Analysis of cytokinin-induced maize leaf developmental changes and interacting genetic modifiers

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Analysis of cytokinin-induced maize leaf developmental changes and interacting genetic modifiers

by

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A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

Major: Genetics

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ABSTRACT

Four distinct segments define the maize leaf along the proximal-distal axis: the proximal sheath and the distal blade, which are separated by the ligule and auricle. The process of maize leaf morphogenesis, patterning and development requires the coordination of a number of proteins and hormones. The semi-dominant gain-of-function mutant *Hairy sheath frayed1* (*Hsf1*) disrupts a number of leaf growth parameters: (i) leaves are shorter and more narrow, (ii) an increased density of macrohairs are apparent on the abaxial sheath and adaxial blade and (iii) the normal proximal-distal leaf pattern is disrupted, resulting in outgrowths, or prongs, of ectopic sheath, auricle and ligule from the margins of the normal leaf blade. The underlying cause for the *Hsf1* phenotype is a missense mutation near the binding pocket of *Zea mays Histidine Kinase1*, a cytokinin (CK) receptor, which results in CK hypersignaling. To investigate the role of cytokinins, a class of plant growth regulatory hormones, in leaf morphogenesis and development, we conducted exogenous hormone germination assays and double mutant analysis of genes acting downstream of CK signaling. Our results confirm the *Hsf1* phenotype is due to an increase in CK signaling and is more sensitive to CK. CK treatment affects leaf growth, cell identity and proximal distal patterning in a dose-dependent manner at specific developmental stages. Genetic diversity underlies CK responses in a diverse selection of CK-treated maize inbred lines, as well as in other species. *Hsf1* alters proximal-distal leaf patterning in a specific manner and double mutant analysis revealed genetic interactions with genes downstream of CK signaling. *Hsf1* prong formation is enhanced by dominant *knotted1*-like homeobox (*knox*) mutants and low GA levels, although *knotted1* itself does not appear to be required for prong formation. Double mutants with *aberrant phyllotaxy1* and *tassels replace upper ears1* reveal dosage-dependent phyllotaxy effects.
CHAPTER I
GENERAL INTRODUCTION

One of the most important processes in developmental biology is morphogenesis, or the developmental process regulating the shape and size of an organism. In plants, leaf morphogenesis and development is especially important, as leaves usually serve as the major photosynthetic surface, allowing for the conversion of carbon dioxide into sugars.

Leaf morphogenesis and development has been well characterized in a number of species, including maize [1]. The process of maize leaf morphogenesis and development requires the coordination of a number of proteins and hormones. The regulation and expression patterns of key transcription factors involved in leaf patterning, such as knotted1, have been studied extensively [2-9]. A number of plant hormones, including gibberellic acid (GA), brassinosteroids, auxin and cytokinin (CK) have also been shown to regulate the initiation and shape of maize leaves [10-12]. The role of CK in maize leaf morphogenesis and development, however, has not been fully investigated.

The maize Hairy sheath frayed1 (Hsf1) mutant is a CK hypersignaling mutant resulting in disruptions in normal leaf development [13]. A number of aspects of the leaf are affected in Hsf1, including leaf size and leaf patterning — Hsf1 leaves have outgrowths of ectopic tissue from the margins of the normal leaf blade [14]. Thus, the Hsf1 mutant provides an opportunity to study the role of CK signaling in the establishment of leaf patterns in maize. In order to address this problem, a series of investigations have been conducted on the effects of exogenous CK on developing maize seedlings as well as analysis of the genetic interactions that occur downstream of CK signaling.
Maize leaf development requires coordination of knox genes and hormone levels

Maize leaf development begins as leaf primordia are initiated from the collection of pluripotent cells, called the shoot apical meristem (SAM). Leaf primordia arise in a repeating pattern, or phyllotaxy, from the flanks of the SAM. Incipient leaf primordia are marked in the SAM by downregulation of knotted1-like homeobox (knox) transcription factors [15]. knox proteins regulate levels of many plant hormones in the SAM to control the SAM’s indeterminate or determinate state. knox proteins control CK levels in some species by directly regulating CK biosynthesis genes [16-18], and regulate GA levels in maize by directly activating a gene responsible for GA catabolism [19]. When knox genes are expressed in the SAM, a high CK and low GA state results in the SAM maintaining an indeterminate state [16]. When knox genes are downregulated, a low CK an high GA state can allow for the change to a determinate state, and thus the initiation of a leaf primordia [19]. Loss-of-function mutants for knotted1, the founding member of the knox gene family, have a reduced SAM size and, depending on the inbred background, may not initiate more than two leaves [2]. This suggests knox genes are important for meristem maintenance.

While knox genes play a major role in meristem maintenance, they also affect proximal-distal leaf patterning. As the initiating leaf primordia cells divide and expand, they begin to be patterned along three axes: lateral-medial, dorsal-ventral and proximal-distal [20]. The proximal-distal axis is defined in the maize leaf by the proximal sheath, which wraps around the culm, and the distal blade, which serves as the primary area for photosynthesis. Separating the two is an epidermal fringe, called the ligule, and two wedge shaped auricles. Specification of these compartments requires regulation of knox genes. When knox genes are ectopically expressed in the leaf, proximal-distal patterning is affected. Dominant gain-of-function class 1 knox mutants
Knotted1, Liguleless3, Gnarley1 and Roughsheath1 express their respective knox genes in the leaf, resulting in proximal sheath, auricle and ligule appearing in the distal leaf blade [4, 6, 21, 22]. One allele of knotted1, Kn1-DL, expresses knotted1 in the distal margins of the leaf blade, resulting in prongs of sheath emerging from the distal margins [3]. These mutants show that ectopic knox expression can result in proximal-distal leaf patterning defects.

Another aspect of maize leaf morphogenesis and development affected by hormone levels is the arrangement, or phyllotaxy of initiating leaves. The maize mutant aberrant phyllotaxy1 (abph1) has an altered phyllotaxy – instead of one leaf at every node initiating on opposite sides of the SAM, the abph1 mutant initiates two leaves at each node, 90 degrees from the previous leaves [23]. The causative gene for the abph1 phenotype is Zea mays Response Regulator3 (RR3), a CK-responsive negative regulator of CK signaling and a positive regulator of auxin accumulation [24]. The loss-of-function mutant has a larger SAM and reduced expression of PIN1, an auxin transport gene [12]. Thus, when RR3 is unable to balance CK and auxin levels, an altered phyllotaxy can occur.

**Hairy sheath frayed1 alters leaf development through CK signaling**

Hairy sheath frayed1 is a semi-dominant gain-of-function mutant that has pleiotropic effects on maize leaf development [14]. As the name suggests, Hsf1 has an increased density of macrohairs on the sheath as well as the adaxial blade. The “frayed” portion of the name refers to a characteristic proximal-distal patterning defect—Hsf1 leaves have ectopic projections, or prongs, of proximal sheath, auricle and ligule from the margins of the distal leaf blade. In addition, Hsf1 leaves are shorter and narrower than wild type (WT) sibling leaves. Although the ectopic prong aspect of the Hsf1 phenotype is similar to the dominant knotted1 mutant, Kn1-DL,
Hsf1 is not a knox gene. The gene underlying the Hsf1 mutation is Zea mays Histidine Kinase1 (ZmHK1), a CK receptor. The phenotype is due to a specific missense mutation near the CK binding domain of ZmHK1, causing a tighter binding affinity for CKs. This is hypothesized to cause CK hypersignaling in the Hsf1 plant [13].

ZmHK1 is part of a two-component CK signaling system in plants. As CK is perceived by the binding domain, ZmHK1 autophosphorylates and transfers the phosphate to response regulators through a histidine phosphotransfer protein [25]. Type-A response regulators, which include RR3, negatively regulate CK signaling [24]. Type-B response regulators positively regulate downstream CK signaling through DNA binding activity [26].

The idea that the Hsf1 phenotype is due to CK hypersignaling is intriguing, as CKs are plant growth hormones involved in a number of regulatory processes. CK has been shown to be required for meristem maintenance in a number of species, as CK deficient mutants have reduced SAM sizes [27]. CK is also involved in adventitious bud formation, release of buds from apical dominance, delay of senescence and leaf expansion [28]. Exogenous CK has been shown to induce macrohairs on mature maize leaves [29]. Since the Hsf1 mutant exhibits leaf size and an increase in macrohair density, along with proximal-distal patterning defects, the mutant could also give insight into further CK regulatory processes.

The role of CK and CK signaling in leaf development

The Hsf1 mutant provides an opportunity to study leaf morphogenesis and development in two ways. First, the effect of CK signaling on leaf morphogenesis and development has not been fully characterized. Traditionally, CK is thought to increase leaf expansion, but the Hsf1 mutant has shorter and narrower leaves than WT siblings, which warrants further investigation.
Hsf1 has been shown to be ZmHK1, part of a two-component system with RR3. To examine the role RR3 plays in negatively regulating CK signaling and controlling phyllotaxy, it would be informative to combine Hsf1 with the RR3 loss-of-function mutant, abph1.

Second, the altered proximal-distal pattern of the Hsf1 leaf allows for the investigation of molecular signals that specify the compartments of the maize leaf outside of their normal context. Hsf1 leaves have ectopic proximal tissue emerging from the margins of the distal leaf blade. Similar patterning defects are seen in dominant knox mutants. This raises the question of which, if any, knox gene is expressed in the mutant margins, and what causes it to be misexpressed.

In order to more fully understand the role of CK in morphogenesis and development a series of experiments were conducted. First, to understand the role CK plays in determining the shape and patterning of leaves in maize seedlings, a germination assay was developed. This assay involved germinating maize inbred kernels in exogenous CK for a set period of time, transplantation, growth for approximately three weeks, followed by observations of morphological changes in the mature leaf. Second, double mutant analysis was conducted with a number of maize mutants. Prospective genes were previously identified from a transcriptome study of developing mutant prongs. Other interesting patterning and hormone mutants were also combined with Hsf1 to investigate genetic interactions. The results of these two studies are presented in the following chapters.

**Thesis organization**

This thesis contains the general introduction (Chapter 1), two chapters in journal format (Chapters 2 and 3), and a general conclusion (Chapter 4).
Chapter 2 is an investigation into the effects of CK on leaf development in maize seedlings. My contributions include the development and execution of a CK germination assay on a number of inbred and mutant lines of maize, sorghum and rice, double hormone treatments on maize, and gene expression analysis on CK treated maize leaves. Portions of this work will be submitted as pieces of future journal articles or on their own.

In chapter 3, the proximal distal leaf patterning defects in Hsf1 leaves were analyzed, followed by double mutant analysis to investigate the role other genes play in prong formation. My contributions include the morphological analysis of the Hsf1 mutant leaves, as well as the morphological analysis of double mutants. Portions of this work will be submitted as parts of larger journal articles currently being written.

References


CHAPTER II

EXOGENOUS CYTOKININ PRODUCES SIMILAR LEAF GROWTH PHENOTYPES AS THE MAIZE HAIRY SHEATH FRAYED1 MUTANT

Abstract

Four distinct segments define the maize leaf: the proximal sheath and the distal blade, which are separated by the ligule and auricle. The semi-dominant gain-of-function Hairy Sheath Fayed1 (Hsf1) mutant has been shown to disrupt this pattern, resulting in ectopic projections, called prongs, of sheath, auricle and ligule arising from the margins of the distal leaf blade. Hsf1 also decreases leaf length and width, and results in an increase in macrohair density on the abaxial sheath and adaxial blade. The gene underlying the Hsf1 mutant is a cytokinin receptor Zea mays Histidine Kinase1 (ZmHK1). Previous analysis has revealed the phenotype is the result of missense mutations near the cytokinin-binding pocket, resulting in increased binding affinity for the plant growth regulatory hormone, cytokinin (CK). To confirm that the Hsf1 phenotype is the result of increased CK signaling, we performed a series of germination assays in the presence of exogenous CK. We found that that the three characteristic Hsf1 phenotypes were able to be reproduced. Further CK treatments were performed to determine the developmental timing of each aspect of the phenotype. A number of inbred lines, including a subset of the Nested Association Mapping (NAM) lines, were treated to assay for genetic diversity in CK responses. Treatments of low concentrations of CK also showed the Hsf1 mutant is more sensitive to CK. Double hormone treatments were conducted to assess the response to high concentrations of gibberellic acid (GA). qRT-PCRs of CK treated leaves showed no change in GA metabolism genes.
Introduction

The maize leaf comprises four distinct segments along the proximal-distal axis. The sheath, which wraps around the culm, is most proximal, and the blade, which is the main photosynthetic organ, is most distal. Both are separated by an adaxial epidermal fringe, the ligule, and the auricle, a hinge-like segment that allows the blade to bend away from the stalk [1].

Leaf development begins in the shoot apical meristem (SAM) where they arise as primordia on the flanks of the SAM. Incipient leaf primordia are marked in the SAM by down-regulation of class I *Knotted1*-like homeobox (*knox*) transcription factors [2], and an accumulation of the plant hormones auxin and GA. *knox* genes are expressed throughout the meristem. *Knox* genes have been shown to regulate two plant hormones, cytokinin (CK) and gibberellic acid (GA), to maintain the meristem in an indeterminate state. *Knox* genes directly regulate GAs by repressing a *GA20oxidase* gene, responsible for a step in GA biosynthesis [3], or by activating *GA2oxidase* genes, responsible for inactivating bioactive GA [4]. In Arabidopsis, *knox* proteins have been shown to activate cytokinin biosynthesis [5]. High CK and low GA are required for the SAM to maintain an indeterminate state [6]. The downregulation of *knox* genes results in high GA and low CK, thus allowing for the change to a determinate state. *Knox* genes are not normally expressed during development of monocot leaves, though *knox* expression has been shown to occur in the primordia of species with dissected leaves [7].

The SAM in the quiescent maize embryo has initiated about six leaf primordia [8], each at their own stage of development, called the plastochron. The leaf primordia closest to the SAM
is the most recently initiated and is at the first plastochron of development, or the P1. The outer leaf is the oldest and will be the first leaf (L1) to emerge from the coleoptile after germination.

As leaves divide and expand, leaf shape is controlled by plant hormones. Auxins and CKs, which promote cell proliferation, are highest in the division zone at the base of the leaf, and GAs, which promote cell elongation and differentiation, are active in the transition between the division zone and the expansion zone [9]. The most distal portion of the developing leaf is the oldest, and the most proximal portion is most recently differentiated.

The first five or six leaves to emerge from the coleoptile are considered to be juvenile phase. Juvenile phase leaves are glabrous, or lack macrohairs, and are coated in epicuticular wax. Adult phase leaves lack epicuticular wax, and are marked by the presence of macrohairs. Macrohairs, which normally appear in single files along bulliform cells, have been used as a marker of adaxial surface [10]. Three different types of hairs appear on the maize leaf: macrohairs, microhairs, and prickle hairs [11], but the prominent single-celled macrohair is a marker of adult phase. There will be several transitional leaves between the two phases that share sectors of both juvenile and adult phases [12].

The semi-dominant, gain-of-function Hairy sheath frayed1 (Hsf1) mutant provides a unique opportunity to study the molecular signals involved in leaf patterning. Hsf1 has been shown to disrupt adult leaf development in three specific ways: shorter, narrower leaves, increased abaxial sheath pubescence and projections of ectopic proximal tissue (sheath, auricle and ligule) emanating from the margins of distal leaf blade [13]. These projections, or prongs, of ectopic tissue allow for the study of cell patterning signals outside of their normal context.

The gene underlying the Hsf1 phenotype is a cytokinin receptor histidine kinase, Zea mays histidine kinase1 (ZmHK1). Previous investigations have revealed that specific missense
mutations cause an amino acid change near the binding pocket of the CK binding domain. This leads to a tighter binding affinity for cytokinin, and a state of cytokinin hypersignaling [14].

*ZmHK1* is part of the CK signaling two-component regulatory system. When CK binds to the binding pocket of the cytokinin receptor, *ZmHK1* autophosphorylates, then transfers the phosphate to a response regulator (RR) protein thorough a histidine phosphotransfer protein. Two types of RR proteins have different functions. Type-B RRs positively regulate CK signaling through DNA binding activity [15, 16]. Type-A RRs are rapidly transcribed in response to CK signaling and function as negative regulators [17].

CK has previously been implicated in many aspects of leaf development. It has been shown to induce macrohairs in a number of species [18], regulate compound leaf development [19], and affect leaf size [20]. To further study the effects of cytokinin on maize leaf development and cellular identity we devised a simple exogenous cytokinin germination assay. Imbibed maize kernels were germinated in the presence of exogenous cytokinin for a specific length of time followed by planting and growth in a greenhouse. After the first four leaves of the seedlings emerged and fully expanded we recorded phenotypic data on a number of traits: leaf size, macrohair presence, and proximal-distal patterning defects. To better understand the biological response to CK, we performed separate experiments adjusting the concentration of treatment, the length of treatment, and the inbred lines that were treated. Experiments were also performed on sorghum and rice to survey CK responses in other species.
Materials and methods

**Genetic stocks**

Maize inbred lines were obtained from the USDA Plant Introduction Center and increased by sib pollinations. The maize inbred lines include B73, A619, A632, Mo17, W22, B97, CML52, CML69, Ki11 and P93. The sorghum line Tx430 was obtained from Erik Vollbrecht and the rice cultivar Kitaake was obtained from Bing Yang.

**Plant hormone stocks**

6-Benzylaminopurine (6-BAP) powder from Sigma Aldrich was first dissolved in 10 drops of 1N NaOH, and brought to a concentration of 10 millimolar with sterile distilled water. A parallel water control stock was also made with 10 drops of 1N NaOH. These stocks were further diluted to achieve the desired hormone treatment concentrations. Gibberellic acid potassium salt powder from MP Biomedicals was dissolved in sterile distilled water.

**Seed hormone treatments**

Maize, sorghum and rice kernels were surface sterilized with two 5-minute washes of 80% ethanol, followed by two 15-minute washes of 50% bleach. The kernels were rinsed five times in sterile distilled water. Kernels were allowed to soak in distilled water overnight before beginning hormone treatments. 20 kernels were placed embryo-side down on two paper towels in a petri dish, then covered with two more paper towels. Each petri dish was filled with 15 mL of hormone treatment or control solution. Dishes were sealed with parafilm and kept in the dark at room temperature. After treatment, the germinating kernels were rinsed in distilled water and
planted in small pots (size) in soilless potting medium. Seedlings were grown in the greenhouse with supplemental lighting under standard maize growth conditions. Leaf measurements were performed after the fourth leaf collar had fully emerged from the whorl after about three weeks of growth.

**Leaf measurements**

Individual leaves were removed from plants and three components were measured. Sheath length is defined as the site of insertion at the base of the sheath to the farthest point of sheath adjoining the ligule. Leaf length is defined as the most proximal point of blade, which may overlap with the auricle, to the distal tip. Leaf width was measured margin to margin at half of the leaf length. Leaf measurements were analyzed in using JMP PRO 12 software. A student’s t-test was used to determine significance for when making two comparisons, and Tukey’s HSD test was used to determine significance for more than two comparisons.

**Quantitative real-time PCR**

Following a 6-day 100 μM CK treatment as described above, the first, second and third leaves were dissected and collected from the emerging coleoptile. RNA was isolated according to the Life Technologies TRIzol RNA isolation procedure. RNA concentration and purity was assessed using the NanoDrop ND1000 spectrophotometer in the Iowa State Protein Facility. 1 μg RNA was treated with DNase according to the Promega protocol. Reverse transcription was performed using Clontech RNA to cDNA EcoDry Premix. Quantitative RT-PCR was performed using SsoFast SybrGreen supermix chemistry on Applied Biosystems StepOne Plus Real-Time
PCR machine. ΔCT and ΔΔCT values were calculated in Microsoft Excel according to the Livak method.

**Glue slide impressions**

A thin line of Krazy Glue Maximum Bond cyanoacrylate glue was applied to a Fisherbrand Superfrost Plus microscope slide. The adaxial blade of leaf one was pressed firmly into the glue for about 30 seconds, followed by immediate removal of the blade. The slides were imaged on an Olympus BX60 light microscope.

**Results**

**The cytokinin hypersignaling mutant Hsf1 alters specific aspects of maize leaf development**

The *Hsf1* mutant alters distinct aspects of maize leaf development. *Hsf1* plants have an altered proximal-distal pattern of leaf growth characterized by the formation of prongs—new organ-like structures consisting of sheath, auricle and ligule tissue emerging from the distal leaf blade margin. *Hsf1* leaves have an increased number and broader spatial pattern of macrohairs suggesting epidermal cell fate is also altered (Fig. 1). It is the presence of the prongs and the increased “hairiness” which prompted the name of this mutant—*Hairy Sheath Frayed1*. A third aspect of development, leaf size, also appears altered in this mutant as the leaf subtending the top ear in mature plants is shorter and narrower than leaves from wild type (WT) sib plants [14]. To further define how *Hsf1* altered leaf development, a more detailed morphological characterization was performed.
**Hsf1 reduces seedling leaf size**

At maturity, leaves from *Hsf1* plants are shorter and narrower than their WT counterparts [13, 14]. *Hsf1* seedlings also appear smaller than their WT siblings, with leaf size apparently reduced in the mutant (Fig. 2 A, B). In order to characterize differences in leaf size, the length of the sheath and blade and the width of the blade of the fully expanded third leaf (L3) of *Hsf1* and WT seedlings were measured and compared. All three parameters of *Hsf1* L3 were reduced compared to WT sib plants. *Hsf1* L3 sheath was reduced 23%, blade length was reduced 19% and blade width was reduced 14% (Fig.2 C-E). Therefore, *Hsf1* also reduces leaf size in seedlings.

**Epidermal cell fate is altered in Hsf1 mutants**

In maize, macrohairs are a characteristic indicative of phase change [21]. Adult leaves are marked by macrohairs on the edge of the abaxial sheath and the adaxial blade, while juvenile leaf blades are glabrous and covered in epicuticular wax. The *Hsf1* mutant alters this pattern by promoting increased macrohair initiation not only on the abaxial sheath and adaxial blade but also on the auricle and blade margins of juvenile, transitional and adult leaves (Fig. 3 A, B) Glue slide impressions of the abaxial surface and margin of L1 show macrohairs present at the margin (Fig. 3 C, D). Macrohairs are present on *Hsf1* leaves from the first leaf that emerges from the coleoptile, indicating a change in epidermal cell fate.

**Proximal-distal leaf patterning is disrupted in Hsf1**

A distinguishing characteristic of *Hsf1* is the “frayed” leaf, which is caused by ectopic projections of sheath, auricle and ligule (prongs) from the margins of the distal leaf blade. To
determine how early in development prongs begin to form, the leaf number where prongs first appeared was recorded in *Hsf1* seedlings. Prongs first appeared on L5 in about 15%, on L6 in 35%, on L7 in 40% and on L8 or later on the last 10% of plants tested (Fig. 4 A). To understand when these prongs formed in development, *Hsf1* seedlings were dissected and shoot apices consisting of the SAM and attached leaf primordia were sectioned. Prongs were first visible as a scalloped edge along the blade margin (Fig. 4 B) on P4 to P6 primordia (Fig. 4 C). In transverse sections of leaf blades at these stages, developing prongs appear as thickened margins, ending in a pronounced point in contrast to the rounded edges of normal margins (Fig. 4 D). These results indicate prongs are first visible as early as P4 but their initiation must begin earlier in development to result in visible changes at these stages.

**Cytokinin treatment alters leaf development similar to *Hsf1***

Previous results support the hypothesis that *Hsf1* mutations cause hypersignaling of CK which leads to altered leaf development. To test this idea, we treated wild type inbred plants with exogenous CK by germinating B73 kernels in 10 μM 6-BAP. After six days of treatment, germinating seedlings were planted and leaf development phenotypes were measured after the fourth leaf collar was visible, after about three weeks of growth. The first three leaves emerging from the coleoptile all showed a reduction in sheath length, blade length and blade width (Fig. 5 A, B). For example, L3 sheath length was reduced 16%, blade length was reduced 12% and blade width was reduced 24% (Fig. 5 C-E). These leaf size changes followed the same general trend of reduced growth as in the *Hsf1* mutant, but were not to the same degrees as the mutant. The mutant sheath and blade length had greater percentage reduction, while CK treated B73 leaves had a greater percent reduction in blade width.
In addition to leaf size reduction, CK treatment of B73 seeds altered epidermal cell fate. Macrohair density increased on the abaxial sheath and auricle, the adaxial blade, and the margins of CK-treated B73 seedling L1 (Fig. 6 A, B, D, E), similar to Hsf1 seedling leaves. L1 of treated seedlings appeared to have a higher density of macrohairs than Hsf1, but this was not measured directly. In contrast to Hsf1, macrohairs also initiated on the abaxial leaf blade and around the ligule, which began to resemble a collection of macrohairs (Fig. 6 C, F). Macrohairs became less prominent on the second and third leaf, mostly visible on the abaxial sheath, auricle, and the distal tip and margins of the blade. By L4, macrohairs, if they were visible at all, were only present at the margins of the distal tip of the blade.

A hallmark of the Hsf1 mutant is the appearance of prongs on the blade margins. After six days of CK treatment, prongs developed on L4 in a low number of B73 seedlings (Fig. 6 G, H). At 10 μM BAP, just over 17% of the seedlings developed 1 or 2 prongs on the margins on L4 (Fig. 6 I), with no prongs forming on subsequent leaves. In contrast, prongs rarely, if ever, developed on L4 of Hsf1 seedlings. As noted above, the earliest leaf on which prongs first appeared was L5. Overall, treatment of inbred seeds with exogenous CK recapitulated three prominent Hsf1 developmental alterations: reduced leaf size, increased pubescence and the formation of prongs.

**CK treatment alters leaf development in other grasses**

To determine if other grasses were responsive to CK inducible leaf growth effects, both normal sorghum and rice were treated with exogenous CK. Similar to the CK treatment of maize, seeds of sorghum inbred Tx430 were treated for six days with 10 μM BAP. Germinating seedlings were then planted and leaf measurements were done on L1 to L3 after L4 was fully
expanded, about three weeks. CK treatment of sorghum resulted in a reduction in sheath and blade length and blade width for some, but not all leaves (Fig. 7 A). L1 sheath and blade length reductions were not significant, along with L2 sheath length. Reductions were significant in L3 sheath length, L2 and L3 blade lengths and in all three blade widths. L3 sheath and blade lengths were both reduced about 15%, and L3 blade width was reduced about 8% (Fig. 7 B-D). Maize leaves treated at this concentration, as previously reported, were reduced 16%, 12% and 24%, respectively. Macrohairs, which were not present on water treated leaves, were also induced on the abaxial sheath and abaxial and adaxial blade of the first two leaves (Fig. 7 E, F). Although leaf size was reduced and pubescence was increased, prong formation was not observed with 10 μM CK treatment of sorghum. Two subsequent treatments were conducted at 100 and 1000 μM CK in an attempt to induce prongs, but no proximal-distal patterning defects were observed. At these higher concentrations, however, leaf size was further reduced. Thus, two of the three leaf development alterations observed with CK treatment of maize were observed in sorghum.

To test the CK responsiveness of rice, treatment of the Kitaake line of rice was performed similar to maize and sorghum. After germination in 10 μM BAP for six days, sheath and blade length were measured. Blade length was not measured. Leaves treated at this concentration appeared to be shorter, but the difference was not statistically significant. Treatments at 100 and 1000 μM were also conducted to determine if leaf size changes were possible. At 100 μM, rice leaves showed a reduction of growth in L3 sheath length (Fig. 8 A, B). There was no significant decrease in blade length when comparing the 100 μM BAP treatment to the water control. There was, however, a significant difference in blade length between the 100 μM BAP and 1000 μM BAP treatment (Fig. 8 C). Despite increasing the concentration of 6-BAP to 1000 μM BAP, no
prongs or macrohairs were detected on rice leaves. Thus, treatment of the Kitaake line of rice only resulted in one of the three developmental changes seen in maize.

**CK treatment showed dose dependent effects on leaf development**

*Increasing CK concentrations had larger effects on leaf development*

Since increased concentrations of CK treatment exacerbated sorghum and rice leaf growth reductions, we hypothesized the same would be true in maize. Treatments of 100 and 1000 μM were conducted to investigate the degree by which the three developmental parameters were affected.

At 100 μM CK, leaf size was further reduced. L3 sheath length was reduced 48%, L3 blade length was reduced 60% and L3 width was reduced 53% (Fig. 9 A-C). While the amount of macrohairs appeared to increase on L1, macrohairs became more apparent on subsequent leaves. Macrohairs showed a strong response at 100 μM, as they were detected on the sheath of L1, L2 and L3. In comparison, 10 μM treatments did not always initiate macrohairs on L2 or L3 sheath. The area in which macrohairs were initiated was also affected by increased concentrations. At 10 μM, macrohairs are sparsely seen on the abaxial blade of L1, but appeared at a higher density at 100 μM.

Prong formation was also increased in seedlings treated with 100 μM CK. As mentioned previously, germinating seeds in 10 μM BAP for 6 days resulted in nearly 20% of seedlings developing prongs on L4. Increasing the concentration to 100 μM BAP resulted in nearly 90% of seedlings developing prongs on L4 (Fig. 6 I).

At treatment levels of 1000 μM BAP, transplanted seedlings began to appear stressed. Leaves emerging from the coleoptile failed to unroll and the tips of leaves soon began to rot.
Subsequent leaves had difficulty emerging past the enclosed leaf one, and some seedlings died as a result. This prevented reliable data collection of leaf size. Leaves treated at this high concentration produced macrohairs, but leaf quality prevented further data collection. Prongs were also induced in the plants that survived to the L4 stage. Therefore, higher concentrations of CK have a greater effect on the three leaf parameters demonstrating a dose dependent response to the hormone treatment.

**Reduction of CK treatment concentration had reduced effects on leaf development**

After seeing a greater reduction of leaf size at high concentrations of CK, we wanted to find the lowest concentration that would still have a measurable effect on leaf size. Treatments of 0.1, 1.0 and 10 μM CK were conducted and the first three leaves were measured. Leaves from plants treated with 0.1 did not show a reduction in sheath or blade size of the first three leaves. Treatment with 1 μM reduced sheath length in L1 and L2 and blade length in L2, however, all other measurements on those leaves and L3 were not significantly different than controls (Fig. 9 D-F). Macrohair initiation was not observed at 0.1 μM, but a few short macrohairs could be detected around the auricle in seedlings treated with 1 μM. Seedlings treated with either 0.1 or 1.0 μM did not produce prongs.

**CK treatment affects development during specific developmental windows**

Since 100 μM CK six-day treatment resulted in consistently strong effects on leaf growth, macrohair density and prong initiation, we wanted to investigate if shorter treatments would still result in leaf changes. Replicated sets of seeds were treated with 100 μM for 30 minutes, 1, 2, 4,
8, 24, 48, 72 and 96 hours. Seeds were rinsed and transplanted following treatment, and then L3 was measured after the fourth leaf collar had fully emerged.

In general, leaf growth decreased as treatment time increased. A 2 hour treatment was not sufficient to reduce L3 sheath and blade length, however it was enough to reduce L3 blade width. A 4 hour treatment was enough to significantly reduce L3 sheath and blade length (Fig. 9 J). While a 4 hour treatment was needed to have an effect on L3, L1 size appeared to be affected at shorter treatment durations. L1 blade length and blade width, but not sheath length, showed a size reduction after a 2 hour treatment. As treatments approached 96 hours, there was no sign of a leaf reduction plateau. For L3 blade length we had anomalous means for 24 hour water treatment and 48 hour CK treatment, as they did not follow the trend from surrounding time point treatments. 24 hour water treatment was significantly reduced compared to both 8 hour and 48 hour water treatments, and 48 hour CK treatment was greater than the 24 hour and 72 hour CK treatments. Overall, these data indicated that a 4 hour 100 μM BAP treatment is the minimum time needed to significantly reduce L3 sheath length, blade length and blade width.

At 100 μM CK, macrohairs were present on the L1 sheath and blade after as little as 30 minutes of treatment (Figure 9 G-I). However, only 85% of plants developed macrohairs on L1 at that time point. No plants had macrohairs on L2 or L3 after the 30 minute treatment. After a 1 hour treatment, macrohairs were present on all L1s, 90% of L2s and 18% of L3s. After a 24 hour treatment 100% of L1s showed macrohairs and 75% of L3s. At 72 hours 100% of L3s had macrohairs on the sheath or auricle. Our data indicate macrohairs can be initiated after as little as 30 minutes of CK treatment, and increasing treatment time resulted in macrohairs initiating on later leaves.
Prong initiation was also assessed using 100 μM CK treatments for different time periods. Proximal-distal patterning defects were affected by the duration of the CK treatment. After treatment for 8 hours, just under 10% of the plants formed prongs. Treatment for 24 hours resulted in 12% of the plants with prongs, while 48 hours induced 29%, and the 72 hour treatment induced prongs in about 75% of the seedlings (Fig. 9 K). No seedlings treated with 4 or fewer hours produced prongs. Therefore, 8 hours was the minimum CK treatment period necessary for prongs to form and increasing treatment times resulted in a higher percentage of seedlings forming prongs.

**Hsf1 is more sensitive to the CK treatment**

Because the *Hsf1* phenotype is thought to be the result of a tighter binding affinity for cytokinins in the mutant receptor, we hypothesized that *Hsf1* would be more sensitive to the CK treatment. To test this, we performed six-day CK treatments at two concentrations: 10 μM, because it is enough to significantly change leaf size in WT, and 0.1 μM, which is not enough to cause leaf size changes in WT. To distinguish *Hsf1* plants from WT sib plants, PCR genotyping was used to detect a size polymorphism in the *Hsf1* allele.

While 0.1 μM CK treatment had no effect on WT leaf size, it did reduce the size of *Hsf1* leaves (Fig. 10 A-C). *Hsf1* L3 sheath was reduced about 19%, while L3 blade was reduced 36%. L3 blade width was reduced 12%. *Hsf1* growth parameters were also affected by germination in 10 μM 6-BAP. When treated with 10 μM BAP for six days, *Hsf1* leaves showed a 44% reduction in sheath length, a 44% reduction in blade length and a 15% reduction in blade width. Thus, treatment with 0.1 μM CK resulted in an increased reduction in leaf size in *Hsf1* but elicited no response in WT sib seedlings indicating the mutant was more sensitive to CK.
The effect of the 10 μM CK treatment on macrohair and prong formation was also assessed to determine increased sensitivity to the hormone. Of the control Hsf1 plants, none showed prongs on L4, fewer than 10% had a prong on L5, about 25% had a prong on L6, and the rest had their first prong appear on L7 or later leaves (Fig. 10 D). In contrast, of the CK treated Hsf1 plants, 58% had prongs first on L4, 10% had prongs on L5 and the other 30% had not yet initiated a prong by L7 (Fig. 10 D, E). Likewise, Hsf1 plants treated with CK also had a noticeable change in macrohair density (Fig. 10 F-I). While Hsf1 plants are normally pubescent on the abaxial sheath, adaxial blade and margins of L1, the CK treatment expanded the range of macrohairs to the abaxial leaf blade, which also occurred on CK-treated WT leaves. Macrohairs appeared to be longer than the macrohairs which formed on either the 10 μM CK treated WT or water treated Hsf1 macrohairs, though this should be confirmed with careful measurements. Additionally, the treated seedlings, both WT and Hsf1, appear to have accumulated more anthocyanins in the sheath, although this was not quantified in this experiment. Because Hsf1 plants showed a response to 0.1 μM CK, and initiated prongs on earlier leaves than untreated sib mutant plants, these results indicated that Hsf1 is more sensitive to CK.

**Genetic variation exists for cytokinin treatment responses**

Since rice, sorghum and maize all showed different CK responsiveness, we wanted to assess differences in CK responses in genetically diverse lines of maize. To do this, CK treatments were conducted on a number of inbred lines to assess any differences in the extent of leaf size reduction compared to B73. In addition to the common inbred lines A632, A619 and W22, a number of Nested Association Mapping (NAM) lines were also selected. Together, the NAM lines were chosen to represent as much genetic diversity in maize as possible [22]. Out of
the 25 NAM lines, we selected six based on differences in their mature leaf dimensions from the leaf architecture dataset at Panzea.org [23]. Lines identified with the longest and shortest average leaf length and widest and narrowest leaf width were B97, CML52, CML69, Ki11, Mo17 and P39 (Fig. 11 A). Seeds from all lines were treated for six days in 10 μM BAP and the third leaf was measured, similar to the previous experiments described above.

The inbred lines showed a diversity of leaf size responses to CK treatment. A619 had the greatest reduction of sheath length at 37%, and P93 showed the smallest reduction at about 8% (Table 1). A619 also had the greatest reduction of blade length at 45% while CML69, which had the longest mature leaf blade length and width of the inbred lines tested, had the smallest reduction in blade length at 11%. Interestingly, B73 showed the second smallest reduction of blade length at about 12%. CML52 had the greatest decrease of blade width at 35% and CML69 showed the smallest reduction at 11%.

The 10 tested lines also showed variable macrohair responses on L1. A632 was the only line with macrohairs on L1 abaxial sheath without treatment. At 10 μM BAP, macrohairs were induced in all tested inbred lines except W22 and B97. Comparison pictures of macrohair initiation on L1 sheath suggest CML52 and CML69 had a greater density of macrohair initiation, but this was not measured (Fig. 11 B-K).

Very few of the lines showed an effect on proximal-distal patterning. Besides the low number of B73 seedlings that produced prongs, A619 and CML69 also showed patterning defects—although neither formed prongs. Two of 12 A619 treated seedlings were observed with the sheath displaced distally along the outer edge of the auricle on L3 (Fig. 11 L). The CML69 inbred displayed a new patterning defect that was distinct from that of B73. Instead of outgrowths on the margins of the blade, CML69 had outgrowths protruding perpendicular to the
lamina. These outgrowths appeared on both abaxial and adaxial surfaces, parallel with the midrib. The CML69 outgrowths were also distinct in that they are not prongs of sheath, auricle and ligule, but appear to be leaf margin (Fig. 11 M-O). This ectopic leaf margin was observed on L3 and L4, and appears as a continuous outgrowth from sheath to blade. These margins can also contain all four compartments of the leaf: sheath, blade, ligule and auricle. Sometimes two of these margins flank what appears to be midrib. Sectioning of an outgrowth on the abaxial blade revealed the vasculature of the ectopic leaf margin is facing the opposite direction as vasculature of the true leaf (Fig. 11 Q-S). These outgrowths appeared on 80% of treated CML69 plants and not in the water treatment.

The wide array of responses in leaf size, macrohair initiation and outgrowth formation to CK treatment suggests there is also genetic diversity in CK responses in different maize lines, which could be exploited to uncover new CK response genes.

**Cytokinin treatment reduces the effects of gibberellic acid treatment**

Since the hormone gibberellic acid (GA) has also been shown to regulate leaf growth [9, 24] in maize and other plants, we were interested in characterizing its interaction with cytokinin on leaf size. Previous studies indicated CK generally represses GA accumulation through the action of class I *knox* transcription factors [6]. Our unpublished transcriptome study has revealed that *Hsf1* ectopically expressed a class I *knox* gene in developing prongs [25]. Therefore, we expected CK treatment might reduce GA-induced leaf growth responses. To investigate the interaction of these two hormones, double hormone treatments were conducted on germinating B73 kernels with different concentrations of CK and GA.
Seed treatments with 100 μM GA resulted in increased sheath and blade lengths, but reduced blade widths on L2 and L3 in 3-week-old seedlings. Blade lengths were increased 45% and 17% on L2 and L3, respectively. When the 100 μM GA treatment was combined with 10 μM BAP, the GA-induced leaf size increase was blocked. In fact, L2 and L3 blade length in GA+BAP treated seedlings were no different than the BAP treatment alone (Fig. 12 A, B). Treatment of maize seeds with CK+GA suggests CK can inhibit GA growth responses.

To further explore the interaction between GA and CK on leaf size, GA treatments were conducted on Hsf1 plants. Treatment of Hsf1 and WT sib plants with 100 μM GA resulted in blade length increase in both genotypes, but no difference in sheath length. Both L2 and L3 blade lengths were significantly longer in GA treated plants than water treated. Hsf1 L2 and L3 leaves treated with GA increased 48% and 40%, respectively (Fig. 12 C, D).

To assess if GA catabolism or biosynthesis genes were induced by 10 μM BAP treatment, we performed a qRT-PCR on developing leaves. B73 kernels were germinated in the same manner as the growth assays. At the end of the six-day treatment, RNA from leaves 1, 2, and 3 from two plants were collected and pooled to create a biological replicate, for a total of seven treated replicates and seven water control replicates.

Two GA2-oxidase genes, which are involved in GA catabolism, and two GA20-oxidase genes, involved in late steps of GA biosynthesis, were selected for analysis due to their expression in maize leaves according to the qTeller expression query tool [26]. To confirm that the CK treatment led to increased cytokinin signaling, ZmRR3, a type-A response regulator that is rapidly induced in response to CK [17], was used as a control. While ZmRR3 transcripts were increased more than 400-fold in treated samples, there were no detectable expression differences in any of the GA2ox and GA20ox genes. These results suggest that, although the CK treatment is
increasing CK signaling in these tissues, it is not affecting expression of these four GA genes in these stages of leaf development.

Discussion

The \textit{Hsf1} mutation has multiple effects on plant growth and development, including specific effects on (i) leaf growth, (ii) leaf epidermal cell fate and (iii) leaf patterning. Analysis of the \textit{Hsf1} mutation indicates this pleiotropic phenotype is caused by specific missense mutations localized near the CK binding pocket of the \textit{ZmHK1} CK receptor which alter CK binding affinity and lead to CK hypersignaling in developing leaves [14]. To further understand how CK signaling controls these three aspects of leaf development altered by \textit{Hsf1}, a germinating-seed hormone assay was developed. In this assay, imbibed seeds are germinated in the presence of different concentrations of hormone for defined time periods. During those times, the 4-5 leaf primordia already present in the maize embryo perceived and responded to the hormone treatment. Responses to the hormone treatment on growth and development were reported in the first four leaves of a three-week-old seedling. In this way, effects of CK treatment on leaf growth, cell fate and patterning were analyzed.

\textbf{CK affects leaf growth similar to \textit{Hsf1}}

We found that CK treatment affects leaf growth similar to \textit{Hsf1}. Like \textit{Hsf1}, CK treated seedlings exhibited shorter sheath and blade lengths and narrower blades. Macrohairs were initiated on L1-L3, despite being juvenile leaves. Prongs of ectopic sheath, auricle and ligule were also induced on L4 at the blade margin. The gene underlying \textit{Hsf1} has been shown to be a cytokinin receptor histidine kinase with a conformational change near the ligand binding pocket,
resulting in CK hypersignaling [14]. Our CK treatment results confirm that the Hsf1 phenotypes can be caused by CK hypersignaling.

There are seven ZmHK proteins in maize [27] and the Hsf1 phenotype is the result of hypersignaling of only one. Since our treatments were not specific in targeting ZmHK1, the other six cytokinin receptor histidine kinases likely increased signaling in response to exogenous CK. Our results may give insight into the strength or specificity of Hsf1 hypersignaling, since the phenotype is observed when only one of the ZmHK proteins is hypersignaling.

The effects of CK treatment differ from Hsf1 in a number of ways. First, macrohairs initiate in a larger domain in CK treated leaves. Macrohairs are present on the abaxial sheath, auricle and adaxial blade of juvenile leaves in Hsf1, but CK treatment also induced them on the abaxial blade. Second, prongs appear on L4 in treated plants, but their first appearance in Hsf1 seedlings is on later leaves. Both of these results suggest there is a threshold for CK responses. Sustained CK treatment at high concentrations may be able to cross that threshold, while constant CK signaling in Hsf1 may not. Thirdly, size reductions of CK treated leaves do not happen to the same extent as leaf size reduction in the Hsf1 mutant. This is probably due to the developmental stage of the leaf in the embryo. Presumably Hsf1 is constantly signaling throughout development, while our treatments act as a one-time CK “pulse.”

Other grasses are differentially affected by CK treatment

Sorghum and rice were competent to respond to exogenous CK with some differences from maize. Sorghum first showed a leaf size difference at 100 μM, while rice required a 1000 μM treatment. Sorghum, which is more closely related to maize than rice, was able to induce
macrohairs on the sheath and blade, while no macrohair differences were noticed in rice. Prongs were never seen in either species, despite treatments up to 1000 μM BAP.

There are a few possible reasons for the reduced CK effect in rice and sorghum. Rice has also been shown to initiate leaf primordia at a slower rate than maize [28]. Maize shoot apices will be surrounded by about five to six developing leaf primordia, while rice will have no more than three. Fewer leaves in the germinating embryo will be exposed to the exogenous cytokinin, and fewer may be at a competent stage for a response. Lastly, both sorghum and rice lines could contain a suppressor of prongs, or lack an essential gene for prong formation. Some maize inbred lines, like W22, appear to suppress prong formation (unpublished data), and it is possible these rice and sorghum lines are similar.

**CK treatment showed dose dependent effects on leaf development**

Increased concentrations of CK in six-day treatments showed increased leaf growth responses. Increasing the concentration of CK treatment 10-fold to 100 μM resulted in a further reduction in leaf size. At this concentration, treated leaves were half the length and width of untreated leaves. Increasing the concentration to 1000 μM, however, was detrimental to the seedlings, as they appeared stressed and leaves began to decompose.

Mac罗hairs were also initiated on later leaves at higher concentrations. In addition to this, the density and domain of macrohairs increased as the concentration increased. *Hsf1* is pubescent on the abaxial sheath and adaxial blade on leaves 1 through 3, despite those leaves having juvenile identity. CK treatment replicated this macrohair arrangement, but expanded the area to include the abaxial leaf blade. This shows that CK is able to induce adult phase characteristics on juvenile sheaths and blades. The presence of hairs on the abaxial leaf blade could also indicate
misregulation of dorsal-ventral epidermal identity specification signals, however, it is important to note that some inbred lines, like CML69, have visible macrohairs on the abaxial L1 blade.

Higher concentrations of CK resulted in a higher percentage of seedlings developing prongs. Six-day 100 μM treatment resulted in almost 90% of plants producing prongs on L4. Concentrations of 1 and 0.1 μM were too low to have a significant effect on leaf size or prong initiation, though short macrohairs were sparsely present.

**CK treatment affects development during specific developmental windows**

Leaves responded to CK treatment differently depending on their stage of development. Macrohair initiation, for example, only required 30 minutes of 100 μM treatment to be seen in seedlings. Leaf size changes required at least four hours of treatment to be significant, and sizes decreased as treatment time increased. Prong formation required a minimum of eight hours treatment, but prongs were initiated on 75% of seedlings after 72 hours of treatment.

At the time of treatment, 5-6 leaves will have initiated from the meristem of the maize kernel embryo. The first leaf to emerge from the coleoptile would be the most developmentally mature and farthest from the SAM in the embryo, while the fourth leaf to emerge would be much closer and less differentiated. Our treatment time results indicate that these different leaf stages are differentially competent to respond to the CK signal. As we pulse the embryo with different lengths of CK treatment, we are able to see different degrees of responses.

The increase in macrohair formation as treatment time increases could be the result of the competence of that particular developmental stage. Leaf primordia at a later stage of development (P5-P6) are competent to induce macrohairs, while leaf primordia at earlier stages, (P2-P3) are not fully competent. The fact that only the distal tip of L4 shows macrohairs supports
this idea, as that area would have been the most mature part of a developing P1 leaf. Since maize leaves divide at the base and mature basipetally, this suggests that epidermal cells must be at a certain level of differentiation before becoming competent for macrohair initiation. Proximal blade cells that have not reached that level of differentiation are unable to initiate hairs, while cells in the more mature distal portion are able to respond.

Successive leaves also show a differential leaf shortening response to germination in CK. The first leaves to emerge, which are also developmentally older leaves, are reduced by a greater percentage than leaves that are developmentally younger. This effect could be a response to the “pulse” of the CK treatment. The entirety of L1 may be exposed to CK treatment, while only a distal portion of L3 may be exposed.

**Hsf1 is more sensitive to the CK treatment**

*Hsf1* appears to be more sensitive to exogenous CK treatment, supporting the hypothesis of a higher CK binding affinity. When treated at 0.1 and 1.0 μM, WT plants showed no leaf growth effect. In contrast, *Hsf1* seedlings showed a significant reduction when treated at both concentrations. Low CK concentrations had an effect on *Hsf1* without affecting WT siblings. CK treatment also pushed prong formation earlier in *Hsf1* plants. More than 50% of *Hsf1* plants treated with 10 μM first formed prongs on L4 while most control *Hsf1* plants first formed prongs after L7. Macrohairs on treated *Hsf1* seedlings appeared to be longer than both water treated *Hsf1* and 10 μM BAP treated WT seedlings. These heightened CK responses indicate that *Hsf1* is more sensitive to CK treatment than WT.
Genetic variation exists for cytokinin treatment responses

Treatment of select inbred lines suggests a wide diversity in maize growth response to CK treatment. Leaf growth was reduced across all tested inbred lines, though the percentage of reduction was variable. Following treatment of 10 μM BAP, sheath length reductions ranged from 8% to 37%, blade reductions ranged from 11% to 45%, and width reductions ranged from 11% to 35%.

Macrohair initiation was also variable across the tested inbred lines. Some inbred lines, like A632 were already pubescent on L1 in the water treatment. We identified two lines that did not show a macrohair response at this concentration: W22 and B97. In this experiment, macrohairs were scored by presence or absence, so we cannot report degree or density of macrohair initiation. Sheath images of CML52 and CML69, however, suggest some lines had a greater macrohair response.

Proximal-distal patterning defects were not expected to be large at 10 μM CK treatments since only a small percentage of B73 seedlings produced prongs at this concentration. Two lines showed proximal-distal patterning defects at this concentration, A619 and CML69. A619 sheath displacement was similar to effects seen on Hsf1 leaves in the B73 background. CML69 produced a new proximal-distal and dorsal-ventral patterning defect in response to CK treatment. These ectopic leaves are attached to the true leaf in between the margin and the midrib, which is unlike the Hsf1 proximal-distal patterning defect. Treating the other inbred lines with a higher concentration of CK might be also provide more information about their ability to produce prongs.

The NAM inbred lines provide a unique opportunity to map genes associated with phenotypes. We have identified two NAM inbred lines with interesting CK responses, B97,
which does not form macrohairs after treatment, and CML69, which forms ectopic leaves on L3 and L4. Performing CK treatment on the NAM recombinant inbred lines could be informative to determine the genes responsible for the B97 macrohair suppression and CML69 patterning response.

**Cytokinin treatment reduces the effects of gibberellic acid treatment**

The balance between CK and GA levels has been shown to be important for meristem maintenance and shoot development [6]. Transcript levels for *lg3*, a class-I *knox* gene, are increased in *Hsf1* developing prongs. Other genes in this class regulate both CK and GA levels. Germination in GA and CK allowed us to investigate how high CK and GA levels interact to affect leaf growth. The results of exogenous treatment with both hormones indicated that the CK response was stronger than the GA response. While both single hormone treatments had significant effects on leaf length (CK resulted in shorter blades and GA resulted in longer), the combination of the two hormones was not different than the CK treatment alone. This result is consistent with CK activity antagonizing GA accumulation. To further test this, we performed GA treatments on *Hsf1*. In contrast to the double hormone results, GA treatment of *Hsf1* produced longer blade lengths. Hypersignaling of *ZmHK1* in *Hsf1* is not enough to reduce the effect of exogenous GA, suggesting a CK dosage effect. High doses of CK treatment are enough to reduce the effects of GA, but *Hsf1* hypersignaling does not cross that signaling threshold.

To delve deeper into the possibility of effects on GA regulation at the transcript level, we performed a qRT-PCR on leaves from CK treated seedlings. Our qRT-PCR results indicate that CK treatment does not affect GA catabolism or biosynthesis genes in the early development of the first three leaves. Because we only collected leaves 1-3 from a six-day treated plant, this does
not rule out the possibility that CK treatment could affect GA regulation later in development. In fact, we have preliminary evidence that \textit{Hsf1} affects GA regulation at specific developmental stages. It is also possible that other \textit{GA2oxidase} and \textit{GA20oxidase} genes are being misexpressed, since the qRT-PCR was only probing for genes that were previously shown to be expressed in leaves. \textit{Liguleless3}, a class-I \textit{knox} gene, has been shown to be expressed in developing prongs, which appear on L4 or later in treated plants and \textit{Hsf1} seedlings [25]. Since class-I \textit{knox} genes regulate GA catabolism [4], we could expect to see differential expression of \textit{Ga2oxidase} genes in \textit{Hsf1} in L4 or later leaves.

\textbf{Future use of the germination assay}

The techniques and results from the CK germination assay could provide a firm starting point for further investigations of leaf growth and patterning alterations caused by CK signaling as well as other plant hormones. The \textit{Hsf1} mutant is a useful model to study genes required for leaf patterning because leaf patterning is occurring ectopically, outside of the normal context. Previous work in our lab to collect developing prongs relied on mass dissections to find prongs at the desired stage in development. That work relied on visible markers, such as enlarged blade margins, in order to confirm patterning defects and may have missed any earlier developmental changes required for prong initiation. With this assay we are able to reliably produce prongs on L4, thus allowing us to trace developmental signals before a prong phenotype is visible.

Similarly, the CK assay would be a useful tool to study the molecular determinants of macrohair initiation, especially considering that CK treatments resulted in macrohairs on the abaxial blade. This simple germination assay could help identify and screen for macrohair initiation mutants at an earlier stage, instead of waiting until the adult leaves emerge.
Figure legends

**Figure 1.** *Hsf1* alters proximal-distal patterning. The wild type maize leaf, left, has a normal proximal-distal pattern. Sheath is most proximal and blade is distal. The auricle and the ligule separate these two. The *Hsf1* leaf, right, shows altered proximal-distal patterning. In addition to the normal compartments, there are projections, or prongs, of sheath auricle and ligule emanating from the margin of the normal leaf blade. The *Hsf1* sheath also has a higher density of macrohairs than the WT sheath. Scale bar = 2 cm.

**Figure 2.** *Hsf1* seedlings are smaller than WT. A. (A) WT seedling and (B) *Hsf1* seedling at about three weeks after planting. The *Hsf1* seedling is noticeably shorter than WT. (C-E) Leaf measurements from leaf 3 (L3) of WT (left) and *Hsf1* siblings (right) after the fourth leaf had fully emerged, approximately three weeks after planting. (C) Sheath length, (D) blade length and (E) blade width at half of the blade length. Each dot represents one leaf and the green bar represents the mean. * indicates p-value<0.05.

**Figure 3.** Macrohairs present on *Hsf1* seedling leaves. (A) L1 of a WT seedling and (B) L1 of *Hsf1* sibling seedling. WT siblings are glabrous on juvenile leaves while *Hsf1* seedlings have macrohairs on the abaxial sheath, auricle, adaxial blade and blade margins. (C, D) Glue slide impressions of the abaxial surface and margin of L1. (C) WT L1 blade and margin are glabrous while (D) *Hsf1* sibling leaves have hairs on the margin.

**Figure 4.** Prongs first appear early in development. (A) The first leaf where prongs develop in *Hsf1* seedlings was recorded up to the emergence of the tenth leaf. (B) Hand dissected 25-day-old *Hsf1* seedling, revealing scalloped margins of leaf 6 (triangles), and the first visible sign of developing prongs. (C) The earliest stage prongs are visible is between P4 and P6 primordia. Developing *Hsf1* plants were hand dissected to determine the stage at which prongs are first visible. (D) Toluidine blue stained transverse section of *Hsf1* where initiating marginal prongs are evident in both P5 and P6 (arrows). Developing prongs appear as thickened margins in transverse sections. The innermost leaf is P4 and the outermost leaf is P6.

**Figure 5.** CK treated seedlings are smaller than water treated. (A) Water treated B73 seedling and (B) B73 seedling treated with 10 μM BAP. (C-E) Leaf 3 measurements of water treated (left) and *Hsf1* treated seedlings after approximately three weeks. (C) Sheath length, (D) blade length (E) blade width at half the blade length. * indicates p-value<0.05.

**Figure 6.** CK treatment induces macrohairs and prong formation. (A, B, D, E) Glue slide impressions of L1 abaxial blade and margin from two-week-old seedlings. (A) Abaxial blade of water treated L1. (B) Abaxial blade and margin of water treated L1. (D) Abaxial blade of 10 μM BAP treated L1. (E) Abaxial blade and margin of 10 μM BAP treated L1. (C) L1 ligule of water treated seedling, which takes on the appearance of a collection of macrohairs. (F) L1 ligule of 10 μM BAP treated seedling. (G) L4 of a water treated plant. (H) L4 of 10 μM BAP treated plant with two prongs visible. (I) The percentage of plants where prongs were induced at 10 μM BAP (left) and 100 μM BAP (right). N>12 for both populations.
**Figure 7.** CK treatment reduces leaf size and induces macrohairs in sorghum seedlings. (A) sorghum seedlings treated with water (left) and seedlings treated with 100 μM BAP treated (right). (B-D) L3 measurements from water treated (left) and 100 μM BAP treated (right) seedlings after the fourth leaf had emerged. (B) L3 sheath length, (C) L3 blade length and (D) L3 blade width at half the blade length. (E) L1 of water treated seedling and (F) L1 of 100 μM BAP treated seedling. * indicates p-value<0.05.

**Figure 8.** Rice leaves are shorter at high concentrations of CK treatment. (A) Water treated (left), 100 μM BAP treated (center) and 1000 μM BAP treated (right) rice seedlings. (C) Sheath length measurement for water treated, 10 μM, 100 μM, and 1000 μM BAP treated rice seedlings. (D) Blade length measurement for water treated, 10 μM, 100 μM, and 1000 μM BAP treated rice seedlings. * indicates p-value<0.05.

**Figure 9.** CK concentration and treatment length affects leaf growth response. (A, D) L3 sheath length, (B, E) L3 blade length and (C, F) L3 blade width at half of the blade length. (A-C) L3 leaf measurements for water treated (left), 10 μM (center) and 100 μM BAP (right) treated seedlings. (D-E) L3 leaf measurements for water treated (far left), 0.1 μM (center left), 1 μM (center right) and 10 μM BAP (right) treated seedlings. (G-I) The percentage of leaves where macrohairs were induced at separate lengths of 100 μM BAP treatment. (G) L1, (H) L2 and (I) L3. N>12 for each time point. (J) L3 blade length after different treatment time length for water (right of red line) and 100 μM BAP (left of red line). The earliest significant difference in leaf length between corresponding water and 100 μM BAP treatment times was after a four hour treatment. * indicates p-value<0.05. (K) The percentage of plants that developed prongs after different lengths of 100 μM BAP treatment. N>12 for all groups.

**Figure 10.** *Hsf1* is more sensitive to CK treatment. (A-C) L3 measurements of *Hsf1* seedlings treated with water (left) and 0.1 μM BAP (right). (A) L3 sheath length, (B) L3 blade length and (C) L3 blade width at half the blade length. (D) The earliest appearance of prongs in water treated *Hsf1* seedlings (blue) and 10 μM BAP treated seedlings (red). (E) Water treated three-week-old *Hsf1* seedling (left) and 10 μM BAP treated *Hsf1* seedling. Arrows indicate prongs. (F) L1 of water treated WT B73 seedling, (G) L1 of water treated *Hsf1* seedling, (H) L1 of 10 μM BAP treated WT B73 seedling and (I) L1 of 10 μM BAP treated *Hsf1* seedling.

**Figure 11.** Treatment of inbred lines reveals diversity in CK responses. (A) Mature blade width compared with mature blade length of Nested Association Mapping (NAM) inbred lines. Selected lines are labeled. (B-K) Macrohair initiation showed variable responses to CK treatment. Water treated (B, D, F, H, J) and 10 μM BAP treated (C, E, G, I, K) L1 leaves are shown for (B, C) CML52, (D, E) Ki11, (F, G) P39, (H, I) CML69 and (J, K) B97. (L) L3 of 10 μM BAP treated A619 seedling. Arrow indicates sheath displaced along margin of blade. (M-S) Leaf patterning defect in 10 μM BAP treated CML69 seedlings. (M) L4 adaxial sheath and blade. Ectopic margin is seen to the right of the midrib. Arrow indicates ectopic ligule. (N) L4 abaxial sheath and blade. Two ectopic margins are attached to the abaxial surface. (O) L4 abaxial sheath and blade with ectopic margins emerging from the blade and sheath surface. Arrow indicates an apparent blade sheath boundary of the ectopic margin. (P) Increased magnification of the ectopic margin from (O). Ectopic margin contains sheath (right) and blade (left) separated by both a ligule (white triangle) and an auricle (black triangle). (Q) Toluidine blue stained
paraffin section of the sheath seen in (O). (R-S) Increased magnification of the boxed veins in (Q).

**Figure 12.** GA treatment effect is reduced by CK treatment, but not by Hsf1. (A) B73 L2 blade length measurements of water treated (far left), 10 μM BAP (center left), 100 μM GA (center right) and 10 μM BAP + 100 μM GA (far right) seedlings. (B) B73 L3 blade length measurements of water treated (far left), 10 μM BAP (center left), 100 μM GA (center right) and 10 μM BAP + 100 μM GA (far right) seedlings. (C) L2 blade length from water treated WT (far left), 100 μM GA WT (center left), water treated Hsf1 (center right) and 100 μM GA treated Hsf1 (far right). (D) L3 blade length from water treated WT (far left), 100 μM GA WT (center left), water treated Hsf1 (center right) and 100 μM GA treated Hsf1 (far right). * indicates p-value<0.05.
Tables

Table 1. Inbred lines show a diversity of leaf size reductions in response to 10 μM CK treatment.

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Figure 1. *Hsf1* alters proximal-distal patterning.

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CHAPTER III
ANALYSIS OF ENHANCERS OF HAIRY SHEATH
FRAYED1 LEAF PATTERNING DEFECTS

Abstract

The maize leaf is composed of four compartments: the proximal sheath, the distal blade, and the ligule and auricle, all polarized along the proximal-distal axis. The semi-dominant gain-of-function \textit{Hsf1} mutant has altered proximal-distal patterning, resulting in outgrowths of proximal tissue, called prongs, from the margins of the distal leaf blade. The underlying cause for this phenotype is a missense mutation in \textit{Zea mays Histidine Kinase1} (\textit{ZmHK1}), a receptor for the plant growth hormone cytokinin. This results in cytokinin hypersignaling and altered proximal-distal patterning. Misexpression of class I \textit{knoted-like homeobox (knox)} genes also disrupt proximal-distal patterning. Many \textit{knox} genes have been shown to directly regulate CK and gibberellic acid (GA) levels in the meristem. To test interactions between \textit{Hsf1} and \textit{knox} gain-of-function mutants, the leaf morphology of double mutants was analyzed. Certain \textit{knox} mutants enhanced the \textit{Hsf1} phenotype in specific ways. Notably, the \textit{Liguleless3} (\textit{Lg3-O}) mutant caused more \textit{Hsf1} blade to be converted to proximal tissue. Analysis of prong formation was also conducted in \textit{Hsf1} double mutants with the \textit{knoted1} (\textit{kn1}) loss-of-function allele. Our results indicate \textit{kn1} is not required for the formation of prongs. Though not a class I \textit{knox} gene, the TCP transcription factor \textit{Wab1} represses prong formation in combination with \textit{Hsf1}. \textit{Hsf1} double mutant analyses were also conducted with \textit{abph1}, which codes for a negative regulator of cytokinin signaling, and with \textit{tru1}, a \textit{blade-on-petiole (bop)} homolog that regulates lateral branching. Both \textit{abph1} and \textit{tru1} revealed gene dosage effects and altered phyllotaxy in double
mutants. *an1*, a loss-of-function mutant involved in GA biosynthesis, showed an increase in prong formation when combined with *Hsf1*. These double mutant analyses give insight into genes functioning downstream of CK signaling.

**Introduction**

The maize leaf can be divided into four distinct compartments, the sheath, blade, auricle and ligule. The leaf compartments are arranged in a consistent proximal-distal pattern. The blade is the most distal portion of the leaf and the sheath is the most proximal. In between are the auricle and ligule [1]. A number of maize mutants have been identified that alter this proximal-distal pattern. To give insight into the genes involved in leaf patterning, we analyzed the cytokinin hypersignaling mutant *Hsf1* in conjunction with other patterning and hormone mutants.

*Hairy sheath frayed1* disrupts proximal-distal leaf patterning

The semi-dominant gain-of-function mutant *Hairy sheath frayed1* alters proximal-distal leaf patterning ([2]. Ectopic projections, or prongs, of sheath, ligule and auricle appear on the margins of the normal leaf blade. The gene underlying the *Hsf1* phenotype is *Zea mays histidine kinase1* (*ZmHK1*), a cytokinin receptor histidine kinase (ref needed). *ZmHK1* is part of a two-component cytokinin signaling system. Missense mutations in *Hsf1* change amino acids near the cytokinin binding pocket. A change in protein conformation is thought to alter the CK binding affinity of ZmHK1, resulting in CK hypersignaling [3].

CK signaling is relayed by a two-component signaling system. After CK binds to a histidine kinase cytokinin receptor, the receptor autophosphorylates then transfers the phosphate to a phosphotransfer protein [4]. This protein then transfers the phosphate to a response regulator.
There are two types of response regulators: type-A, which negatively regulate CK signaling [6], and type-B, which have transcriptional activating domains. Type-B response regulators positively regulate CK signaling through transcriptional activation [7].

**Overexpression of class I knox genes cause leaf patterning defects**

Class I *knotted-like homeobox (knox)* genes are transcription factors involved in meristem maintenance and leaf patterning. Ectopic expression of these genes in the leaf blade results in disruptions of the blade/sheath boundary and leaf patterning defects, revealing their role in leaf patterning [8-10]. Distal expression of *kn1* has been shown to cause *Hsf1*-like prongs to form at the margins of the distal blade [11]. *lg3*, a related class-I *knox* gene, is expressed at high levels of developing *Hsf1* prongs [12].

**knotted1**

*knotted1* is a homeobox domain transcription factor normally expressed in the shoot apical meristem (SAM) that is used as a marker for meristematic tissue. *kn1* is down regulated as incipient leaf primordia begin to form [13]. *kn1* loss-of-function mutants have reduced meristem function, showing *kn1* is required for meristem maintenance [14]. *kn1* has been shown to regulate the balance of a number of growth regulatory hormones in the meristem, including GA metabolism [15], brassinosteroid catabolism [16] and cytokinin biosynthesis [17, 18].

*kn1* gain-of-function mutations result in ectopic expression of *kn1* in the distal leaf blade, causing leaf patterning defects [8]. One allele, *Kn1-DL*, results in ectopic *kn1* expression in the distal margins and midrib of the leaf blade, inducing the formation of outgrowths of sheath, auricle and ligule from the blade margin [11], similar to *Hsf1* prongs.
**liguleless3**

`liguleless3` (lg3) is a class I `knox` gene found to be highly expressed in developing prongs [12]. lg3 is normally expressed in the meristem and not in leaf blades. lg3 has been shown to be coexpressed with other class I `knox` genes, such as `kn1` and `rs1` [19]. Double mutant analysis of `knox` loss-of-function mutants suggests there is an unequal redundancy in `knox` function [20]. The `liguleless3` gain-of-function mutation, Lg3-O, results in ectopic lg3 expression in the developing leaf blade. Ectopic sheath tissue disrupts the blade sheath boundary by distally displacing the ligule medially along the midrib. Loss-of-function lg3 mutations have no obvious phenotypes [19] suggesting that other `knox` genes overlap in their functions and expression domains.

**rough sheath1 and gnarley1**

Rough sheath1 (Rs1) and Gnarley1 (Gn1) are dominant gain-of-function mutants of two duplicate class 1 `knox` genes [9, 10, 21]. Both mutants have phenotypic similarities in that the blade-sheath boundary is disrupted and ligule is displaced by ectopic sheath. In Rs1, a disorganized sector with both sheath and auricle identity appears in the ligule region. Ligules are displaced throughout the sector, sometimes appearing as if there are two parallel ligules [22]. Gn1 is characterized by sheath displacement distally into the blade, taking the place of auricle. In contrast to displacement of the ligule along the midrib in Lg3, the Gn1 ligule is distally displaced laterally towards the margins [10]. Normally both of these genes are expressed in the meristem and are their transcripts are not detected in leaves [21]. Similar to lg3, both rs1 and gn1 loss-of-function alleles show no obvious phenotype [10, 20], again suggesting there is redundancy in function for many of the class-I `knox` genes.
**wavy auricle in blade1**

While *wavy Auricle in blade1 (wab1)* is not a *knox* gene, its dominant mutant, *Wab1*, does produce similar leaf patterning disruptions, namely large patches of auricle within the blade lamina at the proximal end of the leaf blade [23]. *wab1* encodes a TCP (*TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR*) family transcription factor not normally expressed in leaves [24, 25]. *Wab1* leaves are narrower than WT sibling leaves, indicating it may play a role in constraining growth. Normally, *wab1* is expressed between meristems and lateral organs and has been implicated in repressing the growth in the axil of inflorescence branches.

*Wavy auricle in blade-SGL* is another dominant gain-of-function mutant that has an almost identical leaf patterning phenotype to *Wab1*. The gene underlying the *Wab-SGL* phenotype is unknown.

**Meristem function**

**aberrant phyllotaxy1**

*abph1* encodes a cytokinin inducible type-A response regulator [26]. Response regulators negatively regulate CK signaling as part of the two-component CK signaling system. The recessive *abph1* loss-of-function mutant has a larger meristem, allowing two leaf primordia to initiate at each node producing a plant with altered phyllotaxy [27]. This suggests *abph1* has a role in constraining meristem size in response to CK activity. Some leaf-patterning effects that have been reported in *abph1* mutants, such as double midribs, especially in L1, but also appearing on subsequent leaves.
A maize blade-on-petiole gene was identified as being differentially expressed in developing prongs [12]. In Arabidopsis, loss of BOP1 results in ectopic blade emanating from the petiole altering proximal-distal patterning [28]. BOP1 represses the expression of knox genes at the base of the leaf by activating a LATERAL ORGAN BOUNDARY-domain gene, ASYMMETRIC LEAVES2 [29]. BOP1 has also been shown to play a role in inhibiting bract formation [30]. In maize, recessive mutations in tassel replaces upper ear1 (tru1) results in elongated lateral branches with tassels replacing the ear. tru1 is a maize homolog of the Arabidopsis BLADE-ON-PETIOLE1 (BOP1) transcription factor [31] that normally represses ear shank elongation.

**Hormone biosynthesis**

anther ear1

In Arabidopsis, gibberellin biosynthesis can be directly repressed by knox genes [32], which can also activate cytokinin biosynthesis [18]. CK and GA have also been shown to have antagonistic activities that are important in tomato leaf development [33]. In the maize SAM, low GA and high CK levels are required maintenance of an indeterminate state [34].

In maize the anther ear1 (an1) gene encodes an enzyme that functions early in GA biosynthesis and its loss of function results in a GA-responsive dwarf phenotype [35]. Demonstrating the importance of GA accumulation for cell division and cell expansion, l leaves in an1 mutants are shorter and wider than WT siblings.

Materials and methods

Genetic stocks

Two *Hsf1* alleles, *Hsf1*-1595 and *Hsf1*-1603, were obtained from the Maize Genetics Cooperative stock center. *Hsf1*-AEWL was isolated from an EMS mutagenesis of A619 (E.Vollbrecht). *Abph1* was obtained from Dave Jackson. *Lg3-O*, *Wab-SGL* and *anl* were obtained from the Maize Genetics Cooperative Stock Center. *Kn1* loss-of-function, *Kn1-DL* and *Wab1* were obtained from Sarah Hake. *Kn1-O*, *zmbri1-RNAi* and *zmbin2-RNAi* were obtained from Phil Becraft and *trul* was obtained from Erik Vollbrecht.

Leaf measurements

Counting from the tassel, the second, fourth and sixth leaves were removed and measured from each plant. Sheath length was defined as the distance from the point of insertion of the leaf on the culm (stem) to the ligule, which marks the blade/sheath boundary. The blade length was defined as the distance from the ligule (blade/sheath boundary) to the distal tip of the blade. The blade width was measured from left-to-right margin of the adaxial blade surface at 25%, 50% and 75% of the leaf blade length. Percent prong margin was calculated by summing the length of margin that had prong identity on both sides of the entire leaf, and dividing it by twice the sheath length and blade length measurements. Statistical analysis on *Hsf1* prong distribution was performed with help from the Ignacio Alvarez-Castro from the Iowa State statistic department.
Results

**Hsf1 prongs form in a patterned manner**

*Hsf1* leaves form prongs on both margins of the leaf blade. We observed that prongs on one margin of the leaf appeared to have prongs near the same location on the opposite margin (Fig. 1 A). This observation prompted us to determine if prongs are formed in a symmetric pattern. To determine this, the first, second and third leaves above the top ear were collected from about 20 plants from the three *Hsf1* alleles: *Hsf1-1603*, *Hsf1-1595* and *Hsf1-AEWL*–in the B73 background. Blade lengths were measured, along with the start and stop points of every prong along both margins.

**Leaves closer to the tassel have more prongs**

Our results showed that the number of prongs on a leaf is dependent on the leaf’s location on the shoot. The first leaf above the ear had the fewest prongs, while the third leaf above the ear had the most prongs. This was consistent for all three alleles. For the first leaf above the ear, only eight out of 20 *Hsf1-AEWL* plants had prongs. This was similar for the other two alleles, as six out of 19 *Hsf1-1595* plants, and four out of 21 *Hsf1-1603* plants formed prongs on that leaf. The number of prongs per leaf increased as the leaf number on the plant increased. For the third leaf above the ear, *Hsf1-AEWL* and *Hsf1-1595* had 16 out of 19 leaves with prongs and *Hsf1-1603* had 19 out of 21 leaves with prongs (Table 1).

The absolute number of prongs, however, was not necessarily a good measurement, as prongs vary in size from as little as one mm, to more than 11 cm. Knowing this, we wanted to determine the percentage of blade margin that is made up of ectopic sheath, auricle and ligule. To do this, individual start and stop points along the margin for each prong were determined and
used to obtain a length, along the proximal-distal axis, for each prong. These lengths, from both
the left and right side of each blade, were summed and divided by two times the total blade
length. This number, the percent prong margin (PPM), represents the proportion of normal
margin that is occupied by tissue having prong identity.

After excluding leaves with no prongs, the first leaf above the ear had an average PPM of
2.7 for *Hsf1-1595*, and 1.4 for both *Hsf1-1603* and *Hsf1-AEWL*. The second leaf above the ear
had a PPM of 1.8, 1.6 and 1.3 for *Hsf1-1595*, *Hsf1-AEWL* and *Hsf1-1603*, respectively. The third
leaf above the ear had a larger PPM for all three alleles with about 7.4 for both *Hsf1-1595* and
*Hsf1-AEWL* and 4.6 for *Hsf1-1603* (Fig. 1 B-D). None of the differences between alleles were
significant. This shows that not only do leaves closer to the tassel have more prongs but also a
higher percentage of their margin is occupied by prongs than lower leaves.

**Larger prongs tend to form at the base of the leaf**

Because prongs seem to appear most frequently at the base of the blade, we were
interested to find out if there was a relationship between prong size and location on the blade. To
investigate this, we compared the size of the prongs on the third leaf above the ear with the
position of the prong along the leaf blade margin. This was done by first determining the average
prong size. The average prong size was a little over 1 cm in length for all three alleles on both
sides of the blade (Fig. 1 E). Although the most prongs were under 6 cm, one prong was
observed at a length of 11 cm on a *Hsf1-1595* leaf. Large prongs like this are rare and are
characterized by a mass of sheath at the proximal end of the blade that trails off distally as a
hairy fringe along the margin. Next we calculated the midpoints, or points halfway between the
start and stop position of each prong, to determine the location of prongs along the blade. Our
results indicated that 50% of all prong midpoints appear roughly between 15% and 40% of the blade’s length (Fig. 1 F). Finally, we converted the prong lengths to a relative prong size by dividing individual prong lengths by the length of the blade on which they appeared. This number was compared with the midpoint location of each prong. This allowed us to visualize where, along the blade, larger and smaller prongs formed (Fig. 1 G). Our results suggest a negative relationship between prong size and distance along the leaf, indicating that larger prongs formed at the proximal base of the leaf and smaller prongs formed more distally. It is also interesting to note that prongs never formed in the distal 30% of the leaf blade. These results were consistent for all three alleles.

**Hsf1 phenotypes are modified by class I knox mutants**

After determining a baseline for prong location and size for the three alleles of *Hsf1*, we wanted to investigate genetic interactions that could alter this pattern. Obvious candidates investigated first were the class I *knox* genes. Ectopic expression of these genes has been shown to alter proximal-distal patterning, resulting in displacement of sheath, auricle and ligule into the leaf blade, producing phenotypes distinct from *Hsf1*. In fact, transcriptome analysis indicated that a class I *knox* gene, *liguleless3* (*lg3*), was one of the most differentially expressed in early developing prongs [12]. Because the three alleles had similar prong patterns, we chose to restrict our double mutant analysis to one allele, *Hsf1-1603*. Counting from the tassel, the second, fourth and sixth leaf were collected from each plant and prong size and location was determined as described above for *Hsf1* alleles. These data, however, also take into account prongs that form on the sheath, and so sheath length was included in the calculation of PPM.
**Liguleless3 enhances Hsf1 prong formation and alters leaf size**

Since *lg3* was identified as being one of the highest differentially expressed genes in developing *Hsf1* prongs, *Hsf1* was crossed with the semi-dominant *Lg3-O* mutation. In this gain-of-function mutant, the *knotted1-like* LG3 protein is ectopically expressed at the blade-sheath boundary. This results in a distal displacement of sheath and ligule into the blade centered close to the midrib. The ligule, marking the blade-sheath boundary, is displaced along the center of the midrib (Fig. 2 A). If the LG3 protein plays an important role in prong formation, we would expect an increase in prong formation or larger prongs.

The *Hsf1* and *Lg3-O* double mutant plants showed an enhanced effect on prong formation. Prongs appeared larger and had more sheath identity (Fig. 2 B). Compared to *Hsf1*, a higher percentage of the double mutant margins were converted to prong (Fig. 2 C). *Hsf1* siblings had about 4 PPM, while double mutants had 18 PPM. In addition to taking up more of the margin, prongs extended farther medially into the lamina towards the midrib (Fig. 2 D). To quantify this, the distance from the most medial prong edge to midrib was measured. Prongs on double mutant plants had a smaller distance between midrib and prong base (Fig. 2 E). This result suggests that the ectopic expression of LG3 in the *Lg3-O* mutant enhances prong size and that the size increase is due to the prong consuming more of the lateral blade lamina.

The *Lg3-O* mutation also has an effect on leaf size. Compared to WT sib plants, leaves from *Lg3-O* plants have 16% longer sheaths, 13% shorter blades and a 15% increase in blade width. When combined with *Hsf1*, *Lg3* had similar effects on leaf length, but not width: *Lg3* results in a sheath length increase of about 12%, and a decrease of blade length of 14%. *Lg3* does not result in a reduction of blade width in *Hsf1* (Fig. 2 H-J). While both *Lg3* and *Hsf1* single
mutant leaf blades show a similar reduction in blade length, the double mutant leaf blade is shorter than both single mutants.

In addition, double mutants showed a few other novel phenotypic effects. Occasionally blade tissue proximal to the prong continued to grow out around a prong near the midrib, resulting in a wing of leaf blade (Fig. 2 F). Also, midribs at the base of the blade in the double mutant appeared to be distorted, growing in a serpentine manner instead of straight. The presence of macrohairs on the abaxial Hsf1 sheath highlights the amount of sheath displacement occurring along the midrib in Lg3–O mutants. The abaxial epidermal cells with sheath identity extended farther distally than the adaxial ligule appeared to be displaced (Fig. 2 G).

_Gnarley1 and Rough sheath1 also enhance Hsf1_

Given that ectopic lg3 enhanced prong formation in Hsf1, we were interested to find out if other dominant, gain-of-function class I knox mutants produced similar effects. To test this idea, the class I knox gain-of-function mutants Gnarley1 and Rough sheath1 were crossed to Hsf1.

In Gn1 mutants, the sheath proliferates into the blade, the auricle is greatly reduced and replaced by sheath, and the ligule is distally displaced on the lateral sides of the midrib (Fig. 3 A, B). Analysis of leaf size showed the Gnarley1 mutation alone did not have a significant effect on sheath length, blade length or blade width compared to wild type siblings.

In Gn1, Hsf1 double mutants, we found Gn1 enhances the Hsf1 phenotype. When combined with Hsf1, Gn1 results in a dramatic reduction of all three leaf size parameters: sheath length was reduced by 25%, blade length by 32% and blade width by 21%. Gn1 also had a dramatic effect on prong formation. Prongs in the double mutant were enhanced in a similar way
to Lg3. Prongs often initiated in the sheath and extended into the blade margin, sometimes completely eliminating the presence of the auricle (Fig. 3 C, D). Prongs reiterated the Gn1 phenotype in that they did not have a clear auricle at the blade-sheath boundary (Fig. 3 E, F). Double mutants also had a higher PPM, with approximately 25 PPM in Hsf1, Gn1 plants compared to 10 PPM in Hsf1 alone (Fig. 3 G). Similar to Lg3, the double mutant midrib did not always appear straight, but made serpentine bends near the base of the leaf.

*Rough sheath1* is also characterized by an over proliferation of sheath, auricle and ligule into the blade at the blade-sheath boundary (Fig. 4 A, B). This mutant is characterized by a mosaic of all three tissues instead of a forming a clear boundary between the blade and sheath [22]. On some leaves, two parallel lines of ligule were seen. Sheath length in Rs1 plants was significantly reduced when compared to normal siblings. By itself, Rs1 reduced sheath length by about 47% but blade length and width were not affected.

When combined with Hsf1, the Rs1 and Hsf1 double mutants showed a reduction in sheath length of about 41%, similar to Rs1 alone, but did not significantly impact blade length or blade width. Double mutant prongs reflected the same blade-sheath disruption seen in Rs1, both with the large mosaics of sheath and auricle tissue at the blade-prong boundary, and the occasional appearance of two parallel lines of ligule (Fig. 4 C, D). Double mutants also had twice the PPM 16, compared to 8.7 in Hsf1 siblings (Fig. 4 G). These results also indicate Rs1 enhanced the Hsf1 prong phenotype.

Similar to Lg3 and Hsf1 double mutants, the presence of macrohairs revealed information about how Rs1 disrupted patterning by acting as markers of sheath identity. Macrohairs appeared on the abaxial midrib, indicating sheath had been displaced past the blade-sheath boundary. Macrohairs were also observed on some sectors of the rough adaxial sheath in double mutants,
indicating a possible dorsal-ventral patterning effect inside of the mosaic of proximal-distal tissue proliferation (Fig. 4 E, F).

**Distal expression of knotted1 reduces Hsf1 leaf size**

The gain-of-function mutations Lg3, Gn1 and Rs1 result from ectopic expression of class I knox genes at the blade-sheath boundary. All three had an enhancing effect on Hsf1. Next we asked if expression of a knox genes in the distal blade could also enhance Hsf1. knotted1 (kn1) was the first class I knox gene discovered. Gain-of-function kn1 mutants are due to ectopic expression of kn1 in the blade causing displacement the blade-sheath boundary and over proliferation of tissue with proximal identity between the major veins of the blade forming “knots” [8]. The Kn1-DL allele is unique in that this allele causes kn1 to be expressed at the distal tips of leaf blades, resulting in ectopic sheath, auricle and ligule forming at the margin [11]. These ectopic Kn1-DL outgrowths are phenotypically similar to Hsf1 prongs. The distal expression of kn1 resulted in different leaf phenotypes depending on the location of the leaf. For example, on the second leaf from the tassel, Kn1-DL is expressed in the distal midrib, which truncates and bifurcates the leaf. On the sixth leaf from the tassel, prongs appeared on the distal 30% of the blade (Fig. 5 A). These prongs are different from those in Hsf1 primarily due to their location along the leaf blade since Hsf1 prongs appeared only in the proximal 70% of the blade. Because of the similarity in phenotype but difference in location of prongs, we were interested to determine how prong formation was affected in a Kn1-DL, Hsf1 double mutant

Double mutant leaves appeared much shorter than either single mutant (Fig. 5 B, C). Prongs appeared on the sixth leaf from the tassel and leaf length was not significantly different in both Kn1-DL and Hsf1 single mutants. In the double mutant, however, leaf length was reduced
by about 20-30% compared to either single mutant (Fig. 5 D). This reduction appeared to be caused by ectopic sheath tissue forming knots distally in the midrib, which, similar to upper Kn1-DL leaves, severely truncated blade length.

The \textit{Hsf1}-prong phenotype was not affected to the same degree like it was by the other dominant, gain-of-function \textit{knox} mutants. On the sixth leaf from the tassel, there appeared to be no difference in PPM between \textit{Hsf1}, \textit{Kn1-DL} and the double mutant, despite the fact that \textit{Hsf1} and \textit{Kn1-DL} both form prongs in non-overlapping domains of the blade (Fig. 5 E). This is probably due to the truncation of the distal portion of the blade in the double mutant. Upper leaves in the double mutant also show a reduction in leaf blade size. These results indicate that the prongs at the proximal end of the blade, which are caused by \textit{Hsf1}, are not affected by distal expression of \textit{kn1}, but that \textit{Hsf1} affects midrib expression of \textit{kn1}, which resulted in truncated leaves.

\textit{Knotted1} is not required for prong formation

\textit{knotted1} normally functions in meristem maintenance and it is expressed in all above ground meristems in plants [36]. Analysis of the transcriptome of early forming prongs revealed significant overlap with the expression modules found in initiating organ primordia [12]. Since all above ground organs arise from meristems, we were curious to see if \textit{kn1} was required for formation of prongs in \textit{Hsf1}. To test this we crossed \textit{Hsf1} to a \textit{kn1} loss-of-function mutation, \textit{kn1-L4}. Plants homozygous for \textit{kn1-L4} have reduced inflorescences, leaf initiation defects and can be shootless depending on genetic background [14, 37]. A testcross population was made that segregated equal ratios of \textit{kn1-L4} homozygotes, \textit{kn1-L4} heterozygotes (which are phenotypically wild type), \textit{Hsf1} plus \textit{kn1-L4} homozygotes, and \textit{Hsf1} plus \textit{kn1-L4} heterozygotes.
Genotyping was performed to confirm segregation of $kn1-L4$, and to correlate it with the characteristic $kn1-L4$ reduced tassel and ear phenotype. Double mutants showed no difference in their ability to form prongs (Fig. 6 A, B). The PPM of $Hsf1$, $kn1-L4$ double heterozygotes and $Hsf1$, $kn1-L4/kn1-L4$ was not different (Fig. 6 C). There was also no leaf blade size difference between $Hsf1$ and the double mutant. Thus, $kn1$ appears to not be required for the initiation and development of prongs in $Hsf1$. Which class I knox gene, if any, might be required for prong formation requires further investigation.

**Wavy auricle in blade1 reduces Hsf1 prong formation**

While not a knox gene, $Wab1$ is a dominant gain-of-function mutation that disrupts leaf patterning in a similar way to other dominant knox mutants. $Wab1$ is a TCP transcription factor not normally expressed in leaves [24], and the dominant mutant leaves are characterized by ectopic patches of wavy auricle interspersed through the lamina at the proximal end of the blade. We were interested to test if $Hsf1$, which is also characterized by ectopic auricle at the margins of the blade, interacts with $Wab1$. In single $Wab1$ plants, sheaths are about 5% longer than WT, while the blade is 16% shorter. Mutant blades are wide at the base, but quickly narrow; so width is reduced 40% when measured at the midpoint of the blade (Fig. 6 D).

Aspects of both the $Hsf1$ and $Wab1$ phenotypes are modulated in the double mutant. Instead of large sectors of wavy auricle at the proximal end of the blade typical of single $Wab1$ plants, double mutant plants had smaller patches. In contrast to the $Wab1$ single mutant, the leaf blades of the double mutant contained multiple thin striations of auricle or sheath tissue that extended distally along the blade. Single $Wab1$ mutants may have one or two larger striations appearing in the blade (Fig. 6 E). The prong phenotype of double mutants is also greatly reduced.
The PPM in double mutants is reduced by 60%. Prongs appear primarily as hairy fringes of sheath along the margin instead of large “sheathy” outgrowths. These results indicated that \textit{Hsf1} and \textit{Wab1} show a reciprocal repression of phenotypes.

\textit{Hsf1} prong formation is enhanced by \textit{Wavy auricle in blade-SGL}

Superficially, \textit{Wab-SGL} is similar in phenotype to \textit{Wab1}, having large patches of wavy auricle in the blade and a narrow distal blade width. \textit{Wab-SGL} showed no difference in sheath or blade lengths compared to wild type and mutant blade width was reduced about 20% at the midpoint of blade length.

Although \textit{Wab-SGL} is similar to \textit{Wab1}, it had a different interaction with \textit{Hsf1}. \textit{Hsf1} suppressed the \textit{Wab-SGL} wavy auricle phenotype, while \textit{Wab-SGL} enhanced the \textit{Hsf1} prong phenotype. The characteristic ectopic patches of auricle in the blade were nearly eliminated in double mutants, appearing only as the occasional bubble of auricle. The fact that every leaf does not show the \textit{Wab-SGL} phenotype made individual double mutant leaves difficult to phenotype. In some leaves, wavy auricle was reduced to sectors of clear membranous tissue situated between marginal prong and normal blade. The double mutant did not show the same thin striations of auricle visible in \textit{Wab1} double mutant leaves. Instead, larger chlorotic stripes appeared in the leaf blade distal to marginal prongs (Fig. 6 F). The PPM was increased in the double mutant more than 120%, from 6.8% to 15.6% (Fig. 6 G). These interactions indicate that \textit{Hsf1} was enhanced by \textit{Wab-SGL}, while \textit{Hsf1} suppressed the characteristic wavy auricle in blade \textit{Wab-SGL} phenotype.
**Hsf1 uncovered dosage effects in other meristem mutants**

*aberrant phyllotaxy1* negatively regulates CK signaling

Type-A response regulators are rapidly induced in response to CK and negatively regulate CK signaling [38]. In maize, a loss-of-function mutation in the type-A response regulator *ZmRR3* exists [26]. The loss-of-function of *ZmRR3* is also known as the recessive mutant *aberrant phyllotaxy1* (*abph1*), because of the altered phyllotaxy - from alternate distichous to opposite decussate - of homozygous mutant plants [27]. Because a mutant in a negative regulator of CK signaling was available that did not affect leaf patterning, we were interested to assess its interaction with the *Hsf1* mutant. To investigate the role *ZmRR3* (*abph1*) plays in the cytokinin hypersignaling mutant, double mutants with *abph1* and *Hsf1* were made.

Because the *abph1* phenotype is not fully penetrant, a genotyping assay was designed to track the *abph1*-O allele. The *abph1*-O lesion is due to an unknown insertion in between intron 3 and intron 4 [26]. Inverse PCR was performed to amplify a portion of the insertion, which, a BLAST search against the Maize Transposable Elements Database revealed, had an 89% match to a flip family transposable element in the Gypsy superfamily. Primers were placed in both exon 3 and in the insertion to allow for PCR genotyping and tracking of the *abph1*-O allele.

A testcross population was made that segregated equal ratios of *abph1* homozygotes, *abph1* heterozygotes (which are phenotypically wild type), *Hsf1* plus *abph1* homozygotes, and *Hsf1* plus *abph1* heterozygotes. Double mutants heterozygous for *Hsf1* and homozygous for *abph1* resulted in a small plant with a disorganized, bushy phenotype, which resembled the homozygous *Hsf1* phenotype [3]. Many meristems formed with no dominant SAM, resulting in multiple shoots with short and narrow leaves (Fig. 7 F-H). Some of these leaves had discontinuous ligules (Fig. 7 J) while others appeared to be the fusion of two leaves (Fig. 7 K).
The multiple shoot/bushy leaf phenotype was present at germination, and made it difficult for leaves to emerge from the kernel. This difficulty, combined with their small, leafy stature, did not make this genotype viable in the field. These results indicated that loss of \textit{abph1} function enhanced \textit{Hsf1}, confirming that \textit{abph1} negatively regulates cytokinin signaling.

Surprisingly, during analysis of the double mutants, we observed plants that genotyped as heterozygous for both \textit{Hsf1} and \textit{abph1} but also showed a mutant phyllotaxy (Fig 7 D). Normally the aberrant phyllotaxy phenotype, opposite decussate, is only present in the homozygous \textit{abph1} plants (Fig 7 B, C); \textit{abph1} heterozygotes initiate leaves in the normal alternate distichous phyllotaxy (Fig. 7 A). However, when plants were heterozygous for both \textit{Hsf1} and \textit{abph1}, 50% developed two leaves at each node (Fig. 7 D, E). Some of these plants reverted back to a normal phyllotaxy as they developed, but others remained opposite decussate throughout their development. As these plants developed, their \textit{abph1} phenotype was enhanced. For example, in addition to the opposite decussate phyllotaxy, some plants began to form leaves with two midribs (Fig. 7 M). At subsequent nodes, these leaves were wider with two distinct distal tips, as if two leaves had fused together. The shoot apex on one such plant bifurcated, forming two stalks, with each terminating in its own tassel (Fig. 7 I). A small number of seedling leaves in \textit{Hsf1} plants heterozygous for \textit{abph1} also showed lateral-medial patterning defects. The blade-sheath boundary, which is marked by the ligule and auricle, was occasionally displaced on one side of the midrib, with one side a few centimeters past the other (Fig. 7 M). These results suggest that \textit{abph1} negatively regulates CK signaling, but one copy of \textit{abph1} is not sufficient to regulate CK hypersignaling.
**tassels replace upper ears1** also regulates meristem activity

Analysis of the transcriptome of early forming prongs indicated that a *bop* transcription factor was up-regulated relative to developing margin tissue with no prongs [12]. In Arabidopsis, BOP1 regulates leaf architecture by regulating meristem activity [39]. Loss-of-function *bop1* mutants contain leaflet-like organs projecting from the petiole.

To investigate the role *bop* transcription factors might play in prong formation, we analyzed double mutants with *Hsf1* and a *bop* homolog, *tassel replaces upper ear1* (*tru1*). *tru1* loss of function mutations are characterized by elongated ear shanks with a male tassel replacing the female ear but not leaf patterning defects were described [31].

A population segregating heterozygous *tru1*, homozygous *tru1*, *Hsf1* plus heterozygous *tru1*, and *Hsf1* plus homozygous *tru1* was generated. *Hsf1* and *tru1* phenotypes (hairy sheaths and elongated ear shanks) segregated as expected, and genotypes were confirmed by PCR. As *Hsf1* plants developed, both heterozygous and homozygous for *tru1*, novel phenotypes appeared. As leaves emerged from *Hsf1* plants, many began to transition into an *abphl*-like phyllotaxy. The first 14 nodes of *Hsf1* plants produced the expected one leaf per node but upper nodes appeared to initiate two or even three leaves (Fig. 8 A). Carefully removing these leaves revealed that the third leaf was growing from an extremely short internode (Fig. 8 B, C). Leaves on the nodes transitioning from an alternate phyllotaxy to opposite showed a gradient of leaf defects. Early/lower leaves began to show double midribs with the midribs separating farther in subsequent leaves. Some leaves appeared to have one or two blade-like leaflets emerging 90 degrees from the true leaf, resulting in a trifoliate appearance (Fig. 8 D). These leaflets appeared to have a short midrib in the center of proximal lamina, but the midrib did not continue distally
throughout the entire blade (Fig. 8 E). Some of these outgrowths were attached to the proximal end of the blade, while others were attached to the upper distal portion of the sheath (Fig. 8 F).

In double mutants, the tassel rachis appeared fasciated, or thicker, than either the single mutant tru1 or Hsf1 siblings. Small leaves subtended lower tassel branches (Fig. 8 G, H) and bracts subtended some spikelet pairs. A small number of spikelets were observed to have an abnormal number of florets, but this was not quantified.

Most interestingly, the altered phyllotaxy and thickened rachis were observed on Hsf1 plants both that were both heterozygous and homozygous for tru1. This was confirmed both by the elongated ear shank phenotype typical of homozygous tru1 mutants, and genotyping for an insertion polymorphism in the tru1 allele. Thus, novel interaction phenotypes were observed between Hsf1 and tru1 and these novel phenotypes occurred in both homozygous and heterozygous tru1 genotypes.

A novel, discontinuous ligule phenotype was observed on seedlings homozygous for the tru1 mutation. Similar to the abph1 ligule phenotype, ligules on one side of the midrib did not connect with the other, as one side was displaced a few millimeters distally. This was observed in the first four leaves of all homozygous tru1 plants, but the normal continuous ligule was observed on all later leaves. Heterozygous tru1 seedlings did not show this discontinuous ligule phenotype, suggesting this phenotype had recessive inheritance. When crossed with Hsf1, both the tru1 heterozygous and homozygous seedlings showed the discontinuous ligule phenotype (Fig. 8 I, J). This indicated that tru1 has a function regulating lateral-medial symmetry on early leaves and that normally one copy of tru1 was sufficient to promote a normal, continuous ligule. In a Hsf1 background, one copy was no longer able to promote normal ligule development.
Overall, these results suggest *tru1* has a function affecting meristem size or leaf initiation, similar to the function of *abph1*. In a cytokinin hypersignaling background, the loss of one or two functional *tru1* alleles resulted in aberrant leaf initiation and development.

**Hsf1 interacts with mutants affecting other plant growth hormones**

Lower GA levels result in enhanced prong formation

*Hsf1* is a cytokinin hypersignaling mutant, and cytokinin has been shown to interact with gibberellic acid. High GA and low CK levels have been shown to be detrimental to meristem activity [34]. In mutants with ectopic *knox* expression, reduced GA levels have been shown to promote meristematic activity [40]. The *an1* gene encodes a protein involved in GA biosynthesis, and the recessive loss-of-function mutant is characterized by dwarf stature, short and wide leaves, and the production of anthers in the ears [35]. Since *lg3*, a class I *knox* gene, is ectopically expressed in *Hsf1* prongs, we hypothesized that reducing GA accumulation may enhance prong formation in *Hsf1*. To test this, a population segregating *Hsf1* plus homozygous *an1*, *Hsf1* plus heterozygous *an1*, homozygous *an1*, and heterozygous *an1* was made. When compared to *an1* heterozygotes, which appear WT, homozygous *an1* leaf blades are reduced by 18% and blade width increased by 11%. *Hsf1* plus homozygous *an1* leaf blades were 30% shorter and 18% wider than *Hsf1* plus heterozygous *an1* (Fig. 9 A-D).

Reduced GA levels also affected the formation of prongs. *Hsf1* leaves homozygous for *an1* had a 44% increase in PPM (Fig. 9 E). Even though they took up a larger percentage of the margin, these prongs were not as large as other *Hsf1* double mutants. These prongs mostly consisted of long thin fringes of hairy sheath at the margins of the blade (Fig. 9 D). Surprisingly, PPM was also somewhat increased in the *Hsf1* plus heterozygous *an1* plants. Plants *Hsf1* plus
heterozygous an1 had leaves with 13 PPM while other experiments with Hsf1 in the same genetic background and two functional an1 alleles had between 5 and 10 PPM. Ideally, PPM should be measured in a population segregating Hsf1 and Hsf1 plus heterozygous an1 to confirm this observation. Overall, the increase in percent prong margin in Hsf1 plus homozygous an1 indicated that reduced GA levels enhanced prong formation.

Discussion

While CK has been shown to affect leaf development and shape in other species [41-43], especially through the action of knox transcription factors [44, 45], an analysis of the specific way maize leaves are affected had not been performed. Hsf1, a cytokinin hypersignaling mutant, provided an opportunity for such an analysis. A detailed analysis of the proximal-distal leaf patterning alterations was conducted. These ectopic projections, or prongs, of sheath, auricle and ligule from the normal blade margin were measured along mature Hsf1 leaves, and the pattern of appearance was determined. In addition, potential genes downstream of CK signaling, and that alter leaf patterning, were analyzed along with Hsf1. These results give insight into the genetic mechanism behind the Hsf1 mutant phenotype.

*Hsf1 leaves form in a patterned manner*

To better understand the effects of CK hypersignaling in the leaf blade, we analyzed the spatial distribution of prong formation in Hsf1 leaves. Prong locations were measured along the first, second and third leaf above the ear from three Hsf1 alleles. We found leaves closer to the tassel tend to form more prongs than leaves near the ear. Less than half the leaves directly above the ear had any prongs at all, while the third leaf above the ear had prongs in the majority of
plants. Next the percentage of margin with prong identity (PPM) was calculated, and the three alleles showed similar results: the third leaf above the ear had a higher PPM than the first and second. There were no significant differences between the three alleles.

To further analyze the spatial distribution of prong formation, the location and size of prongs was considered. We were interested to find out if there were locations along the blade that had higher or lower density of prong formation. In addition, larger prongs seemed to form at the proximal end of the leaf blade. Prong size was determined from the measurements and compared with the location along the leaf blade. Prongs were found all along the blade margin, except for the most distal 30% of the blade, which never showed any prong formation. The area with the highest prong density was between 15% and 40% of the blade length. Larger prongs tend to be found at the proximal base of the blade and smaller prongs appearing distally along the blade. These results show that prong formation does follow a pattern, and that the three alleles, *Hsf1*-1595, *Hsf1*-1603 and *Hsf1*-AEWL operate similarly. These results show the signals underlying prong formation are not present uniformly along the leaf margin, but may appear in specific areas along the proximal distal axis.

*Hsf1* is enhanced in double mutant leaves

After determining a baseline for prong formation patterns, double mutant analysis was conducted. Genes for this analysis were selected using a number of criteria. *lg3*, *ZmRR3*, and *tru1* were chosen because they, or their homologs, were differentially expressed in developing *Hsf1* prong margins compared to normal WT margins [12]. In addition to *lg3*, other class I *knox* genes and leaf patterning mutants were chosen because these genes play a role in meristem maintenance and their dominant gain-of-function mutants alter proximal-distal leaf patterning.
The *an1* mutant, whose underlying gene is involved in GA biosynthesis, was chosen because of the antagonistic relationship between GA and CK in meristematic tissue.

Three class I *knox* genes enhance the *Hsf1* prong phenotype

*knotted1* is normally expressed in the meristem and is absent in developing leaves [13]. In addition to proximal-distal leaf patterning defects, ectopic *knox* gene expression is correlated with increased leaf lobing, ectopic meristem initiation and leaf dissection [46]. These factors make *kn1* and other *knox* genes attractive potential modifiers of *Hsf1* prong formation.

*liguleless3* appeared in transcriptome analysis as being highly expressed in developing prong margins when compared to wild type margins [12]. To test the role of *lg3* in prong formation, we analyzed double mutants with the dominant gain-of-function *Lg3-O* mutant. Our results indicate *Lg3* enhanced the *Hsf1* prong phenotype. Double mutant prongs were larger, both by taking up a higher percentage of the margin, and by encroaching farther into the lamina towards the midrib. The large prongs that encroach near the midrib occasionally have proximal blade that, instead of being able to expand distally, must expand laterally around the prong. These results show that *lg3* plays an important role in prong formation. Further experiments with an *lg3* loss-of-function allele would reveal if *lg3* is necessary for prong formation.

To investigate the role of *kn1* in prong formation, we made *Hsf1* double mutants with a gain-of-function *kn1* allele, *Kn1-DL*, and with a loss-of-function allele, *kn1-L4*. *Kn1-DL* forms *Hsf1*-like prongs at the distal 30% of blade margins of certain leaves due to distal expression of *Kn1*. Our results show there is no difference in PPM between *Hsf1* and the *Hsf1*, *Kn1-DL* double mutants, suggesting that *kn1* is not expressed in developing prongs. Surprisingly, the double mutant leaves appeared to be truncated as the distal 30% of the blade did not form due to knots
developing in the midrib. This could be the result of \textit{Hsf1} enhancing the ectopic expression of \textit{kn1} in the double mutant midrib. If \textit{kn1} is involved in prong formation, we would expect to see an alteration in prong phenotypes in a \textit{kn1} loss-of-function background. Our results showed \textit{Hsf1} and \textit{kn1} double mutants were still able to make prongs. Additionally, there was no difference in PPM between \textit{Hsf1} and the double mutant. This is clear evidence that \textit{kn1} is not required for prong formation, and is consistent with our observation that the \textit{Kn1-DL} allele did not modify the prong phenotype.

Two other class I \textit{knox} mutants enhanced the \textit{Hsf1} prong phenotype. The \textit{Gn1} and \textit{Rs1} mutants both display proximal-distal leaf patterning defects, with sheath being displaced distally into the blade. When combined with \textit{Hsf1}, both of these mutants reiterated their blade sheath boundary phenotype in the prongs. Double mutants with \textit{Gn1} had larger prongs than \textit{Hsf1} and displayed very little auricle. Double mutants with \textit{Rs1} had prongs with large mosaics of sheath and auricle tissue at the prong-blade boundary and occasionally having two ligules.

\textit{Hsf1} also revealed information about the patterning defects of \textit{Lg3} and \textit{Rs1}. Both feature sheath displaced distally into the blade. Since \textit{Hsf1} induces more macrohairs on abaxial sheath tissue, the presence of macrohairs can be used as a visual marker for sheath identity. \textit{Lg3} sheath displacement along the adaxial midrib can be identified by the ligule. On the abaxial blade, macrohairs appear farther distal along the midrib than the most distal ligule on the adaxial face. Macrohairs also were present at the abaxial \textit{Rs1} blade sheath boundary, indicating a possible dorsal-ventral polarity alteration in the mosaic of sheath and auricle tissue.
Wavy auricle in blade mutants show opposite interactions with \textit{Hsf1}

The dominant gain-of-function \textit{Wavy auricle in blade1} mutant shows patches of auricle at the proximal end of the blade. \textit{Wab1}, which is a TCP transcription factor, has been shown to restrict growth, resulting in narrower leaves. It is normally expressed at the boundary of the meristem and lateral organs, possible repressing growth in the axil [23, 24]. Double mutant analysis with \textit{Hsf1} appeared to show a similar function in prong formation. \textit{Hsf1} and \textit{Wab1} double mutants had a smaller PPM when compared to single \textit{Hsf1} mutants. This indicated ectopic \textit{wab1} expression may be restricting the formation of \textit{Hsf1} prongs.

\textit{Wab-SGL}, another dominant mutant with a similar phenotype to \textit{Wab1}, was crossed with \textit{Hsf1}. Despite having a similar ectopic auricle phenotype like \textit{Wab1}, \textit{Wab-SGL} and \textit{Hsf1} double mutants showed a different phenotype than \textit{Wab1} and \textit{Hsf1} double mutants. \textit{Wab-SGL} double mutants had an increased PPM. The wavy auricle phenotype was also suppressed in double mutant leaves. These results indicated \textit{Wab1} and \textit{Wab-SGL} modified prong formation differently in \textit{Hsf1}–one suppressed it and the other enhanced it. This result also implies \textit{Wab-SGL} not the same gene as \textit{wab1}.

\textit{ZmRR3} negatively regulates CK signaling

As part of a two-component signaling system, type-A response regulators attenuate CK signaling. To assess the role \textit{ZmRR3}, a CK inducible type-A response regulator, plays in \textit{Hsf1} hypersignaling, double mutants were made with the \textit{ZmRR3} loss-of-function mutant, \textit{abph1} [26]. The loss of \textit{ZmRR3} function results in an enlarged meristem, producing two leaves at each node [27]. Double mutants homozygous for \textit{abph1} and heterozygous for \textit{Hsf1} were short and leafy, with multiple shoots with small thin leaves. One \textit{abph1} allele is not normally sufficient for the
formation of an aberrant phyllotaxy. However, in a number of Hsf1 plants heterozygous for abph1, a reversion to the aberrant phyllotaxy of opposite decussate was seen, suggesting in a Hsf1 background, ZmRR3/abph1 exhibits a gene dosage effect. These results strongly suggest that ZmRR3 plays a role in negatively regulating CK signaling.

**Double mutants reveal tru1 plays a role in phyllotaxy regulation**

The BOP1 homolog tru1 normally functions to regulate shoot architecture [31]. The loss-of-function mutant has elongated ear shanks with tassels replacing the upper ears. Transcriptome analysis showed a bop1 homologous transcription factor was highly up-regulated in developing prongs when compared to WT normal margin [12]. Hsf1 plants that were heterozygous for the tru1 loss-of-function allele showed an altered phyllotaxy. These plants transitioned from a normal alternate distichous leaf arrangement to an opposite decussate arrangement. Leaves on these plants had leaflets emerge from the sheath, sometimes with three blade-like projections from one sheath of then with their own midrib or partial midrib. The tassel rachis appeared fasciated and small leaves and bracts subtended tassel branches. Our analysis also uncovered a role for tru1 in regulating ligule formation in the first four leaves. Hsf1 and tru1 heterozygous plants also showed the discontinuous ligule phenotype. These results suggest that, similar to abph1, tru1 plays a role in restricting the meristem in the presence of CK hypersignaling. Our double mutant results highlight the role of tru1, similar to Arabidopsis bop1, in repressing lateral organ initiation [39].
Gibberellic acid levels affect *Hsf1* prong formation

Knox genes regulate GA and CK levels and that balance controls meristem maintenance versus leaf initiation [15, 34, 47]. We hypothesized that *lg3*, which had been shown to be ectopically expressed in developing prong margins [12], might regulate GA levels in developing prongs. To investigate this possibility, double mutants were made with *Hsf1* and *an1*, a gene involved in GA biosynthesis [35]. *Hsf1* and *an1* double mutants showed a higher percentage PPM. The *Hsf1* plus *an1* heterozygous plants also appeared to show an increase in PPM, but that observation should be confirmed. Our results indicated that low GA levels in developing *Hsf1* prongs may result in larger PPM. This suggests that a margin high in CK and low in GA promotes an undifferentiated state, resulting in the production of ectopic proximal structures.
**Figure 1.** *Hsf1* prongs appear in a patterned manner. (A) The third leaf above the ear from an *Hsf1-1603* plant is seen. Projections, or prongs, of sheath, auricle and ligule appear on both margins of adult *Hsf1* leaves. (B-D) Percent prong margin (PPM) for three *Hsf1* alleles are shown for the first leaf above the ear (B), the second leaf above the ear (C) and the third leaf above the ear (D). (E) Boxplots represent the central 50% of prong sizes for both sides of the leaf for each allele. (F) Boxplots represent prong position (midpoint of each prong) along the percentage of blade length. (G) Prong midpoints compared with size of prong for prongs appearing on both margins for three *Hsf1* alleles. The x-axis is the prong midpoint location along the leaf blade and the y-axis is the relative size of the prong. The blue line is a smoothed curve to represent the relationship between size and location.

**Figure 2.** *Lg3-O* enhances *Hsf1* phenotype. (A) The dominant *Liguleless3* gain-of-function mutant results in distal displacement of sheath into the blade, resulting in ligule displacement (arrows) along the midrib. (B) *Hsf1* (left) and *Hsf1, Lg3-O* (right) leaves. *Hsf1* and *Lg3* double mutant prongs appear larger with more sheath identity than *Hsf1* prongs. (C) *Hsf1* (left) and *Hsf1, Lg3-O* double mutant PPM. (D) *Hsf1* prongs (left) and *Hsf1, Lg3-O* prongs (right). (E) The midrib to prong distance for *Hsf1* leaves (blue) and *Hsf1, Lg3-O* (green) on the 14th, 15th and 16th leaves. (F, G) *Hsf1, Lg3-O* double mutant leaves. (F) Large prongs result in proximal blade tissue expanding around the prong. (G) Double mutant leaf with midrib bends, as well as macrohairs extending distally along the midrib. (H-J) Leaf measurements for the 2nd, 4th and 6th leaf above the ear for WT (far left), *Lg3-O* (center left), *Hsf1* and *Lg3-O* (center right) and *Hsf1* (far right). (H) Sheath length, (I) blade length and (J) blade width at half the blade length. * indicates p-value<0.05.

**Figure 3.** *Gn1* enhances *Hsf1* phenotype. (A) The adaxial surface of a mature *Gn1* 6th leaf below the tassel, cut in half along the midrib. The dominant *Gn1* gain-of-function mutant results in distal displacement of sheath into the blade, resulting in a reduced auricle and the distal displacement of ligule (arrows). (B) The abaxial surface of the same mature *Gn1* leaf cut in half along the midrib. (C) The adaxial surface of a mature *Hsf1, Gn1* double mutant 6th leaf below the tassel cut in half along the midrib. (D) The abaxial surface of the other half of the leaf in (C). (E) *Hsf1, Gn1* double mutant leaf. (F) Close-up of the adaxial surface of the prong from (E). (G) The PPM for *Hsf1* (left) and *Hsf1, Gn1* double mutants (right) from the second, fourth and sixth leaves below the tassel. N>12 for each genotype. Scale bar is 2 cm. * indicates p-value<0.05.

**Figure 4.** *Rs1* enhances the *Hsf1* phenotype. (A, B) The *Rs1* gain-of-function mutant results in a disorganized area of sheath, auricle and ligule appearing ectopically in the proximal blade. (A) Adaxial surface of the sixth leaf below the tassel, cut in half along the midrib. (B) The abaxial surface of the other half of the leaf seen in (A). (C) *Hsf1, Gn1* double mutant 4th leaf below the tassel. (D) Close-up of a prong from (C). Arrows indicate ectopic ligule. (E) Adaxial surface of the blade sheath boundary of *Hsf1, Rs1* double mutant sixth leaf from the tassel. (F) Close-up of the ligule area from (E). (G) PPM for *Hsf1* (left) and *Hsf1, Gn1* double mutant (right) leaves. (G) The PPM for *Hsf1* (left) and *Hsf1, Rs1* double mutants (right) from the second, fourth and sixth leaves below the tassel. N>12 for each genotype. Scale bar is 2 cm. * indicates p-value<0.05.
**Figure 5.** Distal expression of knotted1 in Hsf1 further shortens leaf length. (A) The distal portion of the sixth leaf below the tassel from Kn1-DL. (B) The sixth (left), fourth (center) and second leaf (right) below the tassel from a Kn1-DL plant. (C) The sixth (left), fourth (center) and second leaf (right) below the tassel from an Hsf1, Kn1-DL double mutant plant. (D) Blade length of the sixth leaf below the tassel in WT (far left), Kn1-DL (center left), Hsf1, Kn1-DL (center right) and Kn1-DL (far right). (E) PPM for Kn1-DL, Hsf1 and Kn1-DL double mutant and Hsf1 (right). N>6 for all genotypes. * indicates p-value<0.05.

**Figure 6.** knotted1 is not required for prong formation and Hsf1 interacts differently with dominant Wab mutants. (A) The tassel, and second, fourth and sixth leaves from the tassel are shown from an Hsf1, kn1/+ plant. (B) The tassel, and second, fourth and sixth leaves from the tassel are shown from an Hsf1, kn1/kn1 plant. The tassel has a characteristic knotted1 loss-of-function phenotype and each of the leaves shown has prongs on the margins. (C) The PPM for Hsf1 plants heterozygous (left) and homozygous (right) for kn1. N>6 for both genotypes. (D) The sixth leaf below the tassel in WT (top), Wab1 (second from top) Hsf1 (third from top) and Hsf1, Wab1 double mutant (bottom) leaves. (E) The PPM for the second, fourth and sixth leaves below the tassel in Hsf1 (left) and Hsf1, Wab1 double mutant (right) leaves. (D) The sixth leaf below the tassel in WT (top), WabSGL (second from top) Hsf1 (third from top) and Hsf1, WabSGL double mutant (bottom) leaves. (E) The PPM for the second, fourth and sixth leaves below the tassel in Hsf1 (left) and Hsf1, WabSGL double mutant (right) leaves. * indicates p-value<0.05.

**Figure 7.** Hsf1 phenotype is enhanced by abph1 mutant. (A-F) Three-week-old seedlings with different Hsf1 and abph1 genotypes and phenotypes. (A) WT phyllotaxy phenotype; heterozygous for the abph1 mutation. (B) abph1 phyllotaxy phenotype with two leaves at each node; homozygous for abph1 mutation. (C) WT phyllotaxy phenotype; homozygous for abph1 mutation. (D) Hsf1 phenotype and WT phyllotaxy; heterozygous for both Hsf1 and abph1 mutation. (E) Hsf1 phenotype and abph1 phyllotaxy; heterozygous for both Hsf1 and abph1 mutation. (F) Hsf1 phenotype and multiple shoots with short narrow leaves; heterozygous for Hsf1 and homozygous for abph1. (G) Close-up plant from (F). (H) Adult plant heterozygous for Hsf1 and homozygous for abph1. (I) Hsf1 plant heterozygous for abph1 that showed the abph1 phenotype as a seedling. The stalk split a one point to form two. Arrows point to two tassels. (J) Hsf1 plants homozygous for abph1 often display patterning defects, such as ligule displacement (arrows). (K) Hsf1 plants homozygous for abph1 sometimes form fused leaves that may have two midribs. (L) Hsf1 plants heterozygous for abph1 also have ligule displacement defects. Triangles point to the ligule and auricle on separate margins of one leaf.

**Figure 8.** Hsf1 and tru1 double mutants have phyllotaxy and patterning defects. (A) Hsf1 and tru1 heterozygotes began to transition from one leaf per note to two leaves at each node. (B) Upper leaves on Hsf1 and tru1 heterozygotes may appear to have three leaves at each node. (C) Carefully removing the leaves from (B) reveals the third leaf has emerged from a short internode. (D-F) Hsf1 and tru1 heterozygotes also show leaf patterning defects. (D) This leaf has a main blade with two smaller blades attached to the distal end of the sheath. (E) Both leaves shown
have a leaflet attached to the distal end of the sheath. A ligule and auricle separate the leaflets from the sheath. (F) This leaflet is attached to the true blade with a second midrib 90 degrees from the main midrib. (G, H) Tassels from $Hsf1$ and $tru1$ heterozygotes appear fasciated and have leaves subtending the lower branches. Small bracts also subtend some spikelet pairs. (I) Early leaves on $tru1$ homozygotes have a discontinuous ligule phenotype. (J) $Hsf1$ plants that are heterozygous for $tru1$ also show the discontinuous ligule phenotype.

**Figure 9.** $Hsf1$ and $an1$ double mutants have shorter leaves, more prong margin. (A-C) WT, $an1$, $Hsf1 + an1$, and $Hsf1$ leaf measurements from the second, fourth and sixth leaves below the tassel. (A) Sheath length, (B) blade length and (C) blade width at half the blade length. (D) Pictured from the top are WT, $Hsf1$, $an1$, and $Hsf1$, $an1$ double mutant sixth leaf from the tassel. (E) PPM for $Hsf1$ (left) and $Hsf1 + an1$ double mutant leaves. Included are the second, fourth and sixth leaves below the tassel. N>12 for all genotypes. * indicates p-value<0.05.
Tables

Table 1. More *Hsf1* leaves closer to the tassel have prongs.

<table>
<thead>
<tr>
<th></th>
<th>1st leaf above ear</th>
<th>2nd leaf above ear</th>
<th>3rd leaf above ear</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Leaves with prongs</td>
<td>Total leaves</td>
<td>Leaves with prongs</td>
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<tr>
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<td>20</td>
<td>14</td>
</tr>
<tr>
<td><em>Hsf1</em>-1603</td>
<td>4</td>
<td>21</td>
<td>10</td>
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<td><em>Hsf1</em>-1595</td>
<td>6</td>
<td>19</td>
<td>14</td>
</tr>
</tbody>
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References


31. Li, W. and E. Vollbrecht, **Tassels replace upper ears1 encodes a putative transcription factor that regulates maize shoot architecture by multiple pathways.** 2012, Iowa State University.


Figure 1. *Hsfl* prongs appear in a patterned manner.
Figure 2. Lg3-O enhances Hsf1 phenotype.
Figure 3. *Gn1* enhances *Hsf1* phenotype.
Figure 4. Rs1 enhances Hsf1 phenotype.
Figure 5. Distal expression of *knotted1* in *Hsf1* further shortens leaf length.
Figure 6. *knotted1* is not required for prong formation and *Hsf1* interacts differently with dominant *Wab* mutants.
Figure 7. *Hsf1* phenotype is enhanced by *abph1* mutant.
Figure 8. *Hsf1* and *tru1* double mutants have phyllotaxy and patterning defects.
Figure 9. *Hsf1* and *anl* double mutants have shorter leaves, more prong margin.
CHAPTER IV
GENERAL CONCLUSIONS

To investigate the role of CK in leaf morphogenesis and development we conducted a series of CK germination assays to characterize seedling leaf growth responses, and we conducted double mutant analysis of genes enhancing the Hsf1 phenotype. Chapter II showed exogenous CK affects leaf growth parameters in specific ways. Chapter III documented the way in which Hsf1 alters proximal-distal leaf patterning as well as revealing genetic interactions with genes downstream of CK signaling.

**CK germination assay affects leaf growth parameters**

From the exogenous CK germination assay in Chapter II, it became apparent that the Hsf1 phenotype is due to an increase in CK signaling. CK treatment is able to affect leaf growth, cell identity and proximal distal patterning in a dose-dependent manner at specific developmental stages. Genetic diversity underlies CK responses in a diverse selection of maize inbred lines as well as in other species. Our results also show Hsf1 is more sensitive to CK treatment, and that leaf growth changes in the first three leaves do not appear to be caused by CK-mediated regulation of GA metabolic genes.

These findings have a number of implications. While Hsf1 has been shown to perturb proximal-distal leaf patterning, with lg3 highly expressed in developing prongs [1, 2], this analysis is the first to document the effects as a result of exogenous CK. This uncovers an ability of exogenous CK to ectopically induce knox gene expression in the margins leaf blade. Additionally, the novel CK responsive phenotype of CML69 shows these patterning defects may not be limited to the margins of developing blades. The variety of CK responses in a diverse
selection of maize inbred lines, as well as rice and sorghum, reveals genetic diversity in CK responses. The different CK responses of leaves at separate developmental stages suggest a gradient of CK response competence in developing leaf primordia. The increased sensitivity of \textit{Hsf1} to low concentrations of CK is consistent with previous studies showing a tighter binding affinity in the mutant \textit{ZmHK1} receptor \cite{3}. Our qRT-PCR results show leaf size changes in the first three leaves are not due to regulation of GA metabolism genes. This indicates CK signaling is reducing leaf length through other genetic methods.

Our results open a variety of avenues for future research. First, the CK assay itself can be used to reliably and consistently induce prong and macrohair formation at specific doses and treatment lengths, allowing study of those processes in early leaves. The responsiveness of developmental stages could be assessed in more detail. Further investigation is necessary to rule out the possibility that the responses could be due to a delay in CK transport in the treated maize kernel. The differential CK responses in sorghum and rice can be seen as preliminary results—a more robust assessment of CK responses in other species could be conducted to obtain a more complete picture of how they, and other species, respond. The diverse CK responses in the NAM inbred lines opens up the possibility of mapping genes responsible for specific responses. For example, the genes underlying the inability of B97 to initiate macrohairs or the novel CML69 patterning defect could be mapped by treating the recombinant NAM lines.

\textbf{Hsf1 double mutant analysis highlight genetic integrations}

In the \textit{Hsf1} single and double-mutant analysis in Chapter III, we showed that \textit{Hsf1} alters proximal-distal leaf patterning in a consistent way, as well as confirmed genetic interactions downstream of CK signaling. Prongs appear on the margins of \textit{Hsf1} leaf blades on specific
leaves at specific locations. Prong formation is also enhanced by a number of dominant gain-of-function knox mutants, although knotted1 does not appear to be required for prong formation. Opposite prong phenotypes of the Hsf1 and dominant Wab mutants were also uncovered. The interaction of Hsf1 and abph1 highlights the role of RR3 in negatively regulating CK signaling. The phyllotaxy defects seen in Hsf1 and tru1 double mutants are similar to abph1, but also include leaves subtending the lower tassel branches. Hsf1 double mutants with a GA deficient mutant, an1, had a higher PPM than Hsf1 siblings.

These results have a number of implications. First, the specific prong formation pattern in Hsf1 leaves could reveal an underlying gradient of CK responsiveness in the maize leaf margin. The enhancement of the Hsf1 phenotype by the dominant gain-of-function mutant Liguleless3 highlights the role of lg3 in prong formation. Given that both Gnl and Rs1 also enhance the Hsf1 phenotype, it is possible that both also co-regulate lg3 expression. The evidence that lg3 is the primary knox gene involved in prong formation is strengthened by the result that knotted1 does not cause a difference in prong formation or PPM. The disparate Hsf1 and Wab double mutant phenotypes highlight that Wab1 and WabSGL mutants are caused by different underlying genes and function differently. The phyllotaxy defect in Hsf1 and abph1 heterozygotes uncovers a dosage effect for abph1—in an Hsf1 background, abph1 could be seen as having a semi-dominant effect. Phyllotaxy defects and leaves subtending the tassel in Hsf1 and tru1 double mutants suggest tru1 has a role in regulating lateral organ initiation in maize. Finally, the higher PPM of Hsf1 and an1 double mutants gives more evidence that GA levels play a role in regulating prong formation.

There are a number of interesting possibilities for future work when considering these results. First, the enhancement of Hsf1 by Lg3, Gnl and Rs1 should be investigated further.
Neither *gnl*, nor *rl* show up in transcriptome analysis of developing prongs, suggesting that *lg3* may be co-regulated by either or both genes, which could be confirmed by gene expression analysis. Cloning of *WabSGL* could give insight into why it has a different interaction with *Hsf1* when compared to *Wab1*. Meristem measurements of *Hsf1* and *abphl* double mutants should be conducted to give insight into possible SAM size change resulting in an altered phyllotaxy. Spatial expression analysis of *tru1* could be performed to visualize where it is normally expressed in leaf primordia and the SAM. PPM measurements of *Hsf1* and *Hsf1 + an1* heterozygote leaves may reveal a GA dosage effect on *Hsf1* prong formation.

In summary, our investigations were able to further characterize maize leaf morphogenesis and development through a number of methods. First, CK germination assays were able to induce dose-dependent leaf growth changes in specific ways at specific developmental time points. This assay also uncovered a diversity of CK responses in maize inbred lines and in two other grasses, sorghum and rice. Second, the *Hsf1* double mutant analysis confirmed genetic interactions that alter the specific ways *Hsf1* perturbs proximal-distal leaf patterning. These analyses also uncovered novel gene dosage effects on *Hsf1* phyllotaxy. In conclusion, the results contained in this thesis provide a foundation for further investigations into the role of CK in leaf morphogenesis and development.

References


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