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Diversity in Tiller Suppression of Domesticated Cereals: MorphologicalVariance Observed in Maize, Sorghum, and Setaria

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ABSTRACT

The Evolution of Tiller Grain Domestication Shows Diversity: Morphological Variance Observed in Maize, *Sorghum*, and *Setaria*

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Tillers are vegetative branches found in grasses, which develop in early stages of plant life. Located at the base of the central stalk, tillers have agronomical importance by increasing seed production with fewer tillers, or providing alternative forms of biofuel with more tillers. As grains have typically decreased tiller number while undergoing domestication, we explored wild and domesticated strains of varying grains by doing a morphological analysis on tiller development. This thesis shows how the decrease of tillers through in domestication cereals shows diversity not only across maize, *Sorghum*, and *Setaria*, but also between lines of maize and *Setaria* species.

To do so, we first measured axillary bud growth across these grasses and compared bud initiation, growth, dormancy and outgrowth. While maize inbred B73 demonstrated a tiller dormancy pattern by initiating buds, growing buds and then bud dormancy we measured growth in *Sorghum* and *Setaria* to compare and found that although *Sorghum* patterns dormancy similar to maize, *Setaria* had more than one way tiller suppression not previously expected. We look further at *Setaria* buds with a statistical analysis of tiller origin and bud frequency in a wild strain and two domesticated strains of *Setaria*. Furthermore we performed Scanning Electron Microscopy (SEM) to have a clear understanding of bud initiation or lack of initiation in *Setaria italica* (B100) comparing it to its wild ancestor *Setaria viridis*. Because of the diversity in *Setaria*, we re-visited maize tiller domestication by taking bud measurements, performing SEMs and counting bud frequency on other strains of inbred maize. We found that maize also shows diversity in its patterning of tiller domestication.

These results demonstrate that there is diversity in the patterns in which tiller domestication has occurred. This diversity is shown here through differences in tiller bud decisions to initiate or not initiate, or to have axillary buds go dormant post-initiation. Furthermore this variance is shown through differences in bud frequency counts, growth measurements, SEMs, and where tiller branches originate across the grains of maize, *Sorghum* and *Setaria*.

Keywords: developmental genetics, plant morphology, grain domestication, tillers, axillary meristems, teosinte, maize, *Sorghum*, *Setaria*, *tb1*, *gt1*, *tb1-sh*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Tiller development morphology

Tillers are lateral vegetative branches in grasses located at the base of the central stalk. Tillers can be important agronomically by increasing both seed production and biomass for alternative fuel sources. Tillers develop from axillary meristems. A meristem is a group of indeterminate cells that have the potential to grow into differentiated tissues (Wu 2018). Axillary meristems are found in the axil of a subtending leaf. Meristems form primordial leaves and together they can be referred to in one unit as a bud. Axillary buds can grow into vegetative branches such as tillers, aerial branches, or female inflorescences such as an ear of corn (McSteen and al 2008). Tiller branch buds are located specifically at the base of the stalk in the axil of the first few leaves, whereas aerial branch buds originate from leaves higher up on the stalk (Wai and An 2017).

Tillers develop in a three-stage process including axillary bud initiation (Grbic and Bleecker 2000), axillary bud development (Lewis, Mackintosh et al. 2008), and finally axillary bud outgrowth (Ward and Leyser 2004). Tiller number has been modified in various cereals that have undergone independent domestication events (Gross and Olsen 2010) with domestication typically causing a decrease in tiller number (Preece, Livarda et al. 2017). Typically, fewer tillers result in higher seed yield as individual ears of all domesticated grains have higher seed count (Doebley, Stec et al. 1995). A comprehensive knowledge of both the morphological and transcriptomic development of axillary buds, could inform attempts to increase yield or biofuel endeavors based on how genes are manipulated for the desired architectural outcome.(White, Vincent et al. 2012).

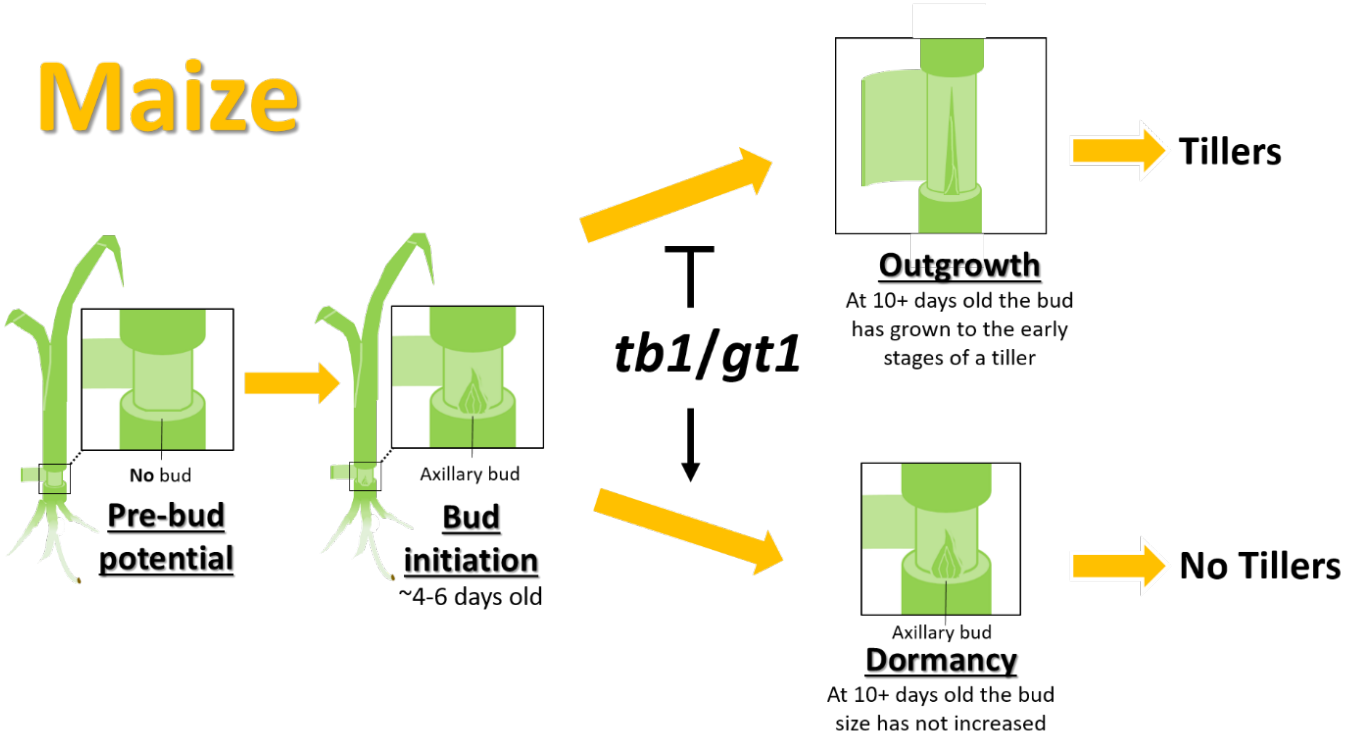

Figure 1 - A flow chart showing the possibilities of axillary bud can suppression. Before a meristem develops the tissue is featureless of a meristem. In time either a meristem will initiate, or it will not. If it does not, tillers do not develop. If the meristem does not become dormant after initiation it will grow out into a tiller. The age of plants are determined in Days After Planting (DAP).

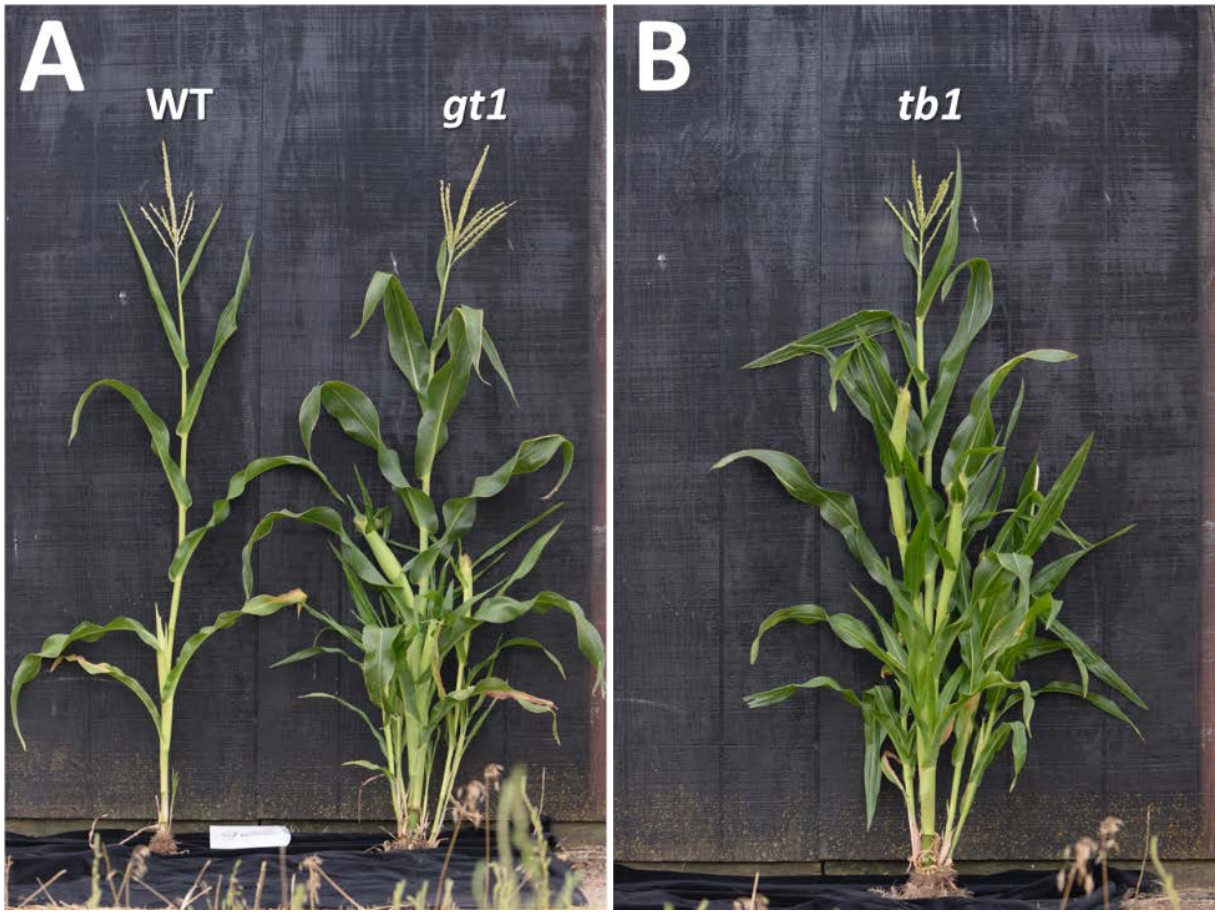

Figure 2 – Wild type inbred maize with tiller mutants *grassy tillers1* (*gt1*) and *teosinte branched1* (*tb1*). (A) Wild type (WT) maize inbred B73 with mutant *gt1* (in B73 background) shows that inbred B73 has no tillers where *gt1* grows many tillers. (B) Mutant *tb1* (in B73 background) grows many tillers.

Genes of maize domestication and tillering

The domestication of maize (*Zea mays* ssp. *mays*) from its wild ancestor teosinte (*Zea mays* spp. *parviglumis*) involved selection for higher seed production and fewer tillers. This domestication event occurred about 9,000 years ago in Mexico (Hake and Ross-Ibarra 2015). Teosinte produces multiple tillers with small ears of ~5-12 kernels per ear (Doebley, Stec et al. 1995). Alternatively, most modern inbred maize does not have any tillers (Hubbard, McSteen et al. 2002) and produces two or three fertile ears each with the number of kernels varying from ~500-800 (Doebley, Stec et al. 1995). In teosinte, the tiller axillary meristem initiates and grows out into a tiller. In inbred maize, the axillary meristem initiates, but then tb1 causes bud dormancy instead of it growing out (Moreno-Pachon 2018). We know of a few genes that control tiller bud dormancy in maize, such as *teosinte branched1* (*tb1*) and *grassy tillers1* (*gt1*). *tb1* is a TCP transcription factor (Horn 2015) that is a negative regulator for axillary meristem growth, and causes meristem dormancy in maize after meristems have initiated (Doebley, Stec et al. 1995). The *gt1* gene is a homeobox transcription factor (Whipple, Kebrom et al. 2011) and also causes meristem dormancy in maize. *gt1* is triggered by environmental factors such as increased shading, which can decrease tillering (Whipple, Kebrom et al. 2011). Furthermore, *gt1* is down stream of and dependent on the presence *tb1* (Whipple, Kebrom et al. 2011). Consequently, inbred maize mutants *gt1*, and *tb1* may grow at least one tiller (Figure 1 and 2).

Separate domestication events could show differences in tiller suppression

Outside of maize, less is known of tiller domestication patterns in other panicoid cereals. There are several sub-families within the Poaceae (grass) family which underwent independent domestication events. Because Poaceae is a large family which consists of about 12,000 species (Christenhusz 2016), the question arose whether or not each of these separate domestication

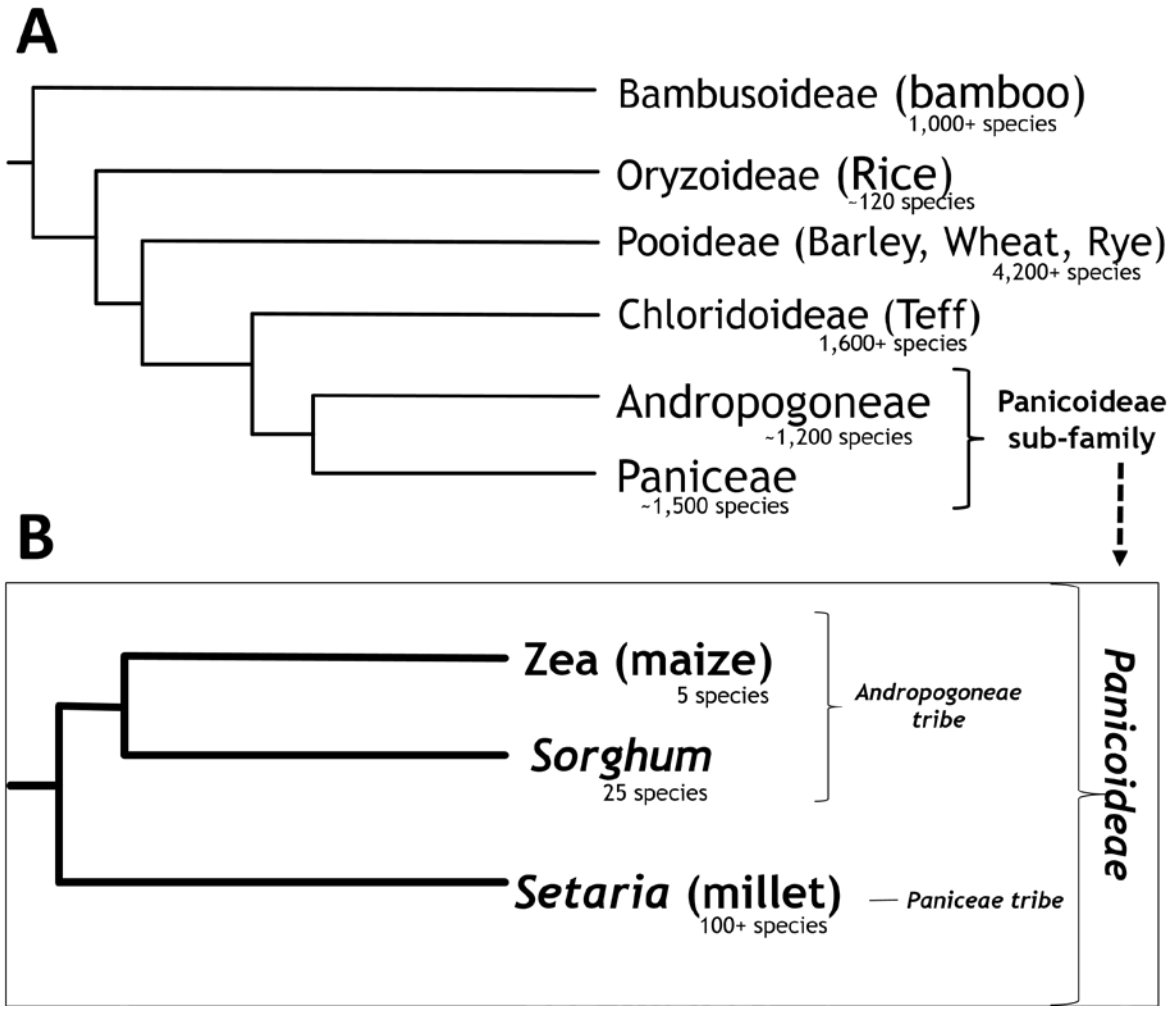

Figure 3 – Poaceae phylogeny of sub-families and tribes. (A) Poaceae family with a few sub-families listed in a phylogenetic tree to show relation. (B) Sub-family Panicoideae showing relation of grains between Andropogoneae and Paniceae tribes. Maize and *Sorghum* are closely related, with *Setaria* as an outside sister group still within Panicoideae.

events suppressed their tillers in the same way that maize did. In this study I examined maize because it is well-studied but also its relatives *Sorghum* in the Andropogoneae tribe, and *Setaria* which is a sister outgroup genus in the Paniceae tribe, which less is characterized. All three of these are in the Panicoid sub-family (Figure 3).

When the genus *Sorghum* underwent domestication it also decreased the number of its tillers (Paterson, Lin et al. 1995, Gross and Olsen 2010). Similar to maize, *Sorghum* initiates tiller buds. However, in domesticated *Sorghum*, the bud becomes dormant. For example, *Sorghum verticiliflorum* has many tillers and produces less seed than domesticated *Sorghum bicolor,* which produces few to no tillers and has high seed yield (Gross and Olsen 2010). These grasses provide an ideal system to determine if domestication targeted similar genetic pathways in the decrease of tillering. Although there is an indication that *Sorghum* follows a similar genetic network in tiller development to that of teosinte and maize (Kebrom, Brutnell et al. 2010), there is insufficient research on the genetics of *Sorghum* tiller suppression to confirm this.

Setaria was also domesticated independently from maize and *Sorghum* (Gross and Olsen 2010)(Figure 3). Besides its close relation to Andropogoneae grasses, *Setaria* is a good model species, with its quick life cycle (612 weeks), and potential for drought-tolerance (Doust, Devos et al. 2004, Li and Brutnell 2011).

Given that *Sorghum* and *Setaria* underwent independent domestication events from each other and also from maize, I wanted to study in detail how domestication took place in these grasses. It was clear that *tb1* and *gt1* have played a role in maize tiller suppression, but I wanted to explore in greater depth how this suppression occurred. Furthermore, I wanted to look at tiller suppression from separately yet related grasses to maize to determine if the means by which these grasses suppressed their tillers was similar or different to maize.

MATERIALS AND METHODS

Plant materials and growth conditions

Some seeds needed to be prepared before they could be planted to ensure a high rate of germination. Maize seeds did not need pre-germination treatment but other seeds were prepared in the following manner:

Setaria italica (A10), *Setaria italica* (B100), *Setaria italica* (yugu1) seeds were frozen in -80˚C for 6072 hours before being planted.

Teosinte fruit cases were clipped with nail clippers to expose seeds. Extracted seeds were immersed into a 10mL tube of 6% Hydrogen Peroxide (H_2O_2) solution (Fisher Scientific, Pennsylvania), completely covered by aluminum foil and placed in a dark room for 24 hours. Seeds were then washed 4 times by soaking seeds in water for 5 minutes each wash before being planted. H_2O_2 solution was prepared by mixing 400 µm of 100% H_2O_2 with 5.4 mL of distilled H_2O. See appendix for detailed protocol.

For the tiller quantity, position and tiller type analysis of *Setaria*, all plants were grown in the BYU greenhouse. Conditions were set at 70% humidity with 16 hour days. Average day temperature was 78˚F while average night temperature was 66˚F.

Axillary bud measurements

Axillary buds originating at the first, second and third leaves (L1, L2, and L3) of *Setaria* were measured every two days for an average rate of growth over time. Measurements were performed by removing the coleoptile, and each leaf layer to reveal the axillary bud under the corresponding leaf. Buds were then imaged with a dissecting microscope and the bud length

determined with the “Basic Annotation” toolbar feature of Leica Application Suite (LAS) software version 3.6.0. Bud length was measured as the vertical height from the top of the prophyll primordia to the base of the bud. Measurements were taken 10 days after planting (DAP) through 20DAP in *Setaria italica* (line B100), 618DAP in *Setaria italica* (inbred line yugu1), and 814DAP in *Setaria viridis* (inbred line A10). Measurements for other grass species were also taken every 2 days as follows: *Zea mays* ssp. *parviglumis* (teosinte), *Zea mays* ssp. *mays* (maize) B73 inbred and maize *grassytillers1* (*gt1*) mutant (B73 inbred background) 6DAP-16DAP, maize A619 inbred10DAP 20DAP, maize *teosintebranched1-ref* (*tb1-ref*) mutant (B73 inbred background) and *tb1-sh* (A619 inbred background) from 8DAP18DAP, *Sorghum verticiliflorum* (PI 300120) and *Sorghum bicolor* (inbred line Tx7000) from 8DAP16DAP. See appendix for detailed protocol.

Setaria analysis of tiller type, position and quantity

Plants were checked daily and emerging leaves were marked with a leaf number and node position as soon as they were large enough to write on with a sharpie. All plants were watered daily with ~150mL water. See appendix for detailed protocol.

DNA extraction

DNA was extracted to determine *tb1-ref* homozygosity when taking bud measurements. To isolate the DNA, 46 cm of leaf tissue was cut from plants. For maize, *tb1-ref* leaves were put in the freeze dryer overnight (-50 C). Post freeze drying, plants were put into the GenoGrinder 2010 (SPEX SamplePrep, New Jersey) and shaken at 1500 RPM for 26 minutes into a powder. Powder was transferred to 1.5mL microtubules and cetyl trimethylammonium bromide (CTAB) buffer was added containing 0.2% β-mercaptoethanol. Chloroform and isopropanol (24:1) was

added to samples and centrifuged to separate DNA from tissue. Aqueous phase was removed from tube and transferred to new tubes. Samples were centrifuged again to consolidate DNA into pellets, supernatant was then removed and pellets were dried in a vacuum dryer for 520min. Next pellets were re-suspended in DNA-ase free H_2O and DNA concentration determined with the NanoDrop^{TM} 2000/2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts), diluted into 25 ng/µL and stored at -20˚C for PCR use. See appendix for detailed protocol.

Scanning Electron Microscopy (SEM)

Axillary buds originating under the first leaf (L1) were isolated for SEM by removing the coleoptile and first leaf of each plant. Buds were left attached to the stem while stems were cut using a razor at a point approximately 2 mm above and 4 mm below the position of the axillary bud. To ensure the side of the stem with the bud was mounted with the bud facing up, the side opposite the bud was removed by making a razor cut parallel to the bud approximately 3mm behind the bud through the entire stem. The portion with axillary buds were fixed using 2% glutaraldehyde for 2 hours up to 4 months. After fixation buds were washed 6 times with 0.3M Na Cacodylate ($C_2H_{12}AsNaO_5$) buffer with 5-10 min per wash. Samples were then post-fixed using 1mL 2% Osmium Tetroxide (OsO_4) in 0.6M Na Cacodylate for no more than 2 hours. Osmium fixative was removed by washing 6 times with distilled water for 5-10 min per wash. Using forceps, samples were then transferred to metal mesh baskets, which then went through a dehydration series consisting of 5-10 min washes in acetone/water concentrations: 10%, 30%, 50%, 70%, 95% and three washes of 100%. Samples were fully dried using a 931.GL Supercritical Autosamdri critical point dryer (CPD) (Tousimis, Maryland) overnight. After critical point drying, samples were mounted on stubs and coated with 1 nm Gold Palladium (80:20) in a Quorum Q150TES (Quorum Technologies, East Sussex, England) sputter coater

before being imaged with an XL30 FEI (TSS Microscopy, Oregon) environmental scanning electron microscope (ESEM), with an accelerating voltage range 100-30kV under high vacuum <20mBar. Working distance ranges varied from 1mm-50µm See appendix for detailed protocol.

TILLER BUD GROWTH DYNAMICS DETAIL THE ROLE OF *TB1*

The difference in tiller growth between wild and domesticated maize

In order to understand in greater detail the regulation of tiller growth in maize, axillary bud measurements were taken during early developmental stages ranging from 6 days after planting (DAP) to 18DAP. Buds from the axil of the first leaf (L1), second leaf (L2), and third leaf (L3) were measured. Bud height was measured from the base to the top of the tallest primordia or prophyll (see materials and methods, Bud Measurements). Measurements were taken in wild maize *Zea mays* ssp. *parviglumis* (teosinte), *Zea mays* ssp. *mays* (maize) inbred B73, maize mutant *gt1* (background B73) and maize mutant *tb1-ref* (background B73) (Figure 4). The data showed differences not just between wild and domesticated lines but furthermore between wild type and mutant lines. Before taking measurements I hypothesized that the maize mutants *tb1* and *gt1* would have similar growth patterns to teosinte because teosinte as well as these maize mutants are known to grow tillers.

Wild type teosinte grows many tillers and thus bud measurements showed a steady growth pattern consistent with a lack of bud dormancy. The L1 bud grew larger and quicker when compared to the L2 and L3 that followed sequentially in size, respectively. Because of these results, I hypothesized tillers to originate from the buds under these leaves.

In inbred maize B73 (which does not grow tillers) buds would initiate and follow a linear growth pattern similar to teosinte until at about 12DAP when buds would go into a state of dormancy, never to continue growth in the time of my measurements. Based on this data I presumed that tiller inhibition occurs in maize generally by a three step process of initiation, growth, dormancy (see figure 4).

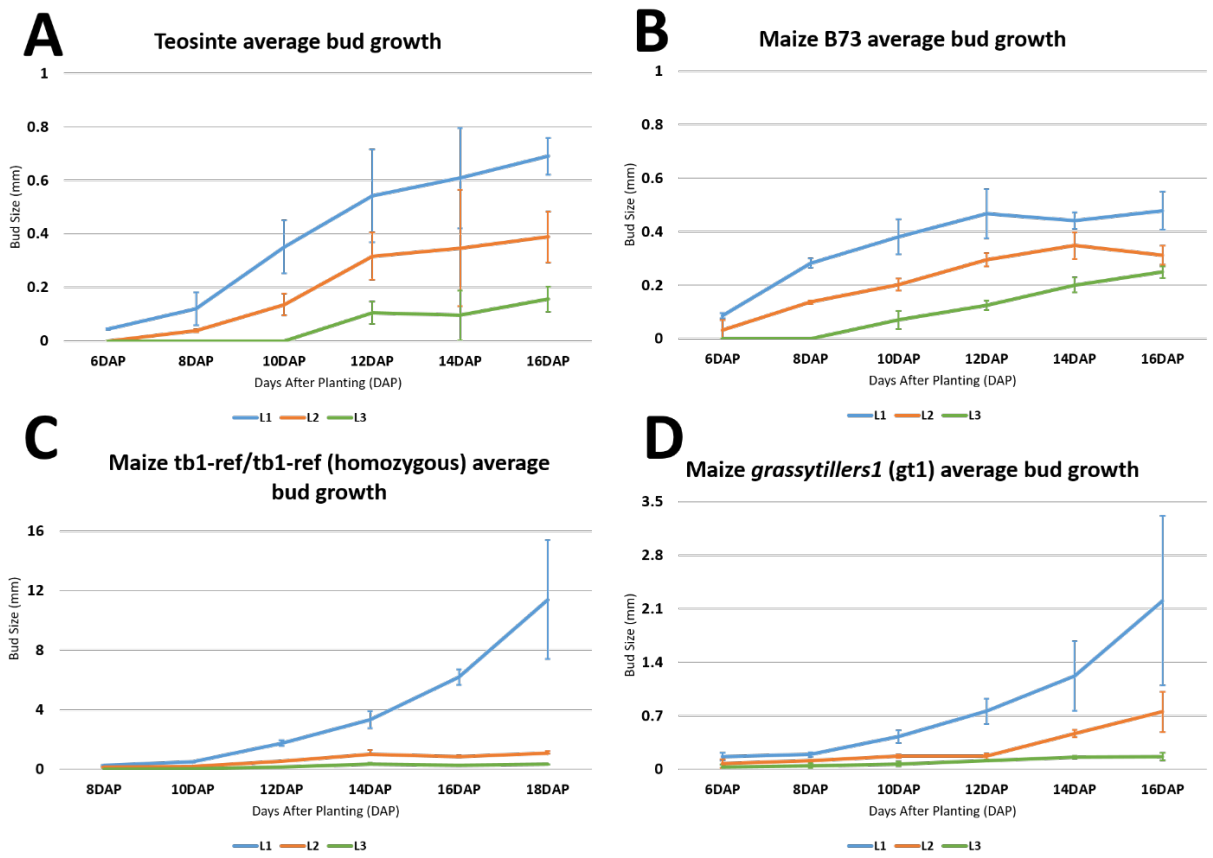

Figure 4-Axillary bud growth in wild and domesticated maize and maize mutants. (A)Wild *Zea mays* ssp. parviglumis (teosinte) average bud growth from 6 days after planting (DAP) to 16DAP with y-axis scale set to 1 mm. The growth shows a steady progression with the bud from the first leaf (L1) being the largest and quickest to grow with the bud from leaf 2 (L2) next and the L3 bud showing the least amount of growth in the 16 days. (B) Domesticated *Zea mays* ssp. mays (maize) B73 axillary bud growth measured from 6DAP to 16DAP. Growth curves show that the L1 bud is the largest and quickest to grow and at 12DAP bud dormancy occurs in the L1 bud. (C) Maize mutant *teosintebranched1* (*tb1*) homozygous (in B73 background) shows a similar growth curve to *gt1* except the y-axis is set to 16mm demonstrating the buds are larger than any of the other growth curves in this figure. (D)Maize mutant *grassytillers1* (*gt1*) in B73 background from 6DAP to 16DAP differs in growth from teosinte and maize B73 in such that the axillary bud growth is exponential. The L1 bud is largest over the 16 day period. The y-axis here is set to 3.5mm showing the buds are significantly larger than in B73.

In maize mutants *tb1-ref* and *gt1*, the axillary buds did not follow the same linear pattern as teosinte, but rather had an exponential growth. This was surprising because originally I believed that knocking out *tb1* and *gt1* would restore tiller growth to what was seen in teosinte. However, the *tb1* and *gt1* alleles in teosinte although weaker in phenotype than in maize B73, are still present in teosinte. These mutant strains therefore showed a complete loss of function causing an exponential bud growth dynamic.

Sorghum's tiller growth patterns similar maize

I wanted to explore if this maize pattern of tiller bud dormancy was conserved in *Sorghum* and *Setaria*. In *Sorghum verticiliflorum* (see Figure 5), buds were measured from 8DAP16DAP (see Figure 5a). Similar to teosinte, there was an initiation and steady rate of growth with the first bud being the largest, the L2 following, and the L3 being the smallest after the 16-day period. However, the rate of growth in *Sorghum* compared to teosinte buds were faster and final bud was larger in the same amount of measured time. Domesticated *Sorghum bicolor* (Tx7000) showed similarities to domesticated maize B73 in that the first bud initiated and went dormant see (Figure 5b). One difference, however, is that the L2 bud outgrew the L1 bud at about 13DAP and continued to grow at a faster rate. Furthermore the L3 bud also surpassed the L1 bud in growth at 15DAP. This data supports that the *Sorghum bicolor* L1 bud initiates and goes dormant much like its relative maize B73. Because domesticated *Sorghum* does grow a few tillers (unlike maize B73 which grows none) (Bruns and Horrocks 1984, Kim, Luquet et al. 2010), I hypothesize that these tillers originate from the buds in the L2 and L3 which surpasses the growth of the L1. After observing *Sorghum*, a close relative of maize, I wanted to see if a more distant relative of maize would behave similarly with bud initiation and dormancy at the L1 bud.

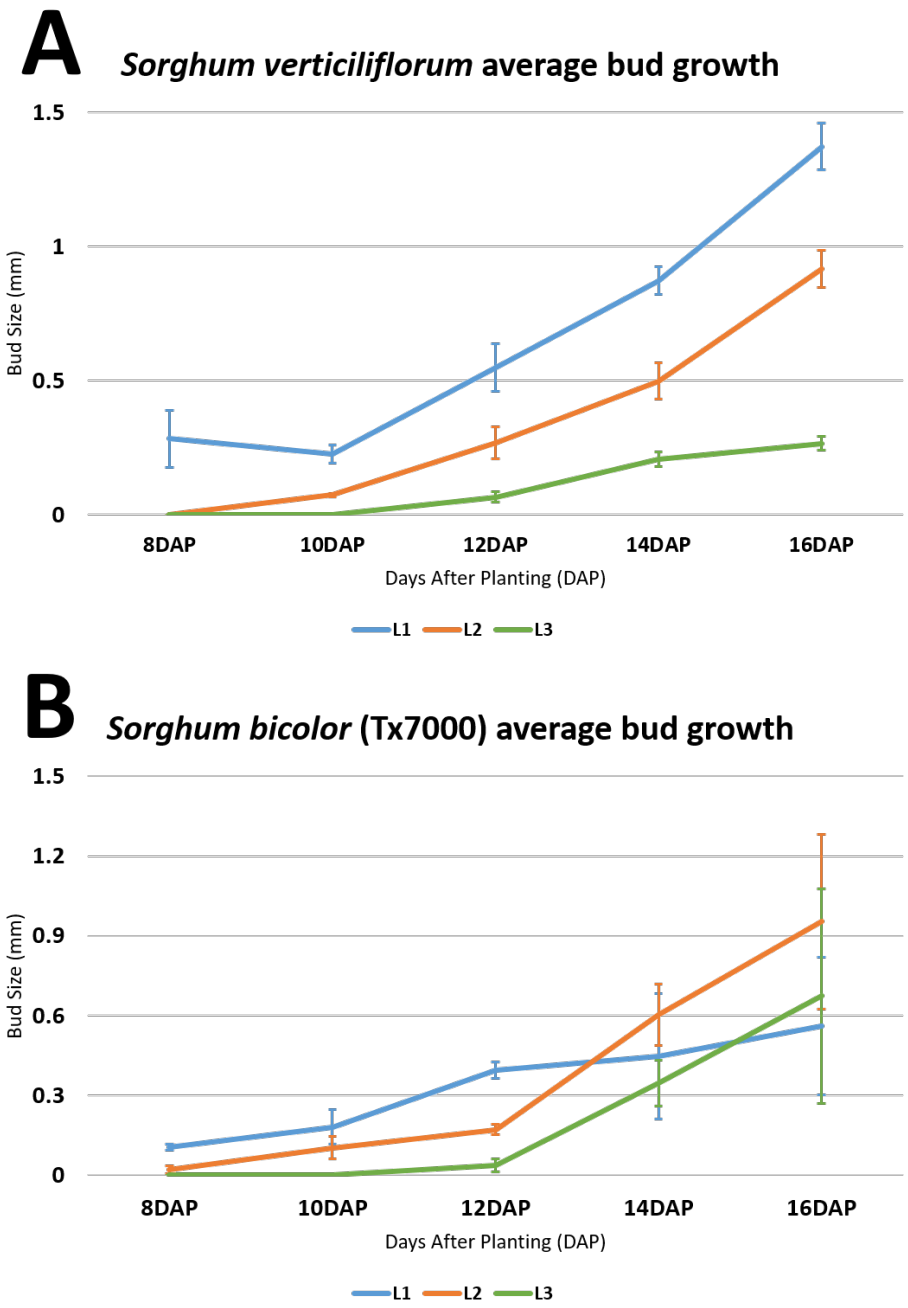

Figure 5- Axillary bud growth in wild and domesticated *Sorghum*. (A) The growth of tiller buds in the axil of leaf 1 (L1), leaf 2 (L2), and leaf 3 (L3) in wild *Sorghum* verticiliflorum from 8 to 16 days after planting (DAP). The L1 bud grows the largest in the 16 day period following with the L2 and then the L3 bud. The y-axis is set to 1.5mm (B) The growth of tiller buds in the axil of L1, L2, and L3 in *Sorghum bicolor* up to 16DAP. The L2 bud outgrows the L1 bud around 13DAP and the L3 follows same growth curve as L2, surpassing the L1 bud at 15 DAP. The y-axis is set to 1.5mm.

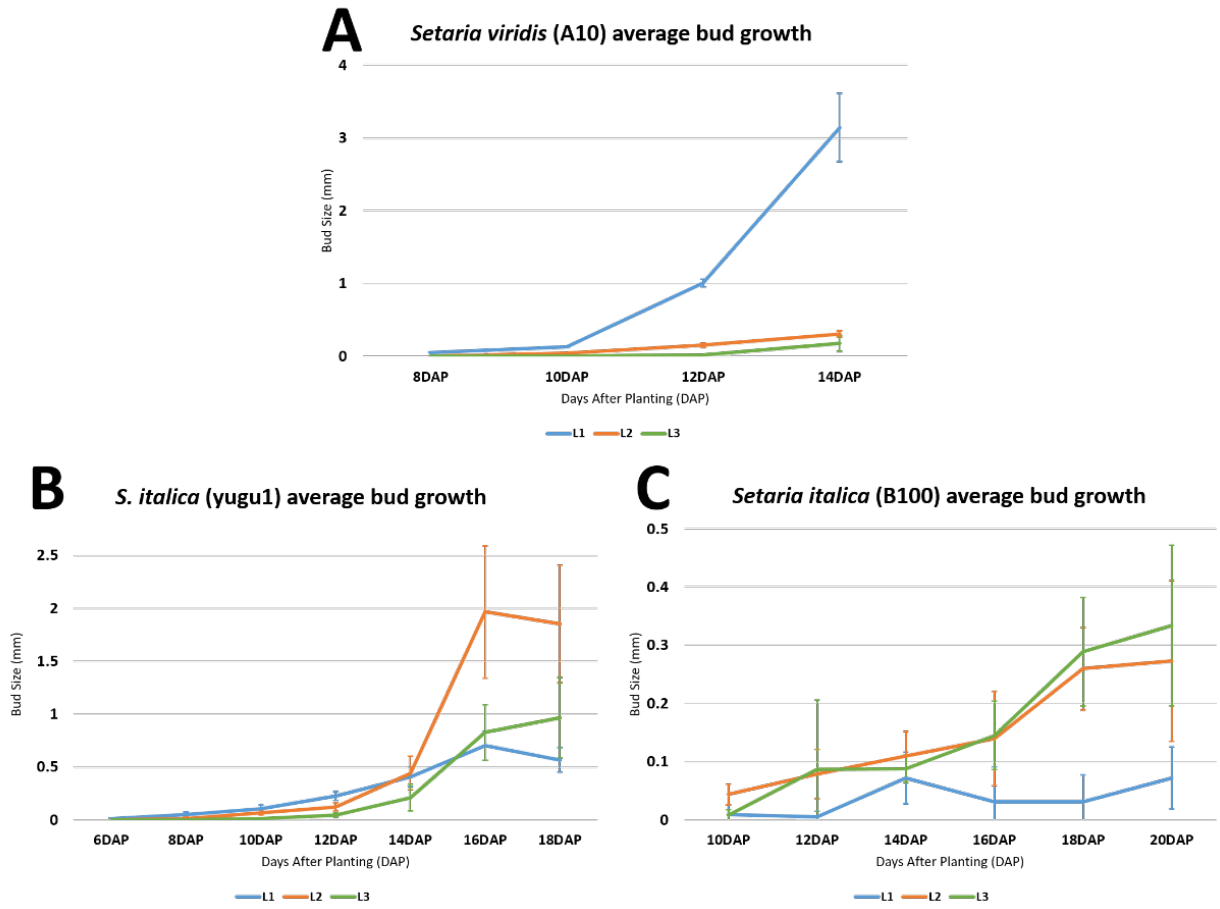

Figure 6- Measurements of axillary bud growth in wild and domesticated *Setaria*. (A) Wild type *S. viridis* (A10) growth from 8DAP through 14DAP and shows the L1 bud grows rapidly into a tiller the first 14 days after planting (DAP) while the L2 and L3 buds are still developing. The x-axis here is set to 4mm. (B) Domesticated *S. italica* (yugu1) bud growth from 6DAP through 18DAP. L2 axillary bud shows significant growth towards tillering the first 18DAP and at 16DAP is about half the size of the A10 L1 bud at 14DAP. The x-axis here is set to 2.5mm. (C) Domesticated *S. italica* (B100) bud growth from 10DAP through 20DAP. L2 and L3 axillary buds grow larger than the L1 bud after 20DAP but it is noted that these buds are significantly smaller than A10 and yugu1. This is shown with the x-axis set at 0.5mm.

Setaria's tiller bud growth shows differences when compared to maize and Sorghum

In order to compare bud growth between maize, *Sorghum* and *Setaria*, measurements were taken in wild type *Setaria viridis* (A10) from 8DAP-14DAP while measurements for domesticated *Setaria italica* (B100 and yugu1) were taken from 6DAP-18 or 20DAP (see Figure 6). Wild *Setaria viridis* has a rapid lifespan (Li and Brutnell 2011) and grows many tillers, and bud measurements reflected this, showing large buds in a short time span. L1 axillary buds exceeded the rate and size of every other L1 bud measured in wild Panicoid cereals. The L2 and L3 buds grew steadily but smaller than the L1 buds. Similar to *Sorghum*, *Setaria italica* (yugu1) also showed the L2 and L3 buds outgrowing the L1 bud suggesting that the few tillers of *Setaria italica* come from those buds and not the L1 bud. *Setaria italica* (B100) was different from *Setaria italica* (yugu1) in that there was not always a L1 bud which initiated. Because of this lack of consistency only those L1 buds that initiated were measured (see Figure 6c). It should be noted that the bud suppression differences between *Setaria italica* (B100) and *Setaria italica* (yugu1) are different. For example, *Setaria italica* (B100) fails to initiate a bud, whereas *Setaria italica* (yugu1) initiates L1 buds which then go dormant. I hypothesize that the network between these lines involves different genes to produce the same result in suppressing tiller outgrowth because the morphology differs. I observed that *Setaria italica* (B100) did not always produce a mature bud, but at what point the bud was suppressed was unclear.

ANALYZING TILLER FREQUENCY, TYPE, QUANTITY AND POSITION IN *SETARIA*

Knowing that there are 12 tillers that still grow from domesticated *Setaria*, I wanted to determine leaf nodes where those tillers originate. To explore this question further, I did a statistical analysis comparing *Setaria viridis* (A10), *Setaria italica* (B100), and *Setaria* italica (yugu1) to determine tiller type, frequency, and location.

Bud frequency counts show that buds do not always initiate in Setaria italica (B100)

From tiller bud measurements I knew *Setaria italica* (yugu1) and *Setaria italica* (B100) behave differently in their suppression of tiller growth. My next objective was to understand how this suppression differed by taking bud frequency counts in *Setaria*. It was obvious that *Setaria italica* (B100) L1 nodes had few buds, but how often those buds occurred was still unknown. Quantifying bud frequency would help us better understand tiller suppression, and therefore I counted buds in *Setaria viridis* (A10), and both strains of *Setaria italica* previously mentioned (see Table 1).

I saw that wild *Setaria viridis* (A10) always produced a bud under the first three leaves. *Setaria italica* (yugu1) consistently produced a bud under the first 3 leaves as well. The measurements in *Setaria italica* (yugu1) show that the first bud went dormant after initiation but a bud was always present (see Figure 6). In *Setaria italica* (B100), a bud only showed up under the first leaf 12.5% of the time, 85% at L2 and 91% in L3. Take note that the L1 numbers include both fully mature buds (12.5%) while 71% includes both mature and bud like structures present which were frequently under developed. I will go into further detail of the range of under-developed initiated buds in the section on Scanning Electron Microscopy.

From the measurements and the frequency counts it was clear that L1 buds in domesticated *Setaria italica* either went dormant (yugu1) or failed to initiate fully mature buds (B100). With this data, I wanted to further understand tiller suppression by determining the nodes at which tillers originate, the types of tillers which each strain produced, and the average number of tillers per strain.

*Table 1- Bud frequency in wild and domesticated Setaria. The frequency of tiller axillary buds occurring in Leaf 1 (L1), Leaf 2 (L2), and Leaf 3 (L3) of Setaria viridis (A10), Setaria italica (yugu1) and Setaria italica (B100). * B100 shows 12.5% of mature buds with 71% of observed buds (including mutated buds).*

	Setaria viridis (A10) Wild	*Setaria italica* (yugu1) Domesticated	*Setaria italica* (B100) Domesticated
Leaf 1 (L1)	100%	100%	12.5% *(71%)
Leaf 2 (L2)	100%	100%	85%
Leaf 3 (L3)	100%	100%	91%

Setaria tiller origin, position, and tiller type

To understand the differences in how tillers grow between wild and the two strains of domesticated *Setaria*, I did a statistical analysis of tiller position, type (primary, secondary or auxiliary), and number of tillers per node (see Table 2). To do so I grew out 30 individuals in each *Setaria viridis* (A10), *Setaria italica* (yugu1) and *Setaria italica* (B100). Each leaf per individual was marked with the leaf number according to when it emerged. All branches which originate from the first leaf axil through the fourth leaf axil were considered tiller branches, and all branches which originate from leaf five or higher are considered aerial branches. When a tiller grew it was marked according to the node of origin. *Setaria viridis* (A10) grew primary, secondary, and auxiliary branches (see Figure 7) while *Setaria italica* (yugu1 and B100) only grew primary branches (see Figure 8). Here I define an auxiliary tillers as one that originates from the same node that a primary tiller originates. Secondary tillers are defined as lateral tillers growing out of primary branches/tillers (Counce, Siebenmorgen et al. 1996, Moulia, Loup et al. 1999).

The number of tillers between wild and domesticated *Setaria* is distinct, confirming previous research that domestication decreases tiller number (Doebley and Stec 1991, Doust, Devos et al. 2004). Based on these statistics I found that wild and domesticated *Setaria* (*Setaria viridis* vs. both lines of *Setaria italica* in this study) differed in that wild *Setaria* had tillers starting to emerge at the 1st leaf (L1) and continued from there, whereas domesticated *Setaria* had all of its tillers originate from the 2nd leaf (L2) or higher. Wild *Setaria* also had secondary and auxiliary tillers while domesticated *Setaria* only had primary tillers. This is evidence that domestication may have not only suppressed tillers at the L1 node but also secondary tiller growth (Table 2).

Table 2- A statistical analysis of Setaria tiller growth. Setaria was grown out to note tiller position, type (primary, secondary or auxiliary), and number of tillers per node. The following table shows difference between wild Setaria viridis and Setaria italica, as well as differences between domesticated Setaria italica lines.

Tiller Characteristic	*Setaria viridis* (A10)	*Setaria italica* (B100)	*Setaria italica* (yugu1)
Total Branches	286	32	58
Total Aerial Branches	44/286 (15.4%)	0	0
Total Tiller Branches	242/286 (84.6%)	32/32 (100%)	58/58 (100%)
1^{o} tillers out of all tiller branches	121/242 (50%)	32/32 (100%)	58/58 (100%)
2^{o} tillers out of all tiller branches	121/242 (50%)	0	0
Auxiliary tillers (a type of 1^{o} tiller)	6/121 (4.95%)	0	0
Tillers with inflorescences	181/242 (63.2%)	0	7/58 (12.1%)
Plants which grew tiller branches	30/30 (100%)	22/30 (73.3%)	(30/30)100%
Plants with only one 1^{o} tiller	0	12/22 (54.5%)	7/30 (23.3%)
Plants with two 1^{o} tillers	0	10/22 (45.5%)	18/30 (60%)
Plants with three or more 1^{o} tillers	(30/30) (100%)	0	5/30 (16.6%)
Percentage of tillers from L1 node	61/243 (25.1%)	0	0
Percentage of tillers from L2 node	68/243 (27.9%)	13/32 (40.6%)	17/58 (29.3%)
Percentage of tillers from L3 node	58/243 (23.8%)	18/32 (56.3%)	26/58 (44.8%)
Percentage of tillers from L4 node	56/243 (23.04%)	1/32 (3.1%)	15/58 (25.8%)
L1 tiller was first to initiate	21/30 (70%)	0	0
L2 tiller was first to initiate (when more than 1 tiller was present)	7/30 (23.3%)	5/10 (50%)	16/23 (69.6%)
L3 tiller was first to initiate (when more than one tiller was present)	2/30(6.6%)	5/10 (50%)	7/23 (30.4%)
L4 was first to initiate (when more than one tiller was present)	0	0	0
L2 tiller only tiller	---	4/12 (33.3%)	0/7 (0%)
L3 tiller only tiller	---	8/12 (66.6%)	3/7 (42.9%)
L4 only tiller	---	0	4/7 (57.1%)
L2-L3 combo when two tillers	---	9/10 (90%)	12/18 (66.6%)
L3-L4 combo when two tillers	---	1/10 (10%)	6/18 (33.3%)
Tillers grew in order (L1, L2, L3, L4)	23/30 (76.6%)	5/10 (50%)	22/23 (95.6%)
Plants with tillers alive at time of adulthood (stem inflorescence)	100%	0	66%

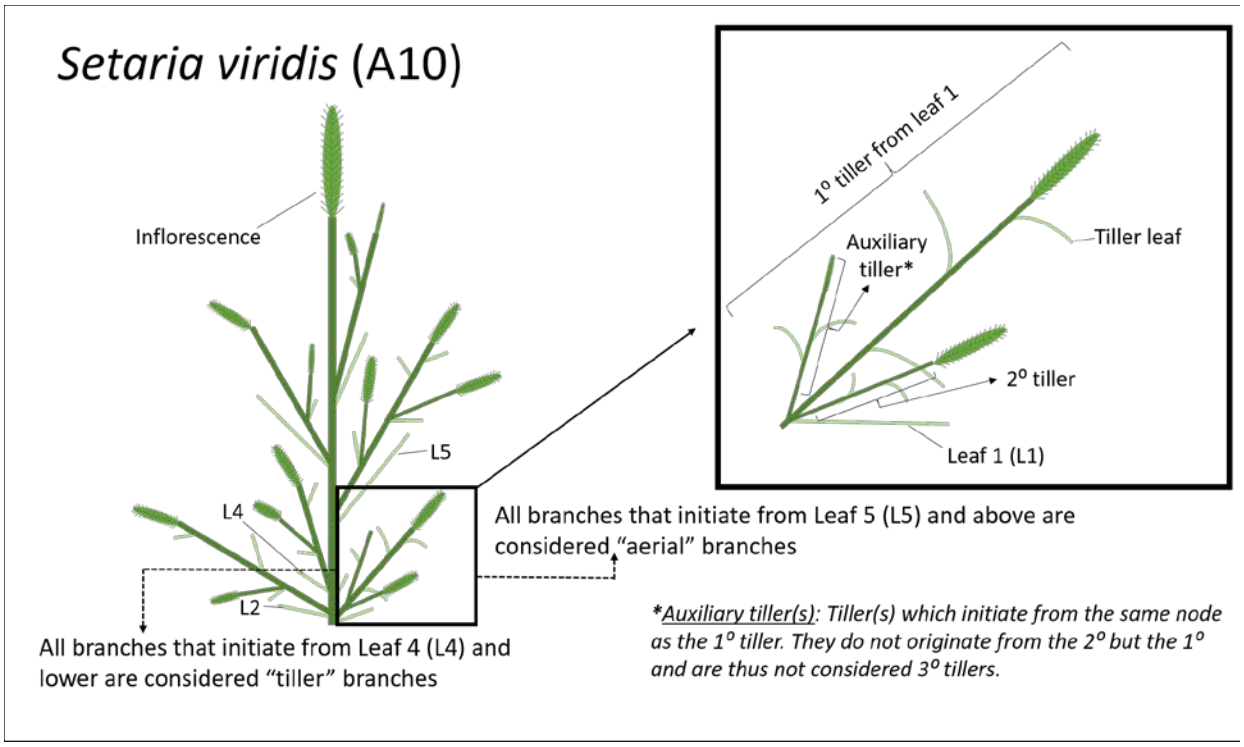

Figure 7- *Setaria viridis* (A10) demonstrating tiller branching. Primary (1˚) tillers originate in the axil between the leaf and the stem. Secondary tillers (2˚) originate between a leaf on the 1˚ tiller and the stem of the 1˚tiller. Auxiliary tillers originate on the original stem located beside the point of initiation and under the same leaf as the 1˚tiller. All branches which originate from Leaf 5 (L5) and higher are considered aerial branches in this experiment, not tiller branches.

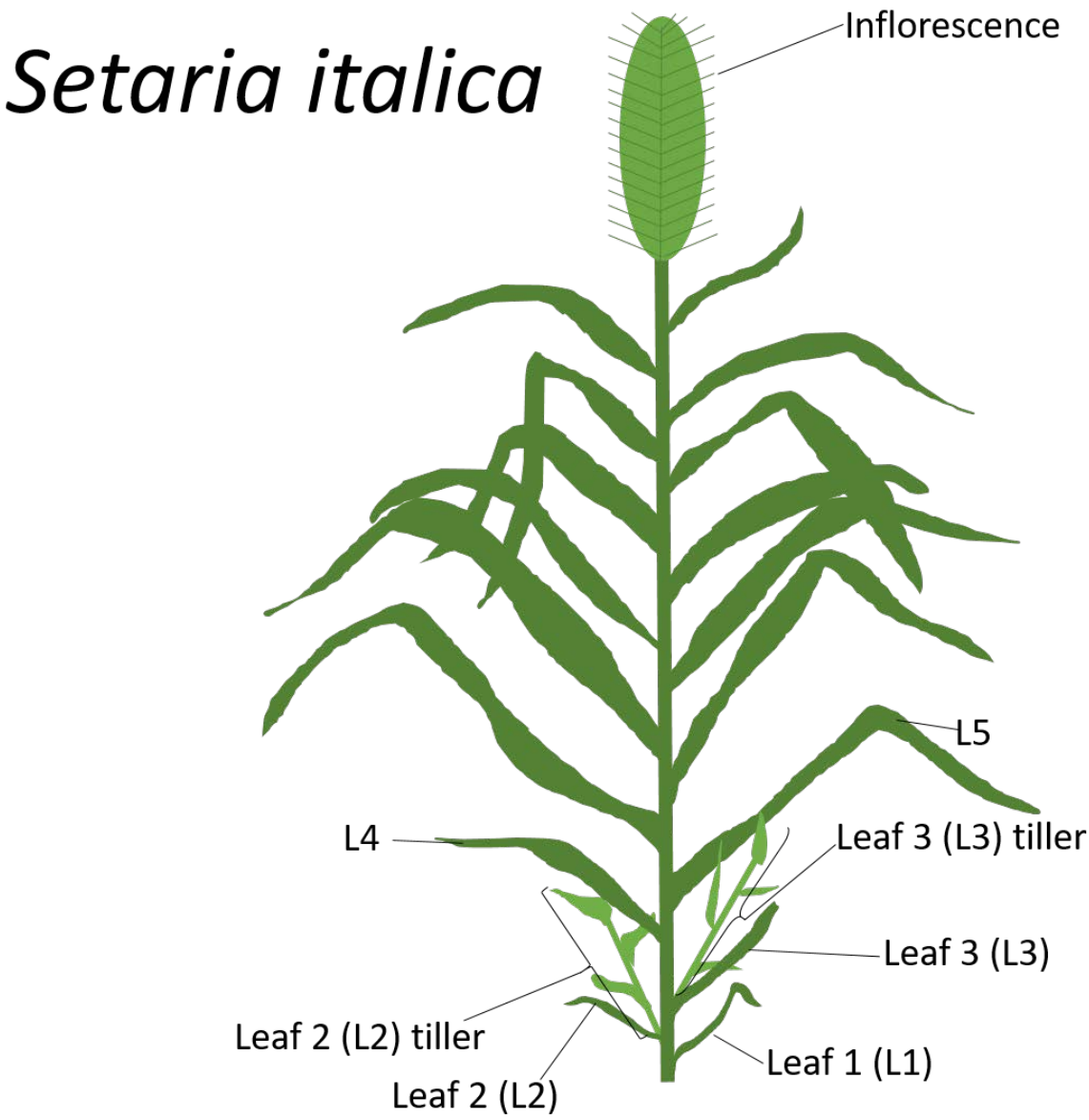

Figure 8 - *Setaria italica* demonstrating tiller branching. All branches which initiated at the leaf 4 (L4) node and below were counted as "tiller branches" all other branches from L5 and higher were counted as "aerial branches" in this experiment. Unlike wild *Setaria viridis*, domesticated *Setaria italica* did not grow secondary or auxiliary tillers.

Within domesticated *Setaria* lines (yugu1 vs. B100) I found that yugu1 was more likely to have 2 tillers whereas B100 was more likely to only grow 1 tiller. When only 1 tiller emerged from a plant in each of the domesticated lines, it was more likely that in yugu1 it came from the 4th leaf and never from the 2nd leaf, while in B100 it came from the 3rd leaf and never originated from the 4th leaf. Again, it should be noted that this data is from the specific conditions in which these plants were grown in the greenhouse and a change of environment in other studies may vary these results (see materials and methods '*Setaria* analysis of tiller type, position and quantity' for precise growing conditions). These differences between *Setaria italica* lines could be due to diversity in how tiller suppression is regulated.

In yugu1 tillers emerged in order of leaves (tiller emerged from L2 first, L3 next, etc.) only 50% of the time whereas in B100 tillers emerged sequentially 95.6% of the time. Lastly, when both lines reached maturity (the main inflorescence started to flower), yugu1 had live tillers which also grew inflorescences whereas B100 had no live tillers at the time of stem inflorescence (see Table 2). This is evidence that tiller suppression varies between these two lines.

With these differences it is not only clear that wild *Setaria* is different from domesticated *Setaria*, but that *Setaria italica* lines also shows diversity between strains. The means in which tiller suppression occurs may not only change between grains of the Poaceae family or the Andropogoneae tribe but furthermore may change even within specific species of domesticated grains.

LOOKING AT *SETARIA* BUD INITIATION THROUGH ELECTRON MICROSCOPY

In order to obtain further data on the initiation or lack of initiation of B100 L1 buds. *Setaria* bud counts showed that there was not always a mature bud, and sometimes there was an abnormal bud, but it was unclear under a light microscope if there was even a small amount of bud cells which had initiated or what an "abnormal" bud may look like morphologically. To answer this question, I decided to explore bud initiation using an electron microscope.

Scanning Electron Microscopy (SEM) images were taken of *Setaria viridis* (A10) L1 axillary buds (Figure 9). Stages starting from 10 DAP through 16DAP are included and these images show the progression of axillary bud growth. As shown and as is typical in axillary buds, the meristem initiates followed by growth of growing primordial leaves (Benkova, Michniewicz et al. 2003, Masiero, Li et al. 2004, Bell 2008) that eventually surround the meristem. These buds continue to elongate during a phyllochron, or time interval between the appearance of leaf tips (Klepper, Rickman et al. 1982), to form a tiller.

SEMs were also taken in B100, where L1 axils from no bud initiation to fully mature bud (Figure 10). Here fully mature bud refers to an axillary bud which contains a minimum of both a meristem and leaf primordia.

The first image shown (Figure 10a) is the L1 node where an axillary bud is expected grow, but shows no evidence of initiation. In the following images (Figure 10b-d) at 18DAP, 8DAP, and 12DAP buds have initiated but not grown to full maturity. Also imaged is a pair of underdeveloped axillary and auxiliary buds at 18DAP (Figure 10e). These buds are abnormal because the primordial leaves seem to have fused with the surrounding tissue

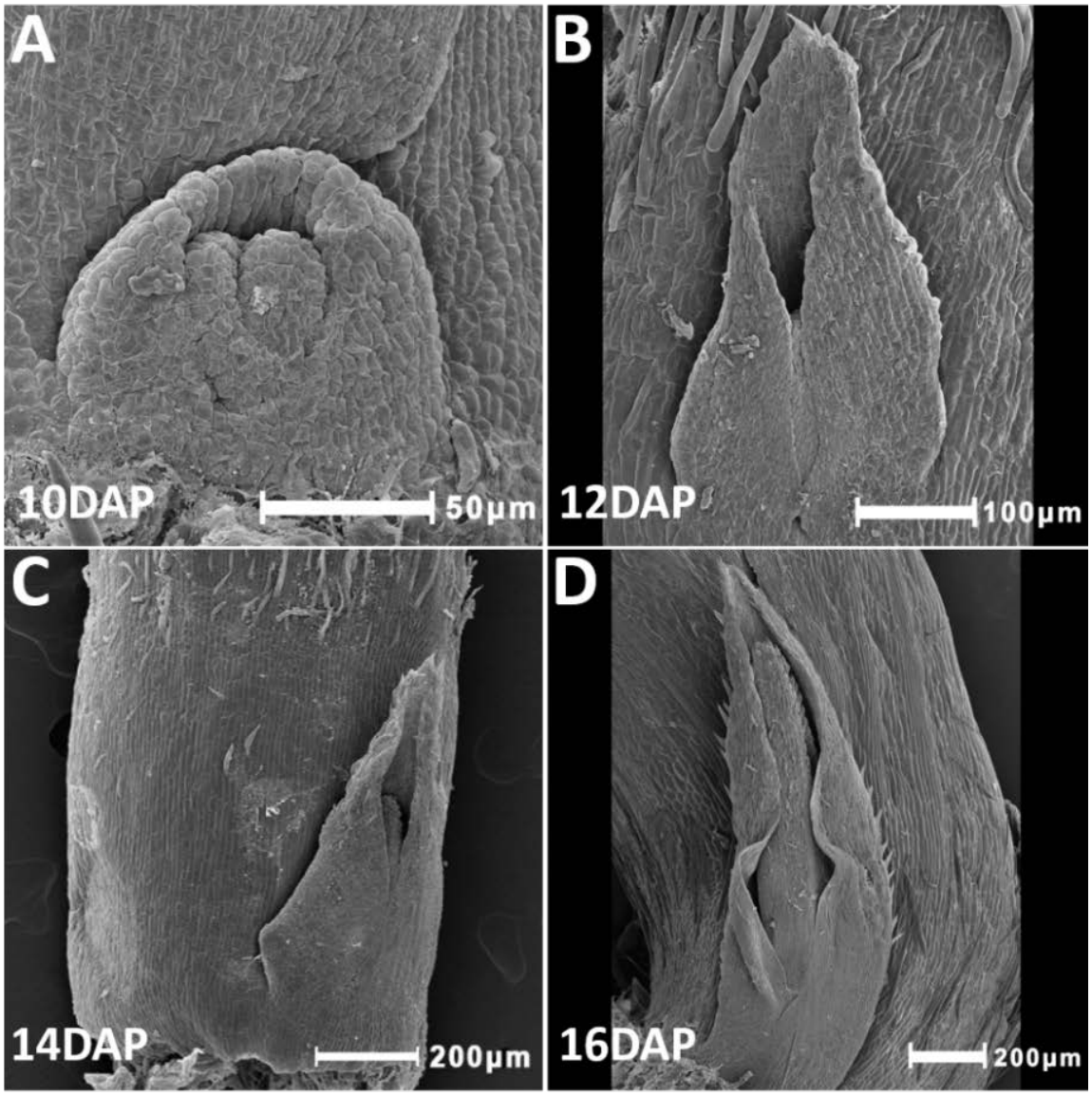

Figure 9- Scanning Electron Microscopy (SEM) images sequencing L1 bud growth in *Setaria viridis* (A10). (A) A10 bud at 10DAP where primordia are growing around exposed meristem. (B) 12DAP bud prophyll exceeds growth of meristem. (C) 14DAP prophyll and meristem continue growth (D) 16DAP bud growth pattern continued.

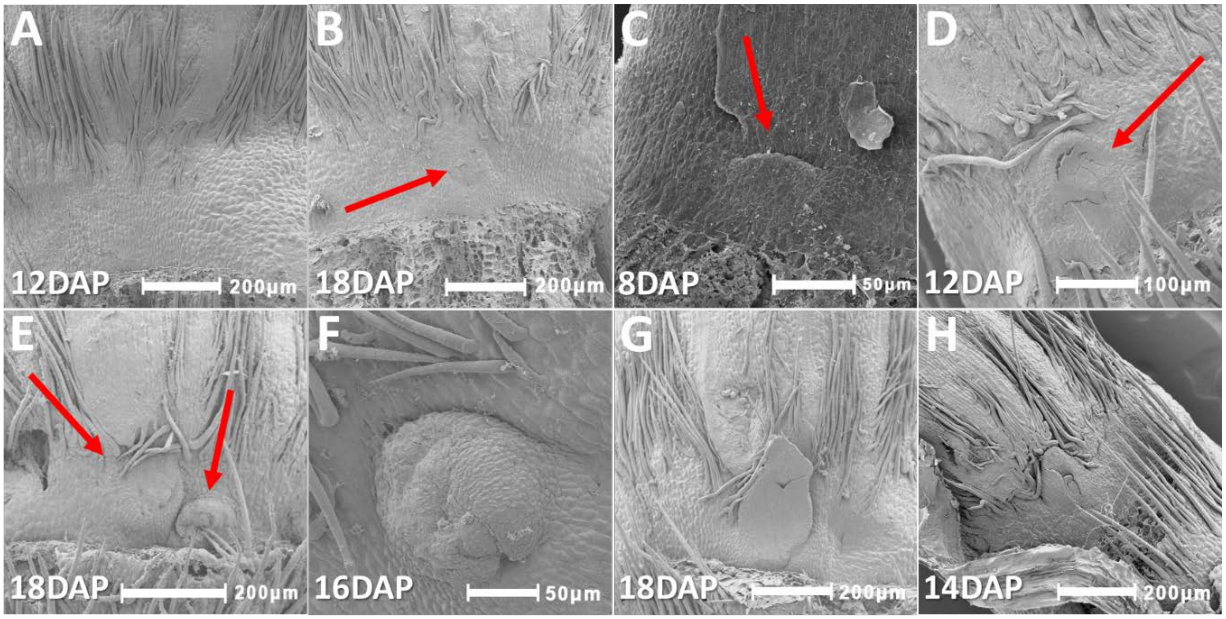

Figure 10- Scanning Electron Microscopy (SEM) developmental series in *Setaria italica* (B100) ranging from no bud initiation to full bud maturity. (A) At 12 days after planting (DAP) there is no sign of bud initiation. (B, C, D) At 18DAP, 8DAP and 12DAP (respectively) there is sign of bud initiation without full maturity. (E) At 18DAP there is sign of an axillary bud and auxiliary bud having initiated without fully maturing. (F-G) At 16DAP and 18DAP (respectively). Further signs of initiation and outgrowth without reaching full maturity. (H) Although small at 14DAP the bud appears to have grown to maturity. *Arrows point to initiated but immature buds that may be difficult to view.

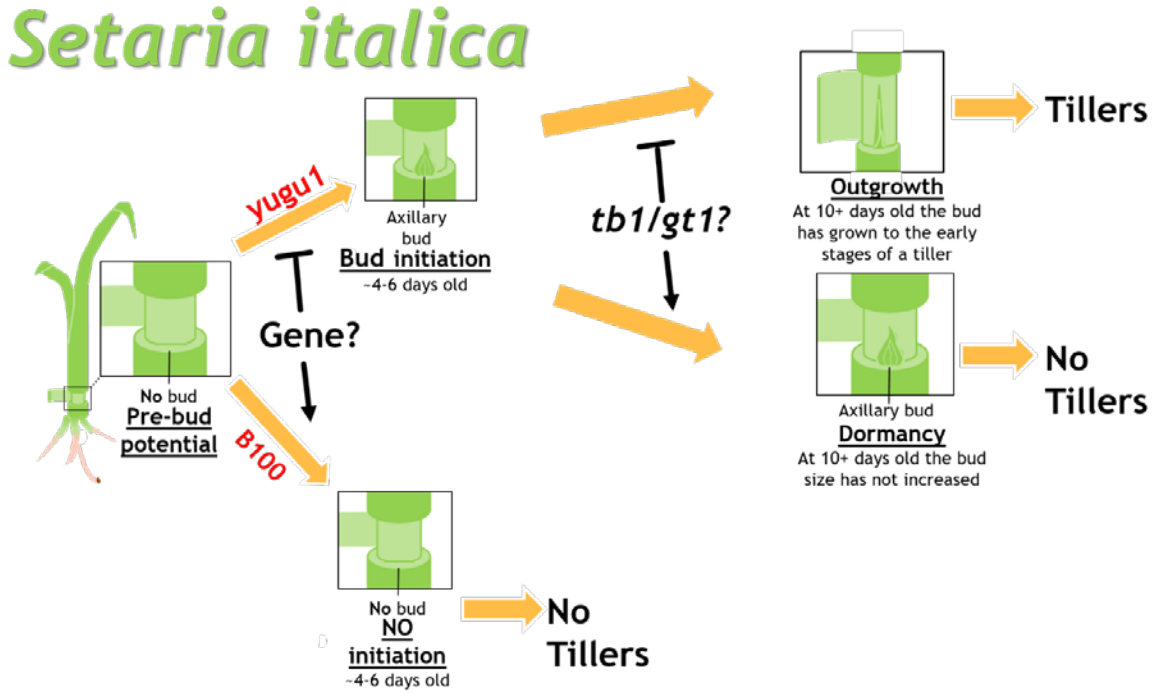

Figure 11- *Setaria italica* possible genetics pathways to tiller suppression. *Setaria italica* (yugu1) demonstrates a similar genetic pathway to maize and *Sorghum* in that buds initiate and then go into a state of dormancy. It is unknown if *tb1* and/or *gt1* play a role in dormancy. *Setaria italica* (B100) on the other hand follows a different pathway of tiller suppression which could involve another gene or genes that prevent bud initiation from occurring.

and it is unclear if there is a meristem present. Other L1 buds have initiated and grew yet larger than shown in previous B100 images but did not reached full maturity (Figure 10f-g). Finally there is a fully matured bud which could be small or dormant at 14DAP (Figure 10h) shows that relatively normal bud formation is possible.

B100 L1 axillary buds may not initiate at all, but even if it does there is no specific stage at which it could stop growing before it reaches maturity. Post-maturity buds were small, and it is still unclear if they went dormant by the time images were taken or were still growing at a slower rate. The data here shows that *Setaria italica* (B100) tiller suppression can occur in a continuum ranging between no bud initiation, failure to properly develop, or bud dormancy after maturity. Therefore, even among domesticated *Setaria* lines of tiller bud suppression mechanisms are diverse.

EXPLORING POTENTIAL TILLER SUPPRESSION PATHWAYS IN MAIZE

Because of the diversity seen in domesticated *Setaria* lines, I wanted to revisit maize to see if there were other means of tiller inhibition in other lines that I had not previously investigated.

Maize inbreds show diversity in bud frequency counts

Based on the diversity seen between domesticated lines of *Setaria italica* (yugu1 and B100), as well as the range of bud suppression seen within *Setaria italica* (B100) alone, I re-visited maize to determine if different inbred lines inhibit tiller buds by a dormancy mechanism. As with *Setaria*, I quantified bud frequency in teosinte, a variety of maize inbreds including A619, W22, Mo17, as well as B73/A619 hybrids (Table 3).

In teosinte, the L1 bud was present at 84%. The reduction of bud presence in teosinte is due to unknown factors. B73 buds were present in all 3 nodes 100% of the time. As was mentioned earlier, B73 buds undergo dormancy post-initiation as a means of inhibiting tiller outgrowth. To find out if all maize inbreds followed B73 in the process of initiation, maturity then dormancy, or inhibited buds by multiple mechanisms like I had found *Setaria*, I counted buds in A619, Mo17, W22 (Table 3). Other lines such as Mo17 had 80% L1 buds present, and W22 had 88% L1 buds present. All lines had 100% present buds in the L2 and L3 axil. From the data it is again clear that the means of tiller suppression is also diverse in maize as evidenced that these other lines do not follow B73 in the pattern of initiation, maturity then dormancy. Rather individuals may or may not initiate buds, while even initiated buds may be abnormal in growth. A619 showed the least amount of bud initiation at a frequency rate of 5%.

A619 exhibits a different tiller suppression pattern than B73

Because A619 buds had the lowest rate of initiation, I investigated the differences between B73/A619 by growing out a B73/A619 F_1 and F_2 population. In the F_1 note that buds were present in the L1 axil at a frequency of 100%, suggesting that bud initiation is dominant over a recessive trait of buds which fail to initiate. Furthermore, a B73/A619 F_2 population showed a ¼ ratio of abnormal or un-initiated buds. Thus my hypothesis was that ¼ of the population would have an abnormal bud phenotype. To test this hypothesis I performed a chi-square test. Out of 35 samples, 9 L1 axils were expected to have abnormal buds and there were 13 observed. The chi-square was 1.78 and with 1 degree of freedom the p-value was 0.182 thus meaning my null hypothesis (that there is no statistical significance) could be rejected. This does not mean the F2 will always segregate into a ¼ ratio, but rather my hypothesis can be accepted for now. In this case, it should be noted that the F_2 bud counts were not a simple present or

Table 3-Bud frequency counts in teosinte and maize inbred lines at 14DAP. Wild teosinte buds were counted as a control but the L1 bud is not 100% due to diversity in this particular line. B73 buds showed 100% frequency in all 3 counted buds. A619 had the smallest percentage of present L1 buds. A hybrid F_1 generation of B73 and A619 showed 100% in all 3 buds while the F_2 generation showed a ¼ ratio of buds no present. Maize inbreds Mo17, W22 and maize mutant tb1-sh (A619) showed L1 bud presence in an 8087% range. All L2 and L3 buds counted in all lines were present. This table shows diversity across maize inbred bud development.

	Teosinte	B73	A619	B73/A619 F_1	B73/A619 F_2	Mo17	W22	Tb1-sh
L1 Bud	30/32 (84%)	36/36 (100%)	2/36 (5%)	36/36 (100%)	51/70 (73%)	24/30 (80%)	30/34 (88%)	26/30 (87%)
L2 Bud	32/32 (100%)	36/36 (100%)	36/36 (100%)	36/36 (100%)	70/70 (100%)	30/30 (100%)	34/34 (100%)	30/30 (100%)
L3 Bud	32/32 (100%)	36/36 (100%)	36/36 (100%)	36/36 (100%)	70/70 (100%)	30/30 (100%)	34/34 (100%)	30/30 (100%)

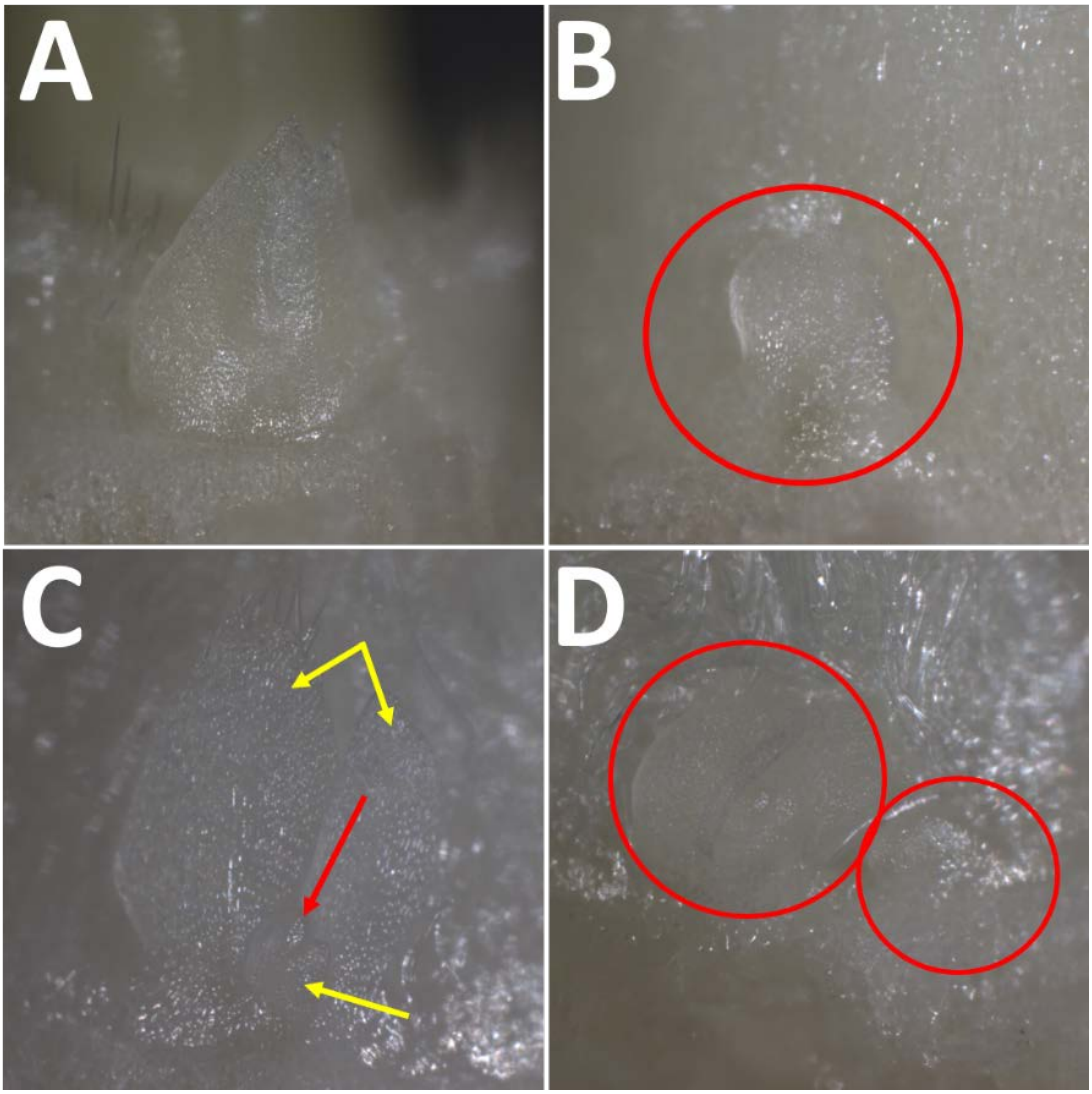

Figure 12-An example of B73/A619 hybrid F_2 abnormal buds at 14DAP. (A) Displays a normal bud at 14DAP. (B) Displays a bud with an unusual prophyll and in which it is unclear where the meristem is morphologically. (C) The red arrow point to a small meristem while the yellow arrows point to 2 sets of primordia; one primordia is small surrounding the meristem, while the other has grown largely at a much faster rate than the meristem, indicating an underdeveloped meristem in 14 days of growth. (D) Displays 2 buds (axillary and auxiliary) at the L1 site.

absent tally. Rather with the F_2 population, there was a variety of irregularities in which I categorized these buds as abnormal. This abnormal ¼ included: buds which did not initiate, unusually small buds, or buds which were abnormal in ways such as deformed primordial growth, underdeveloped meristems compared to a larger surrounding prophyll, and plants with 2 present buds in the L1 axil (Figure 12).

How tb1 behaves in A619

Besides the F_2 population, I wanted to test if mutating *tb1* in A619 would restore bud frequency. I used a weak *tb1* allele called *tb1-sh*, and the bud frequency was indeed restored to 87% frequency (Table 3). This indicates that *tb1* not only controls bud dormancy as has been previously discussed, but it also is involved in bud initiation, which has not been previously known.

Just as was seen in *Setaria*, tiller suppression in domestication maize was diverse. To further explore these lines I took measurements of maize inbred A619 as well as maize mutant *tb1-sh* (A619) (Figure 13).

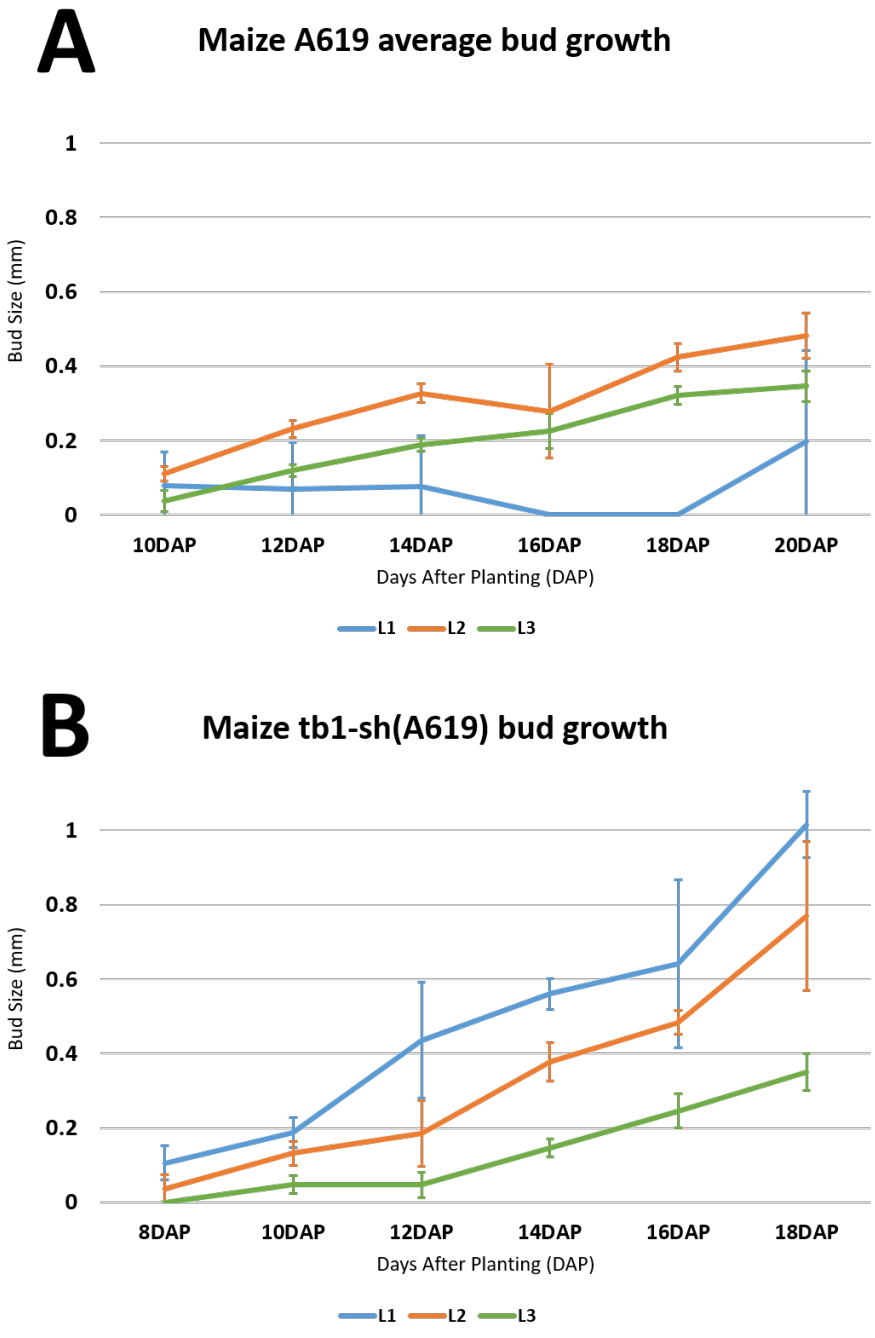

Figure 13 – Domesticated A619 bud growth compared to mutant *tb1-sh* (A619) bud growth. (A) Domesticated A619 bud growth from 10DAP through 20DAP. The bud under the first leaf node grows few to no buds, while buds from L2 and L3 grow steadily. The graph decreases at 16 and 18 DAP from the lack of present buds to measure upon randomly selected individuals. The y-axis is set to 0.6mm. (B) Maize *tb1-sh* (A619) bud growth from 8DAP through 18DAP shows steady growth. The L1 bud grows the fastest and largest with the L2 bud growing the second fastest and largest, and L3 bud growing slowest and smallest. The y-axis is set to 1mm.

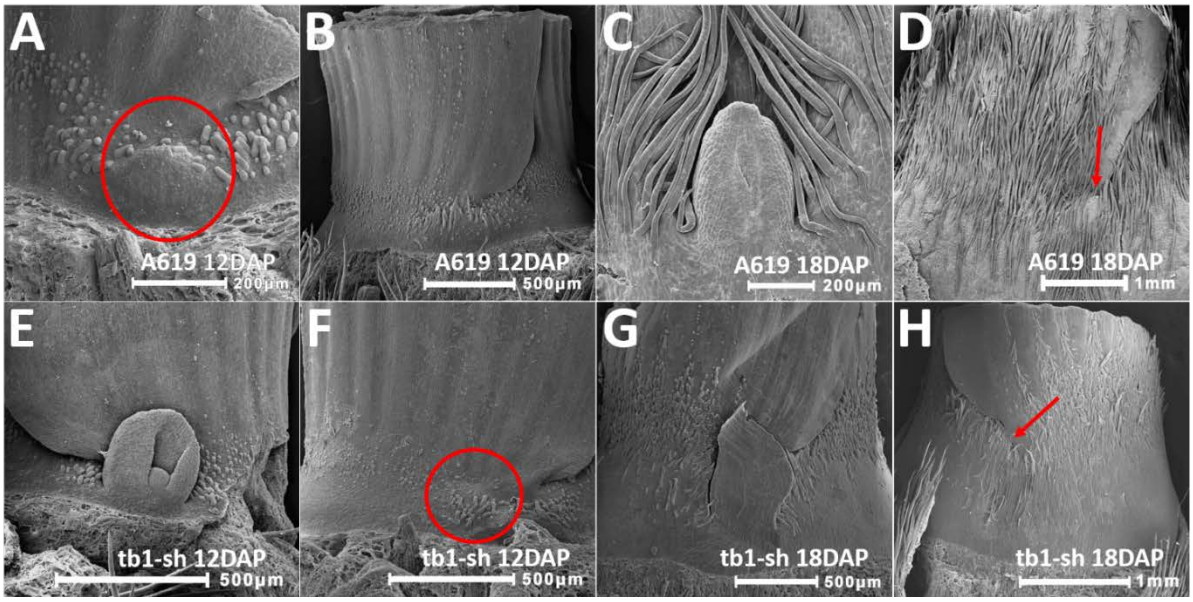

Figure 14 – Scanning Electron Microscopy (SEM) images of maize inbred A619 L1 buds. (A) A619 at 12DAP showing possible bud initiation at L1. (B) A619 at 12DAP without any sign of bud initiation. (C) A619 at 18DAP showing present tiller bud. (D) A619 at 18DAP showing no sign of bud initiation; red arrow indicates place of leaf attachment.

From the measurements of these maize lines it should be noted that because A619 did not always have a bud present at the L1, only buds which initiated were measured. At 16DAP and 18DAP there were no buds to be measured from the samples randomly selected which is why the growth drops to zero (Figure 13a). It is clear from this graph that the L2 and L3 are present and growing. I expect that the growth in the L2 and L3 eventually goes dormant after 18DAP. As mentioned previously, *tb1* is necessary for bud initiation in A619, in order to test its role in bud dormancy so I measured bud growth *in tb1-sh* (Figure 13b). The results demonstrated there was no dormancy in *tb1-sh* as we see continual growth in the L1, L2, and L3 buds. Thus *tb1* is required for both bud initiation and dormancy in A619. It is still unclear if A619 did initiate a bud, would it follow the pattern of B73 in initiation to maturity to dormancy. A further question was if restoration of bud growth in the *tb1-sh* mutant followed the *Setaria italica* (B100) pattern, or if it would look like *tb1-ref* did in its uninhibited growth. To look deeper into bud initiation or lack of initiation at the L1 axis, I took scanning electron microscopy (SEM) images of A619 and *tb1-sh* (A619) to identify further details of bud morphology.

Taking a deeper look at A619 and tb1-sh through SEMs

In order to more clearly understand how tiller buds were suppressed and to what degree the *tb1-sh* mutant restored bud growth, we did a SEM analysis of L1 buds at 12DAP and 18DAP in these lines (Figure 14).

Imaged are examples of A619 with a bud and without a bud at 12DAP (Figure 14a-b) and 18DAP (Figure 14c-d), as well as *tb1-sh* (A619) with and without buds at 12DAP (Figure14e-f) and 18DAP (Figure 14g-h). From the images note that the pattern of A619 follows a similar pattern to *Setaria italica* (B100) where buds do not always initiate, and if they did initiate the bud may be under developed. Furthermore, *tb1-sh* did not fully restore buds (as is evidenced

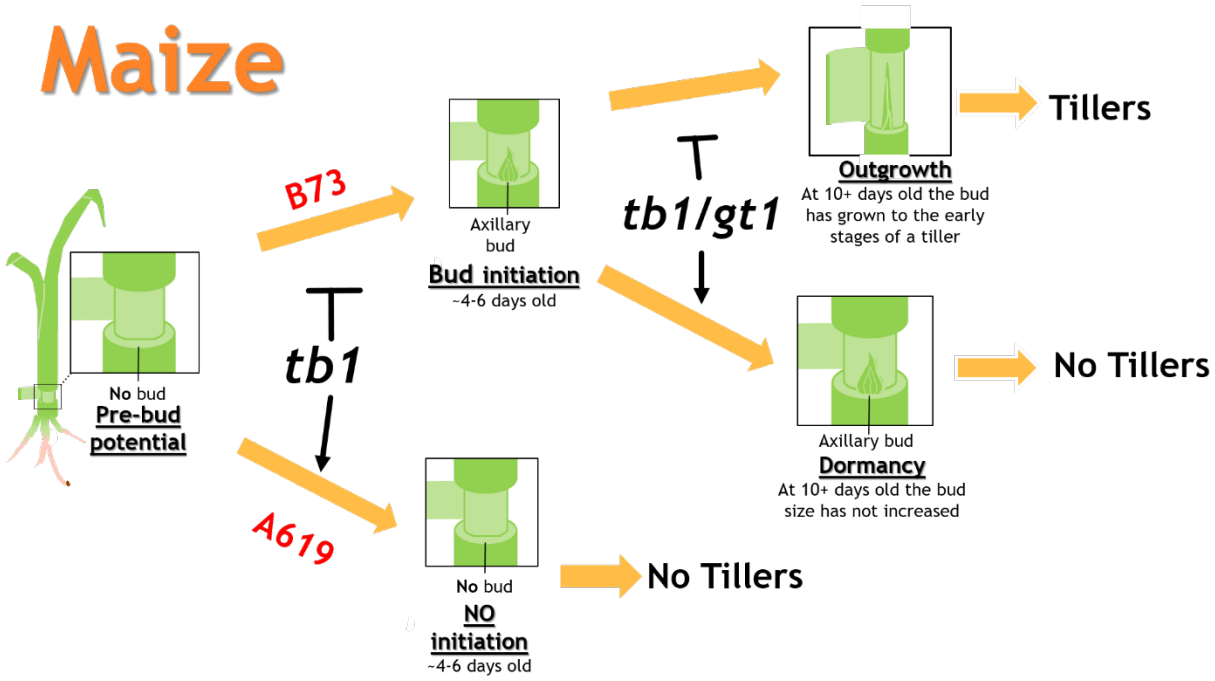

Figure 15 – A comprehensive overview of maize tiller inhibition. In maize B73, *tb1* is involved in bud initiation and later in dormancy. In A619 *tb1* is involved in keeping buds from initiating, thus suppressing tiller growth by a separate means.

with samples which did not grow buds). This is perhaps due to *tb1-sh* being a weaker allele than *tb1-ref*.

Separate maize genetic pathways for tiller inhibition

Based on the morphological data it is clear that maize also inhibits tiller bud growth in more than one way. Just like *Setaria italica* (B100) failed to initiate tiller buds, A619 also fails to initiate buds. Because *tb1-sh* restored bud growth, we also know *tb1* is involved in bud initiation. A comprehensive set of known pathways and genes is portrayed in Figure 15 where *tb1* aids B73 in bud initiation, and then dormancy occurs after *tb1* is expressed again. In A619 *tb1* prevents buds from initiating thus inhibiting tiller growth in a separate pathway.

DISCUSSION

Findings in this thesis include a clearer understanding of bud growth through measurements in different lines of maize, *Sorghum* and *Setaria*. Through taking measurements I learned some lines go dormant to suppress tiller growth, and that some lines do not always have an L1 bud present to measure. From SEM I found that buds do not always initiate in *Setaria italica* (B100) and bud development may range from no initiation through full bud maturity. Data from counting tiller frequency, position and tiller type in *Setaria* also showed that *Setaria viridis* (A10) grows primary, secondary and auxiliary tillers, while *Setaria italica* (yugu1 and B100) only grows primary tillers. Furthermore this same experiment proved that the leaves in which tillers originate differs between domesticated lines of *Setaria italica*. As two patterns of tiller suppression were discovered in *Setaria*, re-visiting maize tiller inhibition also showed two paths of tiller inhibition. When I explored *tb1* in A619 I found that in addition to dormancy, *tb1* is also involved in bud initiation. Finally, by making B73/A619 hybrids, the F_1 generation told me that bud initiation is dominant, and the F_2 generation told me there is a single locus which effects tiller suppression. Taking this work further could entail many potential research projects. Conclusions and ideas are discussed in this section.

An unknown locus controls bud initiation in A619

From the ¼ segregated B73/A619 F_2 generation it is clear that there is an unidentified locus involved in abnormal bud growth. In order to identify this locus, work could be done which includes mapping or fine mapping. Mapping would entail using a screening technique (through dissections) of plants with abnormal bud growth. DNA would need to be collected from the samples which show bud abnormalities, and RFLP markers can confirm differences in

homologous DNA (Ali and Chowdhury 2014, NCBI 2017) . Using QTL mapping and fine mapping would then identify the location of the locus (Wills, Whipple et al. 2013)

grassy tillers1 role in bud initiation is still uncertain

tb1 is frequently studied in B73 backgrounds and in my research, an allele of *tb1* was used to test bud restoration in A619. Because *tb1* in A619 hasn't been thoroughly researched it helped us discover another function of *tb1*, i.e. that *tb1* is involved in bud initiation. To test the role of *gt1* in A619, one could use CRISPR to knock out the gene and record if the mutant effects initiation.

grassy tillers1 role in Setaria is uncertain

Because of the morphological differences between the domesticated lines seen through measurements and SEM in *Setaria*, I wondered what the genotype differences might be. *Setaria italica* (yugu1) behaves similar to maize and *Sorghum* in that the L1 bud initiates and then goes dormant. *Setaria italica* (B100) however, may follow a different path where tiller bud initiation is affected. Furthermore, it is unclear if *tb1* and *gt1* play a role in *Setaria italica* (yugu1) buds going dormant post-initiation but it is a possibility. To explore this one could look for *tb1* and *gt1* orthologs using plant databases (such as phytozome.net), and with CRISPR knock out these genes to observe mutant phenotypes.

Gene activity in Setaria italica (B100) bud initiation and development is still unknown

From B100 we have learned that there is a continuum of whether or not buds will initiate, grow abnormally, or mature. The developmental stage at which this is blocked is still unclear i.e. if there is consistent initiation of a bud.

In maize, the genes *barren stalk1* (*ba1*) and *knotted-1* (*kn1*) have been known to mark bud initiation and bud maturity, respectively. For example, *ba1* activity appears before a bud initiates, thus marking that a bud will initiate (Ritter, Padilla et al. 2002). Post-bud maturity, *kn1* activity is seen to mark that a meristem has successfully matured (Kerstetter, LaudenciaChingcuanco et al. 1997)Because these genes mark the start and end process of axillary meristem development, using them in an *in situ* hybridization could offer more information as to the stage at which B100 bud development is blocked. If *ba1* activity is not present, then there are gene(s) involved in bud development upstream of *ba1*. If *ba1* activity is present but *kn1* activity is not, that will indicate development is blocked between *ba1* and *kn1*. If both *ba1* and *kn1* activity is present, there is an active meristem. *in situ* hybridizations could offer clearer data behind the genetic network in *Setaria italica* (B100).

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APPENDIX

Protocol – Pre-planting seed preparation for Setaria seeds

1. Count out the number of seeds desired for planting and place in a 1.5mL micro tube
2. Place tube in -80˚C freezer for 6072 hours
3. Plant seeds immediately.

Protocol – Pre-planting seed preparation for Teosinte seeds

1. Take teosinte seeds in left hand and using nail clippers clip 35mm off of each of the flat ends of the fruit case.
2. Using your fingernail, separate the two now divided pieces of the fruit case to reveal the seed.
3. Using a pipette add 5.4mL dH_2O and 400µL H_2O_2 to a 15mL Falcon tube.
4. Immerse number of desired seeds into solution and cover completely with aluminum foil.
5. Place in a dark room (I put mine in a closet and covered the tube with a Styrofoam box) for 24 hrs.
6. After 24-hrs take seeds and wash 4 times in H_2O (does not need to be distilled, we used water from the tap) for 5 minutes each wash.
7. Plant seeds immediately.

Protocol – Axillary bud measurements

Before measuring you'll want to have available a program which you can set a camera up to your microscope and then properly measure the length of what is on the stage of the microscope. In this case, and with these instructions we used the Leica Application Suite (LAS) version 3.6.0

1. Use 70% EtOH to clean off dissecting scope work space.
2. Collect 57 plants, remove each plant from the soil and cut roots off. You can take soil off of plants by gently massaging away the dirt. Place all plants in a container of water to keep them from drying out while dissections take place.
3. Remove one plant from the container of water and ensure that you rinse off as much dirt as possible from the lower part of the stem. This helps maintain a clean work space.
4. Holding the plant with forceps, use a razor to remove most of the roots and the whorl of leaves above the stem, leaving the stem and leaf nodes where the buds are located. The amount you remove should be enough to conveniently hold onto the stem while dissecting, but not so much that you risk breaking the stem: approximately 2 mm below the bud and 2mm above the bud.
5. Remove the coleoptile with the needle. You can do so by holding the base of the stem and making a cut vertically down center of the coleoptile (opposite from where the coleoptile margins overlap). You can then remove each side of the now two-piece coleoptile without breaking the stem.
6. Remove the first leaf layer either by unwinding it from the stem by gently twisting the leaf against the natural spiral of the leaf (using the needle), or from slicing it vertically down the leaf 90 degrees away from the region where the bud grows.

7. When the bud is exposed, turn the knob on the microscope from vision *VIS* to *DOC*. This will turn the camera on to the stage of the microscope.

The following steps outline how to use the software to ensure high quality in both photo and bud measurement with the Leica setup:

8. Move the exposure box (pink box) over a dark part of the screen, in order for the light to adjust properly.
9. On the right side of the screen use the top bottom on the left-hand panel to select “Basic Annotation” turned on.
10. Zoom the dissecting scope until the bud is an appropriate size, and note the magnification which can vary from 28 times (1mm500 um) the size. In the upper left corner of the LAS program, set the magnification according to the same magnification that the microscope is at. This will ensure the proper scale for bud measurement.
11. After focusing the image, at the bottom left corner of the LAS program, click “Acquire image”. It will automatically take you to the “browse” tab.
12. Rename the image according to its label.
13. In the “Basic Annotation” box, in the section labelled “Line” click the box that says “Show”. Then using the pull down tab to the right of it, select “Distance line”.
14. Using the mouse, click and drag a line on the screen from the top of the bud’s prophyll or meristem (whichever is higher) to the base. This will measure the bud as the line is drawn. Record measurements in a notebook or spreadsheet.
15. When finished, select the “Process” tab at the top of the screen to save the image. Once processed, the image is automatically saved and you are ready to click the “Acquire” tab to start a new bud measurement.

16. Continue measuring as many buds per leaf layer or per plant as desired (van Emden 2011). (Prevention 2010)

Protocol – DNA Extractions

Protocol from Yuguo Xiao

HT-multiple DNA sample extraction (2 x 96-well block extraction)

1. Take 4~6cm of maize young leaf as material (material 1x 4~6 cm; width: 1 cm, as young as possible), cut the material into **2cm** sections. Don't allow the material stick to the caps. Storage the material at -80°C freezer or
2. Freezer dry the samples at -50C overnight (>12 hours for young seedlings).
3. Grind the material to a fine powder (maize young leaf, 2min; mature leaf, 2~6 min, 1500rpm), Spin down the grinded power (3600rpm, 2min).
4. Add 400ul of CTAB extraction buffer with 0.2% 2-mercaptoethanol (add 100 µl 2-mercaptoethanol into 50ml CTAB buffer**).** Shake with a grinding machine (23min, 1000rpm). Put samples at 65C at least 30min and spin down the bubbles (3600rpm, 2min).
5. Add 300ul of chloroform/isoamy alcohol (24:1), and shake well at 750rpm for 12min.
6. Centrifuge at 3600rpm for 20min (1030min). Transfer 150+150µl upper layer (250~300ul) of aqueous phase to new 96 well blocks. Don't take any part of middle phase. Use an empty rack as mediator in order to move all samples efficiently.
7. Add 1 volume of isopropanol (250~300ul), mix and centrifuge to pellet the DNA at 3600rpm for >30min (in general, >1 hour. Stop and go out for lunch).
8. Remove the isopropanol. Wash the DNA pellets with 70% ethanol (300ul,>10min, 3600rpm).Spin down at 3600rpm, 15min. Remove the ethanol. Spin down the pellet (3600rpm, 2min).
9. Use vacuum to dry the DNA for 520min at high drying rate. Do not over dry your pellet

10. Add 50ul TE (1XT10E1, pH 8.0) containing 10ug/ml RNase A (RNase stock: 10mg/ml. add 100ul 10mg/ml RNase A stock into 10ml 1X TE). Spin down (3600rpm, 2min), Dissolve DNA and digest RNA for 2 hours at 37 °C or at 4C overnight.
11. Measure the concentration of a few DNA samples (In general, 750~3000ug/µl) out of 96 preps. Storage DNA samples at -20 °C.
12. For PCR, dilute DNA samples into 25 (525, or 550) ng /µl, then use 2ul as template for 10ul of PCRs.

Protocol–Scanning Electron Microscopy (SEM)

1. Fixation in 2% glutaraldehyde. Fix tissue for at least 2 hrs. up to months. Glutaraldehyde was made from a 10% solution purchased from Electron Microscopy Sciences (EMS) see *Working Solutions* below for further details.
2. Wash with buffer to remove fixative. Buffer: Na Cacodylate 0.3 molar. 6 washes, 510 min per wash.
3. Post fix (Osmium Tetroxide 2%). Use 1mL 0.6M Na Cacodylate with 1 mL Osmium Tetroxide (OsO_4). Let sit for no longer than 2 hrs.
4. Wash again—WELL (with distilled H_2O) 6 washes, 510 min each wash.
5. Put tissue in metal baskets using forceps.
6. Dehydrate samples using an EtOH or Acetone series (Start with lower concentrations: 10%, 30%, 50%, 70%, 95%, (100% x 3). 510 min each.
7. Critical point dryer (CPD) overnight (see details below).
8. Mount samples using a dissecting microscope and forceps. All tissue samples should be oriented in the same direction, as to not have to re-orient the microscope camera during use. The flat side of the samples should be placed downward with the bud on the opposite end exposed openly and directly in sight of the microscope beam.
9. Coat with 1nm layer AuPd on the sputter coater "Quorum Q150TES". Sputter set to "Timed sputter" which includes a speed of 40mA, 45 seconds and Au:Pd ratio 80:20.
10. Samples are ready for the microscope using the ESEM XL30FEI. Accelerating voltage range 10030Kv and operating pressure at high vacuum.

Working Solutions:

1. 0.6M Sodium Cacodylate—working buffer pH 7.27.4

   Sodium Cacodylate powder was purchased from Electron Microscopy Sciences Inc., Hatfield, Pennsylvania and mixed with distilled water. The pH of the solution was then adjusted to 7.27.4 using HCl or NaOH.

2. 0.3M Sodium Cacodylate—washing buffer pH 7.27.4

   Prepare by mixing 1:1 working buffer with distilled water.

3. 2% Glutaraldehyde: 10% glutaraldehyde was purchased from Electron Microscopy Sciences (EMS) and mixed with 0.06M Sodium Cacodylate buffer to make the 2% buffered glutaradehyde.

Protocol – Critical Point Dryer Protocol (CPD) for SEM preparation

Machine used was the "931.GL Supercritical autosamdri"

1. Check weight of CO_2 tank. Because a full tank is 50 lbs. and sitting on a scale in the lab, the administrator has adjusted the scale reading to inform him of when the gas is low. Once the tank has lost 25 lbs., it is no longer useful for the CPD. A negative value of at least -14lbs means it has sufficient CO_2 for a full run. When value is between -15lbs and -18lbs it is time to change the tank (you'll want a higher value to use). A value of -25 lbs. on the scale means it is empty.
2. Turn machine on
3. Turn gas tank on – open the valve until it stops.
4. Under main menu click "Stasis" (default appropriate)
5. Click "Next"
6. Should see/choose 4 cycles on stasis run with 2 hours per cycle
7. Press "Start"
8. Fill chamber with samples and EtOH or Acetone (depending on what your samples are in)
9. Secure chamber. Tighten the nuts like you would lug nuts on a car tire (star pattern, not in a circle). Snug each bolt down (again in a star pattern) so it doesn't leak and repeat the tightening pattern 23 times. Make it tight, so the bolts cannot move at all.
10. Press "cool". It will drop to 3°C.
11. Log your use into the notebook for billing. No need to worry about changing the tank, the administrator will do that and make note when it is ready to be changed.

12. When it says “Chamber is ready” press “Slow Fill”. It will ask if it is secure, press “confirm”. Then it will continue to CPD on its own.
13. The CPD will go through 4 two-hr. CO_2 stasis washes where the CO_2 will wash through the acetone/alcohol.
14. When finished check to make sure it is complete. Then turn off the gas, press “complete”, turn the machine off and take out samples.