in PE synthesis, such as glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT), have been shown to result in altered flowering time phenotypes. This highlights the significant role that lipid metabolism pathways play in modulating developmental processes in plants ((Nakamura, 2017).

Environmental factors, such as temperature and photoperiod, also exert influence on PE metabolism, thereby impacting flowering time responses. The interplay between lipid homeostasis and environmental adaptation underscores the complexity of the regulatory networks governing plant development (Suh et al., 2022). PE's role in flowering time regulation extends beyond its structural function in cellular membranes. PE-derived lipid signaling molecules, such as phosphatidic acid (PA) and diacylglycerol (DAG), are involved in signaling cascades that modulate gene expression and orchestrate the developmental transitions associated with flowering (Lachezar, 2020).

Furthermore, PE metabolism intersects with other metabolic pathways, including those involved in hormone biosynthesis and stress responses. This intersection further influences flowering time regulation, suggesting that lipid metabolism is integral to a broader network of plant physiological processes (Wan et al., 2020). Understanding these molecular interactions offers valuable insights for targeted breeding strategies aimed at enhancing lipid content in maize kernels, with potential benefits for biofuel production, nutritional quality, and crop resilience.

The regulation of flowering time is a multifaceted process involving various molecular components, including mobile florigen, PC, and PG. Each of these elements plays a distinct yet interconnected role in orchestrating the transition from vegetative growth to reproductive development in plants. Mobile florigen acts as a systemic signal that triggers flowering in response to environmental cues, while PC and PG contribute to membrane dynamics, signaling pathways, and metabolic processes that regulate flowering time (Nakamura et al., 2014).

PC's role in flowering time regulation is particularly significant. It influences the trafficking and localization of mobile florigen from its synthesis site in the leaves to the shoot apical meristem (SAM), where it initiates flower development. Additionally, signaling molecules derived from PC metabolism may directly or indirectly modulate the expression of genes involved in flowering time control, linking PC metabolism to this crucial regulatory process (Jung et al., 2021). This lipid contributes to the integration of environmental signals that regulate flowering time by modulating membrane fluidity and

protein localization (Meng., 2011). Furthermore, PC-derived signaling molecules can affect the expression of genes involved in flowering time control, thereby linking lipid metabolism to the regulation of flowering (Nakamura, 2014).

Similarly, PG-derived molecules, such as jasmonic acid, are implicated in signaling pathways that mediate plant responses to environmental stresses, pathogens, and developmental cues. These signaling pathways intersect with the networks regulating flowering time, thereby modulating gene expression in response to environmental conditions. This positions PG metabolism as a crucial link between environmental signaling, stress responses, and flowering time regulation, enabling plants to adjust their reproductive timing in response to changing environmental conditions (Xu & Zhao, 2021).

Overall, lipid metabolism influences both the production and transport of mobile florigen, as well as the integration of environmental signals that modulate flowering time. By affecting membrane dynamics and signaling pathways, phosphatidylcholine and phosphatidylglycerol contribute to the precise regulation of flowering time, ensuring that plants develop reproductive structures under optimal environmental conditions (Nakamura et al., 2017; Meng., 2011).

Chapter 2

Adaptive Introgression from Teosinte Mexicana Alters Phosphatidylcholine Levels and Influences Maize Flowering Time

Introduction

The domestication of maize stands as a landmark event in agricultural history, characterized by the selection and cultivation of maize varieties with traits advantageous for human use. Among the various biochemical processes that have influenced the evolution of maize, the conservation of enzymes such as Phospholipase A1 (PLA1), particularly the HPC1 isoform, provides valuable insights into the biochemical mechanisms that underlie plant evolution and agricultural development.

Phospholipase A1 (PLA1) enzymes are critical players in lipid metabolism, where they catalyze the hydrolysis of the sn-1 position of phospholipids, releasing fatty acids and lysophospholipids. In maize, the HPC1 isoform of PLA1 has garnered significant attention due to its involvement in various physiological processes, including membrane remodeling, signal transduction, and responses to environmental stress. When found to have a loss of function in highland populations, meaning HPC1 is more functional in lowland environments. The loss of function causes PCs to not be converted to LPCs.

For instance, biochemical investigations have demonstrated that HPC1 plays a significant role in lipid metabolism and membrane dynamics, which are crucial for plant growth, development, and stress tolerance. HPC1 enzymes are localized in various subcellular compartments, such as the endoplasmic reticulum (ER) and plastids, where they participate in lipid remodeling processes essential for maintaining cellular homeostasis and adapting to environmental changes.

Moreover, the interaction between the ZCN8 protein and HPC1 has been a focal point in understanding lipid metabolism regulation in maize. ZCN8 influences lipid metabolism through multiple regulatory mechanisms, including transcriptional control, protein-protein interactions, and epigenetic modifications (Barnes et al., 2022). Recent studies have begun to elucidate the complex network of regulatory mechanisms that connect ZCN8, HPC1, and lipid metabolism. For example, research led by Barnes and Rodríguez-Zapata et al. (2022) identified HPC1 as a key gene affecting phospholipid levels in maize, particularly phosphatidylcholine, which plays a role in flowering time regulation.

Their study demonstrated that the introgression of HPC1 from *teosinte mexicana* into maize led to increased phosphatidylcholine levels and earlier flowering. This adaptation provided a significant advantage in highland environments, linking ZCN8 regulation, HPC1 activity, and lipid metabolism. The findings highlight the importance of lipid signaling in plant development and stress responses, offering new perspectives on the biochemical pathways that have shaped maize domestication and evolution .

The study by Barnes et al. (2022) explored the interactions between ZCN8—a critical flowering time regulator in maize—and specific lipid species, particularly PCs, to elucidate the molecular mechanisms underlying this trait.

Background and Major Findings

The impetus for studying ZCN8-lipid interactions stemmed from a series of key findings we find. We initially identified that variations in the HPC1 gene significantly influenced flowering time in maize. HPC1 emerged as a crucial genetic determinant in the timing of the transition from vegetative to reproductive development, which is a pivotal adaptation trait in plants. Further investigations revealed that HPC1 not only affected flowering time but also modulated the levels of specific phosphatidylcholine (PC) species. This link between HPC1 and lipid metabolism provided a molecular basis for the observed phenotypic variations in flowering time. The work by Nakamura et al. (2014) demonstrated that FT (FLOWERING LOCUS T) proteins in *Arabidopsis thaliana* interact with certain PC species. This interaction plays a significant role in the regulation of flowering time, suggesting that PCs could be integral to the signaling pathways controlling this critical developmental process. Barnes et al. (2022) observed that highland maize varieties, which tend to flower earlier, exhibited an accumulation of the same PC species known to interact with FT in *Arabidopsis*. This observation suggested a possible conserved mechanism across species where PC levels could modulate flowering time through interactions with proteins like FT or its homologs. Based upon the work of Nakamura (2019) ZCN8, the maize homolog of *Arabidopsis* FT, is a well-established

regulator of flowering time. Given the parallels between FT in Arabidopsis and ZCN8 in maize, and the involvement of PCs in FT-mediated flowering regulation, it became critical to investigate whether ZCN8 similarly interacts with PC species in maize. Given these findings, the hypothesis emerged that ZCN8, like FT in Arabidopsis, might interact with specific phosphatidylcholine species to regulate flowering time in maize. This hypothesis was driven by:

The observed effects of HPC1 variation on both flowering time and PC levels. The established interaction between FT and PCs in Arabidopsis. The accumulation of relevant PC species in early-flowering highland maize. The known role of ZCN8 in flowering time regulation. The objective of subsequent experiments was to determine whether ZCN8 interacts with specific PC species, thereby modulating flowering time in maize. This research aimed to uncover a potentially conserved lipid-protein interaction mechanism that could explain the observed phenotypic variations in flowering time and provide new insights into maize adaptation and evolution.

Following the review of Barnes et al. a reviewer suggested we depict the binding of ZCN8 via fat blotting. Following the blotting procedure, no association was observed between the ZCN8 protein and lipids, prompting further investigation using alternative Liquid Chromatography-Mass Spectrometry (LC-MS) methods. LC-MS combines the separating power of liquid chromatography with the sensitive and specific detection

capabilities of mass spectrometry. LC separates complex mixtures of lipids based on their physicochemical properties such as polarity, size, and charge, while MS detects and quantifies individual lipid species based on their mass-to-charge ratio (m/z).

LC-MS operates in two main modes: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI is particularly suited for polar and amphipathic lipids, while APCI is more suitable for less polar lipids. Following separation, lipids eluted from the LC column enter the mass spectrometer, where they are ionized, fragmented, and detected. The resulting mass spectra provide information on lipid identity and structure, allowing researchers to characterize lipid profiles in biological samples with high sensitivity and specificity. Recent studies have refined the crystal structure of Arabidopsis FT, which was used as a model to analyze ZCN8 and compare its PC binding sites. As seen in figure 3 by simulating docking with AutoDock Vina and referencing existing data, we identified potential binding sites for PC34:2 and PC36:2 at sites 2 and 4, as well as 1 and 4, with site 4 being the most favored for both lipid species.

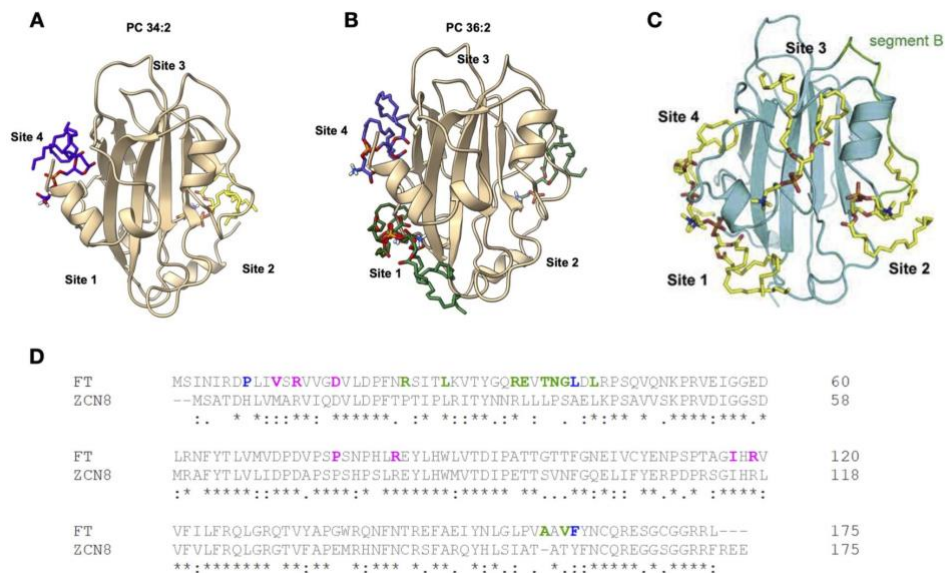


Figure 3: Predicted binding sites of ZCN8 align with binding sites of Arabidopsis FT. Shown are AutoDock Vina ZCN8 - lipid docking interactions of a RoseTTAFold (model of ZCN8 PC 34:2 (A) and PC 36:2 (B) compared with the docking model of PC 36:2 Arabidopsis thaliana FT (C) from Docking was performed on an NMRBox server (D) Alignment of Arabidopsis thaliana FT and Zea mays ZCN8. Residues in blue are present in both docking site 1 and docking site 4 identified in Residues in magenta are present only in docking site 1. In contrast, residues in green are present only in docking site 4. Symbols below the residues indicate level of alignment where * denotes complete alignment, : represents a conserved substitution, and . represents a semi-conserved substitution.

Given the high conservation between maize ZCN8 and Arabidopsis FT, the similarity in predicted binding sites is expected. Subsequently, we produced and purified ZCN8, fused to an SPOT tag in yeast (*Saccharomyces cerevisiae*) cells to study lipid binding in vivo. We then extracted and analyzed the lipids copurified with the recombinant ZCN8 protein using the same lipidomics pipeline applied to maize lipids. As seen in Figure 4, this analysis confirmed the binding of PC34:2 to ZCN8 in all samples, experimentally validating the interaction between ZCN8 and PC species.

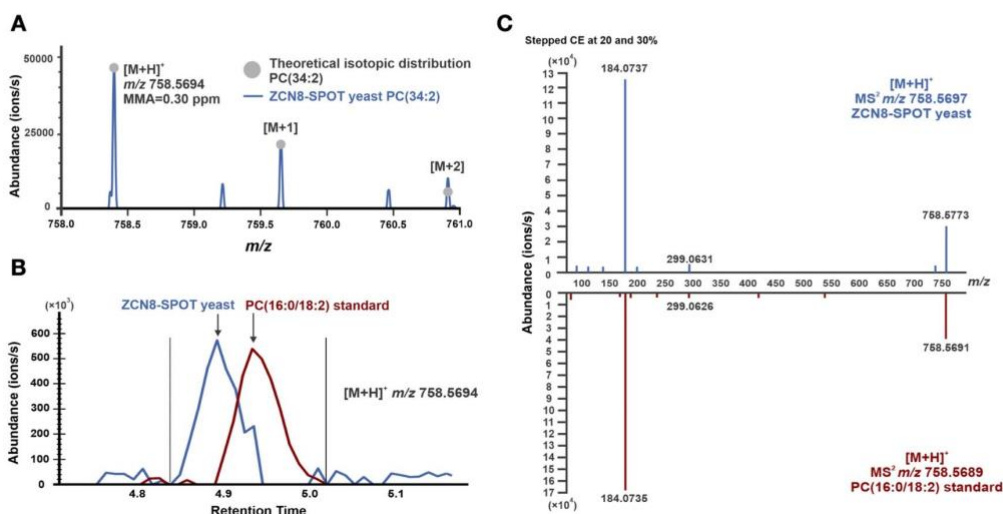


Figure 4: ZCN8 binds to phosphatidylcholine. A Mass spectrum of ZCN8-SPOT yeast sample compared to the theoretical isotopic pattern of PC(34:2) at $[M+H]^+$ m/z 758.5694. The experimental m/z and mass measurement accuracy (MMA) are labeled. The spectrum of ZCN8-SPOT is an average of 16 scans across the chromatographic peak of m/z 758.5694. B Extracted ion chromatogram of m/z 758.5694 from the PC(16:0/18:2) standard and ZCN8-SPOT yeast from two separate injections. C MS2 fragmentation spectra comparison for m/z 758.5694 from ZCN8-SPOT yeast and the PC(16:0/18:2) standard. The comparison data between an authentic standard and ZCN8-SPOT yeast was acquired with the same lipid profiling method as described in the Materials and Methods section for the Thermo Scientific Orbitrap Exploris 480 mass spectrometer with the following modifications: the injection volume was 10 μ L, full scan spectra were acquired from m/z 200 – 1000 and m/z 758.5694 was included in the target mass list for MS2 selection

Following the *in vivo* study of using yeast via heterologous expression to demonstrate the binding of ZCN8 to PC 34:2, the most logical next step was to validate ZCN8 binding to maize lipids. The lipid composition between maize and yeast are vastly different. I sought to validate ZCN8 binding to PC 34:2 by using etiolated maize. Etiolated maize is maize

grown in the absence of light. When maize plants are grown in darkness, they undergo a process called etiolation, where they develop these unique characteristics that are ideal for protoplast harvest. The plants have long, thin, pale leaves and reduced chlorophyll production. In my experiment, I harvested the protoplast from the leaves and transfected them with our ZCN8 construct. I wanted to test whether the endogenous plant lipids would bind to ZCN8. In the early development stages, ZCN8 isn't shown to be found in the leaves of early maize plants.

Methods and Materials

Fat Blotting: This method is akin to Western blotting but focuses on lipids rather than proteins. The process begins with the extraction and separation of lipids from biological samples, typically using thin-layer chromatography (TLC). Lipids are resolved on a TLC plate based on their affinity for the stationary phase and their mobility in the solvent phase. Once separated, lipids are transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. The membrane is then subjected to various detection methods, including staining with specific dyes like Sudan Black or using radiolabeled or fluorescent lipid-binding probes.

Fat blotting is pivotal in studying lipidomics, providing insights into lipid composition, metabolism, and signaling. It is extensively used to analyze lipid alterations in diseases, identify lipid-protein interactions, and monitor changes in lipid profiles under different physiological conditions. The primary advantage of fat blotting is its specificity and sensitivity in detecting lipids. It allows for the detailed analysis of lipid species, offering

valuable data on their relative abundance and distribution. The lipids employed to investigate lipid-protein interactions included PCS 34:2 and 36:4, PS, PI, PA, DOPE, DOPC, PE, PG, LPC, and LPE. Each lipid, at a concentration of 5 micrograms, was diluted in the spotting buffer comprising chloroform, methanol, HCL, and Ponceau S.

ZCN8 Binding to Phospatylcholine: We synthesized a codon-optimized ZCN8 for *Saccharomyces cerevisiae*, tagged with a SPOT peptide tag (Chromotek, Planegg, Germany), referred to as ZCN8-SPOT. ZCN8-SPOT was transformed into the galactose-inducible vector pYES-DEST52 and expressed in the *Saccharomyces cerevisiae* strain FY4. After 8 hours of induction, cultures were harvested and frozen for future protein extractions. We purified the protein using Chromotek SPOT-Cap beads, following their protocol. Briefly, a 50 mL induction pellet was resuspended in lysis buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, pH 8.0, 1 mM DTT, 10 mM MgCl₂, 1x HALT protease and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA), and 1% dodecylmaltoside) and ground in a Genogrinder with glass beads at maximum speed for one minute. This was followed by one minute on ice, repeated for a total of three minutes of grinding. The sample was then incubated on ice for five minutes, vortexed, and incubated for another five minutes. After pelleting, the supernatant was diluted with wash buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, pH 8.0, 1X HALT protease and phosphatase inhibitor, and 0.1% dodecylmaltoside) and added to cleaned, equilibrated SPOT-Cap beads, rotating at 4°C for 1 hour. The beads and lysate were separated by centrifugation at 2500xg for 5 minutes, and the beads were washed three times with 20x bead volume of wash buffer. Protein was eluted with either

SPOT-peptide or 100 mM glycine, pH 2.0. Glycine elutions were immediately neutralized with an equivalent amount of Tris base, pH 10.4. Elutions were frozen, and lipids were extracted and analyzed later. Comparison data between an authentic standard and ZCN8-SPOT yeast were obtained using the same lipid profiling method for the Thermo Scientific Orbitrap Exploris 480 mass spectrometer, with the following modifications: sample injection volume was increased to 10 μ L, full scan spectra were acquired from m/z 200–1000, and m/z 758.5694 was included in the target mass list for MS2 selection. In MSDIAL, lipid identification is based on two approaches: the MSP file and MS/MS identification settings included in MSDIAL, and a post-identification file containing accurate m/z and retention times for a list of lipids. In this study, we used both approaches. Under positive ion mode, the MSP file and MS/MS settings support identification of 51 lipid classes. The post-identification file used was the retention time-corrected MS1-MS2 m/z-rt lipid library previously described. We utilized MSDIAL version 3.40, converting raw data from .d to .abf format using the Reifycs Abf converter. We filtered MSDIAL alignment results based on whether compound intensity was ten times above blank intensity. Filtered data were normalized using Systematic Error Removal using Random Forest (SERRF), based on QC pool samples. Normalized features with a coefficient of variation (CV) equal to or less than 30% among the pools were retained. To curate data for duplicate features, isotopes, and ion adducts, we used MS-FLO. The curated data were further normalized using the sum of all known metabolite signals (mTIC). After processing and normalization, lipid intensities were used for further analysis.

Discussion

The domestication of maize represents a transformative milestone in agricultural history, influenced by selective pressures that favored traits beneficial for human use. The biochemical pathways involved in lipid metabolism and flowering time regulation are central to understanding maize's adaptability and development. This discussion sums up the work from exploring the intricate relationship between ZCN8, phospholipase A1 (HPC1), and the binding of phospholipids. Susila(2021) found that in Arabidopsis, the florigen FT, initiates flowering at the shoot apical meristem. They found that FT, produced in leaf cells, can be kept in reserve at low temperatures by binding to a membrane phospholipid, limiting its mobility. This binding is reduced when temperatures rise, allowing FT to move and promote flowering. This can be contributed to the rigidity of the lipid cell. Building upon that work, Nakamura(2014 & 2017) did a study that demonstrates that FT binds specifically to phosphatidylcholine (PC) in vitro. Increasing PC levels in transgenic plants accelerates flowering, while reduced PC levels delay it, indicating a correlation between PC levels and flowering time. Early flowering is associated with increased FT activity and expression of FT-effector genes. Enhancing both FT and PC in the shoot apical meristem further stimulates flowering, whereas loss of FT function diminishes the effects of increased PC. Additionally, specific PC molecular species fluctuate diurnally, with night-dominant species not favoring FT binding; elevating these species during the day delays flowering. The authors suggest that FT interacts with varying PC species throughout the day to promote flowering. Barnes et al.(2022) proved this study to be true in maize. We noticed better plant fitness in highland

maize grown in Mexico than lowland maize. We also noticed the same PCs as mentioned by Nakamura had a greater accumulation in the highland environment. Which we contributed to a more functional copy of HPC1 in lowland environments, the loss of function HPC1 copy has more PCs that are not converted to LPCs. Next we found significant associations between HPC1 expression in aerial tissues and various flowering-time traits, comparable to known flowering-time genes like ZCN8 and ZmRAP2.7. In Arabidopsis, FT interacts with PC and PG based on temperature and location. Given that ZCN8 is a homolog of FT, it may accelerate flowering in highland maize. We modeled ZCN8's structure based on improved Arabidopsis FT data and identified potential PC binding sites. We then produced and purified ZCN8 from yeast, confirming its ability to bind to PC34:2 through lipid analysis, thus demonstrating that ZCN8 effectively interacts with specific lipid species.

Nakamura's studies underscore the significance of ZCN8 in facilitating the transition from vegetative to reproductive phases, a pivotal adaptation trait in plants. By influencing flowering time, ZCN8 contributes not only to plant fitness but also to agricultural productivity. The parallel between FT and ZCN8 suggests a conserved evolutionary mechanism through which lipid signaling can modulate flowering responses, particularly in varying environmental contexts. Nakamura's work highlights how genetic variation in ZCN8 can lead to phenotypic differences in flowering time, suggesting that adaptations in flowering mechanisms have profound implications for maize cultivation in diverse climates. Building upon Nakamura's foundational work, a critical hypothesis has emerged: ZCN8 interacts with specific phosphatidylcholine species to modulate

flowering time in maize. This hypothesis is supported by multiple lines of evidence, including the established interaction of FT with lipid species in *Arabidopsis* and the identification of lipid profiles associated with flowering time in highland maize.

Initial attempts to visualize the binding of ZCN8 to lipids using fat blotting did not yield conclusive results, prompting further investigations through Liquid Chromatography-Mass Spectrometry (LC-MS). LC-MS offers a highly sensitive approach to analyzing lipid-protein interactions by separating complex lipid mixtures and detecting individual lipid species based on their mass-to-charge ratio. The use of advanced techniques such as molecular docking simulations has allowed for the identification of potential binding sites for phosphatidylcholine species on ZCN8, revealing that specific lipids may indeed play a role in modulating ZCN8 activity.

The importance of lipid metabolism in flowering time regulation has been illuminated by the work of Barnes et al. (2021), which delves into the role of phospholipase A1, particularly the HPC1 isoform, in modulating lipid profiles associated with flowering. HPC1 is instrumental in hydrolyzing phospholipids, influencing the levels of various lipid metabolites, including phosphatidylcholine (PC). Barnes et al. demonstrated that variations in the HPC1 gene affect phospholipid levels, which are linked to maize flowering time. Specifically, the introgression of HPC1 from *Zea mays*' wild relatives resulted in increased PC levels and earlier flowering times in highland varieties.

This connection between HPC1 and ZCN8 indicates that lipid composition is not merely a byproduct of metabolic processes but a critical regulatory component influencing flowering time. The interplay between these two proteins reveals a sophisticated network

where lipid signaling modulates developmental transitions, suggesting that alterations in lipid metabolism can directly affect key phenotypic traits. The findings that I contributed to in from Barnes et al. also extend the understanding of conserved lipid signaling mechanisms across species. By establishing parallels between the interactions of FT with lipids in *Arabidopsis* and ZCN8 in maize, a broader framework emerges that connects lipid metabolism with flowering time regulation in diverse plant species. This conservation suggests that lipid-protein interactions are fundamental to flowering time control, emphasizing the evolutionary significance of lipid dynamics in plant development (Dominguez et al., 2024).

Nakamura's utilization of various methodological approaches, including genetic analysis and molecular docking simulations, further elucidates the functional relationships among ZCN8, HPC1, and lipid species. Identifying potential binding sites for specific phosphatidylcholine species on ZCN8 highlights the sophisticated regulatory mechanisms at play. Although initial attempts to visualize these interactions using fat blotting were inconclusive, the subsequent application of Liquid Chromatography-Mass Spectrometry (LC-MS) demonstrated the efficacy of advanced analytical techniques in characterizing lipid profiles and their interactions with proteins.

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