# THE MU TRANSPOSONS OF ZEA MAYS AND THEIR USE IN DETERMINING GENE FUNCTION: CELLULOSE SYNTHASE-LIKE D GENES IN PLANT AND CELL DEVELOPMENT

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To my beautiful wife, Maggie, for her always-appreciated love, support, kindness and fascinating dinner-time conversations!

ACKNOWLEDGMENTS

I wholeheartedly thank my lab colleagues, past and present, for advice and friendship, especially Wayne Avigne, Brent O'Brien, Andrea Eveland, Li-Fen Huang, Christian Restrepo, Joe Collins, Maggie Paxson, Emeka Ibekwe, and Nana Ankumah. Additionally, I thank the founders of the UniformMu maize population, including Don McCarty, Karen Koch, Mark Settles, and Curt Hannah. I thank the members of my committee, which included John Davis, Don Huber, Karen Koch, Don McCarty, Gary Peter, and Wilfred Vermerris, all of whom have provided much-appreciated input and advice. I also acknowledge and thank the faculty and staff of the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR), including Byung-Ho Kang, Bill Farmerie, Karen Kelley, Kim Backer-Kelley, Neal Benson, David Moraga, and Savita Shankir. My fullest appreciation also goes out to my co-authors, including Gary Peter, Don McCarty, and Anne Sylvester, with whom I look forward to continued collaborations. I acknowledge and thank Bob Meeley for providing access to the Pioneer Trait Utility System in Corn (TUSC) maize population, as well as a wonderful opportunity to experience working at Pionner Hi-Bred International. I call special recognition to work done cooperatively with Christian Restrepo and Christy Gault, who contributed greatly to the characterizations of Mu transposons presented here. I acknowledge Sue Latshaw, Andrea Eveland, and Christain Restrepo for their invaluable help in developing and assisting in the 454-based sequencing experiments conducted in this work. I express my deepest appreciation for my family, who have always given me their love and support, including my parents, Ruthie, Charlie, Shannon, and Wayne, my brother and his wife, David and Keri Anne, and my wonderful bride, Maggie. Finally, I

want to thank my advisor, Karen Koch, for years of encouragement and motivation, and for being an excellent scientific mentor.

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# THE MU TRANSPOSONS OF ZEA MAYS AND THEIR USE IN DETERMINING GENE FUNCTION: CELLULOSE SYNTHASE-LIKE D GENES IN PLANT AND CELL DEVELOPMENT

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December 2010

Chair: Karen Koch
Major: Plant Molecular and Cellular Biology

The two-fold goal of work presented here was, first, to test hypotheses for biological roles of the enigmatic *Cellulose Synthase-Like D* (*CslD*) gene family, and second, to test key aspects of a global resource for such research by examining the extent, diversity, and evolution of the maize Mutator (Mu) transposable elements. The Mu transposons comprise one of the most active mutagenic systems in plant biology, often inserting into genic regions, where they disrupt gene function. Not only is the Mu system a powerful evolutionary force, but also a valuable tool for defining biological roles of genes. Work here reveals new aspects of Mu transposon evolution and behavior by defining positions and relatedness of Mu elements in inbreds of both maize and its wild ancestor, teosinte. Results also demonstrate the diversity within both maize and teosinte, and will aid interpretations of future genetic studies. The surprisingly diverse and numerous Mu12-like elements identified here will also aid expansion of maize functional genetic resources such as that of the UniformMu maize population, used here to investigate the *CslD* gene family of maize. The *CslD* genes comprise one

of the few subfamilies of the Cellulose Synthase superfamily for which biochemical activity remains unclear. The CSLD proteins have domains characteristic of β-linked, cell-wall polysaccharide synthases, and are expected to produce a cell-wall structural component such as cellulose or a hemicellulose backbone. Plants with Mu transposon insertions in *CslD5* were deficient in root hair elongation, indicating a role in tip-growing cells. Plants with insertions in *CslD1* had pleiotropic phenotypes that arose from defective cell division during leaf development. This was evident in fewer cells per leaf and defects in cell size and shape at the earliest stages of leaf development. Cell files were disrupted, cell shapes and sizes were abnormal, and cross-walls between newly-divided cells were incomplete. Collectively, these data define a previously unexpected role for a cell-wall biosynthetic gene, *CslD1*, in cell division, where its contribution involves some aspect of cell plate formation, a process fundamental to all of plant biology.

# CHAPTER 1
# INTRODUCTION

Cell walls provide protection and structural support for whole plants and individual cells. It is through the modification and biosynthesis of cell walls that plant cells grow and their ultimate shapes are determined. Cell walls are also critically important for many aspects of human life, from nutrition to raw materials and industrial feedstocks. They represent the most abundant renewable resource on the planet (Pauly and Keegstra, 2008), and have received much recent attention as sources of transportation fuel in the form of cellulosic ethanol (reviewed by Schubert, 2006 and Sticklen, 2008). Despite their central importance to plant biology and human needs, the manner in which plant cell walls are synthesized and regulated remains poorly understood.

In all plant cell walls, cellulose is the major load-bearing molecule. It is composed of linear β-(1,4)-linked D-glucan chains, and is produced at the plasma membrane via multi-subunit protein complexes called rosettes (Kimura et al., 1999; Delmer, 1999). Within rosettes, Cellulose Synthase (CESA) proteins operate as processive glycosyl transferases that catalyze formation of individual cellulose glucan chains (Pear et al., 1996; Arioli et al., 1998). Collective action of CESA proteins as subunits of multi-protein complexes (rosettes) result in large multi-chain cellulose microfibrils. These cellulose microfibrils are embedded in a matrix of crosslinking hemicelluloses (non-cellulosic polysaccharides composed of diverse other sugars), pectins, and cell-wall proteins (McCann et al., 1990; Carpita and Gibeaut, 1993; McCann and Roberts, 1994; Willats et al., 2001).

Hemicelluloses include the glucans, xylans, and mannans. The various forms of these polysaccharides are made up of at least fourteen different monosaccharides

joined by diverse glycosidic bonds (Scheible and Pauly, 2004). Hemicelluloses are typically highly substituted with various sugar sidechains, potentially associated with a multitude of biological functions yet to be identified (Coutinho et al, 2003). Assembly of these hemicelluloses requires a massive host of enzymes (Sheible and Pauly, 2004; Coutinho et al., 2003). Resulting structures indicate that any of them could interact with cellulose via non-covalent bonds, and they are thus considered likely modulators of cell wall structural properties (Carpita, 1996; Cosgrove, 2005). Hemicelluloses are normally produced in the Golgi apparatus (Delmer and Stone, 1988). In addition to their potential roles in moderating cell wall properties and growth dynamics, hemicelluloses can also serve as energy storage molecules, and commonly do so in developing seeds of diverse species. For example, nasturtium seeds store xyloglucan (Edwards et al., 1985), and galactomannans are common in legume seeds like guar and fenugreek (Reid, 1993; Dhugga et al., 2004). Finally, hemicelluloses, or fragments derived from them, can play major roles in intercellular signaling and in regulating responses to external stimuli (McDougall and Fry, 1990; Takeda et al., 2002; Pilling and Höfte, 2003).

Cell wall composition can vary markedly among plant species and between different tissues in a single plant (Popper and Fry, 2003; Lynch and Staehelin, 1995). One major taxonomic division within flowering plants lies between the commelenoid monocots (including maize, other grasses, bromeliads, and gingers), and non-commelonoid monocots (lilies, orchids, and most others). Commelenoids have a distinctive Type II cell wall, whereas other flowering plants have Type I cell walls (Harris and Hartley, 1980; Carpita and Gibeaut, 1993). This difference is characterized by major qualitative contrasts between the two types of primary cell wall (Carpita, 1996). In

Type I cell walls, the major polymer that interlinks cellulose microfibrils is xyloglucan (Hayashi et al., 1989; Redgwell and Selvendran, 1986), whereas in Type II cell walls, this role is filled by feruloylated glucuronoarabinoxylans (GAX) (Gubler et al., 1985; Carpita, 1996). Also, the Type I cell walls of cereals and other grasses (poaceae) contain abundant (1,3),(1,4) mixed-linkage β-D-glucans (Carpita, 1996; Smith and Harris, 1999), hypothesized to aid wall loosening during cell elongation by interacting with, and influencing properties of GAX molecules (Buckeridge et al., 2004). In addition, Type I cell-wall structure includes diverse and abundant pectins (Jarvis et al., 1988; Jarvis, 1994), compared to Type II cell walls, which instead, feature prominent phenolics such as hydroxycinnamates (Harris and Hartley, 1980; Iiyama et al., 1990; Rudall and Caddick, 1994).

Major qualitative differences in cell wall composition are also commonly observed between even closely related plant species. For instance, the relative proportions of cellulose, hemicelluloses, and lignin differ dramatically between rice straw and wheat straw (Lynd et al., 1999; Jin and Chen, 2007; Pauly and Keegstra, 2008). Additionally, cell wall composition can change radically during growth of individual tissues. In maize coleoptiles, for example, mixed-linkage glucans can accumulate to high levels during elongation (up to a third of total hemicelluloses), then drop to less than detectable quantities as the tissue matures (Meier and Reid, 1982; Carpita, 1984; Gibeaut and Carpita, 1993; Kim et al., 2000; Carpita et al., 2001; Derbyshire et al., 2007). Indeed, wall composition can vary around a single cell, such as in trichoblasts in Arabidopsis roots, where distinct cell-wall microdomains lead to specific areas with unique properties (Freshour et al., 1996). The wide range of plant cell wall compositions, growth-rates,

and chemical properties reveal the complex and highly-regulated nature of their biosynthesis (Zhong and Ye, 2007). With major differences between diverse cell walls, more than a single model species will be needed to fully elucidate the biosynthesis and function of this important plant cell component.

In addition to its position as one of the world's most agriculturally-important crop species, maize provides an excellent model species for genetic studies. Its genome is essentially diploid, its generation time is relatively short, the male and female flowers are easily isolated, and the large seeds are amenable to long-term storage. The amount of diversity represented within the various maize lines is extensive, and has been instrumental to developing the productivity and versatility of modern domesticated varieties (Duvick, 2002; Wei et al., 2007; Gore et al., 2009; Springer et al., 2009; Schnable et al., 2009). Despite the large size of its genome (approximately 2.3 gigabases with over 32,000 genes [Schnable et al., 2009]), genetic studies in maize have proven successful (McCarty et al., 1989; McCarty et al., 2005; McCarty and Meeley, 2009). The availability of numerous genetic resources, including the large Activator populations (Kolkman et al., 2005; Bai et al., 2007), the TILLING project (Targeting Induced Local Lesions IN Genomes) (Weil and Monde, 2007), the mapped recombinant inbred lines like the Intermated B73 x Mo17 (IBM) population (Lee et al., 2002), the Nested Association Mapping (NAM) lines (Yu et al., 2008), and various Mutator-based insertional mutation collections (see below), make maize an attractive species for genetic studies and a good candidate for a model species in the economically-important grasses.

Transposon mutagenesis in maize has been an invaluable tool for elucidating gene function, allowing roles for genes of interest to be examined individually in loss-of-function mutants (Walbot, 2000; Carpita and McCann, 2002; Brutnell, 2002; McCarty et al., 2005; Settles et al., 2007). Transposable elements (TEs) are DNA sequences that can physically change positions within the genome, or replicate themselves (and other TEs), generating copies for insertion elsewhere (Chandler, 1992). All together, transposon-derived sequences make up a large portion of most eukaryotic genomes, comprising about 85% of the maize genome (Schnable et al., 2009). Transposable elements are also a major driving force of genome restructuring and evolution (Wessler, 2001). When a TE inserts into or near a region of DNA that codes for an RNA, the sequence disruption typically (but not always) leads to loss of that RNA's function (Chandler, 1992), allowing researchers to elucidate gene functions by observing plants in which single gene disruptions have occurred (Brutnell , 2002). Transposon mutagenesis provides an avenue for dissecting complex and poorly-understood biological processes, such as the biosynthesis and regulation of plant cell walls, on a gene-by-gene basis.

# CHAPTER 2
# IDENTIFICATION AND CHARACTERIZATION OF THE MU TRANSPOSONS IN VARIOUS MAIZE INBREDS AND THEIR TEOSINTE ANCESTORS

## Introduction

Transposons were discovered in the 1940s by Barbara McClintock, who's studies in maize first defined the concept of small genetic "elements" that couls physically move (or transpose) within a genome (McClintock, 1947). These transposons could excise from one location and re-insert essentially anywhere in the DNA of an organism, leading to them being coined 'jumping genes' by the popular press. Transposons can also replicate during transposition and often lead to natural mutations when they insert into otherwise functional genes.

Following discovery of transposons, the Robertson's Mutator (Mu) transposable elements were identified as a particularly interesting system with high transposition rates that often led to unstable mutations (Robertson, 1978). Since then, Mu elements have become perhaps the most widely-utilized tool for genetic studies in maize (McCarty et al., 1989; McCarty et al., 2005; Settles et al., 2007). These elements make up a large, diverse family, characterized by highly-conserved, terminal inverted repeat (TIR) sequences about 215 nucleotides long, positioned at each termini and oriented in opposite directions (Brutnell, 2002). The TIR sequences are critical for transposon functionality, as they contain the binding sites for the transposase enzyme that catalyzes transposition (Raizada and Walbot, 2000; Lisch, 2002). When a Mu transposon inserts into a given site, a 9-bp target-site duplication (TSD) occurs, and when a Mu excises, it typically leaves this 9-bp TSD "footprint" (Barker et al., 1984; Schnable et al., 1989; Creese et al., 1995).

The transposase enzyme is coded for by genes contained in the autonomous Mu element in maize, MuDR, which, in addition to its TIRs, contains two genes, *mudrA* and *mudrB*, which code for enzymes MURA and MURB, thought to function together as a transposase (Chomet et al., 1991; Lisch, 2002). This transposase is considered to be responsible for the transposition of both autonomous and nonautonomous Mu elements. Nonautonomous Mu elements typically far outnumber autonomous ones (Dietrich et al., 2002; Liu et al., 2009). Nonautonomous elements contain conserved TIRs, but their internal sequences are typically highly diverse, non-functional fragments, apparently derived from captured host sequences (Lisch, 2002). The wide variety of Mu elements in maize have been grouped into 12 classes (Mu1 through Mu12) based on order of discovery and sequence similarity (Dietrich et al., 2002). The class originally designated as Mu9 is now known to include the autonomous MuDR element with its functional transposase-encoding genes (Hershberger et al., 1991; Walbot and Rudenko, 2002;). Previous data indicated that Mu's 1-9 represented the majority of transposition activity (Dietrich et al., 2002; Liu et al., 2009), and these have classically been used in transposon-tagging studies. The Mu1-9 classes also all have similar enough TIR sequences to be amenable to a single set of molecular tools (such as PCR primers), unlike the Mu10 and Mu12 classes.

The numbers and locations of Mu elements can vary widely among maize lines, changing with MuDR activity and genetic background. However, previous estimates have suggested around 50-100 Mu elements in stable maize inbreds (Liu et al., 2009). Mu elements have a high rate of forward mutation when an active MuDR is present, and commonly occur in very high copy numbers in those lines (Brutnell, 2002). Indeed, the

Mu transposons are the most active of all DNA transposons currently known in plants (Alleman and Freeling, 1986; Lisch, 2002). Mu transposons exhibit a trans-genetic mode of action (Lisch et al., 1995; Settles et al., 2004), unlike other TEs (such as the Ac/Ds system) which show tendencies for transposition into linked site (Brutnell, 2002). Mutator elements do, however, preferentially insert into genic sequences (Cresse et al., 1995; Fernandes et al., 2004; Settles et al., 2004; Liu et al., 2009; Vollbrecht et al., 2010), and typically into 5`-regions of genes (Dietrich et al., 2002; Liu et al., 2009). The prevalence of inserts in these targets increases the likelihood of loss-of-function mutations. Mutator transposons reportedly also show some degree of sequence-dependent preference for insertion sites (Creese et al., 1995; Dietrich et al., 2002), although these elements are generally regarded as having the potential to insert anywhere in the genome, regardless of their starting positions.

These characteristics make Mu transposons ideal for genetic resources aimed at identifying a broad array of unique insertion sites. Several large-scale mutagenesis programs have successfully employed Mutator to identify transposon-induced mutations in genes of maize. The most prominent include the Trait Utility System in Corn (TUSC) program developed at Pioneer Hi-Bred International, Inc (Bensen et al., 1995; Meeley and Briggs, 1995; McCarty and Meeley, 2009), the Maize Targeted Mutagenesis (MTM) effort, at Cold Spring Harbor Laboratories (May et al., 2003; Slotkin et al., 2003), the RescueMu resource developed at Stanford University (Walbot, 2000; Raizada et al., 2001), and the UniformMu population developed at the University of Florida (Yong et al., 2005; McCarty et al., 2005; Settles et al., 2007).

The UniformMu population (University of Florida) was generated by introgressing a Mutator-active line (Robertson's Mutator with acive MuDRs and a *bronze1-mutable* [*bz1-mum9*] color marker) into the isogenic, W22 inbred line (McCarty et al., 2005). Presence of the *bz1-mum9* gene results in a spotted aleurone for MuDR-active seeds, providing an easily selectable marker for MuDR activity (Chomet et al., 1991). Repeated backcrosses of UniformMu plants to their W22, wildtype parents allows the population to be maintained with a highly uniform background and a steady mutation frequency. This strategy also prevents build-up of parental Mu insertions to an undesirable level and helps assure that new mutations comprise a significant portion of the total Mu inserts in each generation (McCarty et al., 2005). A database of detailed pedigrees for thousands of lines also allows researchers to trace the ancestry of families of interest. Together, these result in the UniformMu population being ideal for genetic studies, with i) a high and steady transposition rate, ii) a relatively low number of mutations per line, iii) a uniform background for phenotypic comparisons, iv) a traceable pedigree for each plant, and v) an easily selectable marker for MuDR activity. Nonheritable, somatic transpositions are avoided by exclusively selecting stable, non-mutagenic seeds that lack an active MuDR transposase. These "Mu-off" kernels are identified by their bronze kernels not displaying a spotted aleurone and are used in reverse genetic projects.

Release of the maize genome (B73 inbred) (Schnable et al., 2009), allowed the use of the highly-conserved TIR sequences from Mu elements to be identified bioinformatically (reported here). Additionally, PCR primers were designed that anneal to the highly-conserved TIR sequences and extend outward from either end of a given

Mu element. This approach has previously been widely successful in identifying gene mutation sites via either forward genetic screens (using non-specific, degenerate primers) (Settles et al., 2004), or reverse genetic screens (using gene-specific primers) (Bensen et al., 1995; Meeley and Briggs, 1995; Penning et al., 2009; McCarty and Meeley, 2009). More recently, sequencing approaches have become feasible, with the advent of cost-effective, high-throughput sequencng technologies (from 454 [Margulies et al., 2005], to Illumina [www.illumina.com] and Solid [Smith et al., 2010]). Transposon insertion sites can be sequenced (again using TIR-specific and random primers) in large numbers of mutants from mutagenic populations such as UniformMu (McCarty et al., 2005). It is now possible, by taking advantage of the properties and behavior of Mu transposons, to generate resources comparable to the highly-successful, T-DNA insertional mutant lines for Arabidopsis. The latter have provided an invaluable resource for genetic and functional analyses in that model species (Alonso et al., 2003).

Domestication of maize began approximately 10,000 years ago, and began the divergence of this crop species from its wild ancestor, teosinte (reviewed by Doebley, 2004). Data indicate that the origin of current-day maize traces back to a South-Central region of Mexico, the Balsas River region, home of the *parviglumis* subspecies of teosinte (Matsuoka et al., 2002; Fukunaga et al., 2005). Doebley and coworkers (Iowa State University), have isolated varieties of *teosinte parviglumis* from both Balsas and Jalisco regions and used these to generate a number of teosinte inbred lines (TIL). Five of them, designated TIL-1, TIL-11, TIL-14, TIL-15, and TIL-17 have been used in the

present study. The TIL-1, TIL-15, and TIL-17 inbreds originated from the Balsas region, whereas TIL-11 and TIL-14 originated from the Jalisco region.

Maize breeders have developed elite inbreds as powerful tools to create commercial hybrids based on maximal “combining ability” of the parents. The resulting heterosis, or hybrid vigor, is fundamental to the yield potential achieved for maize, and for the uniformity within crop stands (reviewed by Lippman and Zamir, 2007; Springer and Stupar, 2007). Maize inbreds have thus been widely studied from applied to basic levels, with classic lines including B73 (Iowa), Mo17 (Missouri), and W22 (Wisconsin).

By indentifying the Mu transposons from various maize inbreds, differences between the inbreds can be highlighted, as well as their relatedness to one another. The contribution of Mu transposons to diversity between maize inbreds and between maize and teosinte has potential to be large given their elevated activity in some lines, their high affinity for genic sequences, their capacity for gene disruption, and their abundance. Here, the location and classification of the majority of Mu transposons in B73, W22, Mo17, as well as five inbred teosinte lines, derived from two distinct regions of Mexico, are reported. In addition to the most commonly-studied “canonical” Mu elements (Mu1-Mu9), the Mu10, Mu11, and Mu12 elements have been identified and mapped to the B73 reference genome. Interestingly, Mu12-like elements comprised the majority of all Mu elements, and represent a diverse family of Mu transposons with a previously-unrecognized variability and abundance in diverse maize and teosinte inbreds.

The original convention of classifying Mu elements as Mu1-Mu12 based on sequence identity has become challenging in the face of the extensive diversity among

Mu elements identified in the B73 genome. Full-length sequences of the 179 Mu elements identified here showed that the most of them did not match any of the previously-described Mu classes, especially when considering the Mu10, Mu11, and Mu12 groups. We suggest here, based on abundance and diversity of internal sequence for these elements, that the Mu10s, Mu11s, and Mu12s each comprise entire classes of elements separate from the Mu1-9 group. These analyses thus allow Mu elements to be grouped into one of four Mu subfamilies based on their distinct TIR sequences. The "canonical" Mu's include Mu1-Mu9 (and others with highly-similar TIR sequences), the Mu10 and Mu12 groups refer to elements with TIR sequences most closely matching the previously-described Mu10 and Mu12 TIRs reported by Dietrich et al.(2002), and the Mu11 group includes inserts with one TIR being similar Mu10 and the other TIR being similar to the canonicals.

## Results

### Mu Insertions Identified Bioinformatically in the B73 Genome

To explore and capture the diverse sequences of Mu transposons in the B73 inbred, its genome (version 2, release 4a.53) (maizesequence.org) was examined via sequential BLAST analyses using the terminal 150 bp of TIR sequences, first from previously-identified Mu elements, then using the most divergent Mu sequences obtained from prior analyses (Fig. 2-1). Each new TIR identified this way was used to build a Mu-TIR database that included genome location and Mu sequence, and which was used to select the most divergent TIRs as queries for each additional BLAST analysis. Novel TIR sequences continued to be identified until searches yielded no unique results. Using this approach, 300 TIR sequences were identified in the B73 genome, corresponding to 179 unique Mu insertions (Table 1). Of these, 25 were

canonical Mu's (Mu1-Mu9), 17 were classified as Mu10's, 10 were classified as Mu11's, and 127 were classified as Mu12's (Table 1). Two distinct, oppositely-oriented TIRs were identified for only 76.25% of these elements, suggesting either the degradation of TIR sequence through mutation (and thus some TIRs not being recognized in BLAST-based searches) or the absence of some TIRs from the current genome due to gaps or anomalous arrangements in the B73 genome (assembly in progess).

**Phylogenetic Relationships between the B73 Mu Transpsons**

Phylogenetic analyses using 150 bp of each TIR sequence identified through BLAST analysis of the B73 genome revealed the diversity of Mu elements (Fig. 2-2). As expected, the distinct classes of Mu elements grouped into mutually-exclusive groups according to their TIRs, with the canonical Mu's (1-9), Mu10's, and Mu12's each occupying their own positions in the phylogenetic tree (Fig. 2-2), and with Mu11's grouping with both the canonicals and Mu10s. Often, the two arms of a single Mu insert had divergent sequences and thus grouped in different sub-clades (Fig. 2-2). This degree of asymmetry was counter to expectations, and is thought to be atypical of transposons (Brutnell, 2002; Lisch, 2002). These elements with divergent TIRs have been termed "heteromorphic" Mu elements, of which there appear to be at least five independent groups in B73, including the Mu11s.

**The B73 Inbred Contains some MuDR-like Sequences**

Being a stable inbred line, B73 is not expected to contain the autonomous MuDR transposase, and no exact matches to the published MuDR sequence were detected in our analyses (Table 2-2). However, some of the Mu elements in B73 have very high similarity to MuDR and are likely MuDR derivatives. None of these MuDR-like, canonical Mu's appear to have retained the capacity to encode functional proteins since

their putative *mudra* and *mudrb* coding sequences included mutations that would lead to premature stop codons or contained large deletions.

Interestingly, many of the Mu10 elements identified in B73 had internal sequence very similar to MuDR, but divergent TIRs. Although most of the *mudra* and *mudrb* coding sequences of these Mu10s were disrupted, at least one appeared to be intact (Fig. 2-3). This Mu10, designated Mu4K in these analyses, included full-length sequences apparently cabable of encoding both MURA-like and MURB-like proteins (Fig. 2-3). Normal intron-exon borders were intact and, if processed, would lead to translation of proteins highly similar to MURA and MURB (Fig. 2-3). These putative proteins would be 82% and 79% identical at the amino acid level to MURA and MURB, respectively (Fig. 2-3).

**Mu-anchored Sequencing to Identify Mu Insert Locations in Maize and Teosinte Inbreds**

Over 43,000 reads were obtained from 454-based sequencing of thirty independent libraries, each constructed using anchor primers to one of the three classes of Mu elements (canonicals, Mu10s, and Mu12s) in one of the ten *Zea mays* inbreds examined (3 maize and 5 teosinte) (Appendix A). From the resulting reads, 15,348 Mu insert sites were identified by aligning Mu-flanking sequences with the B73 genome. Among these mapped inserts, two flanks were identified for between 20-50% for most libraries, thus we estimate that these data represents an average of approximately 25-60% coverage of the total Mu inserts in these inbreds. Complete appraisal of the Mu inserts by these methods is not currently feasible due to degeneration of TIR sequence and PCR-based variation, though deeper sequencing will provide greater coverage.

Results from Mu-TIR-anchored sequencing of the three maize inbreds (B73, Mo17, and W22) are summarized in Table 2-3, and those from the five teosinte lines are summarized in Table 2-4. All reads from each library were mapped to the B73 reference genome as noted above. Of the current-day maize Inbreds, Mo17 contained the highest numbers of all three classes of Mu transposons (Table 2-3). For the five teosinte inbred lines, the results were more variable, with TIL-11 having the most canonical Mu's and TIL-17 having the most Mu12-like elements (Table 2-4). Reads that originated from opposite arms of the same Mu insertion (one from each TIR) were determined as such by examining their proximity on the reference genome and by the presence of matching 9-bp target-site duplications (TSDs). The number of inserts for which two flanking sequences were recovered from each library was subtracted from the number of uniquely-mapping reads to obtain the total number of Mu elements identified from each library (Tables 2-3, 2-4, column 4). In order to estimate the coverage from each of these libraries, the percentage of elements identified with both flanks was divided by that for which both arms were detectable in the B73 genome using bioinformatic approaches (Tables 2-3, 2-4). Each Mu class was treated separately. The rough estimates of Mu inserts from each class in a given inbred (Tables 2-3, 2-4, column 8) is based on BLAST-analysis alone, and thus represents a general approximation.

Expression of these estimates as proportions of the total Mu presence from each Mu class showed remarkable consistency between inbreds (Fig. 2-4). With the exception of the teosinte lines derived from the Jalisco region (TIL-11 and TIL-14), the canonical Mu elements are estimated to make up between 12 - 15%, while Mu12-like

elements account for between 75 - 82% of the total Mu's. Jalisco-derived teosinte lines had a higher proportion of canonical Mu's at the expense of Mu12s (Fig. 2-4). The B73 maize inbred appears to contain a higher propotion of Mu10 elements than any other group, with around 13% of its total Mu elements being classified as Mu10-like (Fig. 2-4).

### Comparative Analysis of Mu Elements and Insert Sites in Maize and Teosinte

A number of observations can be made by diagramming each Mu insert from the three maize inbreds (Fig. 2-5) and the five teosinte lines (Fig. 2-6) onto the B73 physical map. First, the Mu elements were not distributed in a completely random manner throughout the genome. Distinct areas of some chromosomes were devoid of detectable Mu elements, whereas other areas had a relative overabundance. The latter were typically near the ends of chromosomes (Figs. 2-5; 2-6). However, patterns of Mu-insert distribution vary greatly among the inbreds (Figs. 2-5; 2-6). Additionally, there was no obvious pattern of any two of the maize inbreds sharing more Mu inserts than the other. No immediate inter-relational patterns are thus indicated by these data for the three maize lines. Finally, some classes of Mu elements tended to group together in some areas (Figs. 2-5; 2-6). Importantly, the large majority of Mu elements detected in this study appear to be unique to a single inbred (Figs. 2-5; 2-6).

## Discussion

### Bioinformatic Analysis of B73 Reveals Diversity

Previous estimates for the number of "background" Mu inserts in inbred maize lines were between 20 and 50 (Liu et al., 2009). Until recently, such approximations were based on canonical Mu's (1-9) alone, and even when Mu's 10-12 were identified (Dietrich et al., 2002), the extent of their abundance was not recognized. Here we show that the total number of Mu elements in B73 is closer to 200, and that some inbred

teosinte lines may have closer to 500 (Tables 2-1, 2-3, 2-4). The majority of Mu transposon sequences in all the maize and teosinte inbreds tested here are Mu12-like elements. This abundance is intriguing when considering how little these elements have been utilized in gene tagging experiments. One possibility is that Mu12 elements could be have limited activity in current mutagenic maize lines (such as the UniformMu population). Alternatively, their activity may have gone undetected thus far due to a lack in tools for their detection. Primers designed based on flanking sequences from the canonical Mu's would be unlikely to anneal to TIR sequences from a Mu10 or Mu12 element.

Several factors could contribute to the presence of Mu sequences in the maize genome that did not have two readily-identifiable, oppositely-oriented TIR sequences (23%) (Table 2-1). First, despite being close to completion, the B73 genome is not yet fully assembled (Schnable et al., 2009). Gaps remain where sections have not yet been positioned and oriented, and large sections of DNA may not yet be correctly arranged. A number of the Mu sequences examined here terminate at gap sites, providing the likelihood that some of the single-armed Mu sequences here might actually have an unrecognized complement TIR elsewhere in the dataset. Others will likely be completed as the genome sequence becomes more refined. Actually, the repetitive nature of internal Mu-element sequences may complicate their arrangement during genome assembly and/or lead to gaps in the sequence, a scenario that seems likely given the disproportionate number of elements that terminate in gaps. Assembly of repetitive DNA sequence has long plagued genome sequencing projects (International Human Genome Sequencing Consortium, 2001), and Mu elements might be a

contributing factor of that in maize. Alternatively, Mu elements with a single detectable TIR might have lost all or part of one arm due to deletion or translocation events, or through the accumulation of enough small mutations over time to compromise recognition by BLAST using queries of 150-bp TIR sequences. Further decrease to the threshold of BLAST e-value cutoffs used here might reveal some additional TIRs, but these would be difficult to distinguish from non-Mu-derived DNA sequence.

**Homomorphic Versus Heteromorphic Mu Elements**

Heteromorphic elements (those with divergent TIRs) may reveal important clues about Mu transposon evolution and behavior. The Mu11 elements were first described as having one TIR similar to a canonical Mu and one TIR like that of a Mu10 (Dietrich et al., 2002). Ten such Mu11's were identified in the B73 genome by BLAST analyses (Table 2-1). Additional Mu's that are currently represented by one-armed sequences may also belong to this class. The mechanism by which such Mu elements might arise, with two very different arms, deserves some discussion. It seems almost certain that for a Mu11, one arm originated from a canonical Mu while the other arose from a Mu10. One possibility is that a transposase attached to distal arms of two, physically-close, but unrelated Mu elements, starting the amplification of hybrid Mu element, the progenitor of the Mu11 class. Alternatively, the insertion of a Mu10 inside of a canonical Mu (or vice-versa) could lead to the formation of a hybrid Mu11-like element. A similar mechanism could possibly have given rise to some of the Mu12 elements that contain two very distinct arms (connected by red, dashed lines in Figure 2-2). However, it is also possible that one arm (or both) simply accumulated changes independently of the other, but maintained transposase recognition sites, and thus activity. If so, the evolving, but still-functional arm would continue to be amplified, and thus give rise to groups of

elements with two different arms. Such arms would group in separate clades in phylogenetic analyses, as observed in Figure 2-2. Heteromorphic Mu's do not necessarily lose their potential to transpose, as multiple instances of heteromorphic Mu amplification are evident by replicated inserts seen in the phylogenetic analyses (parallel dashed lines in Fig. 2-2). At least five independent groups of heteromorphic Mu's have arisen in the Mutator system.

There were also a number of Mu elements for which both TIR sequences were essentially identical, differing by as few as 3 base-pair changes (Fig. 2-2). These elements may represent relatively recent TIR duplication events, as left and right arms have not accumulated mutations since their origination. Therefore, the extent of similarity between arms does not seem related to time since insertion of the element into its current site, but time since a single arm was replicated to make a completely homomorphic element. A mechanism for doing so need not be common, but should allow a single TIR sequence to be duplicated, such that the resulting Mu element have identical left and right arms, oriented in opposite directions. No such pathway is currently known, but during replicative transposition a Mu element is copied before its insertion elsewhere (Shapiro, 1979). During this process, one TIR might pissibly be replicated twice, creating a new Mu element with identical TIRs. Notably, for insertions with near-identical TIRs (such as the Mu12 insertion, Mu9K, in this analysis) the homology between the arms is limited to the TIRs (data not shown), and does not extend into internal sequence. This observation indicates that the TIRs in particular are replicated to generate a new homomorphic Mu element, and not larger portions of the transposon.

**Some Mu10 Elements Have Full-length Putative Transposase Sequences**

The possibility that some Mu10 elements might encode functional transposases is indicated by the high degree of predicted amino acid sequence similarity between putative coding regions of the Mu10 element, Mu4K, and the MURA and MURB proteins of the MuDR transposase (Fig. 2-3). One possibility is that these Mu10 elements function in a similar manner and on similar targets as MuDR, but have remained undetected and uncharacterized because of differences in activity and/or sequence. Alternatively, Mu10 and MuDR elements might have diverged enough to have distinct targets, either mutually-exclusive or overlapping. The autonomous MuDR has been associated with transposition of canonical Mu elements (Mu1-9) (Chomet et al., 1991), but its effects on the more divergent Mu's (10-12) have received little previous attention, and are unknown. Involvement of another transposase is possible, particularly given the presence of the Mu10s, and the divergent sequence of Mu10 and Mu12 TIRs (and transposase binding sites). Either MuDRs, Mu10s, or both could be responsible for transposition of the divergent Mu classes, including the Mu10s, Mu11s, and/or Mu12s. It is also possible that Mu10 elements may be non-functional derivatives of MuDR that have not yet diverged in significant ways. However, as the TIR sequences of MuDRs and Mu10s have accumulated many changes, but the putative coding sequences have maintained homology, their being non-functional derivatives seems unlikely. The observation that predicted protein sequence is more similar than the nucleotide sequence between MuDR and Mu4K suggests selection pressure to maintain protein function (Miyata et al., 1980). Selective pressures that might favor an active transposase are hotly debated (Kidwell and Lisch, 2000; Okamoto and Hirochika, 2001; Rebollo et al., 2010).

**Relationships Between Zea Inbreds are Highlighted by Mu Element Mapping**

While the overall ratios of Mu classes were similar for the inbreds tested here (Fig. 2-4), comparisons of insert locations and overall abundance in each of the maize and teosinte inbreds demonstrated some striking differences between them. First, the greater variation between Mu insert sites in teosinte lines than between the maize inbreds is consistent with the greater diversity of teosinte overall (Fukunaga et al., 2005) (compare Tables 2-3 and 2-4). Only about 10% of the Mu elements detected in the five teosinte inbreds were present in more than one line, as opposed to around 30% for the maize lines. Also, some of the teosinte lines, specifically TIL-15 and TIL-17 from the Balsas region, have abundant Mu's, estimated at over 600 insertions each. In addition, the TIL-11 teosinte line contains approximately twice the number of canonical Mu elements as any of the other maize or teosinte inbreds tested, indicating a higher activity for this particular class in that line (Table 2-4, Fig. 2-6; Fig. 2-4). Some inbreds had areas of especially high insert abundance, such as TIL-17 having 27 unique inserts on the short arm of chromosome 10 (covering 58 million nucleotides), that seem to suggest location-specific differences in insert activity between lines (Fig. 2-6). It is possible that the various inbreds have different chromatin structure and/or other DNA changes that could influence insertion frequency and insert site preference.

Other patterns of Mu insert location were common among all inbreds tested. Certain regions of chromosomes 2, 5, 6, and 9 had high numbers of transposons within relatively small areas in teosinte and maize inbreds (Figs. 2-5, 2-6). Also apparent was a relative abundance of Mu elements at the ends of chromosomes and their relative scarcity near centromeres in both teosinte and modern-day maize inbreds. Additionally, large areas of chromosomes lacking Mu inserts, together with frequent clusters of Mu

elements, strongly indicated that insertion sites were non-random. It is likely that physical characteristics of the DNA, like chromatin structure or DNA modification, influence the likelihood of Mu transposon insertion in a particular site, with more genic regions being less tightly organized and thus better candidates for Mu insertion, as has previously been suggested (Liu et al., 2009; Vollbrecht et al., 2010). This scenario is supported by data shown here, as well as observations that Mu elements target the five-prime regions of genes (Dietrich et al., 2002; Liu et al., 2009). This pattern seems to extend from canonical Mu's to the Mu10 and Mu12 elements (Figs. 2-5; 2-6).

Also apparent were chromosomal regions that appeared to preferentially accumulate insertions of certain classes. For instance, despite Mu10 elements being less abundant than the canonicals or Mu12s, regions on the short arms of chromosomes 2 and 5 contained multiple Mu10 elements within relatively small areas (Fig. 2-5). Other regions contained unusually high numbers of canonical Mu elements or Mu12s, possibly reflecting differences between the Mu classes in their insertion-site preferences. What the underlying causes of these differences might be remains to be determined.

Many of the Mu elements identified here were present in both maize and teosinte (stars in Figs. 2-5, 2-6), and presumably represent ancient insertion events that have been propogated since before the divergence of teosinte and modern maize. Alternatively, these Mu elements could theoretically be independent insertion events in the same exact location, as has been reported, albeit rarely, in other cases (Dietrich et al., 2002). We consider the former suggestion, that of their being ancient insertion events, to be more likely, as the classification of shared Mu's is typically consistent (a

Mu12 insert in teosinte matches a Mu12 insert in maize). While these elements were found throughout the genome, the dispersal pattern was uneven, with small clusters of two or more shared Mu's occurring regularly (Figs. 2-5, 2-6). In particular, regions of chromosome 2 short arm, and chromosomes 4 and 5 long arms had groups of 4 or more Mu elements that appear to be ancient in origin and to have been inherited together. Other regions, such as the short arm of chromosome 4 and the majority of chromosomes 3, 8, and 10 have very few shared Mu elements between teosinte and maize. Interestingly, the Mu elements shared between these subspecies were not over-represented by a certain class of Mu. There were specific canonicals, Mu10s, Mu11s, and Mu12s present in both modern maize and teosinte, in approximately the same ratio as their overall abundance. We had speculated that the large Mu12 class may represent more ancient insertions, but based on these data, that does not necessarily appear to be the case.

## Methods

### Bioinformatic Identification of Mu Elements in the B73 Maize Genome

As diagramed in Fig. 2-1, mining of the B73 reference genome (version 2, release 4a.53) (maizesequence.org) was conducted by repeated BLAST analyses, starting with a single Mu TIR from each class of Mu transposons, (canonicals, Mu10s, and Mu12s). Individual returns from each BLAST output (cut-off at 10e^-6) were annotated and deposited in a database. They were named based on chromosome, then order of discovery (as in Mu1A for the first transposon identified on chromosome 1). This process was repeated, using more divergent Mu TIR sequences, until no additional unique transposons were identified. Genomic sequences surrounding each Mu were then individually analyzed to match paired TIRs (opposite arms from the same element)

based on 9-bp target-site duplications. Matching arms were given a common name and arbitrarily designated left or right arm (as in Mu1AL for the left arm of the first transposon identified on chromosome 1).

**Phylogenetic Analysis of B73 Mu-TIRs**

For each bioinformatically-identified TIR in the B73 reference genome, the terminal 150 bp were trimmed, oriented in the same 5`-to-3` direction, and arranged with their terminal bases oriented 5` (as in 5`-GAGATAA-3`). The 299 TIRs (one was omitted due to truncation by a gap in the reference genome) were aligned using ClustalW (Larkin et al., 2007). A neighbor-joining tree was created with MEGA4 (Tamura et al., 2007) using the pairwise deletion option and 1,000 bootstrap repetitions.

**Mu-flank-anchored 454 Library Construction**

DNA from each maize and teosinte inbred (B73, Mo17, W22, TIL-1, TIL-11, TIL-14, TIL-15, and TIL-17) was randomly sheared and ligated to biotinylated amplicon-B (bioTEG0-ampB) adaptors. Approximately 5 µg of DNA was bound to 50 µL of Strepavidin beads (Dynal, product # 653.05) and immobilized using a magnetic rack (Applied Biosystems, Lot # 0804015). Beads were washed 4 times with 100 µL of washing buffer #1 (10mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0]), and removed from the rack between each wash. The second washes were conducted at 37°C for 5 min. The final washes were transferred to 0.2-mL PCR tubes, where buffer was removed from beads immobilized on a magnetic stand-96 (Applied Biosystems, Lot # 0903014). For primer extension 1 (Fig. 2-7), the following was added for each reaction: 0.33 µL of 10 pmol/ul TIR primer (TIR6 for canonical Mu elements, primer 10.1 for Mu10s, and primer 12.1 for Mu12s, see Table 2-5) (10 pmol/ul), 10 µL 10X NEB ThermoPol buffer (1X = 10mM KCl, 20 mM Tris-HCl [pH 8.8 at 25°C], 10 mM $NH_4 2SO_4$, 2 mM $MgSO_4$,

0.1% Triton X-100 [Sigma Lot # MKBD6639V]), 2 µL dNTPs (10 mM), 85 µL HPLC-grade water, and 2 µL Vent DNA Polymerase (NEB M0257S 2 units/µL). Each reaction tube received 100 µL of this master mix, and were transferred to a thermocycler. The primer extension 1 reaction differed for canonicals (80°C, 2 min; 72°C, 10 min; -1°C/min to 67°C; 67°C, 10 min; 70°C, 6 min), and Mu10s and Mu12s (80°C, 2 min; 70°C, 10 min; -1C/min to 65°C, 65°C, 10 min; 70°C, 6 min). To stop the reaction, 1 µL 0.5 M EDTA (pH 8.0) was added. Tubes were gently vortexed and beads were transferred to the original 1.5-mL tubes. Beads were rinsed 4 times with washing buffer #1, with the second wash at 37°C for 5 min. After the last wash was removed, 125 µL of melt solution (100 mM NaCl, 125 mM NaOH) was added and tubes were incubated on a slow shaker for 10 min to elute the newly synthesized strand. Beads were immobilized and supernatant was transferred to acidified PBI buffer (Qiagen, 625 µL PBI with 4.5 µL 20% fresh aqueous acetic acid). Single-stranded DNA was concentrated and de-salted using MinElute PCR purification kit columns (Qiagen Cat # 28704), before elution with 20 µL of 55°C EB buffer. Beads (containing original DNA) were washed as before and stored at 4°C.

Products from primer extension 1 were then PCR amplified using a 2-step PCR protocol. The following reagents were used for a single reaction: 6 µL 10X PCR Enhancer (Invitrogen Cat # 11495-017), 2 µL 10X PCR amp buffer, 1.5 µL 100 mM $MgSO_4$, 0.5 µL 10 mM dNTP, 0.8 µL 10 µM bioTEG0-ampB primer, 0.8 µL 10 µM Mu primer (TIR 8 for canonicals, 10.1 for Mu10s, 12.1 for Mu12s), 0.2 µL 10 units/µL Taq, and 7.9 µL HPLC-grade water. The 2-step PCR thermocycler settings were: 96°C, 3

min; [96°C, 30s; 60°C, 45s; 72°C, 90s] 9 cycles; [96°C, 30s; 54°C, 30s; 72°C, 90s] 31 cycles; 72°C, 5 min.

The amplified PCR products were then optimized for the desired size as follows. Portions of the amplified libraries (85 µL) were added to 59.5 µL SPRI bead slurry. Solutions were vortexed and incubated at 25°C for 5 min. Beads were immobilized for 15 min and liquid was removed. While tubes remained on the magnet rack, beads were washed 2 times with 200 µL 70% EtOH. After the wash was removed, beads (on rack) were placed in a 37°C incubator for 30 min to allow EtOH evaporation. Size-selected, double-stranded DNA was eluted with 50 µL 10 mM Tris (pH 7.5), 1 mM EDTA (pH 8.0).

Strepavidin beads were prepared for binding by washing twice with 100 µL of 1X B&W buffer (5 mM Tris-HCl [pH 7.5], 0.5 mM EDTA [pH 8.0], 1 M NaCl). Buffer was then added (50 µL of 2X B&W buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), and 2 M NaCl], along with 50 µL of the size-selected PCR product. Tubes were vigorously shaken for 20 min at 25°C to bind beads. The beads were then immobilized and washed twice with 1X B&W, and twice with HPLC-grade water. The biotinylated strands were isolated by adding 125 µL melt solution and shaking for 10 min at 25°C. Beads were again immobilized and the eluted strands were removed (purified and retained for validation analyses). Beads were washed twice with 200 µL HPLC-grade water, twice with 100 µL washing buffer 2 (100 mM Tris-HCl [pH 7.5], 0.5 mM EDTA [pH 8.0]), and twice with washing buffer 1. The second wash was done at 37°C for 5 min. Prepared strepdavidin beads were then resuspended in 50 µL of washing buffer 1.

For primer extension 2, beads (50 µL) were transferred to 0.2-mL PCR tubes, immobilized, and solution was removed. The following was added to each tube: 5 µL 10

pmol/uL sequencing primer (specific for each reaction, see Table 2-5), 10 µL 10X ThermoPol Buffer (NEB), 2 µL 10 mM dNTP, 81 µL HPLC-grade water. Tubes were transferred to a thermocylcler and subjected to the following: 80°C, 2 min; 61°C, 10 min; -1°C/min to 51°C; 51°C, 30 min. Reactions were initiated by adding 5 µL Vent DNA Polymerase (NEB Cat # M0257S) and mixing the solution by gentle pipetting. Temperature was raised to 70°C for 6 min, and 1 µL 0.5 uM EDTA (pH 8.0) was added to stop the reaction. Tubes were gently vortexed and beads were transferred to new, 1.5-mL tubes. Beads were again immobilized and rinsed 4 times with washing buffer 1, with the second was at 37°C for 5 min. The last rinse was removed before adding 125 uL wash solution. Tubes were inclubated at 25°C for 10 min while shaking. Beads were immobilized and newly-synthesized DNA strands (with A- and B-adaptors) were eluted, purified, and concentrated in a final volume of 20 µL using a PCR purification kit (Qiagen Cat # 28704). Single-stranded DNA templates were then sequenced on a 454 GS-20 (Roche Biosciences, Indianapolis, IN) as per Margulies et al., (2005).

**Sequence Annotation and Alignment**

Sequences from 454 runs were trimmed and compiled using custom, Java-based programs (courtesy of Don McCarty). Each sequence was surveyed for correct, 4-base keycodes to assign sub-libraries of origin and to check for sequencing errors (bar-codes included a checksum code). They were each independently used as queries against the B73 reference genome in BLAST analyses in order to map them to specific chromosomal locations. Databases for each inbred and Mu class were assembled based on these BLAST results. Repetitive sequences were discarded. Insertions mapping within close proximity were examined for matching 9-bp target-site duplications and scored as single insertions for the final analyses.

## Accession Numbers

Mu1, X00913.1; Mu1.7, Y00603.1; Mu3, U19613.1; Mu4, X14224.1; Mu5, X14225.1; Mu7, X15872.1; Mu8, X53604.1; Mu9 (MuDR), M76978.1.

Table 2-1. Bioinformatically-identified Mu elements in B73

| Mu Class | # TIRs detected | # Mu elements | % with both arms |
|---|---|---|---|
| Mu (1-9) | 44 | 25 | 76% |
| Mu 10 | 28 | 17 | 65% |
| Mu 11 | 20 | 10 | 100% |
| Mu 12 | 208 | 127 | 64% |
| Total | 300 | 179 | 76.25% |

Numbers of Mu elements from each class detected by BLAST searches of the B73 genome using 150 bp terminal inverted repeat (TIR) sequences. The TIRs were manually curated to determine whether they belonged to the same Mu element based on their relative genomic locations and by matching 9-bp duplications of insert site DNA. *Note that a Mu 11 element with only one arm would be classified with either the Mu10s or the canonicals (Mu1-9) because the Mu11s have heteromorphic TIRs (one like the Mu10s and one like the canonicals).

Table 2-2. Classification of canonical B73 Mu insertions.

| Insert | Length | Class | % Identity | e-value | Score |
|---|---|---|---|---|---|
| Mu1 | 1376 | Mu1 | 100 | 0.0 | 1990 |
| Mu1.7 | 1745 | Mu1.7 | 100 | 0.0 | 2825 |
| Mu3 | 1824 | Mu3 | 100 | 0.0 | 2944 |
| Mu4 | 2015 | Mu4 | 100 | 0.0 | 3947 |
| Mu5 | 1320 | Mu5 | 100 | 0.0 | 2617 |
| Mu7 | 2199 | Mu7 | 100 | 0.0 | 4359 |
| Mu8 | 1410 | Mu8 | 100 | 0.0 | 2795 |
| Mu9 (MuDR) | 4942 | Mu9 | 100 | 0.0 | 6445 |
| | | | | | |
| 1B | 2018 | Mu4 | 99.85 | 0.0 | 3905 |
| 1C | 4805 | Mu9 | 94.82 | 0.0 | 2761 |
| 2A | 4864 | Mu9 | 94.32 | 0.0 | 3406 |
| 2B | 1891 | Mu4 | 91.79 | 1e-071 | 260 |
| 2C | 1890 | Mu4 | 91.79 | 1e-071 | 260 |
| 2D | 1914 | Mu4 | 92.31 | 6e-074 | 268 |
| 3A | 3578 | Mu9 | 94.21 | 0.0 | 2072 |
| 4B | 2018 | Mu4 | 99.70 | 0.0 | 3881 |
| 5A | 1498 | Mu9 | 91.93 | 0.0 | 1292 |
| 6A | 1321 | Mu5 | 99.92 | 0.0 | 2603 |
| 7B | 1134 | Mu7 | 95.57 | 0.0 | 1047 |
| 7C | 2199 | Mu7 | 99.59 | 0.0 | 4270 |
| 8A | 2037 | Mu4 | 92.34 | 3e-085 | 305 |
| 8B | 1621 | Mu7 | 97.26 | 8e-110 | 387 |
| 9A | 1955 | Mu7 | 97.26 | 1e-109 | 387 |
| 10A | 10830 | Mu3 | 98.47 | 0.0 | 1850 |
| UNKA | 1955 | Mu9 | 93.38 | 0.0 | 1984 |
| UNKB | 2078 | Mu4 | 92.79 | 1e-087 | 313 |

To assign the canonical Mu elements identified in the B73 genome to a specific class, each of those for which the entire sequence was available were compared to the eight previously-published Mu elements in a BLAST-based analysis. The upper portion of this table shows the results using the eight published Mu elements, and should be referred to in comparing BLAST scores to those of the individual B73 elements below. Of the 18 full-length canonical Mu elements identified in B73, the majority of them were most similar to Mu4, Mu7, and Mu9. None were most similar to Mu1.7 or Mu8.

Table 2-3. Mu elements identified by Mu-flank sequencing in three maize inbreds

| Inbred | Mu class | Mapped reads | # both flanks | # Mu's | % both flanks | Expected % | Estimated coverage | Estimated # of Mu's |
|---|---|---|---|---|---|---|---|---|
| B73 | (1-9) | 43 | 15 | 28 | 53.6% | 76% | 70.5% | 40 |
| B73 | 10 | 17 | 3 | 14 | 21.4% | 65% | 33.0% | 42 |
| B73 | 12 | 128 | 27 | 101 | 26.7% | 64% | 41.8% | 242 |
| Mo17 | (1-9) | 64 | 23 | 41 | 56.1% | 76% | 73.8% | 56 |
| Mo17 | 10 | 25 | 7 | 18 | 38.9% | 65% | 59.8% | 30 |
| Mo17 | 12 | 150 | 26 | 124 | 21.0% | 64% | 32.8% | 378 |
| W22 | (1-9) | 53 | 19 | 34 | 55.9% | 76% | 73.5% | 46 |
| W22 | 10 | 13 | 4 | 9 | 44.4% | 65% | 68.4% | 13 |
| W22 | 12 | 115 | 22 | 93 | 23.7% | 64% | 37.0% | 251 |

Mu inserts in three maize inbreds were identified through 454-based Mu-flank sequencing. Each inbred and Mu class combination was sequenced with a unique key code to determine their library of origin. Mapped reads were unique TIR-flanking sequences that mapped to specific sites on the B73 genome. The number of these that had both flanks were determined by location on B73 the reference genome and by sequenced target-site duplications. Expected percentages with both flanks was based on bioinformatic results of BLAST analyses on the B73 genomic sequence, and was used in calculating the estimated coverage for each library. Estimated number of Mu's was calculated by dividing the number of Mu's detected by the percent coverage, and refers to the total number of Mu elements of each class that would be expected by these calculations, and can be considered a rough estimate only.

Table 2-4. Mu elements identified by Mu-flank sequencing in five teosinte inbreds

| Inbred | Mu class | Mapped reads | # both flanks | # Mu's | % both flanks | Expected % | Estimated coverage | Estimated # of Mu's |
|---|---|---|---|---|---|---|---|---|
| TIL-1 | (1-9) | 45 | 12 | 33 | 36.4% | 76% | 47.8% | 69 |
| TIL-1 | 10 | 13 | 5 | 8 | 62.5% | 65% | 96.2% | 8 |
| TIL-1 | 12 | 86 | 9 | 77 | 11.7% | 64% | 18.2% | 422 |
| TIL-15 | (1-9) | 41 | 7 | 34 | 20.6% | 76% | 27.1% | 126 |
| TIL-15 | 10 | 15 | 2 | 13 | 15.4% | 65% | 23.7% | 55 |
| TIL-15 | 12 | 169 | 25 | 144 | 17.4% | 64% | 27.1% | 531 |
| TIL-17 | (1-9) | 55 | 15 | 40 | 37.5% | 76% | 49.3% | 81 |
| TIL-17 | 10 | 33 | 8 | 25 | 32.0% | 65% | 49.2% | 51 |
| TIL-17 | 12 | 215 | 30 | 185 | 16.2% | 64% | 25.3% | 730 |
| TIL-11 | (1-9) | 68 | 21 | 47 | 44.7% | 76% | 58.8% | 80 |
| TIL-11 | 10 | 3 | 0 | 3 | 0% | 65% | N/A | N/A |
| TIL-11 | 12 | 61 | 11 | 50 | 22.0% | 64% | 34.4% | 145 |
| TIL-14 | (1-9) | 75 | 20 | 55 | 36.4% | 76% | 47.8% | 115 |
| TIL-14 | 10 | 26 | 10 | 16 | 62.5% | 65% | 96.2% | 17 |
| TIL-14 | 12 | 71 | 8 | 63 | 12.7% | 64% | 19.8% | 318 |

Mu inserts in five teosinte inbreds were identified through 454-based Mu-flank sequencing. Each inbred and Mu class combination was sequenced with a unique key code to determine their library of origin. Mapped reads were unique TIR-flanking sequences that mapped to specific sites on the B73 genome. The number of these that had both flanks were determined by location on B73 the reference genome and by sequenced target-site duplications. Expected percentages with both flanks was based on bioinformatic results of BLAST analyses on the B73 genomic sequence, and was used in calculating the estimated coverage for each library. Estimated number of Mu's was calculated by dividing the number of Mu's detected by the percent coverage, and refers to the total number of Mu elements of each class that would be expected by these calculations, and can be considered a rough estimate only.

Table 2-5. Primers used for 454-based Mu-flank sequencing.

| Name | Primer sequence |
|---|---|
| TIR6 | AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC |
| TIR8 | CGCCTCCATTTCGTCGAATCCCCTS, CGCCTCCATTTCGTCGAATCCSCTT, SGCCTCCATTTCGTCGAATCCCKT, CGCCTCCATTTCGTCGAATCACCTC |
| Mu-direct | CBCTCTTCKTCYATAATGGCAAT |
| 12.1 | YATTTCGTYGAARCCGCAYCCGTCGTGTTTC |
| 12-direct | CCGTCGTRTTTCATAATRBCAAA |
| 10.1 | SCAACGCCTCCRWTTYDTCGAAACCGYKTCTC |
| 10-direct | CTCTBDTGTTTYATAATGGCAAT |

Primers TIR6, TIR8, and Mu-direct were used to amplify canonical Mu elements, while 12.1 and 12-direct were used to amplify Mu12 elements, and 10.1 and 10-direct were used to amplify Mu10 elements. Sequencing primers included a 4-base bar code unique for each library to allow assignment of each read. Code: A (Adenine), C (Cytosine), G (Guanine), T (Thymine), R (Purine [A or G]), Y (Pyrimidine [C, T, or U]), M (C or A), K (T, U, or G), W (T, U, or A), S (C or G), B (C, T, U, or G), D (A, T, U, or G), H (A, T, U, or C), V (A, C, or G).

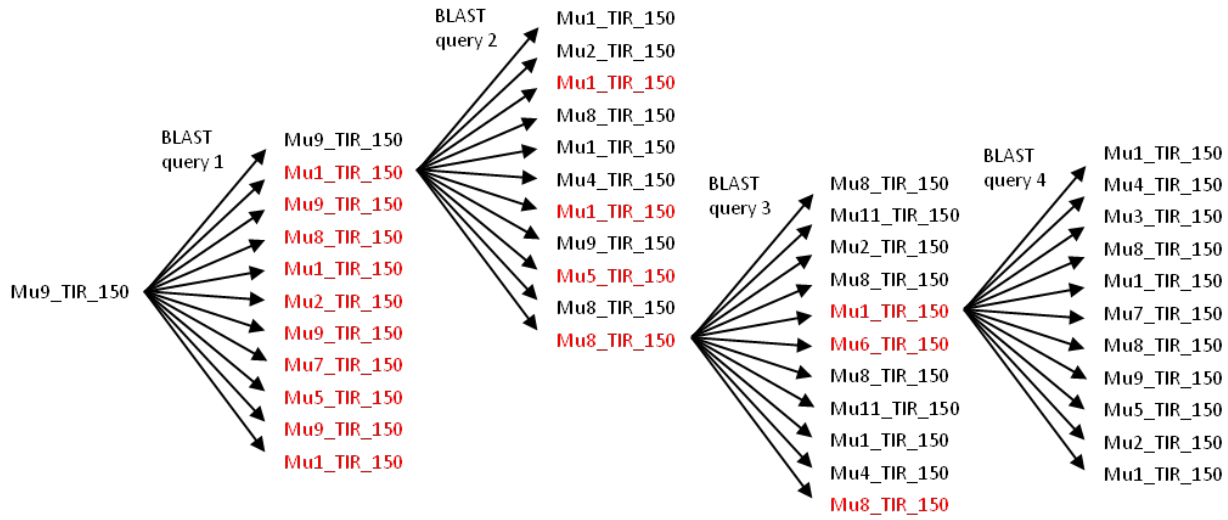


Figure 2-1. One example of a sequential mining track, diagramed here to show progression from an initial BLAST query using a single 150-bp TIR sequence to search the B73 genome. The first query typically returns multiple similar sequences, which are captured and cataloged in a database by genomic location. This initial query is followed by additional BLAST queries using 150 bp from the more divergent TIRs identified, and so on, until no new novel insertion sites are identified. This process was conducted using single initial queries from Mu9, Mu10, and Mu12 elements, and was successful in identifying 300 TIR sequences in the B73 genome. (Black = previously identified; Red = newly identified).

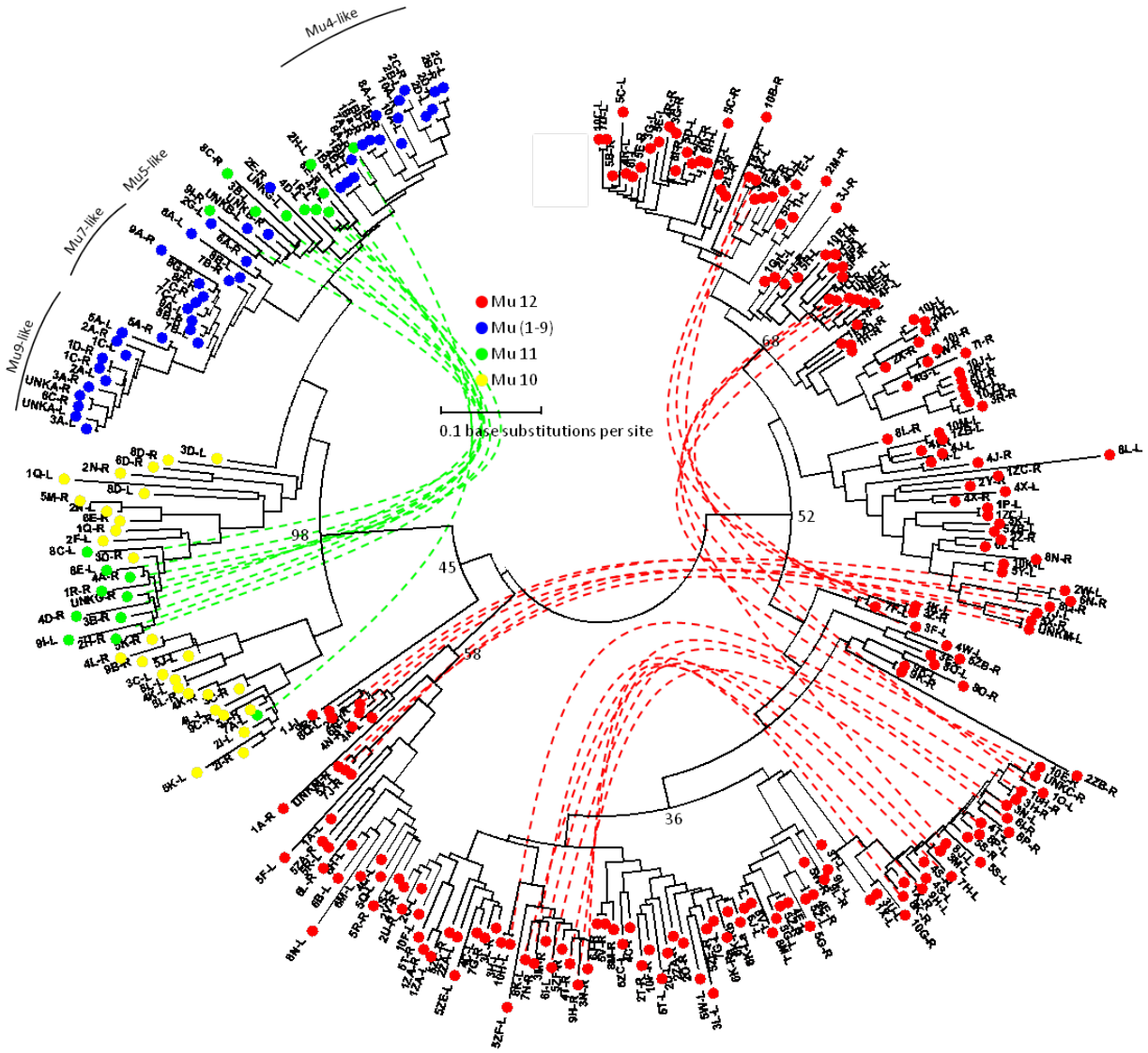


Figure 2-2. Neighbor-joining tree of 299, B73 Mu Terminal Inverted Reapeats. The terminal 150 bases from each TIR of bioinformatically-identified B73 Mu transposons were trimmed, oriented in the same direction, and aligned using ClustalW. The phylogenetic tree was created in MEGA4, using 1,000 bootstrap replications with a pairwise deletion option. The canonical Mu elements are labeled based on the most closely-related, previously-described Mu element. Key bootstrap values are shown. Units are in number of base substitutions per site. Opposite arms of heteromorphic Mu elements are connected by dashed lines.

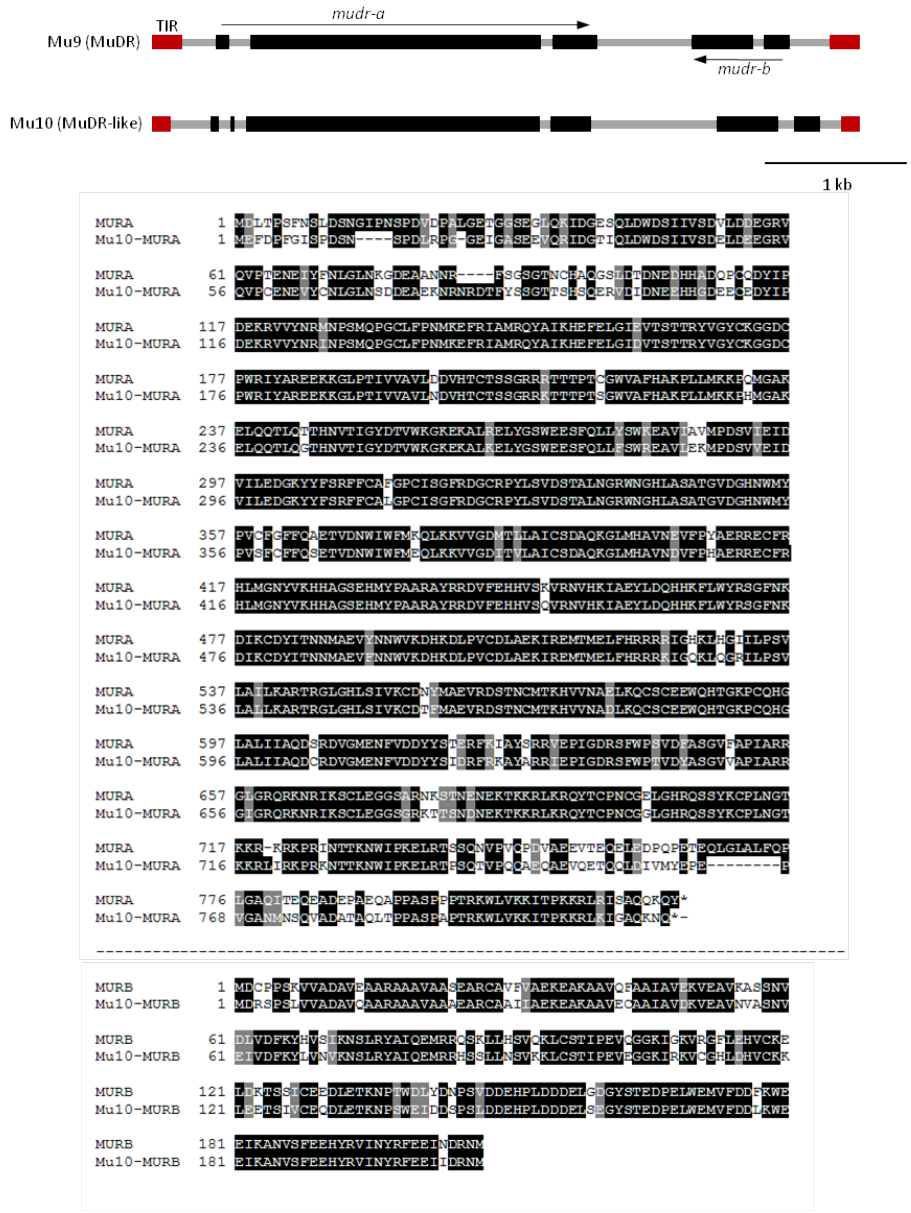


Figure 2-3. Comparison between MuDR and a Mu10 (Mu4K) element from the B73 maize inbred. The overall structure of MuDR and the MuDR-like element are diagramed in the upper portion of the figure, with TIR sequences and putative coding regions of the *mudr-a* and *mudr-b* genes shown. The Mu10 element is the only apparent MuDR-like sequence in the B73 genome with an intact, uninterrupted sequence. Both *mudr-a* and *mudr-b* genes have potential to code for functional proteins, since they contain no mutations that would lead to premature stop codons (unlike the other MuDR-like sequences in B73). Below, protein alignments between MURA and MURB from MuDR (top) and the predicted analogous proteins from Mu10 MuDR-like element (bottom) reveal high sequence similarity (82% identical for MURA, 79% identical for MURB).

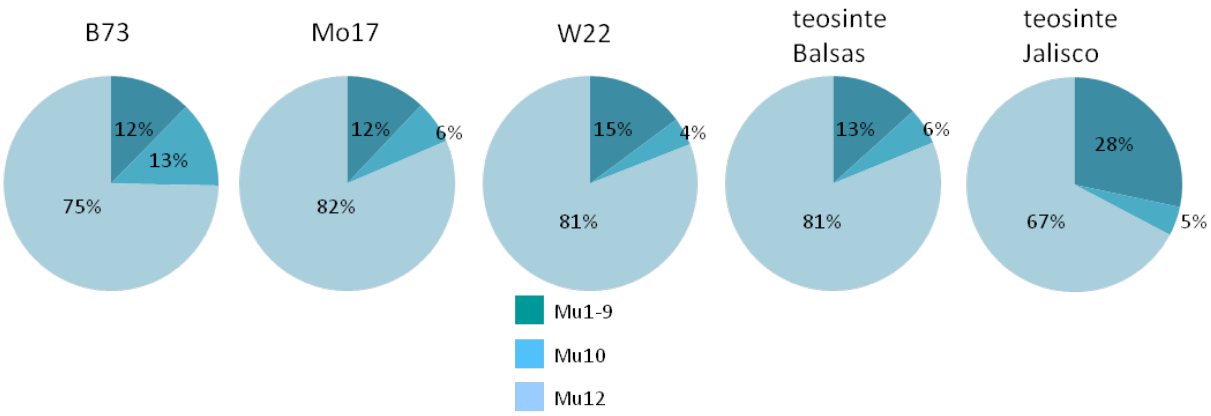


Figure 2-4. Estimated proportions of each Mu class in three maize inbreds and teosinte derived from the Balsas and Jalisco regions of Mexico. Estimates are based on extrapolations of 454-generated Mu-flank sequences summarized in Tables 2-3 and 2-4. Teosinte Balsas represents pooled data from teosinte inbred lines 1, 15, and 17. Teosinte Jalisco represents pooled data from teosinte inbred lines 11 and 14.

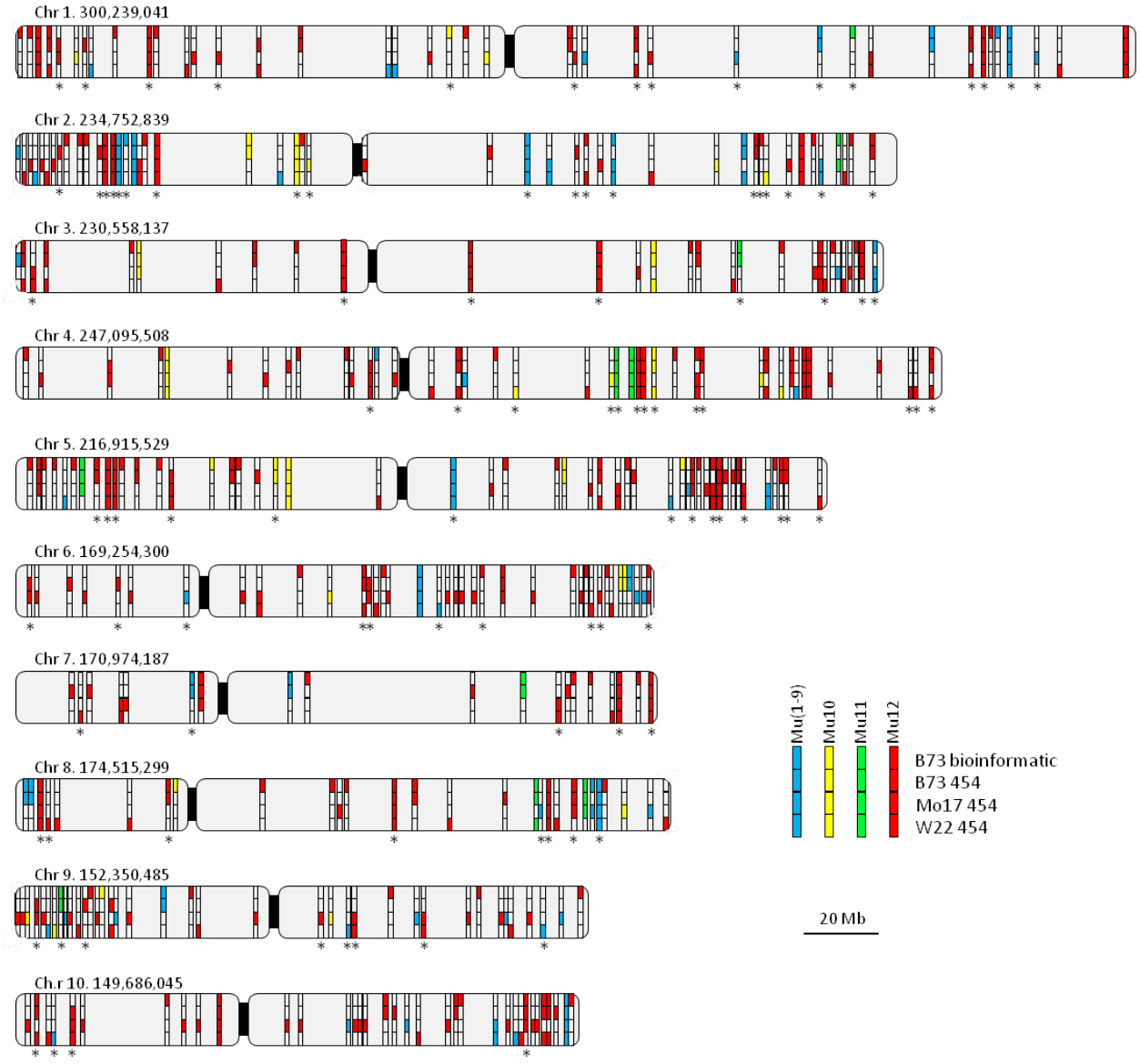


Figure 2-5. Approximate map sites for Mu insertions in three maize inbreds. Insertions were identified either through bioinformatic analysis of the B73 genome (top row), or through Mu-flank 454 sequencing of libraries from the three maize inbreds shown (B73, Mo17, W22 respectively positioned in rows 2, 3, and 4 of each vertical band on chromosomes). Positions on chromosomes are based on BLAST alignments of 454 flanking sequences to the B73 physical map, and are approximate. Above each chromosome diagram are total chromosome lengths (in bp). Inserts shared between at least one maize and one teosinte line are starred.

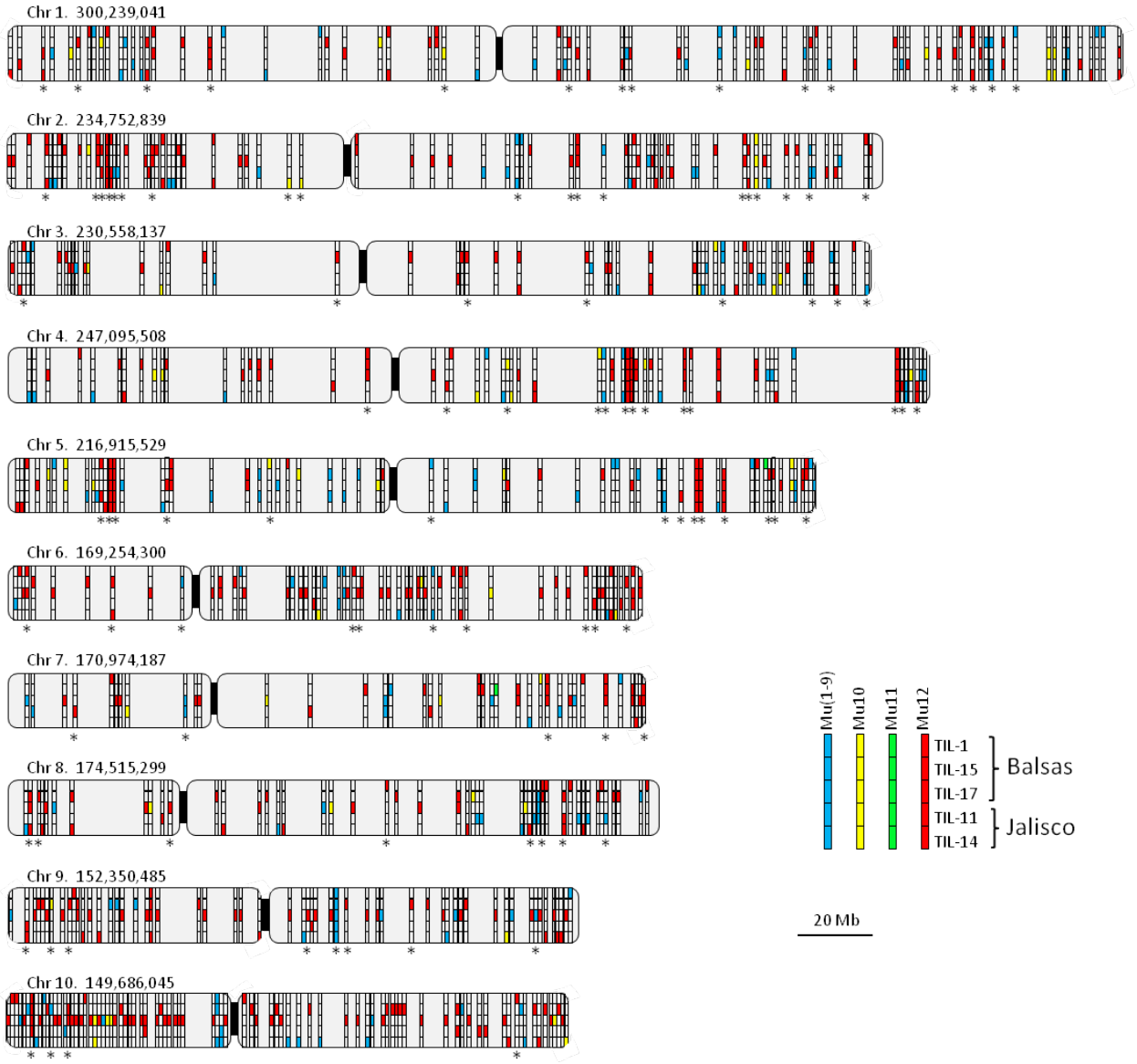


Figure 2-6. Approximate map sites for Mu insertions in five teosinte inbreds. Insertions were identified either through Mu-flank 454 sequencing of libraries from the five teosinte inbreds shown (TIL-1, TIL-15, TIL-17, TIL-11, and TIL-14), respectively positioned in rows 1-5 of each vertical band on chromosomes. Inbred lines 1,15, and 17 originated from the Balsas region (top three rows), and inbred lines 11 and 14 originated from the Jalisco region (bottom two rows). Positions on chromosomes are based on BLAST alignments of 454 flanking sequences to the B73 physical map, and are approximate. Above each chromosome diagram are total chromosome lengths (in bp). Inserts shared between at least one maize and one teosinte line are starred.

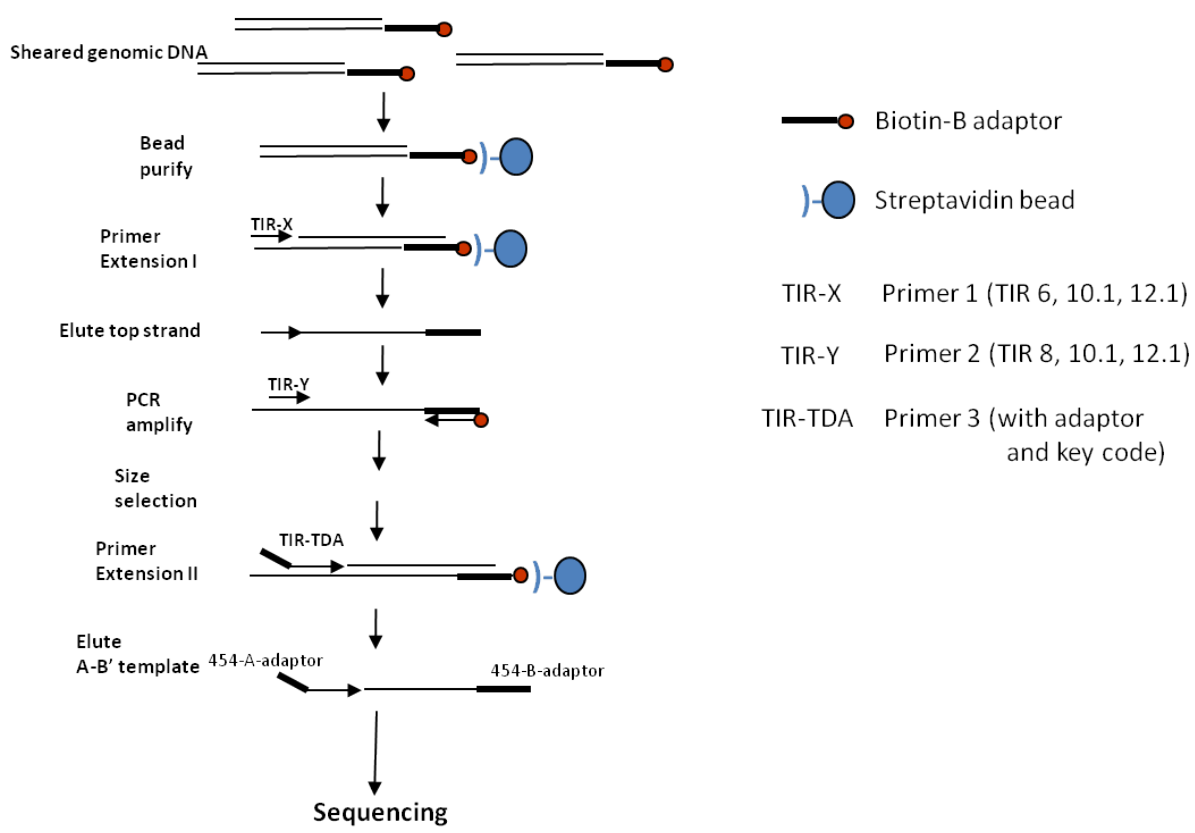


Figure 2-7. Diagramatic representation of Mu-flank 454-sequencing library preparation. Sheared genomic DNA from inbred maize and teosinte lines was used to generate libraries with amplified canonical Mu's (Mu1-9), Mu10s, and Mu12s, separately. TDA primers contained library-specific key codes for assignment of each read to correct sub-libraries. See Table 2-5 for primer information.

# CHAPTER 3
# ANALYSIS OF THE CELLULOSE SYNTHASE-LIKE D GENE FAMILY IN MAIZE

## Introduction

The ancient, highly-conserved *Cellulose Synthase-Like D* (*CslD*) genes encode enzymes required for specific cellular growth processes, yet whose biochemical and cellular functions remain elusive (Richmond and Somerville, 2000; 2001; Favery et al., 2001; Wang et al., 2001; Bernal et al., 2007; 2008; Yin et al., 2009). They form one of ten distinct subgroups in the Cellulose Synthase superfamily, defined by sequence similarity to Cellulose Synthases (CESAs) (Richmond and Somerville, 2000; Hazen et al., 2002; Farrokhi et al., 2006; Penning et al., 2009; Fincher, 2009). All members of the superfamily share characteristics of membrane-bound, processive glycosyltransferases that synthesize beta-linked glycan polymers, such as those of some cell wall polysaccharides (Richmond and Somerville, 2000; 2001), and are included in glycosyltransferase family 2 (Saxena, 1995; Campbell et al.,1997; Delmer, 1999; Coutinho and Henrissat, 1999; Holland et al., 2000). Known products range from cellulose to hemicellulose backbones, and may include additional beta-linked glycan chains (Arioli et al., 1998; Dhugga et al., 2004; Liepman et al., 2005; Burton et al., 2006; Cocuron et al., 2007; Doblin et al., 2009). Despite evidence that the CSLDs are of ancient origin, extending to the earliest non-vascular land plants and possibly before (Roberts and Bushoven, 2007), their contributions to cell wall biosynthesis are yet to be defined.

Of the cellulose synthase-like protein families, CSLDs are the most closely related to the cellulose synthases (CESAs), leading to the suggestion that CSLDs may themselves function as cellulose synthases (Doblin et al., 2001). The CSLDs share the

greatest amino acid sequence similarity with CESAs (40-50% identity) and are of similar, though slightly larger size (Richmond and Somerville, 2001). The CSLDs are also the only members of the cellulose synthase superfamily to have the amino-terminal, RING-type Zn-finger-like domains typical of the CESAs (Richmond and Somerville, 2000). These Zn-finger domains are thought to function in protein-protein interactions, possibly mediating complex formation or protein turnover (Gamsjaeger et al., 2007).

The locations and sizes of introns in *CslD* genes led to the suggestion that this subfamily may be ancestral to the entire Cellulose Synthase superfamily (Richmond and Somerville, 2000; 2001; Yin et al., 2009). Also, *CslD* genes are present in all plant genomes examined thus far, including non-vascular mosses (Richomond and Somerville, 2000; 2001; Roberts and Bushoven, 2007; Yin et al., 2009). In contrast, many of the other *Csl* subfamilies appear in only specific taxa (Farrokhi et al., 2006; Keegstra and Walton, 2006; Vogel, 2008; Penning et al., 2009; Fincher, 2009). Of the five *Csl* subfamilies yet to be assigned a specific polysaccharide synthase role, only *CslD* and *CslE* subfamilies are found in both dicot and monocot genomes (unlike *CslB*, *CslG*, and *CslJ*). This broad taxonomic distribution of the *CslD* genes indicates a conserved function throughout the plant kingdom.

Clues to the biological roles of the CSLDs have been sought by defining their biochemical activity, but this has proven challenging. Heterologous expression studies, while successful in determining the functions of CSLA (Liepman et al., 2005), CSLF (Burton et al., 2006), CSLC (Cocuron et al., 2007), and CSLH (Doblin et al., 2009) proteins, have thus far been unsuccessful for CSLDs. However, based on the

identification of biochemical actions for CSLA, CSLC, CSLF, and CSLH enzymes, the *CslD* genes have been hypothesized to encode hemicellulose synthases (Sandhu et al., 2009). Other important lines of evidence have led to alternate interpretations. Analysis of cell-wall polysaccharides, for example, from key cell types, cell-culture treatments, or genetic perturbations have been consistent with roles for CSLDs in production of either cellulose (Manfield et al., 2004; Li et al., 2009) or hemicellulose backbones (Bernal et al., 2007; Li et al., 2009). A complicating factor is that alterations in cell wall composition are often broadly pleiotropic, making primary effects difficult to distinguish from secondary or other closely-related changes (Orfila et al., 2005; Persson et al., 2007; Bernal et al., 2007; Li et all, 2009).

Another valuable source of information has come from subcellular localizations of CSLD proteins. Although the majority of studies thus far have favored a Golgi apparatus localization, supporting a role in hemicellulose backbone synthesis (Favery et al., 2001; Bernal et al., 2007; 2008; Zeng and Keegstra, 2008; Li et al., 2009), these findings are also consistent with the possible transit of CSLD proteins through these compartments enroute to the plasma membrane, as is the case for CESAs (Kimura et al., 1999; Crowell et al., 2009; Gutierrez et al., 2009). Indeed, recent studies have indicated a plasma membrane locale for CSLD proteins in rice suspension culture cells (Natera et al., 2008) and Arabidopsis root hair cells (Neilson and coworkers, unpublished data, cited with permission). More work is needed to conclusively demonstrate the subcellular localizations for active, endogenous CSLDs.

Until recently, phenotypes from *csld* mutants in multiple species favored roles for these proteins in tip growth (Bernal et al., 2008). The moss, *Physcomitrella patens,*

where tip growth is the predominating form of cell growth, *CslD* genes comprise 46% of all expressed sequence tags from the Cellulose Synthase superfamily (*CesA*s and all *Csl*s) (Roberts and Bushoven, 2007). In higher plants, root hairs and pollen tubes provide classic models for cellular tip growth (Hepler et al., 2001; Cole and Fowler, 2006), but xylem fibers also elongate by intrusive tip growth (Mellerowicz et al., 2001; Samuga and Joshi, 2004). Expression of *CslD2* in developing xylem of *Populus* is thus consistent with its proposed influence on xylem fiber length and its role in tip-growing cells (Samuga and Joshi, 2004). Prior to the work reported here for maize *csld1* mutants, genetic studies of *CslD* genes were consistently interpreted in the context of CSLD contributions to the process of tip growth (Bernal et al., 2008). However, recent genetic studies indicate an alternate and/or additional role for CSLD proteins (Chapter 4; Bernal et al., 2007; Li et al., 2009).

Although *Csl* genes are found in fairly large gene families, and some of these genes may function in overlapping or redundant fashions, many act in a non-redundant, organ-specific, or developmentally-controlled manner. Large gene families for cell-wall biosynthetic enzymes may be related to the complexity and diversity of the many cell wall polysaccharides (Reiter, 2002; Coutinho et al., 2003). By characterizing individual *Csl* gene knockouts in key species, clues to their functions may be revealed, and our understanding of cell wall biosynthesis improved.

## Results

### Bioinformatic Analyses of the *CslD* Genes from Maize, Rice, and Arabidopsis

The availability of fully-sequenced genomes for maize (Schnable et al., 2009; Van Erp and Walton, 2009), rice (Yu et al., 2002; Goff et al., 2002; Hazen et al., 2002), and Arabidopsis (Arabidopsis Genome Initiative, 2000; Richmond and Somerville, 2000)

allowed identification of *Cellulose Synthase-Like D* (*CslD*) gene families in these species (Fig. 3-1). These families were relatively small, with five to six members. Among the cellulose synthase superfamily, the *CslD* subgroup stands out as having the longest coding sequences, as well as particularly small introns (70 to110 bp) and large exons (Fig. 3-1). The *CslD* genes typically have well-conserved intron-exon boundaries, delineating two to four exons. Exceptions included maize *CslD2*, which has the largest intron (546 bp) of the *CslD*s examined. Only three other *CslD* genes had introns longer than 200 bp (Os-*CslD2*, Os-*CslD4*, and At-*CslD3*) (Fig. 3-1). Also, At-*CslD1* had five introns, two more than any other *CslD* gene, and no introns were evident in Os-*CslD5* (Fig. 3-1). Both Zm-*CslD3* and At-*CslD6* had apparent five-prime deletions, with the coding sequence of these genes starting at a point well within what would be the first exon of the other *CslD* genes (Fig. 3-1).

Translation of each full-length *CslD* coding sequence was predicted to produce a protein product from 1000-1250 amino acids (with the exception of the five-prime truncated Zm-*CslD3* and At-*CslD6*) (Fig. 3-2). A high degree of conservation was indicated for numbers and locations of transmembrane domains within the CSLD proteins (predicted using TMHMM [cbs.dtu.dk/services/TMHMM]). These domains are thought to be important for protein conformation and/or localization, and thus function (Somerville, 2006). While the degree of confidence for individual transmembrane domains varied considerably, each CSLD protein sequence was predicted to contain six carboxy-terminal, and two amino-terminal, transmembrane domains (with the exception of At-CSLD5, which was predicted to contain a single amino-terminal domain) (Fig. 3-2). Of the six carboxy-terminal domains, the fourth from that terminus showed the greatest

range in probability scores among the CSLD proteins. Amino-terminal domains showed the lowest probability scores in Zm-CSLD1, At-CSLD5, and Os-CSLD4 (Fig. 3-2).

A key feature of CSLD proteins are the RING-type Zn-finger-like domains they are thought to share with the CESAs (Richmond and Somerville, 2000). To test for the presence/absence of these domains, as well as their similarity to those of CESAs, protein sequences for each of the CSLDs of Arabidopsis, maize, and rice, as well as two CESAs, were examined using SMART (smart.embl-heidelberg.de), a domain prediction web tool (Schultz et al., 1998; Letunic etl., 2009). While this program recognized RING-type Zn-finger domains in both CESA proteins with high confidence, only six of sixteen CSLD proteins contained sequences recognized by this program as having Zn-finger similarity, and those at low probabilities (scored below the threshold for significance) (Fig. 3-2). Direct examination of protein sequence alignments, however, revealed a highly-conserved motif with strong similarity to CESA RING-type Zn-finger motifs in all but four of the CSLD proteins. Exceptions were predicted to carry five-prime truncations (see Fig. 3-3). Eight highly-conserved cysteine residues matching those of the RING domain of the CESA proteins, and oriented in a way compatible with Zn-finger organization (Fig. 3-3), indicate that these CSLD proteins may indeed contain functional domains, despite their not being recognized by SMART.

Active sites for glycosyltransferase family-2 processive glycosyl transferases typically include the D,D,QxxRW conserved residues (Richmond and Somerville, 2000). This motif was readily identified in all of the CSLD proteins examined from maize, Arabidopsis, and rice (Figs. 3-2,3-3). Examination of 35 amino acid residues

surrounding this motif revealed very strong sequence conservation in each of the 16 CSLD proteins and both CESA proteins examined (Fig. 3-3).

Alignment of the CSLD proteins from maize, Arabidopsis, and rice, along with two Arabidopsis cellulose synthases (At-CESA1 and At-CESA7) revealed the extent of conservation among the CSLDs and between the CESAs and the CSLDs (Fig. 3-3). Near-perfect alignment was observed between large portions of these proteins, including transmembrane domains (evident by conserved regions of densely-packed hydrophobic residues), as well as substrate-binding and catalytic sites (Fig. 3-3). More significant changes between CSLD proteins were observed in predicted RING-type Zn-finger motifs, although near-perfect alignment was observed in the cysteine residues noted above (Fig. 3-3).

Phylogenetic analyses revealed three clades in the CSLD subfamily that correspond to three phenotypic classes among *csld* mutants described here and elsewhere (Fig. 3-4). In Arabidopsis, *CslD1* and *CslD4* are implicated in pollen tube growth (Bernal et al., 2008), whereas *CslD2* and *CslD3* act in root hair formation (Favery et al., 2001; Wang et al., 2001; Bernal et al., 2008), and *CslD5* disruption reduces overall plant growth (Bernal et al., 2007). Thus far, mutants of the most closely related *CslD* genes in rice and maize yield phenotypes that appear analogous to those of Arabidopsis. Rice *CslD1* (Kim et al., 2007), and its maize homolog, *CslD5* (Penning et al., 2009), result in root hair-deficient phenotypes. Disruption of rice *CslD4* (closest homolog of Zm-*CslD1* and At-*CslD5*), confer a *narrow-leaf and dwarf1* (*nd1*) phenotype (Li et al., 2009). Similar functional roles are indicated by the reduced-growth phenotypes common to all three of these mutants (Chapter 4, Bernal et al., 2007; Li et

al., 2009). Collectively, data indicate conservation of specific developmental roles for individual CSLD proteins between Arabidopsis, rice, and maize.

**Expression Profiles of the Maize *CslD* Genes**

To evaluate the likely sites of action for each of the maize *CslD* genes, mRNA levels were determined by quantitative RT-PCR for selected maize tissues and developmental stages (Fig. 3-5). Each gene showed a distinctive pattern of expression, with the exception of *CslD3* and *CslD4*, whose patterns differed only in magnitude from one another (Fig. 3-5). Expression of most *CslD* genes was consistently low, compared to that of other genes in these tissues (data not shown). By far the greatest level of expression was that measured for *CslD4* (with *CslD3* close behind) in anthers 2 days before anthesis. The broadest expression profile was observed for *CslD2*, which was expressed in most tissues examined, including above- and below-ground structures, as well as in both vegetative and reproductive organs. In contrast, *CslD1* expression was more specific, having most abundant transcript levels in developing leaf blades and coleoptiles with enveloped young leaves. While *CslD5* mRNA was detectable in diverse tissues, levels were greatest in root hair cells (Fig. 3-5).

By tissue, the *CslD* gene with maximal expression in coleoptiles, shoot apical meristems, and developing leaves was *CslD1* (Fig. 3-5). Young and mature leaf blades were highest in *CslD5* mRNA, whereas midribs showed relatively high levels of *CslD2*, *CslD3*, *CslD4*, and *CslD5* expression. No *CslD* gene was detected at high levels in either mature stems or primary roots. The most highly-expressed gene in both silks and ligules was *CslD2*, whereas root hairs had elevated levels of *CslD5*, and ovaries showed significant expression of *CslD2*, *CslD3*, and *CslD4*. Finally, anthers had very high expression of both *CslD3* and *CslD4* (Fig. 3-5).

**Reverse Genetic Screens for *csld* Mutants**

Single-gene knockout mutations were sought for individual members of the maize *CslD* gene family by reverse genetic screening of around 15,000 lines from the UniformMu maize population (Yong et al., 2005; Penning et al., 2009). Mu transposon insertions were identified in *CslD1* (see Chapter 4) and *CslD5* (see below). No heritable insertions were recovered in *CslD2*, *CslD3 or CslD4*.

Screening identified a Mu transposon in exon 2 of *CslD5* in UniformMu Grid 1 (coordinates X-13 and Y-37), indicating presence of a putative knockout mutant in UniformMu family 02S-1039-15. Seeds from this family were planted, grown, and tested for the presence of a Mu insertion. Results showed a heritable insert in *CslD5* with normal Mendelian segregation patterns in over 200 PCR-genotyped individuals (data not shown). Plants carrying the Mu insertion were back-crossed to W22 inbred progenitors for an additional three generations to establish the *csld5-1* line. Above-ground characteristics of plants homozygous for this mutation showed no visible abnormalities in plant height, growth rate, flowering time, or accumulated biomass (data not shown). However, examination of *csld5* mutant seedlings grown on germination paper revealed a near-complete lack of root hairs (Fig. 3-6).

Linkage between the root hair-deficient phenotype and the *Mu* insert in *CslD5 was* confirmed by testing for the phenotype in an Ac-insertional allele with a transposon inserted upstream of the *csld5-1* allele in exon 1 (accession [AC027037] was obtained from the Ac/Ds collection at Cornell University, courtesy of Tom Brutnell [Kolkman et al., 2005]). Mutants from this line (established as *csld5-2*) were phenotypically identical to those of the *csld5-1* line (data not shown). Offspring of reciprocal crosses between

*csld5-1* and *csld5-2* parents consistently showed the root hair-deficiency, conclusively linking disruption of *CslD5* with the phenotype.

**Analysis of *csld5* Mutants**

In-depth examination of primary roots from seedling-stage, *csld5* mutant and wildtype plants revealed that in the mutants, root hairs initiated but usually failed to elongate (Fig. 3-6). Although typical wildtype root hairs grew to around 600 µm, those of *csld5* mutants seldom elongated much after initiation, and in the rare instances of substantial growth, reached only around 300 µm (Fig. 3-6). From SEM analyses, it is estimated that as few as one in fifty root hairs grew to longer than 100 µm in *csld5* mutants. The majority of *csld5* mutant root hair initials appeared swollen and bulging, often wider at the base than typical wildtype root hairs (Fig. 3-6). Only root hairs appeared to be affected, since other root cells, including epidermal and internal cells, appeared to be normal in *csld5* mutants (Fig. 3-6). Under all growing conditions tested (from field to greenhouse and germination trays) plus a range of stresses, *csld5* mutant plants behaved no differently from wildtype plants as far as growth rates and flowering times. Above ground phenotype and biomass accumulation did not differ (data not shown).

**Root-hair Transcriptome Profiling**

Specificity of the root-hair phenotype in *csld5* mutants raised broader questions about distinctive features of gene expression and cell wall biosynthesis in this cell type. To obtain more global data on transcripts in these cells, we profiled mRNAs of root hairs from seedlings of W22 and B73 inbreds, and from their F1 hybrid progeny. We used three-prime anchored, 454 transcript sequencing, which allowed quantitative measurements of relative transcript levels without alignment to a reference genome

(each sequence read could be immediately assigned identity due to its 3`-anchor) (Eveland et al., 2008). A single titration run (1/16 plate) on a 454, GS-20 sequencer (Roche Biosciences, Indianapolis, IN) yielded 79,448 sequences corresponding to 10,618 unique transcripts, 6,273 present as 2 or more reads. Annotation of the 100 most highly-expressed genes based on similarity to previously-described genes, indicated that many of the most-abundant mRNAs were involved in amino acid metabolism, cell wall modification, and redox regulation (Fig. 3-7). Maize *CslD5* was not among the genes identified in this screen, presumably because of low expression.

## Discussion

### The *CSLD* Genes are Highly-conserved Among Diverse Species

The cross-species conservation of *CslD* gene copy number, intron-exon boundaries, gene size, and protein domains (Figs. 3-1, 3-2) are indicative of long-conserved function and strong selection against mutations. Compared to *CesA7* and other *CesA*s, *CslD*s have remarkably few introns for such large genes (Fig. 3-1). Intron number and position can be important to gene regulation in a number of instances (Clancy and Hannah, 2002; Jeong et al., 2007; Rose et al., 2008), but whether differences between *CslD*s and *CesA*s reflect functionally-significant aspects remains unclear. Either way, the similarities between *CslD* genes and *CesA* genes are consistent with a close relationship between these two gene subfamilies and have been used to support suggestions of a similar catalytic function (Doblin et al., 2001; Richmond and Somerville, 2001). Truncations to the five-prime regions of Zm-*CslD3* and At-*CslD6* result in loss of the RING-type Zn-finger-like domain thought to function in protein-protein interactions, supporting the possibility of these genes being non-functional pseudogenes.

The conservation of transmembrane domain position and number in the CSLD proteins indicates their importance for function (Fig. 3-2). In CESA proteins, these domains are thought to function together in forming a channel through the plasma membrane, through which the elongating glucan chain is excreted into extracellular space (Somerville, 2006; Zhang et al., 2009). Interestingly, the three proteins with amino-terminal transmembrane domains having the lowest probablility of maintaining membrane-spanning capability (based on TMHMM predictions) are the same that result in the reduced-growth phenotypes when disrupted (Zm-CSLD1 [Chapter 4], At-CSLD5 [Bernal et al., 2007], and Os-CSLD4 [Li et al., 2009]). Although mutant phenotypes indicate that these three proteins have an in vivo function, they have either lost one or more transmemembrane domains, or the domains have remained functional while diverging enough to not be recognized by TMHMM.

The active site of the sixteen CSD proteins analyzed here is extremely well conserved. Within the 35 amino acids surrounding the active site, there are only three amino acid differences likely to alter properties at that site (Fig. 3-3). In contrast, the Zn-finger-like domain and the transmembrane domains both showed considerably more variation between CSLD proteins (Fig. 3-3 and 3-2, respectively).

### Conservation of Developmental Roles are Indicated by Phenotypic Similarities Across Taxa

Comparative phylogenetic analysis, combined with mutant phenotypes described here and elsewhere, indicate ancient functional divergence and highly-conserved developmental roles for sub-groups within the *CslD* gene family (Fig. 3-4). When mutant phenotypes from maize, rice, and Arabidopsis were overlaid on a neighbor-joining tree,

CSLD clades corresponded to distinct classes of phenotypes. These were: (i) root-hair-defective, (ii) male-transmission-defective, or (iii) reduced-growth (Fig. 3-4).

Several additional aspects of this association included, first, that all of the *csld* mutants identified thus far have visible phenotypes (Favery et al., 2001; Wang et al., 2001; Bernal et al., 2008, 2009; Kim et al., 2007; Li et al., 2009; Penning et al., 2009). Second, the associations shown in Figure 3-4 persist despite fundamental structural differences between the primary cell walls of Arabidopsis (Type I walls) and those of rice and maize (Type II walls) (Harris and Hartley, 1980; Carpita and Gibeaut, 1993; Carpita, 1996; Carpita et al., 2001). Roles of *CslD* genes thus apparently transcend major differences in non-cellulosic cell wall constituents between diverse plant species, and individual *CslD* genes appear to have maintained their primary developmental roles. Third, previous results suggested specific functions for CSLD proteins in tip-growing cells (Bernal et al., 2008), but data here and elsewhere indicate broader developmental roles as well.

**Specificity of Expression Argues for Strictly-defined Roles for *CslD* Genes**

Quantitative RT-PCR of individual maize *CslD* genes revealed distinct expression patterns (except for *CslD3*, a predicted pseudogene with a large deletion in its amino terminus) (Figs. 3-1, 3-2). These expression patterns contrast with those of *CesA* genes, which show apparently coordinated expression among defined subsets of the gene family (Burton et al., 2004; Nairn and Haselkorn; 2005). Coordinate expression of *CesA* subgroups is considered consistent with the hypothesis that the CESA proteins act together in functional rosettes. No obvious pattern of co-expression was observed between any of the *CslD* genes, indicating either a capacity to function alone, or at least independently of strictly-defined partners. The possibility remains that different

combinations of CSLD and/or CESA proteins may function together in an as-yet-undefined manner. Nonetheless, the well-defined expression pattern for each of the *CslD* genes is consistent with proposed specificity of function (Fig. 3-5). Also, although genetic studies of *csld* mutants have consistently indicated a role for CSLD proteins in tip-growing cells, the expression profiles of the *CslD* genes suggest potentially broader roles. No obvious tip-growth occurs in cell comprising many of the tissues in which relatively strong expression of one or more *CslD* gene was observed (Fig. 3-5). Developing leaves, silks, and ovaries, for example, have relatively abundant *CslD* gene expression, but few cells in these organs appear to use tip-growth-like mechanics.

The root hair-defective phenotype of cs*ld5* mutants (Fig. 3-6), is consistent with patterns of expression for the *CslD5* gene. Greatest levels of *CslD5* mRNA were observed in isolated root hairs (Fig. 3-5). In contrast, growing primary roots show little expression of *CslD5*, despite containing growing root hairs. The difference between expression in root hairs alone and whole root tips indicates the specificity of expression for this gene, which seems to be essentially limited to a single cell type in growing primary roots.

**Functional *CslD5* is Required for Maize Root-hair Elongation**

The lack of elongated root hairs on *csld5* mutants indicates a role for this gene product in the formation of new cell wall at the growing tip of these classic tip-growing cells (Fig. 3-6). Interestingly, *CslD5* is not required for initiation of root hairs, only their elongation (Fig. 3-6). The process through which a trichoblast cell initiates root hair formation has been extensively studied, and involves intense, coordinated cell wall modification and biosynthesis (Syzmanski and Cosgrove, 2009; Anderson et al., 2010). Wall-loosening enzymes are focused at the site of root hair formation, and new cell wall

biosynthesis is targeted to the site via actin-mediated re-organization of the endomembrane system (František et al., 2000). That *csld5* mutants initiate root hairs indicates that CSLD5 is not essential to this process. One or more other CSLD enyzmes may contribute to the first steps of root hair formation, or perhaps there is partial redundancy, with *CslD5* essential only during the most rapid phase of root hair growth.

In Arabidopsis *csld3* mutants (homologous to maize *CslD5*), root hairs have been reported to burst at the tip, release cytoplasm, and cease growth (Wang et al., 2001). Similar bursting does not appear to occur in root hairs of maize *csld5* mutants. Indeed, SEM analyses of *csld5* root hairs show them to be short, but intact (Fig. 3-6). The mutant root hairs are swollen and uneven compared to wildtype, but they do not appear ruptured (Fig. 3-6). Why some mutant root hairs undergo partial elongation (up to 250 µm), while the majority terminate growth much earlier remains unclear. One possibility is that another CSLD protein is able to partially compensate for loss of CSLD5, or rare instances of root hair growth could occur without the specific polysaccharide contribution of CSLD5. In Arabidopsis, mutations in either *CslD2* or *CslD3* result in aberrant root hairs, indicating either coopertation of those two gene products or limited redundancy (Favery et al., 2001; Wang et al., 2001; Bernal et al., 2008). From both the phylogenetic analysis (Zm-CSLD2 is closest to At-CSLD2 [Fig. 3-4]) and transcription profiles (Fig. 3-5), the most likely candidate for compensating for CSLD5 loss is CSLD2.

Transcript profiles from maize root hairs reveal the complexity of genetic activity in this single cell type (Fig. 3-7). The failure to detect *CslD5* transcript in this analysis is consistent with a relative scarcity of this mRNA compared to the more abundant

transcripts. If an arbitrary “abundance” score is set at detection of 10 mRNAs, at least 1,210 other genes are expressed at a higher level than *CslD5* in this cell type. Presence of a mutant phenotype indicates that expression of this gene at some level is important to normal root hair elongation, but apparently a very small amount of this mRNA is sufficient. In either case, mRNA levels do not necessarily reflect CSLD5 protein levels, which could well be higher if the protein is long-lived.

The most highly expressed genes included many with high homology to genes of known function, and others that were not similar to any previously-characterized genes (thus were annotated as hypothetical or unknown). Genes for cell-wall modification, redox regulation, and amino acid metabolism were predominant. The 50 most highly expressed mRNAs included genes for a beta expansin, an arabinogalactan protein, and two xyloglucan endotransglycosylases (Fig. 3-7). Also in the top 50 genes were those encoding two adenosylmethionine synthases, two methionine adenosyltransferases, multiple transporter proteins, ubiquitin-related proteins, and redox-related proteins (Fig. 3-7).

Also evident in the root-hair transcript profiles were differences in the most abundant mRNAs between the three maize lines tested (B73, W22, and their hybrid). Most genes were expressed at similar levels in all lines (based on read numbers, normalized for total per library). However, there were also multiple cases showing very different expression of certain genes in W22 and B73. For instance, the most highly expressed gene in B73 (described in Figure 3-7 as a hypothetical protein) was expressed at very low levels in W22 (802 transcripts sequenced for B73, compared to 3 for W22). For the hybrid, transcript levels for most genes indicated additive effects,

being detected at levels between that of B73 and W22 (Fig. 3-7). In other instances, non-additive effects were observed, with apparent expression of a given gene in the B73-W22 hybrid much higher or lower, respectively, than in both inbreds (Fig. 3-7). The most highly expressed gene in the hybrid (again a hypothetical protein) ranked much lower in the expression profiles of both inbreds. The reverse was also observed in multiple instances, where a gene not detectable in the hybrid was expressed at relatively high levels in both inbreds.

## Methods

### Bioinformatic Analyses of *CslD* Genes from Maize, Rice, and Arabidopsis

Full-length coding sequences from each of the CSLD genes in maize, rice, and Arabidopsis were compared to genomic sequence to identify exon-intron boundaries. Coding sequences were used to predict amino acid sequences, using a web tool at changbioscience.com/res/rest. Each of these predicted amino acid sequences, as well as that of Arabidopsis Cellulose Synthase A 7 (At-CESA7), was independently analyzed with the protein domain-prediction software SMART (smart.embl-heidelberg.de) to test for presence of RING-type Zinc-finger-like domains. Proteins for which SMART failed to detect RING domains were manually examined after alignment with ClustalW for similar amino acid sequences. Putative trans-membrane domains were identified by TMHMM for each predicted protein (cbs.dtu.dk/services/TMHMM).

### Phylogenetic Analyses

The neighbor-joining tree was created using Mega 4.0 (Tamura et al., 2007; megasoftware.net), with ClustalW-generated alignments of protein sequences predicted from full length cDNAs for each of the CSLD genes from rice, maize, and Arabidopsis. The resulting tree represents 2,000 bootstrap repetitions using the pairwise deletion

option. Nomenclature for proteins encoded by the *CslD* gene family in maize was assigned as per Van Erp and Walton (2009).

**Real-Time RT-PCR**

For each sample, RNA was extracted from approximately 200 mg of tissue, initially frozen in liquid nitrogen, then homogenized in 1.0 mL Trizol (Invitrogen Cat # 15596-018) using a Q-BIOgene FastPrep 120 with Lysing Matrix D (MP Biomedicals Cat # 116913). Samples were incubated for 5 min at 25°C, with frequent vortexing. Chloroform (200 µL) was added and samples were vortexed 15 sec before and after a 1-min incubation at 25°C. Phases were separated by centrifuging 10 min at 15,000 x g, followed by transfer of 200 µL of the aqueous layers to 700 µL of Qiagen RLT buffer (from RNeasy Plant Mini kit, Qiagen Cat # 74904). Ethanol was added (500 µL, 100% EtOH) and samples were vortexed. Half of the resulting volume was used to clean and elute total RNA as per the RNeasy Plant Mini kit (Qiagen Cat # 74904). Resulting RNA was treated with DNase-1 (Ambion Cat # AM1906), and quantified using a BioRad SmartSpec 3000. The cDNA was synthesized using a SuperScript One-Step kit and protocol (Invitrogen Cat # 10928-042).

Levels of mRNA were quantified from selected maize tissues at a range of developmental stages using quantitative Real-time RT-PCR (Step One Plus Real-Time PCR System [Applied Biosystems]). At least three biological replicates were analyzed for each tissue or time point, and for each of these replicates, reactions were performed in duplicate. A given reaction included 10 µL Fast SYBR Green Master Mix (ABI Lot # 1003024), 5.0 µL of cDNA sample (diluted 10x from cDNA reaction), and 100 nM of each gene-specific primer (Table 3-2) in a final volume of 20 µL. The relative abundance of transcripts was normalized with 18S rRNA controls (Taqman Ribosomal

RNA Control Reagents, ABI Lot # 0804133) as in Eveland et al., 2008. Primer pairs for all genes were designed using Primer Express 3.0 (ABI).

**Reverse Genetic Screening**

The UniformMu population was screened using PCR-based assays to identify Mu transposon insertions in each of the maize *CslD* genes as per Penning et al., (2009). Close to 15,000 UniformMu lines were screened using a series of pooled DNA samples. These lines were forerunners of the sequence-indexed materials currently available at MaizeGDB (maizegdb.org; UniformMu.UF-genome.org). For PCR screening, gene-specific primers were used along with TIR6, a Mu-specific primer (Table 3-2). Resulting products were separated on 1% agarose gels, blotted onto nylon membranes, and probed with gene-specific PCR products. Where positive results were obtained in both x- and y-axes (as for *CslD1* and *CslD5*), seeds from the identified UniformMu family were planted, leaf tissue was harvested, and DNA was tested by PCR for segregation of homozygous mutants.

**Growth Conditions for *csld5* Mutant Analyses**

Maize seeds were sterilized by soaking in 15% bleach while stirring for 10 min, then rinsed thoroughly with water. Kernels were allowed to imbibe water overnight and pericarps were manually removed. Seeds were arranged on wet germination paper in glass trays with embryos facing up, sprayed with Captan solution (1% Captan), and covered in plastic wrap. Air was pumped through these trays to prevent $CO_2$ and ethylene build-up, and seeds were allowed to germinate.

**Fixation and Sectioning**

One-cm-long pieces of primary root from 10-day-old seedlings were collected and fixed in FAA (10% formaldehyde [Fisher Lot # 992720], 5% acetic acid, 50% EtOH).

Samples were vacuum infiltrated overnight at 4°C, then shaken at 4°C during a dehydration series using ethanol in PBS (60 min each, progressing from 1X PBS with 30% EtOH to 40%, 50%, 60%, 70%, 85%, and finally 95% EtOH). Samples were stained overnight with eosin in 95% EtOH, followed by four, 1-hr incubations in 100% EtOH and eosin at 25°C. Wax imbedding was initiated by introducing CitriSolv (Fisher Cat # 22-143975) into samples using a series of 1-hr incubations (while shaking) in ethanol with increasing CitriSolv/EtOH content (25/75, 50/50, 75/25,100/0). Paraplast wax chips (Fisher Cat # 23-021-399) (1 g wax/mL CitriSolv) were added to the 100% CitriSolv and incubated overnight at 25°C. Additional wax was added, followed by a 2-hr incubation at 42°C. Samples were transferred to 60°C for 1 hr. Wax was poured-off and replaced eight times before samples were allowed to harden in molds. Sections (10 µm, cut with a Leitz 1512 microtome) were de-waxed with three, 5-min incubations in xylene (Fisher Lot # 083423), then washed twice in 100% EtOH (5 min each), and once in 95% EtOH (3 min). Slides were dried and examined under an Olympus BH2 light microscope.

**Scanning Electron Microscopy**

Primary roots, approximately 5-cm long from *csld5* mutant and non-mutant seedlings, were fixed in FAA (10% formaldehyde [Fisher Lot # 992720], 5% acetic acid, 50% EtOH), dehydrated in an ethanol series 75%, 95%, 100%, and critical point dried (Bal-Tec CPD030, Leica Microsystems, Bannockburn, IL). Dried samples were mounted with carbon adhesive tabs on aluminum specimen mounts, Au/Pd sputter coated (DeskII, Denton Vacuum, Moorestown, NJ), and examined with a field-emission scanning electron microscope (S-4000, Hitachi High Technologies America, Inc. Schaumburg, IL). Digital micrographs were acquired with PCI Quartz software.

**Isolation of Root Hair mRNA**

Maize seedlings were grown as above. When primary roots averaged 5 cm in length (10 days after germination), individual seedlings were suspended in liquid nitrogen until completely frozen. Root hairs were then harvested using a metal spatula to shatter them from the root body and into a small weigh boat of liquid nitrogen. Root hairs from around 150 individual seedlings were pooled to get approximately 100 mg, which were stored at -80ºC until further use. A modified RNA extraction protocol was used to recover adequate amounts of RNA from root hairs. Total root hair samples were added to 200 µL of extraction buffer (50 mM TRIS [pH 8.0], 150 mM LiCl, 5 mM EDTA [pH 8.0], 1% SDS), and ground in liquid nitrogen. Samples were allowed to thaw and immediately transferred to tubes containing 200 µL of 1:1 Phenol-Chloroform, shaken, and placed on ice for 5 min, with periodic mixing. Samples were transferred to 2-mL Phase Lock Gel tubes (Eppendorf, cat # 2302830), and centrifuged (10 min, 10,000 x g, 4°C). After centrifugation, 200 µL of 1:1 Phenol-Chloroform was added, samples were well-shaken, then centrifuged (10 min, 10,000 x g, 4°C). Next, 200 µL of Chloroform was added, samples were shaken then placed on ice for 5 min, with occasional mixing for centrifuging (10 min, 10,000 x g, 4°C). The aqueous layer was poured into a new PHASE LOCK tube (Eppendorf) and 1 mL of TRIZOL (Sigma, Lot # MKBD6639V) was added. Tubes were shaken well for 15 sec and incubated at room temperature for 5 min. After incubation, 200 µL Chloroform was added, tubes were shaken, and incubated at room temperature for 3 min before centrifuging (10 min, 10,000 x g, 4°C). The aqueous layer was transferred to new microfuge tubes and 500 µL of Isopropanol was added. Samples were mixed and placed on ice for 10 min before centrifuging (10 min, 10,000 x g, 4°C) to precipitate RNA. Supernatant was removed by

pipetting, 1 mL 70% EtOH was added, and samples were centrifuged (5 min, 10,000 x g, 4°C). Supernatant was carefully removed by pipetting, pellets were allowed to air dry, and RNA was re-suspended in 30 µL water.

**Three-prime Anchored 454 Sequencing**

Libraries for 454-based sequencing were prepared as per Eveland et al. (2008). Total RNA (5 µg) from root hairs of B73, W22, and the B73 x W22 hybrid was used for cDNA synthesis (MessageAmp II, Ambion, Cat # AM1793) by priming with 6 pmol of biotinylated (T12) B-adaptor oligo (modified from Margulies et al., 2005) (Table 3-2). Purified cDNA (DNA clear, Ambion) was bound to M-270 Strepdavidin Dynabeads (Invitrogen, Cat # 653.05), immobilized on a magnetic tube stand (Applied Biosystems, Lot # 0804015), and digested with *Msp1* (Promega) to create 2-base CG overhangs for adaptor ligation. A-adaptor oligos (modified from Margulies et al., 2005) included 3-base multiplex keys (Table 3-2). Adaptor pairs (top-strand and bottom-strand for each sample [Table 3-2]) were combined and concentrated to 1 pmol/µL in (10 mM Tris, 1 mM EDTA, 50 mM NaCl [pH 8.0]) and annealed by gradual 1°C/min decreases (95°C-4°C, holding at 72°C for 30 min). Adaptors (5 pmol) with the correct multiplex keys were ligated to digested samples. Unligated adaptors were removed by washing beads twice with 1X B&W (2.5 mM Tric-HCL [pH 7.5], 0.25 mM EDTA, 0.5 M Nacl) followed by two washes with water. The template strands were eluted with 100 mM NaOH, neutralized, and concentrated on a Qiagen column (from RNeasy Plant Mini kit, Qiagen Cat # 74904). Sequencing was conducted as per Margulies et al., (2005) on a 454 GS-20 instrument (Roche Biosciences, Indianapolis, IN).

**Analysis of 454-generated Sequence Data**

Trimmed 454 reads were filtered for valid key-code sequences and ligation junction sites (CGG) at 5`-ends. Poly-A tails were trimmed to 6-A's at the 3`-end using custom, java-based programs (courtesy of Don McCarty). Trimmed sequences were assembled using CAP3 (genome.cs.mtu.edu/sas). The nonredundant set of consensus cDNA sequences were annotated by BLASTN searches of cDNA databases for maize. These included publicly available cDNAs on maizegdb.org and IUC, a collection of cDNAs provided by an industry consortium via a user's agreement (maizeseq.org).

**Accession Numbers**

Accesion numbers for each of the gene sequences are At-*CslD1*:AT2G33100.1, At-*CslD2*:AT5G16910.1, At-*CslD3*:AT3G03050.1, At-*CslD4*:AT4G38190.1, At-*CslD5*:AT1G02730, At-*CslD6*:AT1G32180.1, Os-*CslD1*:AC027037.6, Os-*CslD2*:Os06g0111800, Os-*CslD3*:AC091687.1, Os-*CslD4*:AK242601.1, Os-*CslD5*:Os06g0336500, Zm-*CslD1*:GRMZM2G015886, Zm-*CslD2*:GRMZM2G052149, Zm-*CslD3*:GRMZM2G061764, Zm-*CslD4*:GRMZM2G044269, Zm-*CslD5*:GRMZM2G436299, At-*CesA7*: AT5G17420.

Table 3-1. Three prime-anchored 454-sequencing transcript profiles.

| | B73 | B73xW22 | W22 | Combined |
|---|---|---|---|---|
| Total reads | 27,675 | 36,816 | 14,957 | 79,448 |
| Unique transcripts | 5,634 | 6,815 | 3,972 | 10,618 |

Total reads from a single 454 titration run. Each library was key-coded prior to combining for sequencing. Total reads are the number of individual sequences recovered. Unique transcripts are the number of different genes represented based on alignment to cDNA collections.

Table 3-2. PCR primers utilized in Chapter 3.

For Real-Time RT-PCR

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *CslD1* | GCCGCTCACGTCAATGG | CTGGGCATCTTCATGGAGTGT |
| *CslD2* | ACGTCTCCAACTCCCTCTTCAC | CGGCTTCAACAGTGTC |
| *CslD3* | TCCATCGTGTGCGAGTTCTG | CAGCTTTGGCATCTGATCCA |
| *CslD4* | ATGAAGGCCGAGGAGCAGTA | CGCGTGACGCTGTTGAAC |
| *CslD5* | GGGCGCTTCATCAGCTACTC | GGACGTGGTAGTCCTGGAAGTC |

For reverse genetic grid screening

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *CslD1* | AGTTCGTGCACTACACCGTGCACATCC | TGCTACCTGTAAGGACTGAGGATGGCCTG |
| *CslD2* | TCTCACTGTCCCCCGTGACCTTCTGGATG | ACTCTCCCAGGCTGATCCCCGACCACTTG |
| *CslD3* | GTCAAGATGGAGGACCTCGTTGACAAGCC | CTCCGCCATTGCTTCGAAGGTTAGCAGC |
| *CslD4* | CCAACAACAACACCGTCTTCTTCGACGGC | GTCTGCACGATGAAGAAGCCCGAGAAGAG |
| *CslD5* | TTGTTCCTCCCATCCCATCCAGGCTCCTC | TAGCACGCAAGCTTCTCGACGGGGTAGTC |
| TIR6 | AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC | |

For Three-prime-anchored 454 transcript profiling

| | |
|---|---|
| B-adaptor | Biotin-CCTATCCCCTGTGTGCCTTGCCTATCCCTGTTGCGTGTCTCAGTTTTTTTTTTTT T[AGC] |
| B73 top | CCATCTCATCCCTGCGTGTCCCATCTGTTCCCTCCCTGTCTCAG<u>AGC</u> |
| B73 bot. | CG<u>GTT</u>CTGAGACAGGGAGGGAACAGATGGGACACGCAGGGATGA |
| W22 top | CCATCTCATCCCTGCGTGTCCCATCTGTTCCCTCCCTGTCTCAG<u>ACA</u> |
| W22 bot. | CG<u>ATT</u>CTGAGACAGGGAGGGAACAGATGGGACACGCAGGGATGA |
| BxW top | CCATCTCATCCCTGCGTGTCCCATCTGTTCCCTCCCTGTCTCAG<u>ATG</u> |
| BxW bot. | CG<u>CTT</u>CTGAGACAGGGAGGGAACAGATGGGACACGCAGGGATGA |

For sublibrary oligos, 3-bp keys are underlined. BxW: B73xW22 hybrid. bot = bottom.

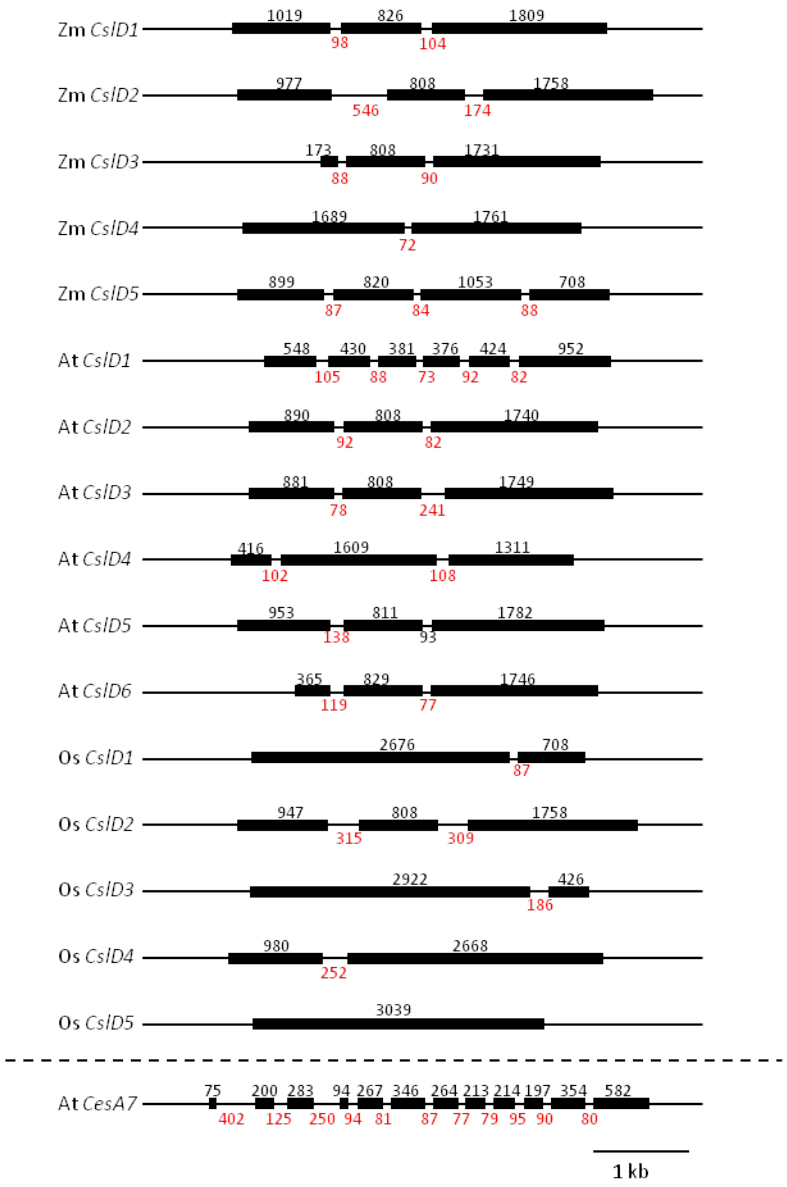


Figure 3-1. *Cellulose synthase-like D* (*CslD*) genes from Arabidopsis, maize, and rice. Each of the *CslD* gene coding sequences from maize (5), Arabidopsis (6), and rice(5), as well as Arabidopsis *CesA7*, are diagramed with exon/intron locations and sizes drawn proportionally. Untranslated regions are not included. Exons are represented by black bars and their length is indicated above each. Intron lengths are indicated in red.

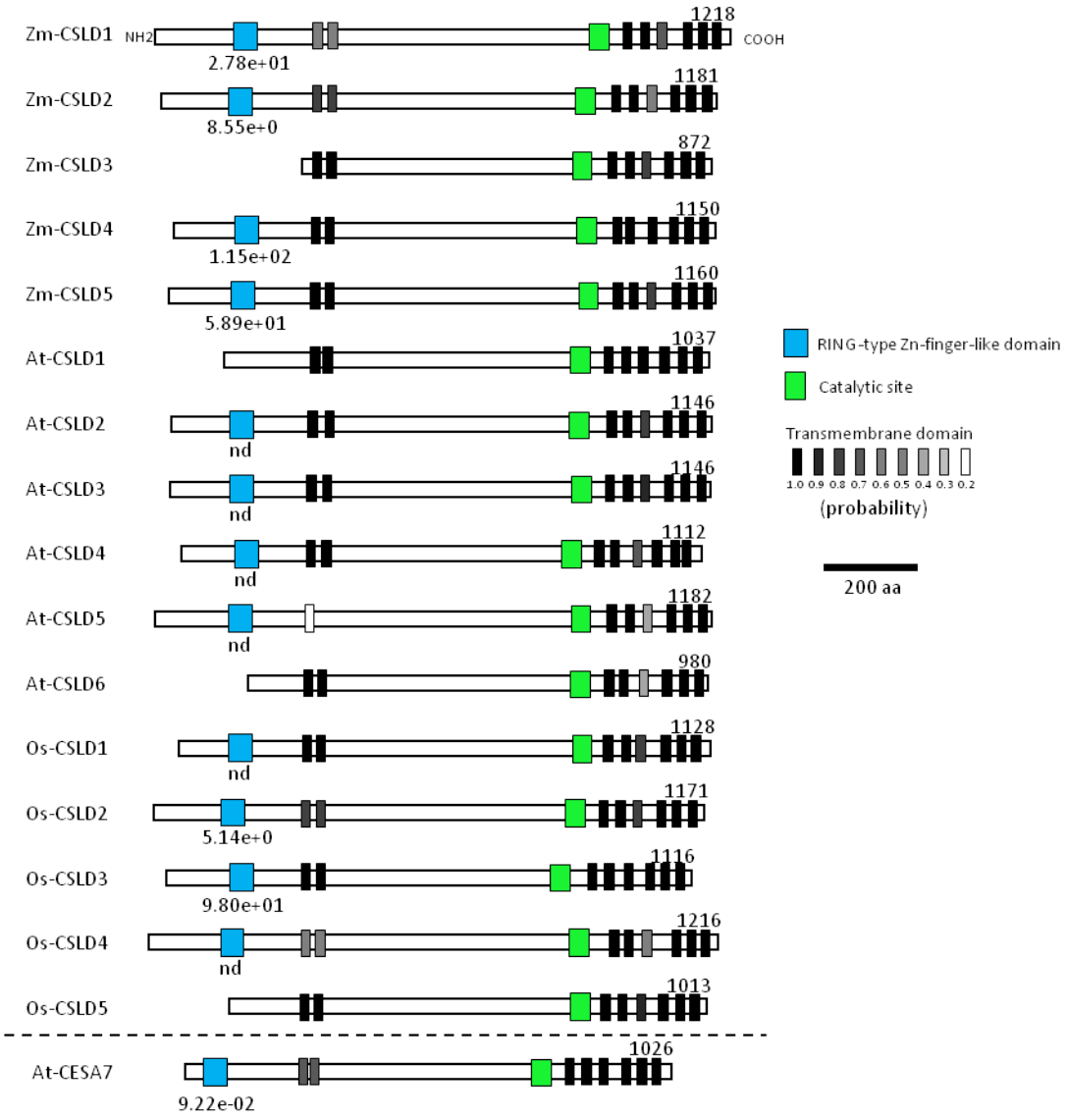


Figure 3-2. Cellulose synthase-like D proteins from Arabidopsis, maize, and rice. All of the CSLD proteins are diagramed as predicted from translation of *CSLD* genes of maize (5), Arabidopsis (6), and rice(5). Protein length is indicated above each diagram. Transmembrane domain predictions were generated using TMHMM (cbs.dtu.dk/services/TMHMM). Color scale shows the probability of a transmembrane domain, based on TMHMM output. The Zn finger-like domains were predicted using SMART (smart.embl-heidelberg.de). E-values (from SMART output) for Zn-finger-like domains are indicated below each diagram, nd = not detected by SMART, but apparent in direct sequence visualization (see Figure 3-3).

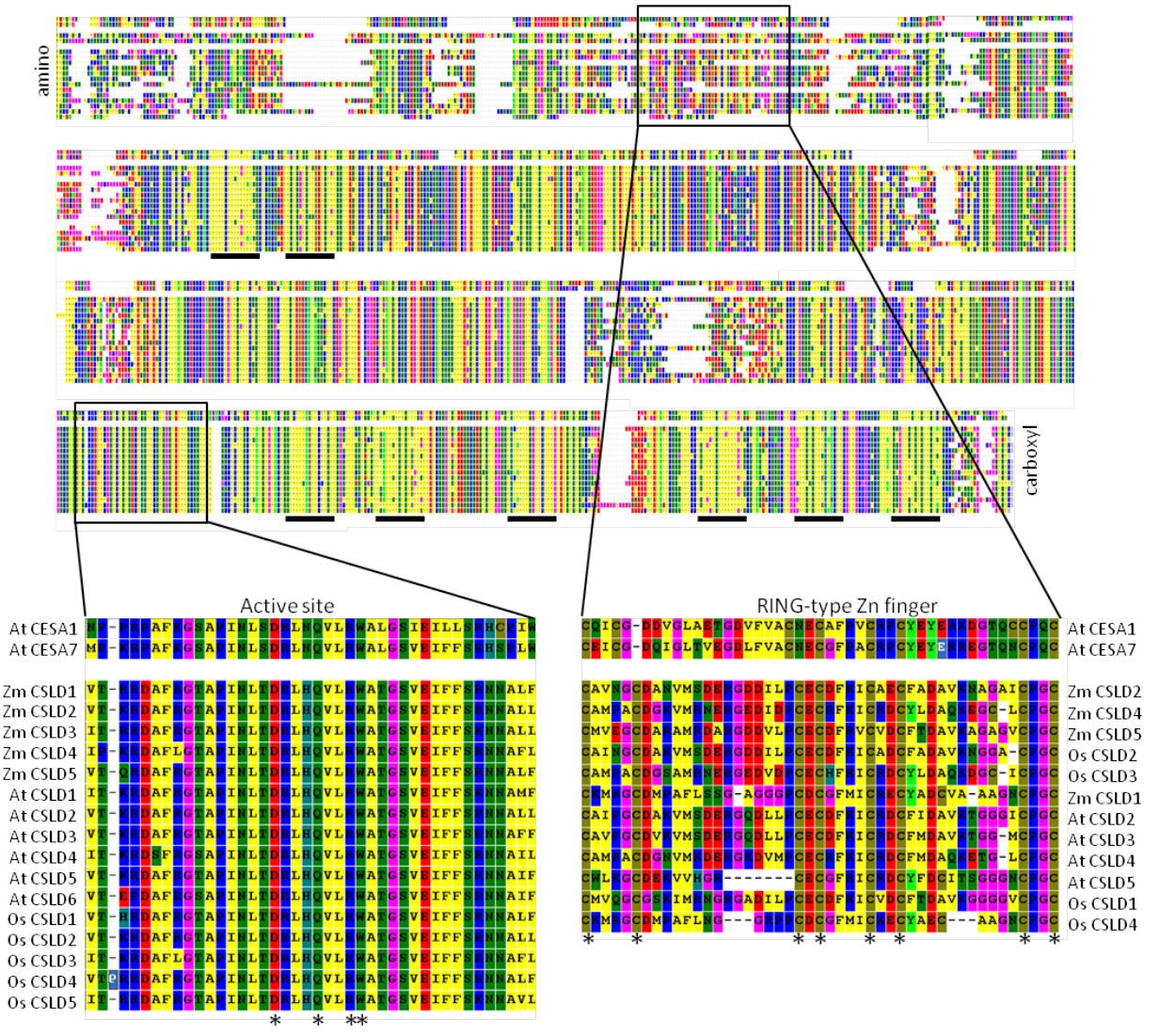


Figure 3-3. Protein sequence alignment and analysis of conserved motifs. All of the CSLD amino acid sequences from maize, Arabidosis, and rice, as well as two Cellulose Synthases (At-CESA1 and At-CESA7) were aligned using MEGA 4.0 (www.megasoftware.net/). Predicted transmembrane domains are underlined. Domains of intrest were expanded for deatailed visualization. Near-complete conservation was observed at the predicted active site of all the CSLD proteins. Amino acids that characterize the GT family 2 processive glycosyltransferase (D, QxxRW) are starred. The alignment showing the RING-type Zn finger motifs was ordered by conservation of the domain, and does not include putative CSLD proteins that lacked this domain. Cysteine residues (expected Zn-binding sites) are starred. The only consistent difference between proteins for which SMART recognized a Zn-finger-like motif , and those it did not, was at position 17 of the RING-type domain (arrow).

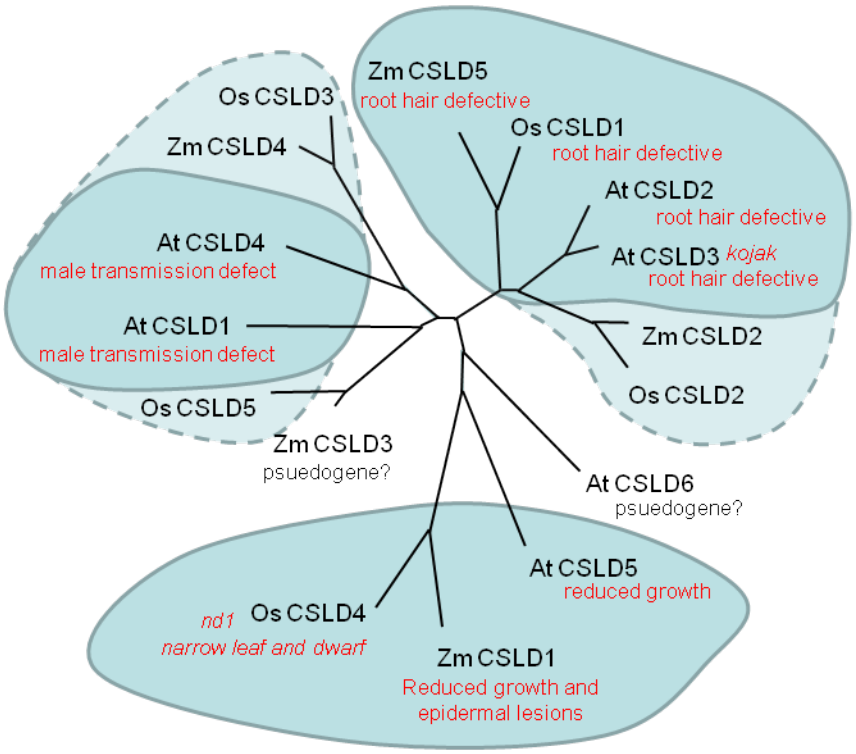


Figure 3-4. Comparison of mutant phenotypes to CSLD protein phylogeny. Neighbor-joining tree of predicted protein sequences encoded by *CslD* genes in maize, rice, and *Arabidopsis*. Reported phenotypes for null alleles are shown in red (Zm-CSLD5 [Penning et al., 2009], Os-CSLD1 [Kim et al., 2007], At-CSLD2 [Bernal et al., 2008], At-CSLD3 [Favery et al., 2001; Wang et al., 2001], At-CSLD5 [Bernal et al., 2007], Os-CSLD4 [Li et al., 2009], At-CSLD1 and At-CSLD4 [Bernal et al., 2008]). At-*CslD6* and Zm-c*sld3* are predicted to be psuedogenes. The tree was created using Mega 4.0 (megasoftware.net/mega.html; Tamura et a., 2007), with 2,000 bootstrap repetitions and a pairwise deletion option. Units are in amino acid substitutions per site.

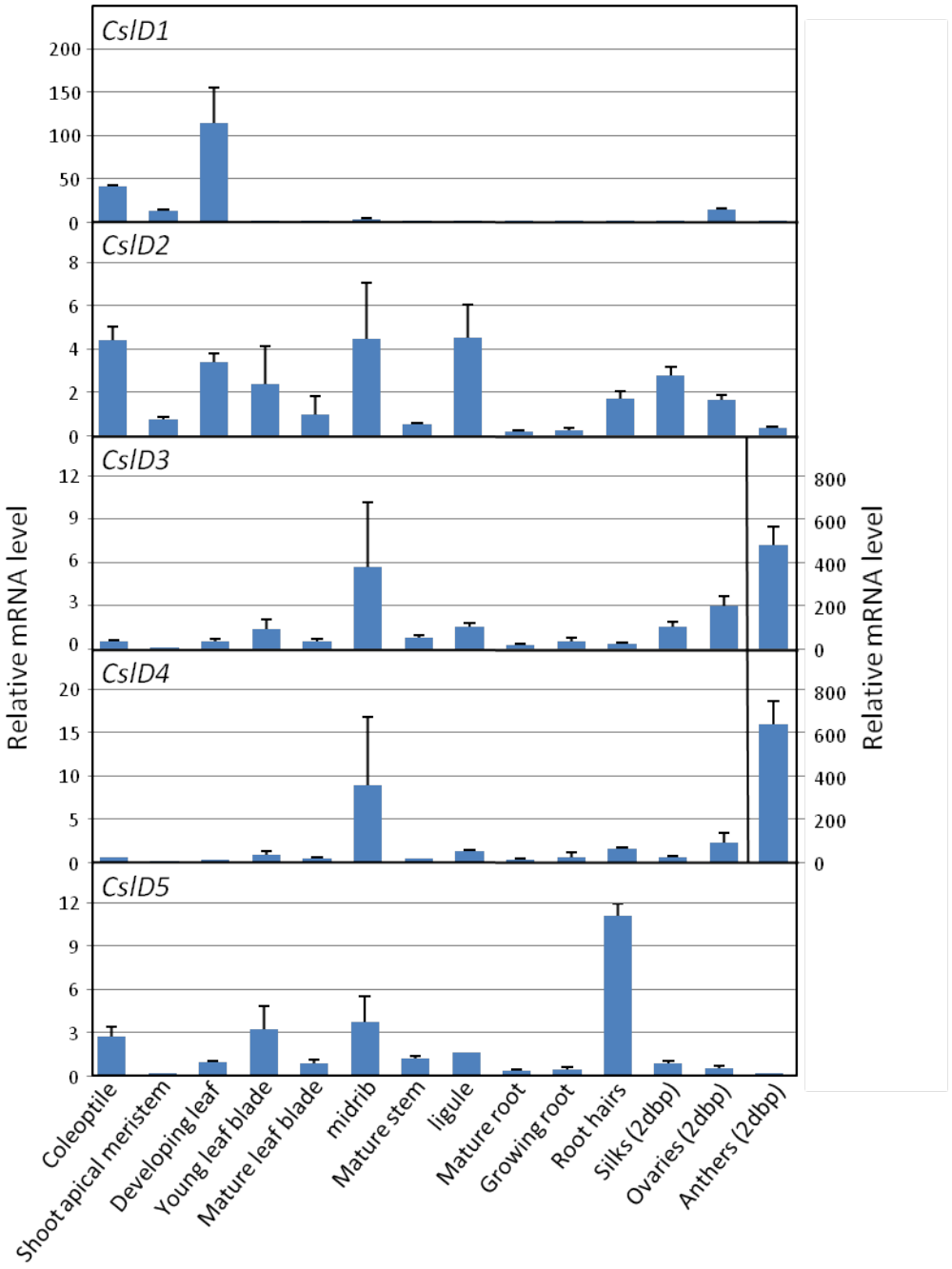


Figure 3-5. Quantitative RT-PCR showing mRNA levels of the maize *CslD* genes in selected tissues and stages of development. Error bars represent standard errors from three biological replicates. Note that the scale bar is different for each gene, and for *CslD3* and *CslD4* expression in anthers (2dbp). dbp = 2 days before pollination.

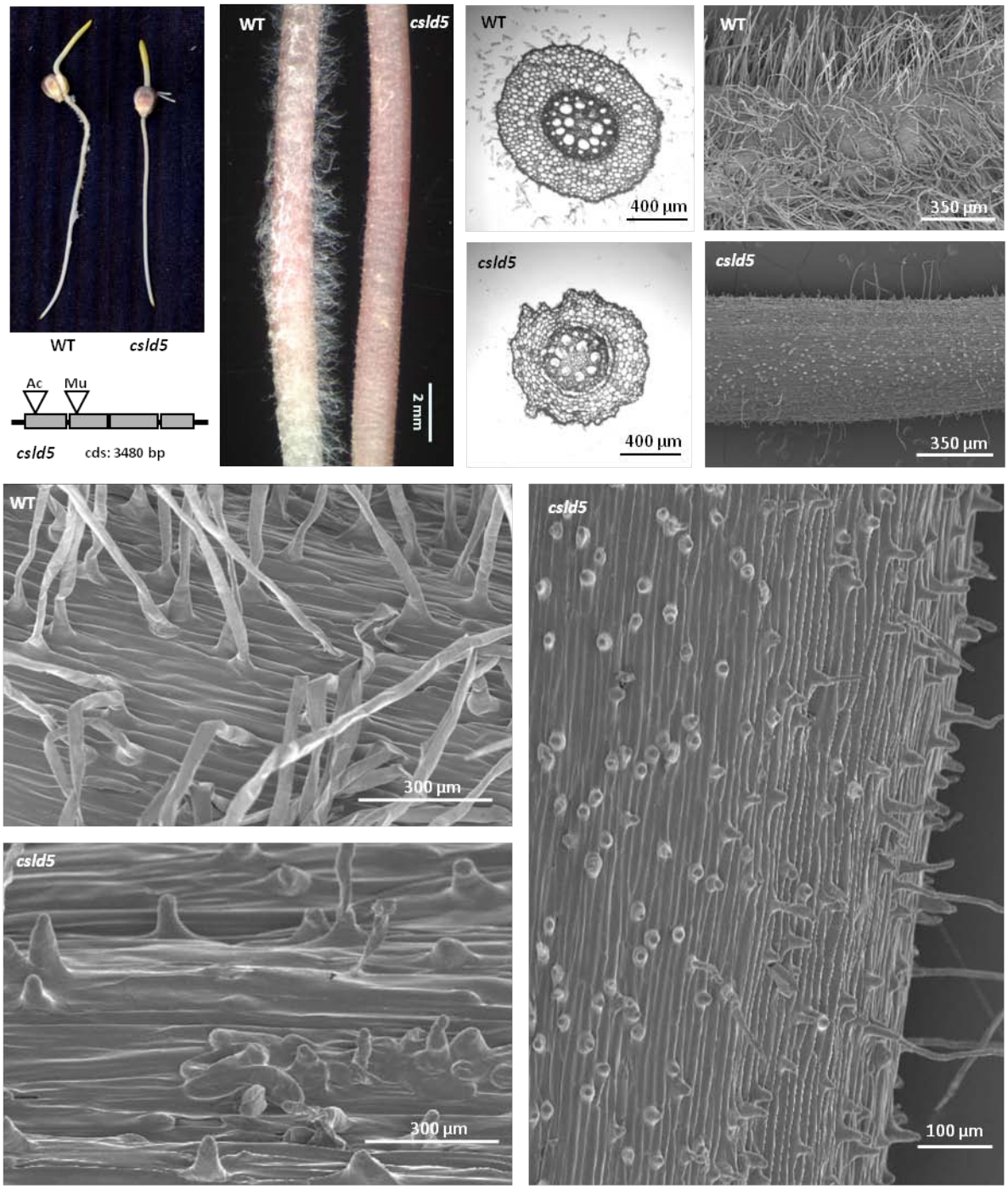


Figure 3-6. The root hair phenotype caused by mutation of *CslD5*. Note that although the *csld5* roots appear hairless, they retain a capacity to initiate-, but not necessarily elongate hairs (see hair initials).

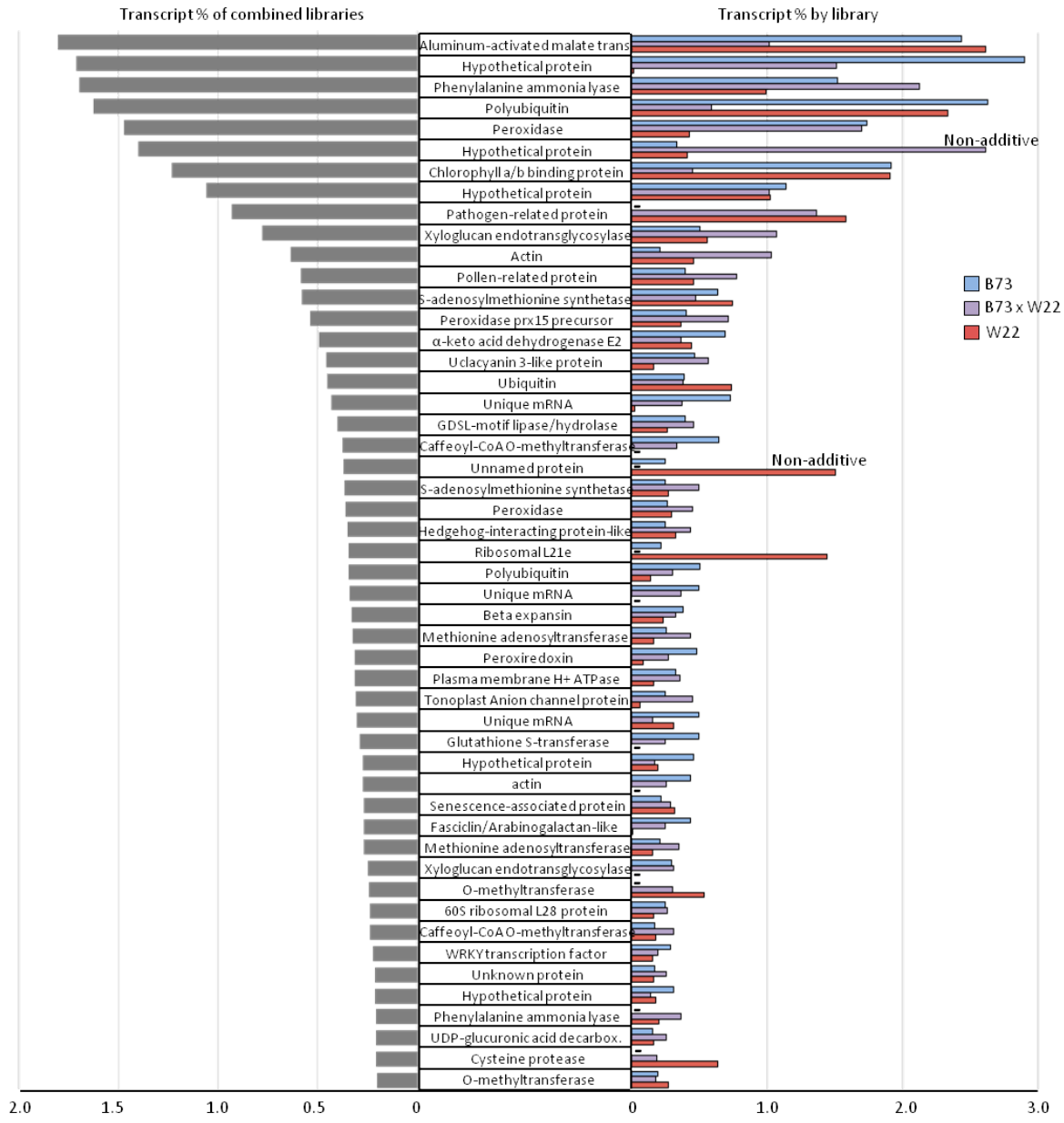


Figure 3-7. Transcript profiles of maize root hairs. Analysis of the 50 most highly-abundant transcripts as quantified by 3`-anchored 454 sequencing revealed genes related to cell wall modification, redox regulation, and amino acid metabolism, among others. Separate libraries were generated using cDNA from root hairs of B73, W22, and their F1 hybrid. Percentage of total reads represented by each gene when the three libraries were combined is charted on the left. Abundance in individual libraries for each gene is charted on the right, and shown as message level in proportion to each library separately.

# CHAPTER 4
# MUTATIONS OF *CELLULOSE SYNTHASE-LIKE D1* DISRUPT CELL DIVISION AND LEAD TO A *NARROW-LEAF WARTY* PHENOTYPE IN MAIZE

## Introduction

Plant form depends on coordination of cell division and selective cell expansion (Sylvester, 2000; Szymanski, 2009). Cell wall deposition is centrally important to both of these processes, forming cross walls de novo during cell division, and excreting new cell wall material and cell wall modifying enzymes during cell expansion (Cosgrove, 2005). Despite its central importance to plant biology, very little is currently understood about cell wall formation during cell division.

Investigations of maize mutants that show aberrant cell divisions, but maintain overall leaf shape, have led to the suggestion that an as-yet-undefined mechanism may maintain of leaf shape (Smith, 1996; Reynolds et al., 1998; Walker and Smith, 2002). For such a mechanism to accomodate aberrantly-shaped cells, some cells must develop in ways that compensate for malformed ones (Walker and Smith, 2002). The basis for such a mechanism remains uncertain.

The rigid walls of plant cells mean cytokinesis by constriction is impossible, and plants have, instead, evolved elaborate mechanisms of cell division. These involve cytoskeletal-based organization of cellular bodies and targeting of cell wall material to a defined division plane. In this process, Golgi- and endocytosis-derived vesicles containing cell wall polysaccharides, structural proteins, and enzymes, are targeted to the division plane, where they merge to form the cell plate (Samuels et al., 1995; Staehelin and Helpler, 1996; Smith 2001; Dhonukshe et al., 2006). As vesicles fuse with the cell plate, this structure expands radially, spreading from the inner cell to the parental walls at the cell boundaries. The position of a new wall, determined by actin-

myosin-dependent alignment of the cell plate, is defined by markers associated with the preprophase band (Gallagher and Smith, 1999; Chaffey and Barlow, 2002; Molchan et al., 2002). Others have highlighted the similarities between cell division and the targeted cell wall deposition seen in tip-growing cells such as root hairs (Bednarek and Flabel, 2002). Callose is thought to be among the first polysaccharides deposited at a new cell wall (Samuels et al., 1995; Hong et al., 2001). However,current unknowns include how cellulose, hemicelluloses, pectins, and structural proteins are delivered, synthesized, and meshed to form a functional wall.

Work presented here provides evidence for an unexpected, but integral role for CSLDs (see Chapter 3) in plant cell division. In *csld1* mutants of maize, defects in cell division are revealed as the probable cause for pleiotropic phenotypes that include reduction in plant growth, altered leaf morphology, distinctive epidermal warts, and changes in cell wall properties of stems. Analysis of leaf-blade epidermis shows that cells in *csld1* mutants are consistently wider and fewer in number compared to wildtype. This result, along with the close association between *csld1* expression and zones of cell division in basal regions of developing maize leaves, suggests a role for CSLD1 in cell division. Other evidence for cell division-related defects includes aberrantly-shaped epidermal cells, disruption of cell files, an abundance of mega-nucleate cells, and misplaced and partially-formed cell walls. These data provide new insights into the function of CSLD proteins during plant growth, expanding the understanding of their roles from that of tip-growth alone, to include cell division and consequent pleiotropic effects on development.

## Results

### An Allelic Series of *csld1* Mutants in Maize Enabled Functional Analysis

Seven independent loss-of-function mutants for the maize *CslD1* gene were identified in reverse genetic screens, including two from the UnifromMu maize population (University of Flroida) and five from the Trait Utilities System in Corn (TUSC) lines (Pioneer Hi-Bred International) (Fig. 4-1). The two UniformMu alleles, *csld1-1* and *csld1-2*, were examined in the greatest depth because of their uniform genetic background (McCarty et al., 2005). Phenotypes of *csld1-1* and *csld1-2* homozygous mutants, as well as offspring from their reciprocal F1 hybrids, were indistinguishable, thus demonstrating a causal role for the dysfunctional Zm-*CslD1* gene. These plants showed overall reduced growth, narrow leaves, and had a rough leaf texture due to warty protrusions from the mature leaf epidermis. Genotypic analysis of over 200 individuals from segregating families showed a 100% correspondence between this phenotype and homozygosity for the *csld1-1* mutation, as well as Mendelian segregation ratios typical of a recessive mutation (data not shown). Finally, the five other transposon insertions in *CslD1* (courtesy of Pioneer Hi-Bred Int.) also showed reduced vegetative growth, narrow leaves, and epidermal warts, regardless of the phenotypic variation and heterologous genetic backgrounds.

### Plant Dry Weight and Organ Width are Reduced in *csld1* Mutants

Overall growth and organ size were reduced in homozygous *csld1* mutants (Figs. 4-2 and 4-3). However, general plant shape, leaf number, and flowering time, were similar for *csld1* mutant and wildtype progeny under field and greenhouse conditions (data not shown). Mean height of mutant plants (to auricle of the uppermost leaf) was only 11% ($p < 0.001$) less at maturity (Fig. 4-2), but more pronounced differences were

evident in both above- and below-ground dry weight (reduced 44% [$p < 0.0025$], and 49% [$p < 0.002$], respectively) (Fig. 4-2). Proportional reductions were evident for all organs examined, including ears, tassels, stalks, roots, and leaves. Length and width of mature leaf blades were also decreased, but disproportionally more so for width (35% [$p < 0.0003$]) than length (10% [$p < 0.0003$]) (Fig. 4-3), giving a narrow-leaf phenotype. The reduction was proportional in all leaves examined, indicating a consistent defect in lateral expansion rather than an ontological effect at specific leaf positions (Fig. 4-3).

Leaf-blade curling (adaxial rolling) was also a consistent feature of *csld1* mutants (Fig. 4-4), and affected all leaves, regardless of position. Blade rolling was most apparent as leaves matured, and occurred regardless of watering regime or growing conditions (field or greenhouse). The amount of leaf-blade rolling was greatest where size and extent of epidermal warts (see below) on the leaf was also maximal. Although leaf rolling in non-mutant maize plants is a common response to water stress, this aspect of the phenotype persisted in well-watered plants. Most likely, it is the leaf epidermis itself which promotes the curling effect, as the abaxial surface of mutant leaf blades accumulate relatively more warty cell clusters, thus extending the abaxial surface and causing the leaf to curl in the opposite (adaxial) direction (see below).

**Epidermal Cells Balloon into Warts on *csld1* Mutants**

The rough texture of *csld1* mutant leaves was due to irregular swelling of epidermal cells and groups of cells (Fig. 4-5). This phenotype was observed along the entire length of mature leaf blades and midribs (Fig. 4-5A), but not on leaf sheaths or stalks (data not shown). Some epidermal cells in warts expanded to many times their normal size (about 75-fold greater volume), and were generally arranged in linear profiles along the longitudinal axis of the leaf blade (Fig. 4-5B). Swollen cells remained

fluid-filled until the onset of leaf senescence (Fig. 4-5C). Warts were present on both surfaces of mutant leaf blades, but were larger and more abundant on the abaxial face (Fig. 4-5D). As noted above, this was likely the cause of the rolled-leaf phenotype (Fig. 4-4). These malformed cells consistently lacked chloroplasts (Fig. 4-5C, E), indicating an epidermal origin. Also, serial sectioning of the leaf revealed that the cells in the warts originated from the epidermal layer (Fig. 4-5F, G). Ballooned epidermal cells in *csld1* mutants often had diameters over 100 µm, at least five times greater than epidermal pavement cells in leaves of wildtype plants (Fig. 4-5G).

Topographical data from SEM analyses and epidermal impressions allowed more specific definition of the surface dimensions and distribution of lesions, as well as analysis of their maximal growth and advanced development. Groups of warty cells were randomly interspersed with normal-appearing regions of leaf epidermis (Fig. 4-6A). Clusters of swollen cells tended to be evenly distributed over large areas, along the entire length of the leaf blade (Fig. 4-6A). Both SEM and optical microscopy of fresh, intact leaves showed that lesions continued to grow until leaves reached full expansion, and that the largest clusters often included swollen cells that had collapsed (Fig. 4-6B, center panel). In other instances cells remained intact, even in lesions greater than 300 µm wide (Fig. 4-6B, right panel). Individual cells in these cluster were identified at least 5-fold wider and 3-fold longer than more standard epidermal cells (Fig. 4-6B, right panel).

**Cells of *csld1* Mutant Leaves are Larger, but Fewer in Number**

We initially hypothesized that epidermal pavement cells in the narrow-leaf mutants might also be narrow (where not ballooning), but the opposite was observed (Fig. 4-7). Examination of epidermal impressions from non-warty areas of mutant and

non-mutant leaves revealed that although there was no significant difference in cell length, there was a consistent increase in width of mature *csld1* mutant epidermal pavement cells ($p < 0.0003$). This 17% greater cell width, together with 35% narrower leaves, indicated that mutant plants had fewer total epidermal cells across their width. This decrease in cell number translated to an estimated 40-45% reduction in epidermal cell divisions compared to wildtype.

Further analyses showed that *csld1* mutant leaf blades were also visibly thicker, with less distance between vascular bundles (Fig. 4-8, top three panels). Fully-expanded *csld1* mutant leaf blades were on average 40% ($p < 0.004$) thicker than non-mutant leaf blades (Fig. 4-8, lower left). To determine whether this increase in thickness was due to larger epidermal cells alone or non-epidermal cells as well, inter-epidermal distance was measured. Sub-epidermal contributions to total leaf thickness were proportional to epidermal contributions, being 43% ($p < 0.002$) greater for *csld1* mutant leaf blades (Fig. 4-8, lower left). The *csd1* phenotype is therefore not limited to the epidermis alone. Additionally, mutant leaves showed a 12% ($p < 0.03$) increase in vascular-bundle density (Fig. 4-8, lower right), an effect also evident from visual appraisal. These data, along with average leaf width (excluding the midrib) of 80.2 mm for non-mutant and 51.1 mm for mutant leaves (Fig. 4-2), were used to estimate the number of vascular bundles across the widest portion of fully-expanded leaves. Resulting values were 601 and 429 vascular bundles per leaf, for non-mutants and *csld1* mutants, respectively (Fig. 4-8, lower right).

### Stalks of *csld1* Mutants Contain Altered Vascular Bundle Number and Cell Wall Properties

Analysis of mutant stalks confirmed that organ width and vascular bundle number were also affected elsewhere in *csld1* mutant plants (Fig. 4-9). Cross-sectional area of *csld1* mutant stalks was reduced by 24% ($p < 0.025$) on average compared to non-mutant stalks (Fig. 4-9). As with leaf blades, total number of vascular bundles was also decreased. At the third internode from the ground, mutant plant stalks had an average of 11% ($p < 0.025$) fewer vascular bundles than non-mutant stalks (Fig. 4-9). Again as observed for mutant leaves, *csld1* mutant stalks had more vascular bundles per unit area than non-mutants, with an average of 2.0 (SE, 0.1) bundle per $mm^2$ compared to 1.7 (SE, 0.1) bundle per $mm^2$ for non-mutants across the entire stalk (Fig. 4-9). This increase was partly due to the relative increase in rind-to-pith ratio in *csld1* mutants, but even in the central pith, greater vascular bundle density was evident.

To further characterize differences between mutant and non-mutant stalks, high-resolution X-ray micro computed tomography (micro CT) was employed on hand-cut stalk sections (Fig. 4-10). Three-dimensional reconstruction of these data revealed a shift in the distribution of cell wall thickness between mutant and non-mutant stalks, with mutant walls in this tissue generally thinner (Fig. 4-10). Additionally, this approach allowed comparison of the density of cell wall material, which was greater for cell walls of *csld1* mutant stalks (as measured by X-ray beam attenuation) compared to non-mutant stalks (Fig. 4-10).

### Cell Wall Sugar Components were not Detectably Altered by Loss of *CslD1* Function

With the goal of determining whether the changes in wall material properties from stem sections observed via micro CT were reflected in global changes in cell wall

polysaccharide composition, we examined cell wall composition from mature leaf blades as well as isolated leaf epidermis of both mutant and wildtype plants. No significant differences in alcohol-insoluble cell wall composition were found between wildtype and *csld1* mutants for either cellulose or sugar subunits of non-cellulosic constituents (Table 4-1). Cell walls from epidermal cells of both genotypes revealed distinctive composition relative to samples from whole-leaf blades. Specifically, epidermal cell walls had less glucose, rhamnose, galactose, and galacturonic acid, but relatively more xylose, compared to whole-leaf blades (Table 4-1).

**Levels of Maize *CslD1* mRNA are Greatest in Regions of Active Cell Division**

In order to view the phenotypes of *csld1* mutants in context of where the wildtype gene is expressed, quantitative RT-PCR was used to determine levels of *CslD1* mRNA across diverse tissues and stages of development (Fig. 4-11). Highest levels of *CslD1* transcript were evident in young, pre-emergent leaves (inside the whorl), with lesser expression in young primary roots and bases of more mature leaves (Fig. 4-11A). To more clearly define the pattern of transcript accumulation during leaf development, staged samples of very young to mature leaves were analyzed. The *CslD1* mRNA was most abundant in tissues containing actively dividing cells, highest in shoots 6 days after germination (Fig. 4-11B). Samples of more mature, single leaves showed that basal portions of blades from expanding leaves 15-25 cm long had the greatest levels of *CslD1* mRNA accumulation. By the time of cessation of cell division in fully-expanded leaves, *CslD1* mRNA dropped to undetectable levels and remained nearly undetectable in the fully-differentiated portions of leaves (Fig. 4-11B).

**Multiple Cell Division Defects are Evident in *csld1* mutant Epidermis.**

Dark-field images of single-cell-layer epidermal peels from mature leaf blades showed that the normally highly-ordered epidermal cells of maize leaves were disrupted in epidermis of *csld1* mutants (Fig. 4-12). Also striking was the extent of apparent cell division anomalies in the mutant epidermis (Fig. 4-12). Cell wall stubs were commonly observed in samples from mutant plants, where cells apparently failed to complete cell division (Fig. 4-12). These incomplete cell walls were observed exclusively in the longitudinal plane (Fig. 4-12). Other frequent occurrences of altered cell shape and misaligned cell walls also implicated cell-division defects as the cause (Fig. 4-12).

To determine when these cell abnormalities could first be detected, fresh, 5-10 cm, immature leaves of *csld1* mutant and wildtype plants were stained with propidium iodide. Confocal microscopy imaging of abaxial epidermal cells from the pre- and post-differentiation zones (determined by the absence/presence of stomata), revealed disrupted cell files and misshapen cells (Fig. 4-13A, B), similar to what was observed in epidermal peels of mature leaves (Fig. 4-12). Cell wall stubs were frequently observed in both pre- and post-differentiation epidermis (Fig. 4-13). Again, they were typically oriented in the longitudinal direction (Fig. 4-13). While many epidermal cells at these stages were approximately twice the normal width (as if they failed to undergo a single division), nearly all cells of *csld1* mutants were visibly larger than wildtype (Fig. 4-13 A, B), supporting the findings that even non-warty, otherwise normal-looking cells, were wider in the mutant (Fig. 4-7). In multiple instances, a series of large epidermal cells were located at positions normally inhabited by two distinct cell files, indicating either failure of consecutive neighboring cells to divide or, more likely, a clonal file derived from a single cell that failed to complete division, as indicated by oppositely-facing cell

wall stubs at either extreme of such cell files (Fig. 4-13C). Three dimensional imaging with confocal microscopy identified a large number of gaps in cell walls that initially appeared complete (Fig. 4-13D), suggesting the many more walls than at first apparent were incomplete.

Because cells with large or multiple nuclei are commonly observed in cell division defective mutants (Smith et al., 1996; Lukowitz et al., 1996; Spitzer et al., 2006), propidium-iodide stained nuclei were examined in immature *csld1* mutant leaves (Fig. 4-14A). Compared to wildtype epidermis of the same stage (pre-differentiation zone of 5-10 cm leaves), *csld1* mutant cells generally had larger-appearing nuclei (Fig. 4-14A). While significant variation in nuclear size was observed even in wildtype tissue, the range in *csld1* mutants was much greater, with cells containing nuclei ranging from normal to approximately four times larger in size (Fig. 4-14A). Large nuclear size was generally correlated with large cell size (Fig. 4-14A). Flow cytometry of nuclei from basal regions of immature leaves showed a small, but significant ($p < 0.05$), increase in ploidy level in *csld1* mutants compared to wildtype (Fig. 4-14B). Relatively more tetraploid nuclei were identified in *csld1* mutants than in wildtype, while no significant differences were observed at any other ploidy level (Fig. 4-14B).

## Discussion

### The CSLD1 Enzyme Appears to Affect a Mechanism Other than Tip-growth

Previous results suggested specific functions for CSLD proteins in tip-growing cells (Bernal et al., 2008), but data here and elsewhere indicate broader developmental roles as well. Disruptions in At-*CslD5*, Os-*CslD4*, and Zm-*CslD1*, in particular (Bernal et al., 2007; Li et al., 2009), lead to reduced overall plant growth without visibly altering classic tip-growing cells (root hairs and pollen tubes). Among the

reduced-growth phenotypes, maize *csld1* is unique in production of visible epidermal warts. This difference might lie in the greater growth and expansion of maize leaves. In other respects, however, commonalities between the reduced-growth phenotypes suggest a shared function for this sub-group of CSLDs.

**The Maize *CslD1* Gene is Essential for Normal Plant Dry Weight and Organ Width**

An underlying basis for the pleiotropic *csld1* phenotype is indicated by the proportional reduction in dry mass (~45%) and size affecting all organs including leaves, shoots, roots, tassels and ears (Figs. 4-2, 4-3, and data not shown). A reduction in cell number was partially compensated by an increase in average cell size, and in epidermis of narrow leaves, expansion was increased only in the lateral direction. These results are broadly consistent with work on other, non-allelic warty mutants in maize that suggest a compensatory mechanism in young leaves which might regulate the balance between cell division and expansion, especially in response to defects that could alter organ shape (such as the disrupted cell divisions and epidermal lesions shown here) (Reynolds et al., 1998). While this is compatible with a primary defect in division rate or total number of cell divisions, indirect effects at the whole-plant level could also reduce dry matter accumulation. The narrow leaves of *csld1* mutants, for example, are likely to result in decreased photosynthetic capacity and the smaller root system may further reduce growth (Figs. 4-3, 4-4). Finally, the epidermis may have a prominent physical role in organ expansion and meristem geometry (Green, 1980; Moulia, 2000) providing additional potential for secondary or tertiary affects of the *csld1* mutations.

## Warty Cells Represent Distinctive and Informative Features of Maize *csld1* Mutants

Warts created by excessive swelling of epidermal cells on *csld1* mutant leaves (Figs. 4-5, 4-6) result from apparent cell division flaws early in leaf development. The epidermal lesions were broadly distributed across leaf blades, with essentially any epidermal cell having potential to swell (including, albeit rarely, stomata or trichomes). This lack of a discernable pattern or position dependence of wart formation is consistent with a random process for determining which epidermal cells have division defects during development (Fig. 4-5).

Whether cell division defects alone are responsible for the entirety of epidermal wart formation remains unclear. Another possibility is that altered cell walls (missing a CSLD1 product) are weakened and less able to withstand normal turgor pressure. Swelling of weakened epidermal cells in the mutant would lead to further weakening (stretching/thinning) of the cell wall, and a still-greater tendency to expand. This abnormal expansion would in turn enhance physical stress on cell walls of neighboring cells, which could account for the lateral spreading that is observed in warty lesions. The suggestion of more-easily-stretched cell walls would be consistent with the larger size of even the non-ballooned epidermal cells as well as the later development of swollen cell clusters.

Possibly-analogous wart-like epidermal swellings were described by Burton et al., (2000) in a VIGS gene silencing experiment in tobacco. Although a *CESA* gene was targeted, conceivably the highly-similar *CSLD* genes may also have also been silenced. In any case, the transgenic tobacco had a reduced stature, chlorotic regions, and “a relatively crisp or crunchy texture… [with]… numerous surface lumps” predominately on

the abaxial side of leaves (Burton et al., 2000). This *csld1*-like phenotype would be consistent with some degree of repression of the Zm-*CslD1* ortholog in the tobacco experiment. Alternatively, if observed lesions did result from down-regulation of CESA genes alone, then this would be consistent with a role for CSLD proteins in cellulose biosynthesis (Doblin et al., 2001).

**Larger, Non-warty Epidermal Cells Suggest a Limitation in Cell Division**

The combination of narrower leaves and wider pavement cells resulted in an estimated 45% fewer epidermal cells across a mutant leaf blade (Fig. 4-7), supporting an early role for CSLD1 in leaf development. The majority of mutant leaf epidermal cells, while wider than normal, do not form warts and have a standard pavement-cell appearance, with typical neighbor-cell boundaries, suggesting a link between CSLD1 and the number of cell divisions in developing leaf epidermis. Similar effects were evident in internal leaf structures, where non-epidermal cells contributed to thicker leaf blades for *csld1* mutants (Fig. 4-8). While examination of cross-sections suggested that leaf vascular bundle number and density was altered in mutant leaves, the size and shape of vascular bundles were generally unchanged (Fig. 4-8). Instead, the greater thickness of *csld1* leaf blades was due to increased mesophyll cell size, indicating a broader role for CSLD1 than epidermal development alone. Alternatively, a less-expanded epidermis might constrain internal structures (mesophyll and bundle sheath cells) into a more limited space, resulting in leaf swelling.

**Cell Walls of the *csld1* Mutant are Thinner and More Dense, but of Normal Composition**

While the overall quantity of cell wall material is reduced in the *csld1* mutant plants by 45%, cell wall composition is largely unaffected (Table 4-1). There are several

alternative possibilities that may account for a lack of detectable differences in cell wall composition between mutant and wildtype leaves, or in epidermal peels alone. First it is possible that the CSLD1 polysaccharide product may normally be present in only very small amounts and thus be masked by more abundant polymers. Alternatively, the CSLD1 product may be synthesized early in development, but not be abundant in mature leaves, such as those analyzed here. Levels of *CslD1* mRNA were maximal in very young leaves and basal portions of fast-growing leaves, the zone most active in cell division (Sylvester et al., 1990; Freeling, 1992), as well as in primary root tips (Fig. 4-11). Another interpretation could be that the CSLD1 enzyme might synthesize a limiting constituent of specialized cell walls, such that when production is limited, other cell-wall polymer synthesis is similarly reduced. In this way, *csld1* mutants may produce less total cell wall, but remain unchanged in relative proportions of individual cell-wall constituents. In this respect, the implication that CSLD1 has an essential role in formation of new cell walls during cell division is intriguing.

High-resolution X-ray micro CT technologies allow for detailed structural analyses of internal regions of intact tissue, including the relative density of cell wall material, based on x-ray beam attenuation (Steppe et al., 2004; Dhondt et al., 2010). Examination of *csld1* mutant and non-mutant stem sections revealed increased wall density and a shift in the distribution of cell wall thickness (Fig. 4-10). These findings suggest an altered architecture for these cell walls that could be independent of a change in composition, and led to the hypothesis that these changes may reflect a relative increase in cellulose crystalinity, with decreased amounts of amorphous cellulose being present in the cell walls of *csld1* mutants.

**Maize *CslD1* has a Role in Plant Cell Division**

The primary evidence supporting a role for CSLD1 during cell division lies in the degree to which *csld1* mutations disrupt the otherwise highly-ordered epidermal cell files (Fig. 4-12). The appearance of incomplete cross walls between what would normally have been two cells, indicate a failure of cells to complete division. This observation may be interpreted in several ways. First, it is possible that the polysaccharide product of the CSLD1 enzyme functions in cell plate formation. If this constituent is limiting, cell plates may be less likely to form correctly or completely. Alternatively, because epidermal cells in *csld1* mutants are abnormally large prior to initiation of cell division (Fig. 4-13), cell plates may be unable to readily span the entire length of a cell, leading to incomplete cell wall formation. Both could also be true. The observation that cell wall stubs were almost universally oriented in the longitudinal direction (Fig. 4-12, 4-13) suggested a bias towards effecting longitudinal cell division defects, rather than lateral divisions. Whether this indicates separate mechanisms for these two types of cell division remains unclear.

Cell-level abnormalities were evident even at the earliest stages of leaf-blade development examined (Fig. 4-13A). Uneven and disrupted cell files, large and misshapen cells, and incomplete cell walls within a cell are all indicative of defects in cell division (Fig. 4-13A, B). Even undifferentiated regions of epidermis contained some cells which occupied a two-dimensional area over 30-fold greater than a standard epidermal pavement cell. The presence of long files of cells being uniformly large (Fig. 4-13C) suggests either the congruent failure of a string of cells to complete cell division, or clonal inheritance, where one mis-divided cell gives rise to abnormally-large daughter cells. Without the support of the highly-ordered, brick-like pattern of wildtype epidermal

cells, the large, misshapen and unorganized epidermal cells in the *csld1* mutants seem prone to excessive expansion during turgor-driven growth. The defects in cell division at the earliest stages of leaf development might account for the entirety of the warty phenotype of the *csld1* mutants. In this way, the polysaccharide product of CSLD1 might have its direct role limited to cell division, and its presence may be essentially transitory.

An interesting aspect of maize leaf epidermis shown here is the differential effect of *csld1* mutations on lateral and longitudinal cell divisions. Only longitudinal divisions seem to be affected by mutations of *CslD1*, possibly indicating a highly-specific role for CSLD1 activity. Supporting evidence includes reduced leaf width (but not length) (Fig. 4-3), reduced cell width (but not length) (Fig. 4-7), and the exclusivity of cell wall stubs being oriented only along the longitudinal axis (Figs. 4-12, 4-13). Additionally, the long files of uniformly un-divided cells demonstrate that in *csld1* mutant epidermis, lateral divisions have the capacity to reach completion across cells two-times their normal width (Fig. 4-13). The same was not evident for divisions parallel to the leaf axis however, and consequences are consistent with the linear, clonal propagation of a single mis-divided cell into a file of subsequently ballooning cells observed here.

Large-nucleate cells have been reported in studies of cell division-defective mutants (Lukowitz et al., 1996). In pre-differentiation zones of *csld1* immature leaves, abnormally large cells were consistently observed to contain larger nuclei than normal (Fig. 4-14A). Compared to confocal imaging, where a large number of cells (estimated at 40-50%) contained excessively large nuclei, flow cytometry showed only a modest increase in endoreduplication (Fig. 4-14B). This result fits with the interpretation that

the large nuclei phenotype predominately affects the epidermis. Even with 50% of epidermal cells undergoing endoreduplication, the relative scarcity of these cells compared to other leaf cells would result in partial masking of the effect when analyzing nuclei from whole-leaf tissues. Whether the increase in endoreduplication and larger nuclei size reflects a response to larger cell size or the arrest of the cell cycle after DNA replication but before nuclear division remains unclear. The arrest of cells at the G2 phase would implicate a cell wall biosynthetic enzyme in feedback to the cell cycle, and seems unlikely. More likely, when a mis-divided cell (with two nuclei) undergoes later divisions, the nuclei would replicate and segregate as tetraploid nuclei, and be passed on as such in future divisions.

**Broader Perspectives**

The unique features of the *csld1* phenotype observed in maize provide insights into the function of the *CslD* subfamily in all plants. Data demonstrate a link between this putative cell-wall polysaccharide synthase and cell division during early maize leaf development, as well as connections between early cell divisions and subsequent organ development. The disproportionate reduction in plant dry weight compared to plant height can be accounted for by the larger but fewer cells, as observed in leaf epidermis (Fig. 4-7), along with thinner cell walls, as indicated by micro CT analysis of mature stems (Fig. 4-10). Together, along with narrow leaves (Fig. 4-3) and smaller stems (Fig. 4-9), these effects could account for the decrease of dry weight by 45% in mutant plants reduced in stature by only 11% (Fig. 4-3). In contrast to previous analyses of *CslD* family members, our results show that maize *CslD1* mutations have a dramatic effect at the whole-plant level and on leaf epidermis in particular. These studies contribute a new dimension to the understanding of CSLD protein function in plants.

Some aspects of the *csld1* phenotype reported here have also been observed in other mutants. Among these are the similar (but less severe) clusters of swollen, epidermal cells reported in the *warty-1* maize mutant (Reynolds et al., 1998). However, the *csld1* mutants described here show distinctive changes in stature, leaf width, and morphology not observed elsewhere. Also, maize *tangled-1* mutants show irregular cell divisions in leaves, but maintain their overall shape and plant architecture (Smith et al., 1996). Initially, the leaf epidermal surface of these mutants may seem similar to that of *csld1*, but in leaves of the *tangled-1* mutants, aberrantly dividing cells are evident in all cell layers, as opposed to the prominent epidermal locale in *csdl1* (Smith et al., 1996; Cleary and Smith, 1998). The *Tangled-1* gene encodes a microtubule binding protein (Smith et al., 2001).

While at first glance it might seem surprising that a CSLD protein would be involved in the development of non-tip-growing cells like maize epidermal pavement cells, upon consideration, cell division involves a strikingly similar process to that of tip-growth. Cell plate formation during cell division has many parallels with the process of tip-growth, including the delivery of cell wall material being focused to a very specific subcellular position via cytoskeletal-mediated organization of the exocytic pathway machinery (reviewed in Bednarek and Falbel, 2002). Other examples of individual proteins from common families functioning in either tip-growing cells or cytokinesis include the formins (Ingouff et al., 2005; Backues et al., 2007) and ROP GTPases (Molendijk et al., 2001; Xu and Scheres, 2005).

## Methods

### Identification of *csld1* Mutants

The UniformMu population was screened using PCR-based assays to identify Mu transposon inserts in Zm-*CslD1* as per Penning et al., (2009). Close to 15,000 UniformMu lines were screened using a series of pooled DNA samples, which were forerunners of the sequence-indexed materials currently available at MaizeGDB (maizegdb.org; UniformMu.UF-genome.org). For PCR screening, *CslD1*-specific primers (AGTTCGTGCACTACACCGTGCACATCC and TGCTACCTGTAAGGACTGAGGATGGCCTG) were used along with the Mu-specific primer TIR6 (AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC). Resulting products were separated on 1% agarose gels, blotted onto nylon membranes, and probed with a *CslD1*-specific PCR product (Fig. 4-1). Positive probe-binding samples were identified at X45:Y4 of the UniformMu Reverse Genetics Grid 6 (of 8 total) (Fig. 4-1). Seeds from this UniformMu family corresponding to these coordinates (04S-1130-27) were grown, and PCR-genotyped to identify individuals homozygous for an insertion in *csld1*. The *csld1-1* allele was identified from this family, and a *csld1-1* line established after three generations of successive backcrosses to the W22 inbred.

A second mutant allele, *csld1-2*, was identified during a visual screen of field-grown UniformMu lines. Its phenotype was indistinguishable from that of *csld1-1.* PCR primers (ACCAGATCCTCTTCCTCCTCGGTTTGC, ACCTTGTTCCTGAGGAAGTCCCTCTTC, GTGGTGATCACGCTGGCATCATTCAG, AGGAGGGCTGATGTAGACCCACAG,) were designed to cover nearly the entire length of the *CslD1* gene and identified a Mu insertion in the third exon. Homozygous recessive mutants of this allele were obtained from segregating progeny from this

family, and used to generate *csld1-2* line after two successive backcrosses into the W22 inbred.

Additional Mu-insert alleles of *csld1* were identified from the The Trait Utilities System in Corn (TUSC) population of Pioneer Hi-bred as per McCarty and Meeley, (2009). Primers used were: ACCAGATCCTCTTCCTCCTCGGTTTGC, ACCTTGTTCCTGAGGAAGTCCCTCTTC, GTGGTGATCACGCTGGCATCATTCAG, AGGAGGGCTGATGTAGACCCACAG, which identified five additional mutant alleles designated *csld1-3* through *csld1-7* (Fig. 4-1).

**Overall Phenotypic Analyses and Size Measurements**

Plant height was measured from soil level to auricle at the base of upper-most leaf blades for 55 field-grown, mature plants (25 mutant, 30 non-mutant). These same 55 plants were used to measure width (at the widest point) and length of leaves at positions three, four, and five (relative to the apex). For dry weight measurements, whole-plant samples (including released root mass) were collected three days after ear harvest and did not include mature ears. Samples were weighed after drying for 4 weeks at 38°C. Below-and above-ground dry weights were determined by separating root masses from the aerial portions of these plants.

Leaf thickness was measured on images from *csld1* mutant and non-mutant sectioned leaf blade pieces (4 each). Non-epidermal contributions were determined by measuring the inter-epidermal space of each of these pieces. Total thickness and inter-epidermal space were reported as averages of 10 independent measurements. Vascular bundles per leaf were estimated by multiplying the average vascular bundle density by the average leaf width (excluding midrib).

For analysis of leaf-blade conformation (adaxial curling), leaf blades were harvested from position three during the anthesis-silking interval of mutant and non-mutant greenhouse-grown plants. Samples included 1-cm sections from the base, middle, and tip of each blade (Fig. 4-4). Excised leaf strips were allowed to assume their natural conformations, and imaged immediately.

**Cell Volume Estimates**

Extent of maximal expansion was estimated for ballooning epidermal cells of the *csld1* mutant by comparing their volume to standard epidermal pavement cells of wildtype plants. Cell were considered to be roughly cylindrical ($V=\pi r^2 \cdot m$), with wildtype pavement cell dimensions approximately 40 µm (diameter) x 200 µm (length) (Figs 4-5, 4-6). Ballooned epidermal cells of blades from *csld1* mutant plants were often as large as 200 um (diameter) x 600 µm (length) (Figs. 4-5, 4-6).

**Epidermal Impressions and Non-warty Cell Size Determination**

Fresh samples from mature leaves of greenhouse-grown plants were cut into 2-$cm^2$ pieces and firmly pressed into Superglue on glass slides. Glue was allowed to dry completely before leaf tissue was removed, leaving detailed epidermal impressions. These were imaged under a light microscope (Olympus BH2) with an RT SPOT camera (Diagnostic Instruments). Average cell length and width were determined by quantifying the total number of cells in a given distance (1.88 x 1.40 mm). Longitudinal and lateral transects were used that did not include warty protrusions.

**Tissue Fixation and Sectioning**

One-cm squares were excised from leaves of greenhouse-grown plants and fixed in FAA (10% formaldehyde [Fisher Lot # 992720], 5% acetic acid, 50% EtOH). Samples were vacuum infiltrated overnight at 4°C, then shaken at 4°C during a

dehydration series using ethanol in PBS (60 min each, progressing from 1x PBS with 30% EtOH to 40%, 50%, 60%, 70%, 85%, and finally 95% EtOH). Samples were stained overnight with eosin in 95% EtOH, followed by four, 1-hr incubations in 100% EtOH and eosin at 25°C. Wax imbedding was initiated by introducing CitriSolv (Fisher Cat # 22-143975) into samples using a series of 1-hr incubations (while shaking) in ethanol with increasing CitriSolv/EtOH content (25/75, 50/50, 75/25,100/0). Paraplast wax chips (Fisher Cat # 23-021-399) (1-g wax/mL CitriSolv) were added to the 100% CitriSolv and incubated overnight at 25°C. Additional wax was added, followed by a 2-hr incubation at 42°C. Samples were transferred to 60°C for 1 hr. Wax was poured off and replaced eight times before samples were allowed to harden in molds. Sections (10 µm, cut with a Leitz 1512 microtome) were de-waxed with three, 5-min incubations in xylene (Fisher Lot # 083423), then washed twice in 100% EtOH (5 min each), and once in 95% EtOH (3 min). Slides were dried and examined under a Olympus BH2 light microscope.

**Scanning Electron Microscopy**

Mature leaf pieces (1-$cm^2$) from *csld1* mutant and non-mutant plants were fixed in FAA (10% formaldehyde [Fisher Lot # 992720], 5% acetic acid, 50% EtOH) , dehydrated in an ethanol series 75%, 95%, 100%, and critical point dried (Bal-Tec CPD030, Leica Microsystems, Bannockburn, IL). Dried samples were mounted with carbon adhesive tabs on aluminum specimen mounts, Au/Pd sputter coated (DeskII, Denton Vacuum, Moorestown, NJ), and examined with a field-emission scanning electron microscope (S-4000, Hitachi High Technologies America, Schaumburg, IL). Digital micrographs were acquired with PCI Quartz software.

**Phloroglucinol Staining and Stalk Measurements**

Cross-sections of stalks from field-grown plants were examined using hand-cut sections (about 5 mm) from mid-way up the second internode (from ground level). Tissue was stained by incubating 45 sec in Phloriglucinol (1% in 95% EtOH), then adding excess 25% HCl. Images were acquired using a RT SPOT camera (Diagnostic Instruments) attached to a Leica MZ 12-5 dissection microscope. Cross-sectional area for these sections was determined using ImageJ software (rsbweb.nih.gov/ij/index.html). Vascular bundle density was determined by dividing the number of vascular bundles per section by the cross-sectional area.

**High-resolution X-ray Micro Computed Tomography Analysis**

Field-grown plants were collected three days after harvesting ears and dried at 38°C for three weeks. Sections (~0.5 cm) from mid-way up the second internode of the conditioned stems (~9% moisture content) were cut using a small band saw and scanned using a Scanco Medical Ag uCT35 instrument (Brüttisellen, Switzerland). Initial measurements were conducted on whole-stem sections at 10-micron resolution. Regions including pith and rind (3 x 4 mm) were hand-cut from the edge of these sections and scanned at 3.5-uM resolution over a 0.88 mm high region for quantitative measurements of cell wall and air space sizes. The 232 slices from each scan were reconstructed into three dimensional images and contoured over whole stems for volumetric analyses. Scans at 3.5 micron and 10.0 micron resolutions were conducted with integration times of 600 microseconds and averaging two times. Both a fixed, common threshold and an adaptive threshold were used to segment cell wall from airspace and volumetric analyses were calculated with an algorithm developed for trabeacular bone (Hildebrand and Ruegsegger, 1997). For rind-only analyses, hand-

drawn contours were used to isolate the vascular bundle-rich region along the edge of the stem prior to 3D reconstruction.

**Epidermal Isolation**

Epidermal peels were manually removed from the abaxial surfaces of fully-expanded leaf blades from greenhouse-grown plants. Each of the peels were harvested as in Figure 4-15. For dark-field imaging, peels were placed on droplets of water on glass slides and allowed to dry. For cell-wall composition analysis, 100 mg of peels were collected, frozen in liquid nitrogen, and stored at -80°C until cell wall extraction.

**Cell Wall Composition Analysis**

Samples from leaves and epidermal peels were ground in liquid nitrogen along with 200 µL of extraction buffer (50mM Tris-Cl with 1% SDS at pH 7.2). Homogenate was transferred to 14-mL polypropylene, round-bottom tubes (Falcon product # 352059) along with 9 mL of extraction buffer, incubated for 15 minutes at 80°C, and centrifuged at 3,500 rpm for 5 min (~2,000 x g) in a swinging-bucket rotor centrifuge (ThermoForma 1LGP). Supernatant was removed with an aspirator, and pellets (water-insoluble cell-wall fraction) were washed, resuspended, and re-pelleted three times in about 10 mL 80°C water. The same process was repeated three times with 50% EtOH at 80°C, followed by three washes with 80°C water. Samples were transferred to 1.5-mL Eppendorf tubes, alcohol-insoluble cell wall fractions were pelleted and dried, and composition was analyzed by the Complex Carbohydrate Research Center (University of Georgia; Athens, GA).

For cellulose content, the alcohol-insoluble cell wall fractions from whole-leaf samples were isolated in the same way, dried for 16 h at 60°C, transferred to 14-mL

polypropylene, round-bottom tubes, and weighed. For each sample, approximately 50 mg of cell wall isolate was used, to which 3 mL 80% aqueous acetic acid and 300 µL 70% nitric acid were added. Tubes were incubated in an oil bath at 110°C and 120°C for 20 min each, to hydrolyze hemicellulose and lignin (from Sun et al., 2004). Samples were allowed to cool, 1.8 mL distilled water was added, tubes were centrifuged for 5 min (~2,000 x g), and supernatant was removed with an aspirator. Celluose was rinsed thoroughly with water (3 times) and 95% EtOH (3 times), and dried for 16 h at 60°C. Samples were weighed and compared for cellulose content as a fraction of alcohol-insoluble cell wall isolate.

**Real Time Quantitative RT-PCR**

For each sample, RNA was extracted from approximately 200 mg of tissue, initially frozen in liquid nitrogen, then homogenized in 1.0 mL Trizol (Invitrogen Cat # 15596-018) using a Q-BIOgene FastPrep 120 with Lysing Matrix D (MP Biomedicals Cat # 116913). Samples were incubated 5 min at 25°C, with frequent vortexing. Chloroform (200 µL) was added and samples were vortexed 15 sec before and after a 1-min incubation at 25°C. Phases were separated by centrifuging 10 min at 15,000 x g, and 200 µL of the aqueous layers were transferred to 700 µL of Qiagen RLT buffer (from RNeasy Plant Mini kit, Qiagen Cat # 74904). Ethanol was added (500 µL, 100% EtOH) and samples were vortexed. Half of this volume was used to clean and elute total RNA as per RNeasy Plant Mini kit (Qiagen Cat # 74904). Resulting RNA was treated with DNase-1 (Ambion Cat # AM1906), and quantified using a BioRad SmartSpec 3000. The cDNA was synthesized using SuperScript One-Step kit and protocol (Invitrogen Cat # 10928-042).

Levels of *CslD1* mRNA were quantified in diverse maize tissues and in leaf blades at a range of developmental stages via Real-time RT-PCR using a Step One Plus Real-Time PCR System (ABI, Carlsbad, CA). At least three biological replicates were analyzed for each tissue or time point, and for each of these replicates, reactions were performed in duplicate. A given reaction included 10 µL Fast SYBR Green Master Mix (ABI Lot # 1003024), 5.0 µL of cDNA sample (diluted 10x from cDNA reaction), and 100 nM of each gene-specific primer (Fwd: GCCGCTCACGTCAATGG, Rev: CTGGGCATCTTCATGGAGTGT) in a final volume of 20 µL. The relative abundance of transcripts was normalized with 18S rRNA controls (Taqman Ribosomal RNA Control Reagents, ABI Lot # 0804133) as in Eveland et al., 2008. Primer pairs for *CslD1* were designed using Primer Express 3.0 (ABI).

**Propidium Iodide Staining**

Immature leaves (10-15 cm) were dissected from whorls of *csld1* mutant and non-mutant plants. The basal portions (2 cm) of these leaves were immediately submerged in a solution of 0.1 mg/mL propidium iodide, and allowed to absorb the dye for 5 min at 25°C. Samples were then rinsed thoroughly in water to remove excess stain and flattened on a glass slide. The abaxial epidermis was imaged using a Zeiss confocal microscope. For visualization of nuclei, the same process was followed, but leaf samples were first fixed in FAA (10% formaldehyde [Fisher Lot # 992720], 5% acetic acid, 50% EtOH), before staining with propidium iodide.

**Flow Cytometry**

The basal 1 cm of immature leaves (2-3 cm) were dissected and finely sliced (~0.5 mm) with a razor blade in ice-cold chopping buffer (4% MOPS [0.5 M, pH 7.2], 9% $MgCl_2$ [0.5 M], 6% $Na_3$Citrate [0.5 M], 0.1 % Triton X-100 [Sigma, Lot # MKBD6639V], 1

mg RNase [Thermo Scientific, Cat # AB-0549], in water). Homogenate was filtered through 50 micron nylon mesh followed by 20 micron nylon mesh, then transferred to a 1.5-mL microcentrifuge tube. Nuclei were pelleted at 1,000 rpm for 3 min and supernatant was discarded. Pellets were resuspended in staining buffer (chopping buffer plus 1% propidium iodide [5 mg/mL]), and incubated at room temperature for 5 min. Nuclei were re-pelleted at 1,000 rpm for 3 min and supernatant was discarded. Pellets were resuspended in 300 µl of staining buffer and analyzed on a LSR-II cytometer (BD Biosciences, San Jose, CA). Nuclei were excited using a solid state laser emitting 100 milliwatts at 488 nm. Forward light scatter and orange fluorescence (575 +/- 13 nm) were collected on up to 5,000 particles per sample. Small particles of debris were gated out using a fluorescence vs forward light scatter dot plot. Peaks were identified on a fluorescence histogram plotted on logarithmic scale and the geometric and median fluorescence values for each peak were calculated. Software used was Diva 6.1.2 (BD Biosciences).

Table 4-1. Cell wall composition of whole-leaf blades and epidermal peels from *csld1* mutant and non-mutant plants.

| | Sample | Ara | Rha | Fuc | Xyl | Man | GalA | Gal | Glc | GlcA | Cellulose |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Whole leaf | WT | 11.43 (0.33) | 0.53 (0.02) | nd | 81.40 (0.01) | nd | 1.36 (0.07) | 0.85 (0.69) | 4.43 (0.41) | nd | 26.6 (1.8) |
| | *csld1* | 11.84 (0.18) | 0.57 (0.05) | nd | 81.18 (1.31) | nd | 1.59 (.016) | 0.80 (0.04) | 4.03 (1.41) | nd | 22.9 (3.1) |
| Epidermal peels | WT | 10.66 (0.54) | 0.31 (0.02) | nd | 85.04 (0.69) | nd | 0.95 (0.29) | 0.65 (0.08) | 2.39 (0.34) | nd | nt |
| | *csld1* | 11.93 (0.63) | 0.34 (0.03) | nd | 84.37 (0.96) | nd | 0.71 (0.05) | 0.69 (0.08) | 1.97 (0.22) | nd | nt |

Alcohol-insoluble residues were prepared from both whole-leaf-blade sections and isolated epidermal strips of greenhouse-grown plants identified as mutant or wildtype by PCR of a segregating family. Analysis of non-cellulosic cell wall sugars was done at the CCRC at the University of Georgia using combined gas chromotography/mass spectrometry (GC/MS) of the Tetramethylsilane (TMS) derivatives of the monosaccharide methyl glycosides produced by acidic methanolysis. Values for non-cellulosic sugars are given as mole percent, with standard error in parentheses. Cellulose content was estimated based on remaining weight after hydrolysis of hemicelluloses and lignin. Cellulose content is given as weight percentage of alcohol-insoluble cell wall fraction, with standard error in parentheses. None of the differences between *csld1* mutant and wildtype samples were statistically significant at $p < 0.05$ ($N = 4$, for each sample). nd = not determined, nt = not tested.

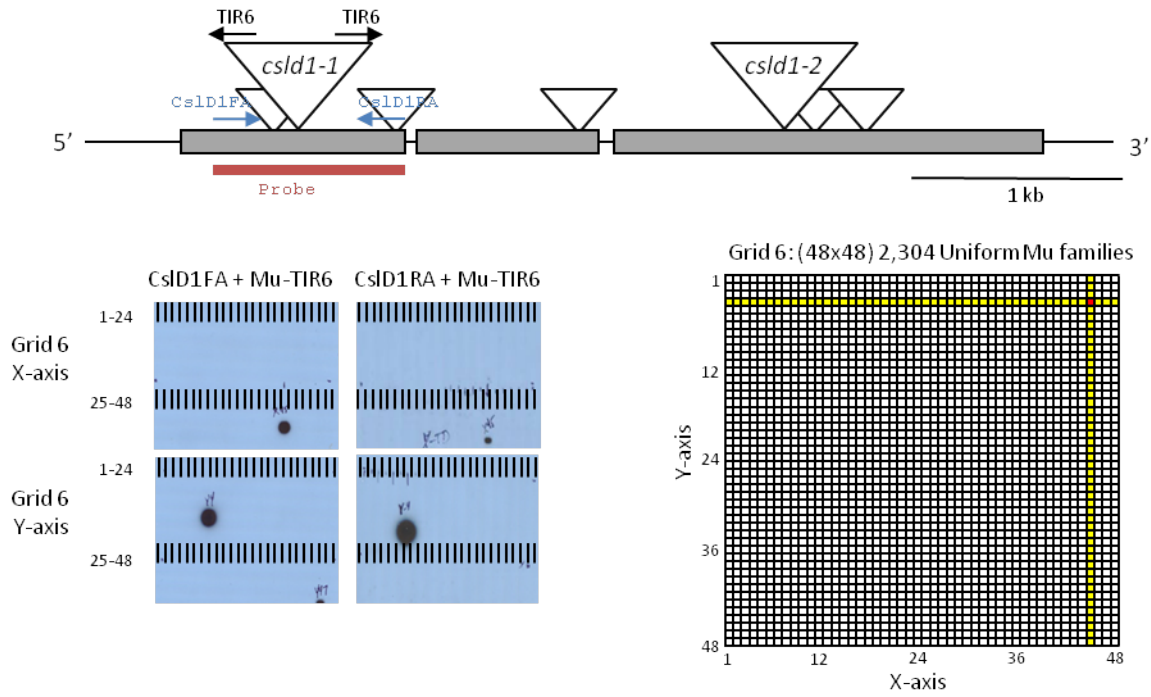


Figure 4-1. Gene diagram of Zm-*CslD1* and location of each of the Mu insertions identified. Large triangles represent the two UniformMu alleles (*csld1-1* and *csld1-2*) and small triangles show locations of TUSC alleles (*csld1-3* through *csld1-7*). Southern Blots are from the original hit in UniformMu Grid 6, with positive probe-binding lanes being identified in X45 and Y4 for both forward and reverse primers along with TIR6 primers (as diagramed above). Right panel shows the intersect in Grid 6 corresponding to UnifromMu family 04S-1130-27.

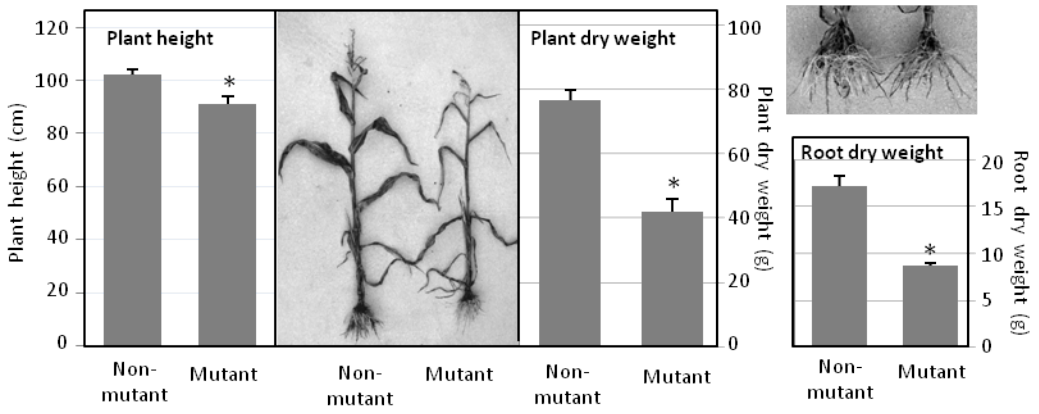


Figure 4-2. Morphology and dry weight of the *csld1-1* mutant. Plant height and dry weight (above- and below-ground) were quantified for field-grown *csld1* mutant and non-mutant plants. Non-mutant plants indluded both wildtype and heterozygous individuals from segregating progeny after 3 back-crosses into the W22 inbred. Height was measured from the soil line to the flag leaf of each plant. Below-ground dry weight was based on recovery of major roots as shown in the image above (root systems were compact in the irrigated sandy field conditions). Mutant plant height was reduced ( av. 9%), and total plant dry weight decreased (av. 45%). Above- and below-ground dry weights were reduced by similar amounts (av. 44% and 49%, respectively). For dry weight measurements, whole plants were sampled three days after ear maturity (40 days post pollination), and did not include mature ears. Significant difference ($p < 0.05$) from WT indicated by *.

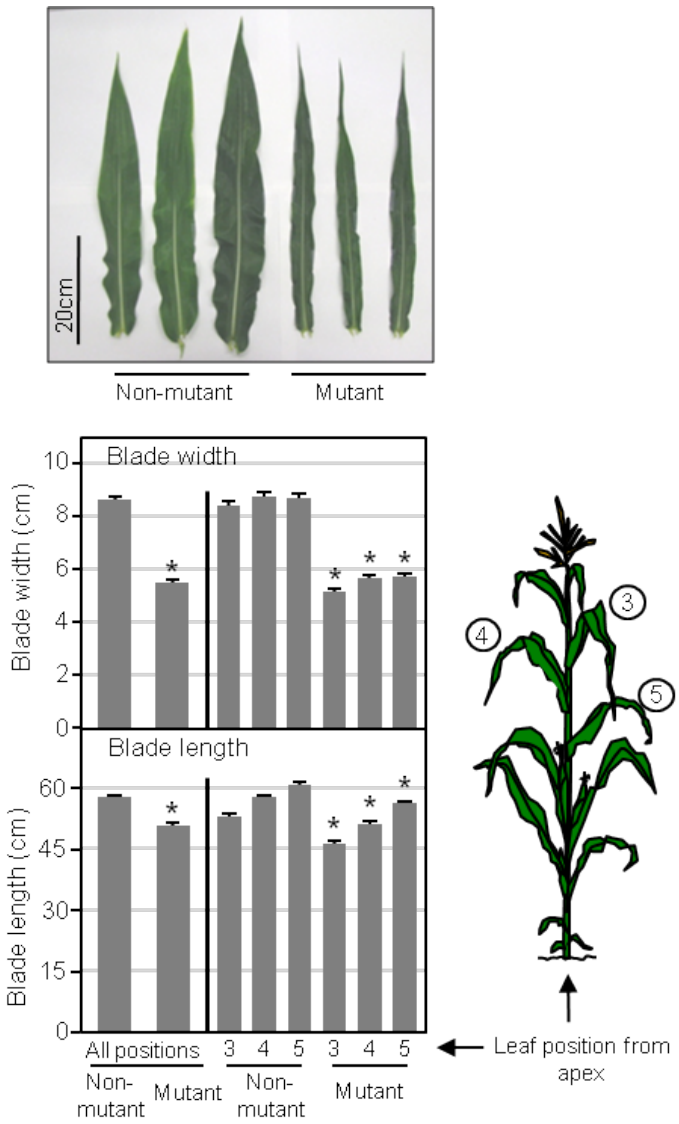


Figure 4-3. The narrow-leaf phenotype of *csld1-1* mutant plants. Leaf blade length and width (at widest point) were quantified for leaf positions 3 through 5 (as diagramed) for field-grown mutant and non-mutant plants. Non-mutant plants included both wildtype and heterozygous individuals from segregating progeny after 3 back-crosses into the W22 inbred. Imaged blades were from leaves in position #3 from the apex. The left-most portion of each graph shows combined data from all leaf positions measured. The leaf blade width-to-length ratio was 27% less for mutant plants. Blade width and length are reduced 35% and 10% respectively, relative to those of non-mutant plants. Data are similar for *csld1-2* (not shown), and visual appraisals of the five other *csld1* mutants. Note: Leaf photo is from greenhouse-grown plants, whereas quantifications are from field-grown plants. Significant difference ($p < 0.05$) from WT indicated by *.

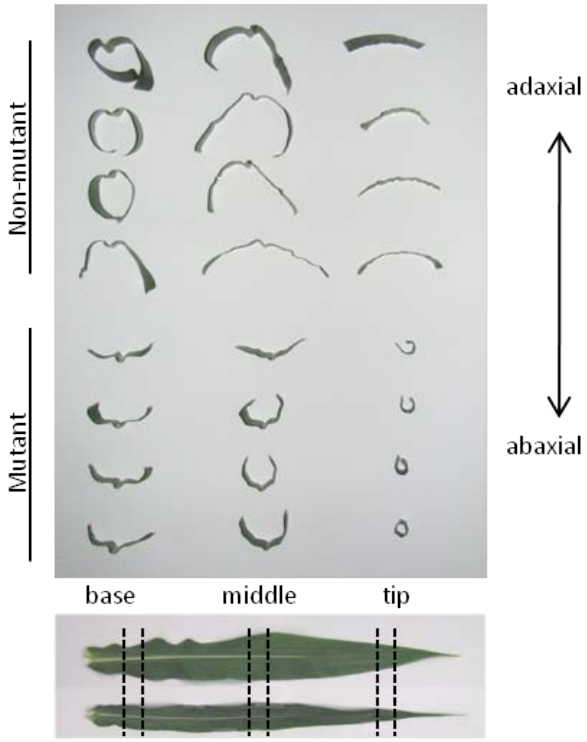


Figure 4-4. Leaf blade curling in *csld1* mutants. Leaf blades of *csld1* mutant leaves curl adaxially compared to non-mutant leaves, which typically flex downward (abaxially). Here, 1-cm sections of mutant and non-mutant leaf blades from well-watered plants were excised and imaged after adopting their natural conformations.

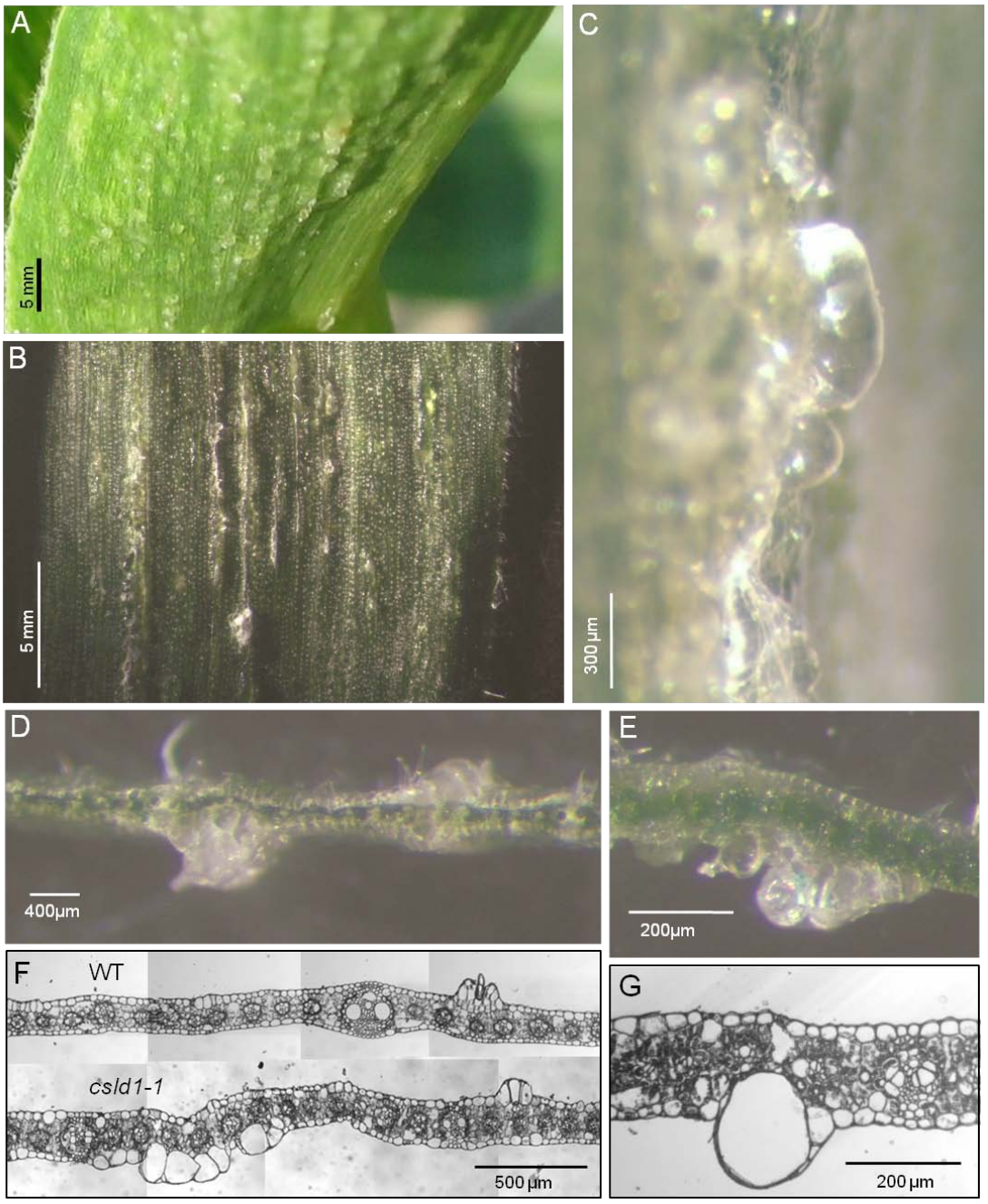


Figure 4-5. The warty phenotype of *csld1* mutant leaf blades. (A-C) Fresh, intact leaves. Epidermal warts were distributed in a non-uniform, apparently random manner across the entire blade and midrib of a mature fully-expanded leaf. (D, E) Fresh-sectioned leaf blades. Swollen lesions were most abundant on the abaxial leaf blade surface. (F, G) Fixed, imbedded cross-sections. Leaf blade interior is less visibly affected than epidermal cells in *csld1* mutants. Some epidermal cells have two-dimensional, cross-sectional areas up to 25-fold greater than normal (r = 100 µm for swollen cell in G). Image in F is composed of overlapping composite images.

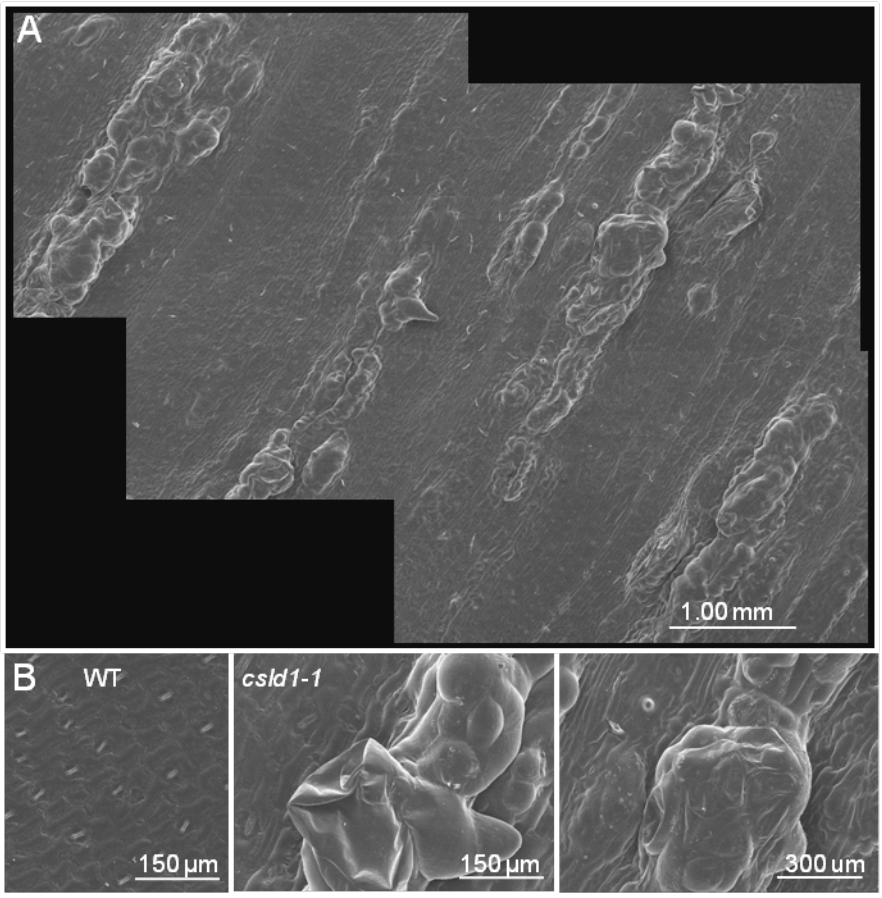


Figure 4-6. SEM of *csld1* mutant and wildtype leaves showing advanced development of epidermal lesions that continue growing after leaves reach full expansion. (A) Composite of overlapping images from of a mature, *csld1* mutant leaf revealing the complexity, dimensions, and distribution of the continually-expanding epidermal lesions. Clusters of swollen cells alternate with less-disturbed areas of epidermis. (B). Contrast between surfaces of wildtype (left panel) and *csld1* mutant leaves showing advanced development of large globular clusters, some having lost integrity and collapsed (center panel), others remaining intact and continuing to expand (right panel, expanded from composite image).

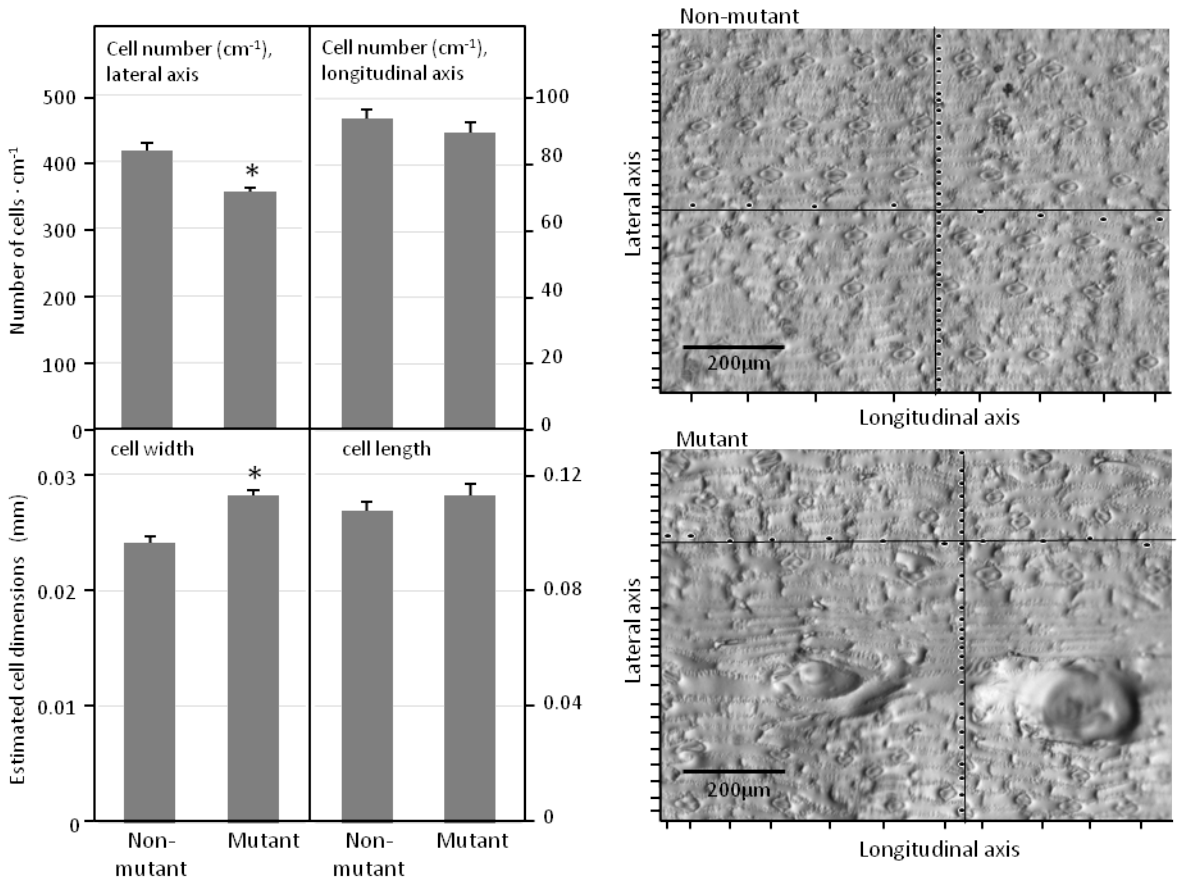


Figure 4-7. Size estimates for epidermal cells of inter-lesion regions on *csld1* mutant and non-mutant leaf blades. Epidermal impressions were taken from abaxial surfaces of fully-expanded leaf blades on mature, greenhouse-grown plants. Non-mutant plants included both wildtype and heterozygous individuals from segregating progeny after 3 back-crosses into the W22 inbred. Cell numbers were quantified along longitudinal and lateral axes of defined length. Mean cellular dimensions were determined by dividing number of cells along an axis by the length of that axis. (mutant N=14; non-mutant N=10; longitudinal axis 1.88 mm; lateral axis 1.40 mm). Axes used for analyses of epidermal cell size on mutant leaves did not include cells in the ballooning protrusions. Significant difference ($p < 0.05$) from non-mutant indicated by *.

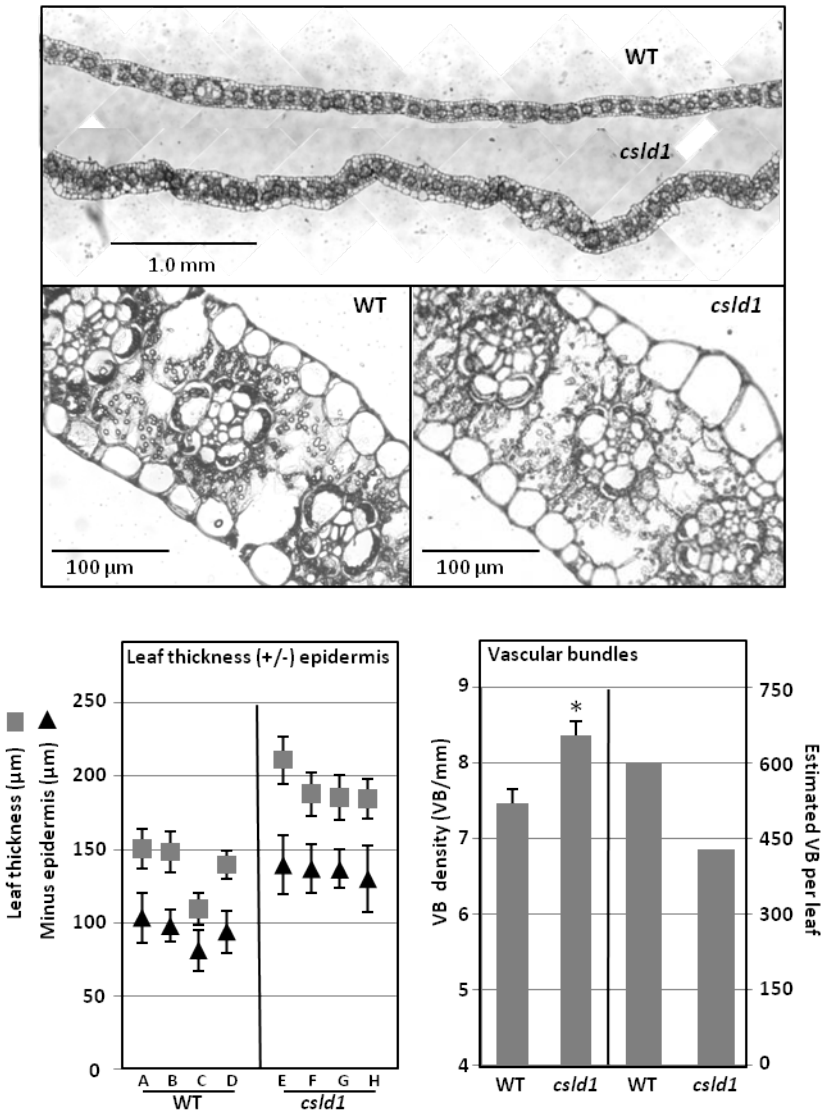


Figure 4-8. Internal structure of *csld1* mutant and non-mutant leaf blades. Sections of fully-expanded leaves from greenhouse-grown *csld1* mutant and wildtype plants showing visible differences in leaf thickness, vascular-bundle density, and mesophyll structure (upper three panels, top panel is a composite of overlapping images). Leaf thickness was quantified with- and without epidermal layers (lower left panel) for eight sections of fully-exapnded leaves labeled A-H. Error bars show SEM for 10 measurements across each leaf. Mutant leaves were a mean of 40% thicker than those of wildtype, and proportional increases were observed for internal tissues alone. Vascular bundle density was also quantified (lower right panel), and used to estimate vascular bundle number per leaf (with mean leaf-width values [exluding midribs] being 80.2 mm and 51.1 mm for wildtype and *csld1*-mutant blades, respectively). Resulting estimates of vascular bundle number per leaf width were 29% less for *csld1* mutants. Error bars indicate standard error. Significant difference between *csld1* and wildtype ($p < 0.05$) is indicated by *.

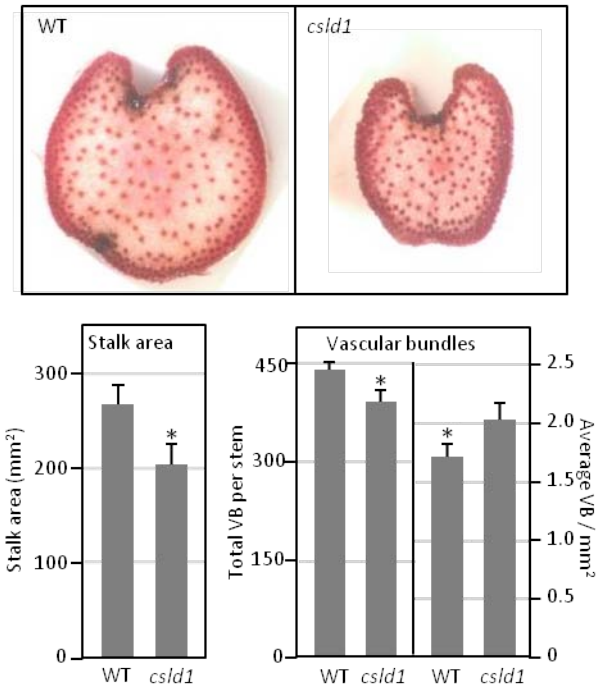


Figure 4-9. Internal structure of *csld1* mutant and non-mutant stems. Stem sections from the third internode of greenhouse-grown *csld1* mutant and wildtype plants (PCR-genotyped). Material was stained with phloroglucinol, imaged, and cross-sectional area was calculated using Image-J. Measurements were collected for 11 WT and 8 mutant stalks. Mutant stalks were on average 24% smaller than those of wildtype. Mutant sections had a mean of 12% fewer vascular bundles, while bundle density was increased by 14%. Error bars indicate standard error. Significant differences between mutant and wildtype ($p < 0.05$) are indicated by *.

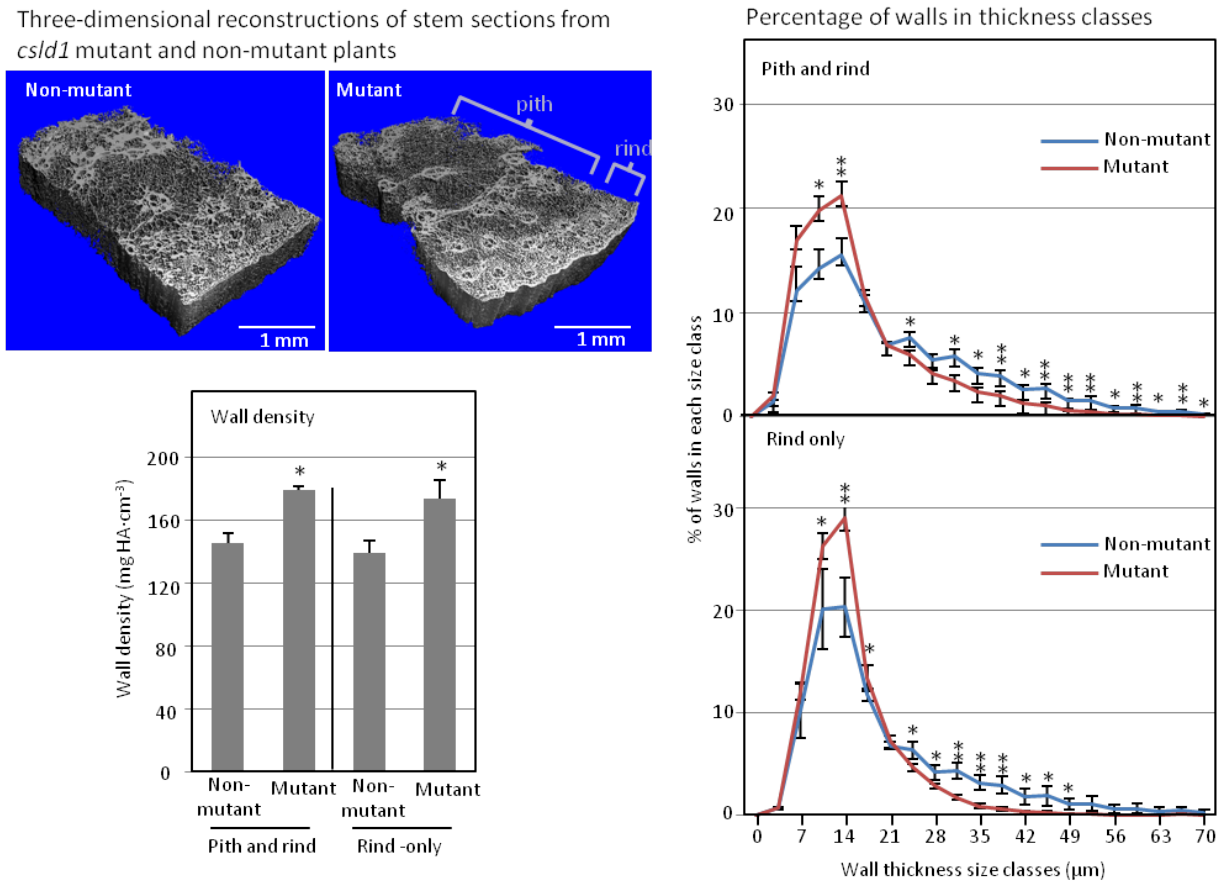


Figure 4-10. High-resolution X-ray micro computed tomography analysis of *csld1* and wildtype stalks. Analysis of stem sections from the third internode of greenhouse-grown *csld1* mutant and wildtype plants using high-resolution X-ray micro-CT. Hand-cut sections approximately 3 x 2 x 10 mm from the edge of mutant and wildtype stems (four each) were scanned at 3.5 µm resolution. Three-dimensional analyses revealed significant differences in density of wall material (lower left) and distribution of wall thickness (right). Mutant stems had more dense, but generally thinner, walls, even when analyses were limited to the rind only. Volumetric analyses were calculated with an algorithm developed for trabeacular bone (Hildebrand and Regsegger, 1997). Error bars represent standard error. Significant differences from WT are indicated, ($p < 0.05$)** and ($p<0.1$)*.

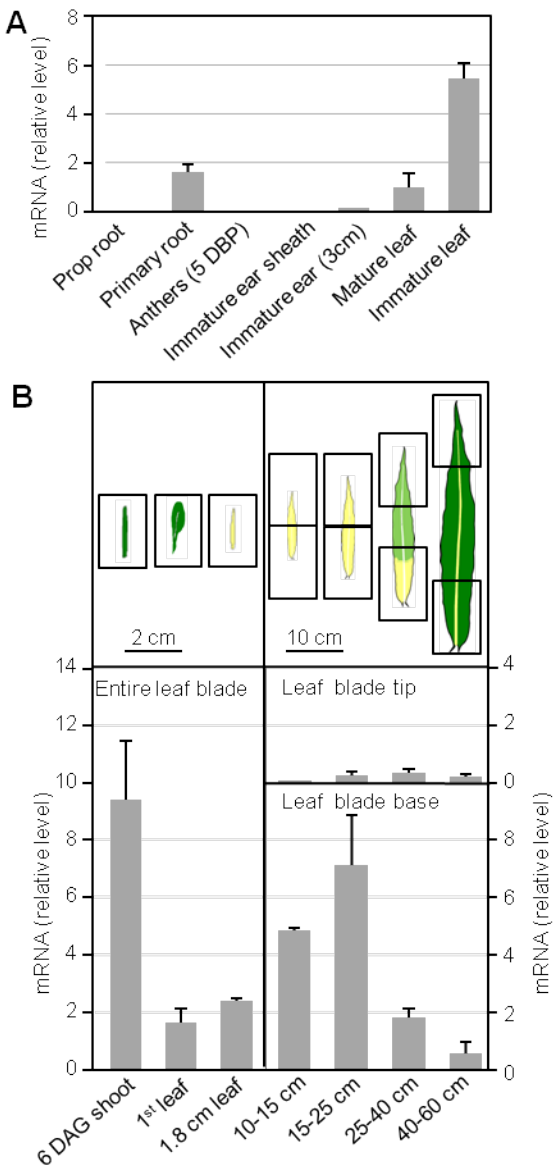


Figure 4-11. Levels of Zm-*CslD1* mRNA in diverse tissues from wildtype plants of the W22 inbred. (A) Expression of Zm-*CslD1* in diverse organs. (B) Zm-*CslD1* mRNA levels in leaf blades and blade-regions at different stages of development. Levels of mRNA was quantified by CyberGreen quantitative Real Time RT-PCR. Three biological replications were analyzed for or each tissue. Note abundance of *CslD1* transcripts in pre-emergent immature leaves. Maximal expression was observed in very young shoots (including coleoptile and enfolded leaves), followed by the basal portion of intermediate-staged developing leaves.

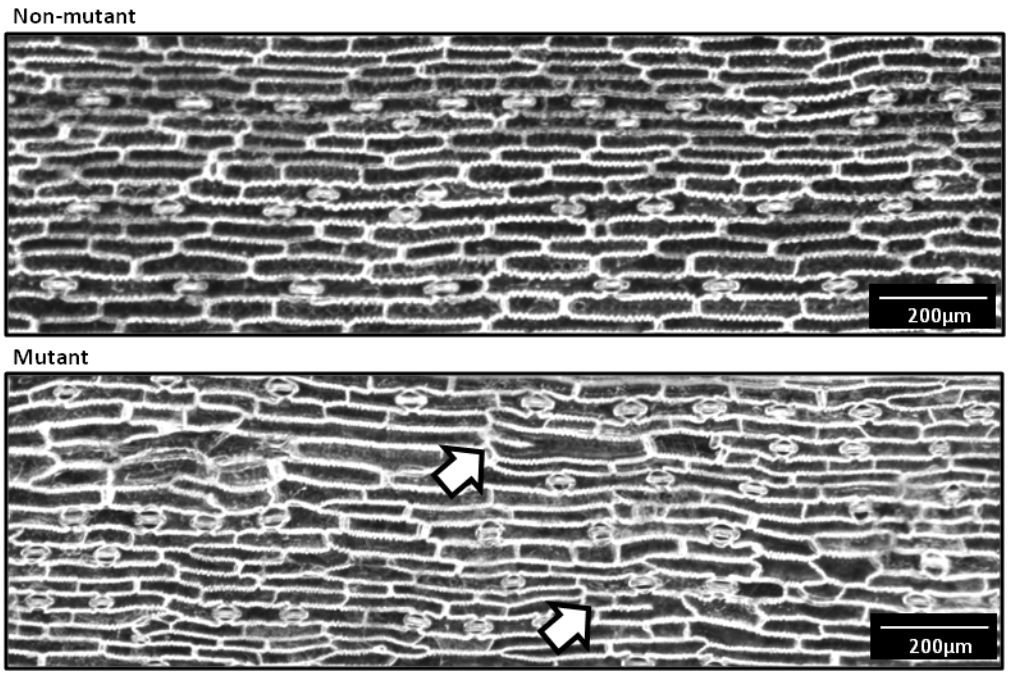


Figure 4-12. Mature *csld1* mutant and non-mutant epidermis revealed apparent defects in cell division. Comparison of isolated epidermis from non-warty areas of mature *csld1* mutant and non-mutant leaves using dark-field microscopy revealed a number of striking defects. Mutant epidermis appeared disrupted and unorganized compared to non-mutant epidermis. There were also numerous cases of misshapen cells and cell wall stubs (arrows), indicating a failure of cells to complete cell division.

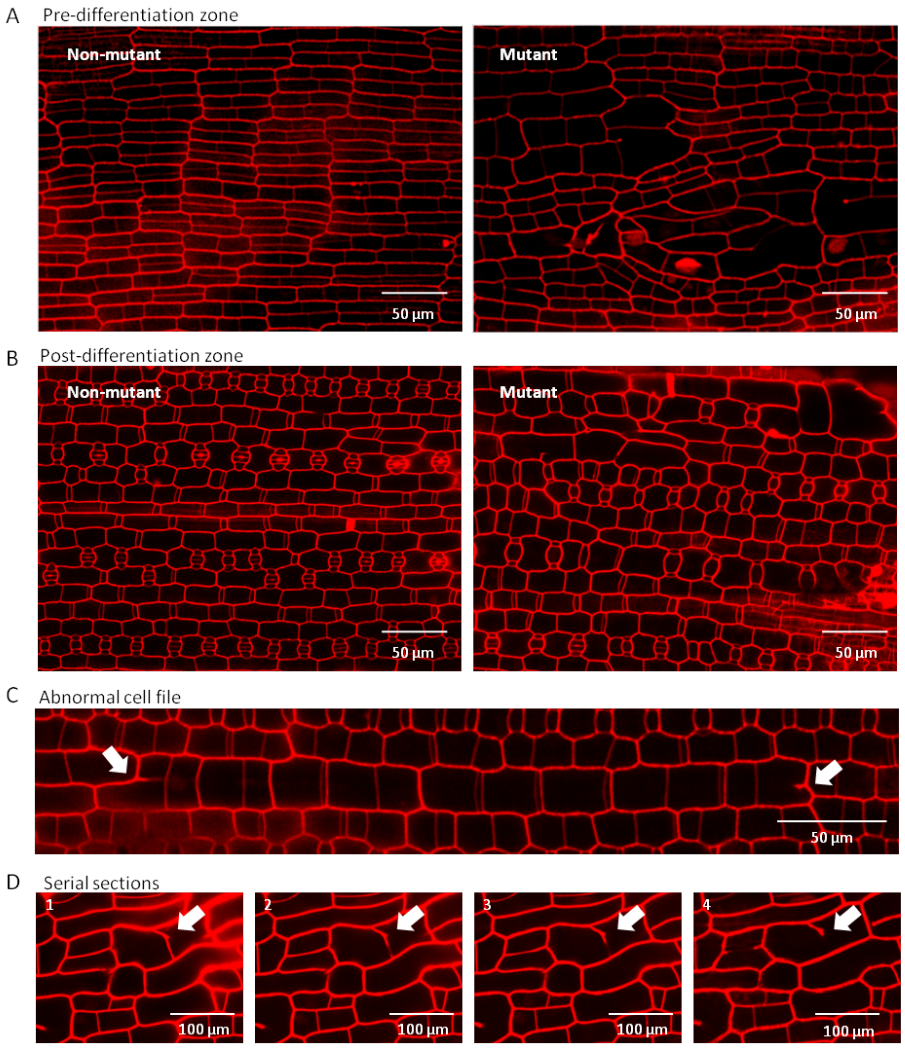


Figure 4-13. Confocal images showing defects early in development of *csld1* mutant leaf epidermis. Propidium iodide-stained cell walls from fresh, immature *csld1* mutant and wildtype leaves. (A) Pre-differentiation zones of basal portions of wildtype leaves contrasting with the abnormal *csld1-1* cell size, shape, and organization. Leaf blades were approximately 10-cm long. (B) Post-differentiation zones showing persistent effects of altered cell division in large, misshapen, unordered cells of the *csld1-1* leaf epidermis. Again, leaf blades were approximately 10-cm long. (C) A typical file of large, irregular cells bounded by individuals with incomplete cell walls protruding from their outer edges, consistent with clonal inheritance of large cell size. (D) Serial optical sections revealing irregular gaps in cell walls (arrow) that occur frequently in epidermal cells of the *csld1* mutant.

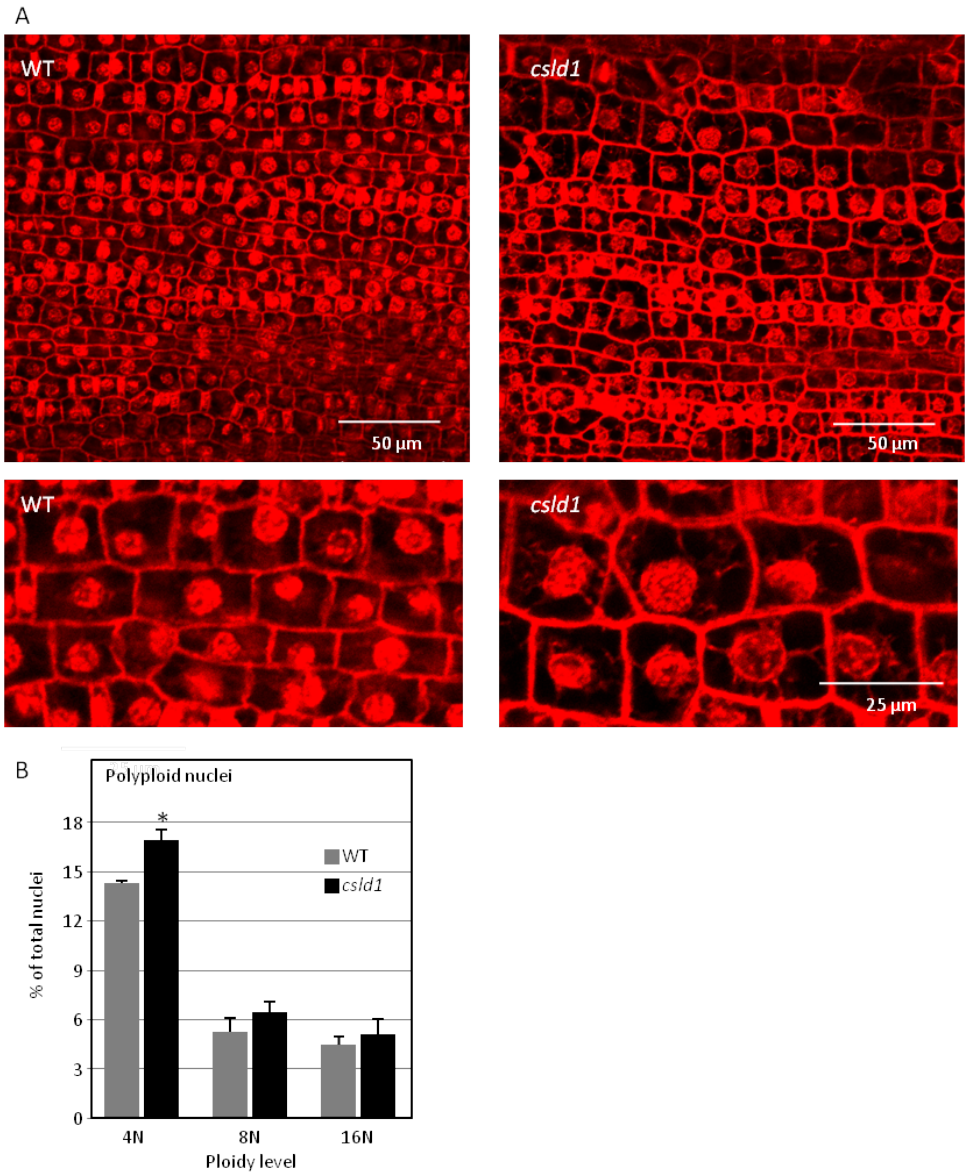


Figure 4-14. Nuclei of immature *csld1* mutant and non-mutant epidermis. (A) Confocal imaging of fixed cells stained with propidium iodide revealed an abundance of large nuclei in *clsd1* mutant epidermis. Larger nuclei corresponded with the larger cell size of mutant epidermis. (B) Percent of total nuclei with 4N, 8N, and 16N. Nuclei from immature tissue from basal portions of *csld1* mutant and wildtype leaves were examined using flow cytometry of isolated nuclei stained with propidium iodide. The majority of nuclei were 2N and are not displayed here. Significant difference between *csld1* and wildtype ($p < 0.05$) are indicated by *, N = 5.

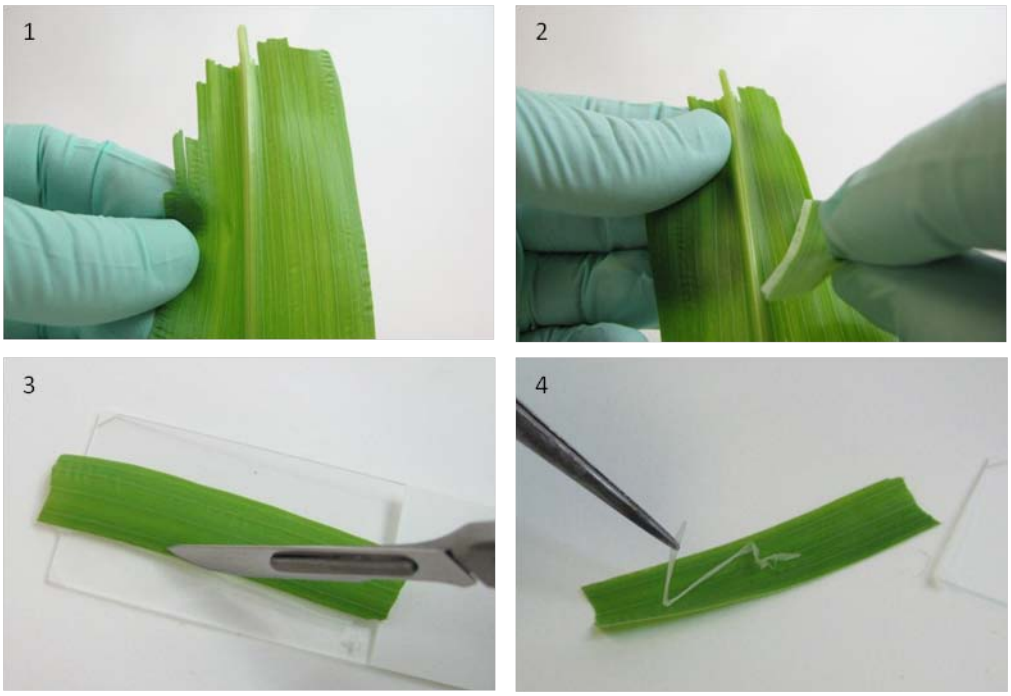


Figure 4-15. Isolation of maize leaf epidermis. 1) Hold a piece of fresh leaf tissue, with the epidermal surface to be isolated facing inward (abaxial surface in this case). 2) Pull back on part of the leaf so that it tears along the longitudinal leaf axis. As a strip of epidermis is revealed, angle the piece towards the main leaf body to maximize epidermal strip width. Typical epidermal strips are between 2-5 mm. 3) Completely remove the leaf piece containing the epidermal strip, hold against a hard surface (such as a glass slide), and slice off clean epidermis using a scalpel. Areas with non-epidermal contaminants should be visible against a white background. 4) Use forceps to transfer epidermal strip to collection tube, or leave strip on glass slide (with a small amount of water) for visualization under dark-field microscopy.

# CHAPTER 5
# CONCLUSIONS AND FUTURE DIRECTIONS

Work presented here revealed a surprisingly large number of Mu transposable elements, with extensive divergence, and widely-varying sites of insertion in maize and teosinte inbreds (Chapter 2). Results have been immediately incorporated into new attempts to harness the mutagenic power of these transposons in the UniformMu maize population (a globally-accessed community resource initiated at the University of Florida [McCarty et al., 2005]). Preliminary results from high-throughput sequencing of UniformMu DNA identified large numbers of apparently unique Mu12 elements that were not previously recognized in this population. The extent of activity by these Mu's was unexpected due to previous reports that detected little activity of Mu10s and Mu12s (Dietrich et al., 2002; Liu et al., 2009). However, previous studies did not utilize Mu12-specific primers for their Mu-flanking sequence generation. Regardless, results here show these to be an abundant, diverse, and active component of the Mu system. A re-appraisal of Mu10s and Mu12s in other mutagenic maize lines may yield results similar to those shown here. We will soon be able to test the degree to which the divergent and abundant Mu12 elements of the UniformMu population are active, and if so, a large number of insertional mutants will become available for the global community of maize geneticists and breeders. Even if the UniformMu population itself does not harbor particularly active Mu12 elements, our current sequencing protocols and materials should allow identification of lines that do have highly active Mu12s. These could be bred or engineered to create future mutagenic populations.

The presence of a previously unrecognized, possibly active transposase in the B73 maize genome is indicated by data shown here (Chapter 3, Figure 3-7). Additional

analyses will be needed to confirm function, but a clear homology and extensive conservation was shown between Mu10 elements in the B73 genome and the known MuDR transposase. Expression of the putative transposase genes in such Mu10 elements would indicate function, although their expression could be low, highly regulated, and/or limited to specific sites or times, thus not easily detectable. As of now, no Mu10-derived transcripts have been deposited in public maize EST collections (data not shown). Another line of future experimentation to test hypotheses for Mu10 activity would be to compare segregation of these (or similar elements) in wildtype and Mu-active populations such as UniformMu. If Mu-active lines could be identified that lacked classical MuDR elements, but which included these Mu10 elements, then this observation would support the hypothesis that some Mu10s encode functional transposases.

Evidence here revealed an unexpected role of a cell wall biosynthetic gene in plant cell division (Chapter 4), yet the specific biochemical mechanism is yet to be defined. Future work on sub-cellular localization of the maize CSLD1 protein may also provide valuable information about its specific biological role in the process of cell division. Transgenic maize lines with YFP-tagged CSLD1 protein driven by the CSLD1 native promoter are currently being generated by the Maize Cell Genomics group (Mohanty et al., 2009). We hypothesize that the CSLD1 enzyme is targeted to, and functional at, the newly-forming cell plate in epidermal cells of developing maize leaves. If so, then the tagged protein may localize to these regions near newly-forming cross walls. New evidence (from Erik Neilson, University of Michigan) also supports action by CSLD proteins in synthesis of cellulose, and a specialized form of cellulose could possibly be

required for cell wall formation at not only the tips of elongating cells such as root hairs and pollen tubes, but also at the cell plate of dividing plant cells.

The increased density and thinner cell walls of the *csld1* mutant stems, as revealed by high-resolution micro CT analyses, highlight another intriguing possibility regarding the function of CSLD proteins. Estimation of the cellulose crystallinity using X-ray diffraction and/or NMR spectroscopy (Park et al., 2010) could provide important evidence for the biochemical and/or biophysical basis for roles of the CSLD1 protein. If the *csld1* mutant walls contain more crystalline cellulose (as implied by results presented here), such data would provide still further resolution of whether CSLD enzymes produce contribute to formation of cellulose with a more amorphous nature.

# APPENDIX A

All mapped Mu insertions in ten Zea inbreds, arranged by inbred, followed by Mu class and chromosome location. TSD: tandem-site duplication.

| Inbred | Mu | chr | start | TSD |
|---|---|---|---|---|
| **B73** | Mu1-9 | chr1 | 215947275 | GTCGGCTGG |
| **B73** | Mu1-9 | chr1 | 245365818 | GTGTTAGGT |
| **B73** | Mu1-9 | chr1 | 245367837 | GTGTTAGGT |
| **B73** | Mu1-9 | chr1 | 264263006 | TTAGGTCGG |
| **B73** | Mu1-9 | chr1 | 264267812 | TTAGGTCGG |
| **B73** | Mu1-9 | chr10 | 146901724 | CTCGATTTG |
| **B73** | Mu1-9 | chr10 | 146912555 | CTCGATTTG |
| **B73** | Mu1-9 | chr2 | 17647 | CTCTCTTTC |
| **B73** | Mu1-9 | chr2 | 61514 | GAAAGAGAG |
| **B73** | Mu1-9 | chr2 | 25300688 | GAGGCTCTC |
| **B73** | Mu1-9 | chr2 | 25302603 | GAGGCCCTC |
| **B73** | Mu1-9 | chr2 | 31723258 | GGAGTGCGG |
| **B73** | Mu1-9 | chr2 | 135007660 | GGCTGGCGG |
| **B73** | Mu1-9 | chr2 | 194078115 | GCCGGGGGC |
| **B73** | Mu1-9 | chr2 | 194082980 | GCCGGGGGC |
| **B73** | Mu1-9 | chr3 | 63759 | CTGCATGGG |
| **B73** | Mu1-9 | chr3 | 63767 | CTGCAGGGG |
| **B73** | Mu1-9 | chr3 | 170233622 | GTCGCCAGC |
| **B73** | Mu1-9 | chr3 | 170235173 | GTCGCCAGC |
| **B73** | Mu1-9 | chr3 | 192454287 | AAATGGATG |
| **B73** | Mu1-9 | chr4 | 159505930 | GCCGTGCGA |
| **B73** | Mu1-9 | chr4 | 165969433 | GGGCCTAGG |
| **B73** | Mu1-9 | chr5 | 17111268 | CAAGGTGGG |
| **B73** | Mu1-9 | chr5 | 17197656 | CAAGGTGGG |
| **B73** | Mu1-9 | chr5 | 116267118 | TCACCCAAG |
| **B73** | Mu1-9 | chr5 | 116268617 | TCACCCGAG |
| **B73** | Mu1-9 | chr6 | 107992293 | CACAAAAAA |
| **B73** | Mu1-9 | chr6 | 161765840 | CTGGTAGTG |
| **B73** | Mu1-9 | chr6 | 161770900 | CTGGTAGTG |
| **B73** | Mu1-9 | chr7 | 48473780 | GCGGGAGAG |
| **B73** | Mu1-9 | chr7 | 48474915 | GCGGGAGGG |
| **B73** | Mu1-9 | chr7 | 72528765 | CTATGCGAT |
| **B73** | Mu1-9 | chr7 | 72530965 | CTATGCGAT |
| **B73** | Mu1-9 | chr7 | 134022998 | CTCCTTAAG |
| **B73** | Mu1-9 | chr8 | 3812868 | GTTGTGCTC |
| **B73** | Mu1-9 | chr8 | 3875319 | GAGCACAAC |
| **B73** | Mu1-9 | chr8 | 138633675 | GTTGTATTC |

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|---|---|---|---|---|
| **B73** | Mu1-9 | chr8 | 151461730 | CTCTCTACC |
| **B73** | Mu1-9 | chr8 | 153725735 | GTTAGTTGT |
| **B73** | Mu1-9 | chr8 | 153727357 | GTTAGTTGT |
| **B73** | Mu1-9 | chr9 | 40134754 | GGCGCCCAG |
| **B73** | Mu1-9 | chrUNKNOWN | 13826404 | TGCAGTACA |
| **B73** | Mu1-9 | chrUNKNOWN | 13829283 | TGCAGTACA |
| **B73** | Mu10 | chr2 | 63630170 | GCACAAACT |
| **B73** | Mu10 | chr2 | 76218121 | TCGAGAGGG |
| **B73** | Mu10 | chr2 | 76222892 | TCGAGAGGG |
| **B73** | Mu10 | chr3 | 32832334 | CTCGCTGCC |
| **B73** | Mu10 | chr4 | 40201165 | TCGAGCGCG |
| **B73** | Mu10 | chr4 | 170887654 | GCGGGCGGA |
| **B73** | Mu10 | chr5 | 70381872 | GTTTTTCGG |
| **B73** | Mu10 | chr5 | 70386843 | GTTTTTCGG |
| **B73** | Mu10 | chr5 | 73323804 | GTCGAAATC |
| **B73** | Mu10 | chr5 | 73327895 | GTCAAAATC |
| **B73** | Mu10 | chr5 | 145977953 | TCCCCTCCA |
| **B73** | Mu10 | chr6 | 160446766 | TCCCACGAG |
| **B73** | Mu10 | chr6 | 160464150 | CTCCTGGGA |
| **B73** | Mu10 | chr7 | 134021621 | TTCTTTAAG |
| **B73** | Mu10 | chr9 | 11643467 | TGGGTTGGG |
| **B73** | Mu10 | chr9 | 11653693 | GGGCTGGGA |
| **B73** | Mu10 | chrUNKNOWN | 5300073 | ATGGGAGTG |
| **B73** | Mu12 | chr1 | 5853336 | CCCGTCAGT |
| **B73** | Mu12 | chr1 | 5855090 | CCCGTCAGT |
| **B73** | Mu12 | chr1 | 9045993 | CTAGATTTG |
| **B73** | Mu12 | chr1 | 10975143 | CTCTCCTCG |
| **B73** | Mu12 | chr1 | 19513553 | CGAGAGCAG |
| **B73** | Mu12 | chr1 | 37644606 | ACCGAATGT |
| **B73** | Mu12 | chr1 | 65294475 | TAACATATC |
| **B73** | Mu12 | chr1 | 65294573 | CGACATATC |
| **B73** | Mu12 | chr1 | 78845541 | TGGACACTA |
| **B73** | Mu12 | chr1 | 78847493 | ATGGACACT |
| **B73** | Mu12 | chr1 | 148130653 | TGGCCCGTG |
| **B73** | Mu12 | chr1 | 148231792 | CACGGGCCA |
| **B73** | Mu12 | chr1 | 164914751 | CTGCTCTAC |
| **B73** | Mu12 | chr1 | 255428941 | GTTTCCCAA |
| **B73** | Mu12 | chr1 | 255431229 | TTTCCTTGG |
| **B73** | Mu12 | chr1 | 259661577 | CCATTTTTT |
| **B73** | Mu12 | chr1 | 297180880 | GAGAGATGA |
| **B73** | Mu12 | chr1 | 297188349 | GAGAGATGA |
| **B73** | Mu12 | chr10 | 4001312 | CCCTCTCCT |
| **B73** | Mu12 | chr10 | 15300760 | CTTGCAATG |

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| **B73** | Mu12 | chr10 | 15308429 | CTTGCAATG |
| **B73** | Mu12 | chr10 | 55963355 | CCTCTAGAG |
| **B73** | Mu12 | chr10 | 97224421 | TGACGAAAC |
| **B73** | Mu12 | chr10 | 97271042 | TGACGAAAC |
| **B73** | Mu12 | chr10 | 99885745 | TGATGGTAT |
| **B73** | Mu12 | chr10 | 116689007 | GCAGGGCAG |
| **B73** | Mu12 | chr10 | 135327980 | GTCAGGGCT |
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| **B73** | Mu12 | chr10 | 138397252 | TCCCAGGAT |
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| **B73** | Mu12 | chr10 | 141729910 | CTCAGAAAG |
| **B73** | Mu12 | chr2 | 9947891 | CTTGGACGA |
| **B73** | Mu12 | chr2 | 24581453 | TCGGTTGCG |
| **B73** | Mu12 | chr2 | 24982189 | GACCTCAAA |
| **B73** | Mu12 | chr2 | 25000049 | TTTGAGGTC |
| **B73** | Mu12 | chr2 | 25001836 | CAAAGGGTC |
| **B73** | Mu12 | chr2 | 37869180 | GGGCACGAG |
| **B73** | Mu12 | chr2 | 126547121 | AGCTGCGGC |
| **B73** | Mu12 | chr2 | 149547189 | TCCATATGG |
| **B73** | Mu12 | chr2 | 149548163 | TCCATATGG |
| **B73** | Mu12 | chr2 | 197215143 | CGCGGGGGC |
| **B73** | Mu12 | chr2 | 229531942 | AGAGGGGGG |
| **B73** | Mu12 | chr2 | 229540123 | AGGAGGGGG |
| **B73** | Mu12 | chr3 | 1603146 | CATGAACCC |
| **B73** | Mu12 | chr3 | 1703348 | GGGTTCATG |
| **B73** | Mu12 | chr3 | 9282491 | ATTTCCCGT |
| **B73** | Mu12 | chr3 | 9286569 | ATTTCCCGT |
| **B73** | Mu12 | chr3 | 64925288 | GATGCCGGC |
| **B73** | Mu12 | chr3 | 64927462 | GATGCCGGA |
| **B73** | Mu12 | chr3 | 79376179 | CCATTTTTT |
| **B73** | Mu12 | chr3 | 89795711 | CTTCAGAGA |
| **B73** | Mu12 | chr3 | 121611818 | GGCGTAACT |
| **B73** | Mu12 | chr3 | 121613607 | GGCGTAACT |
| **B73** | Mu12 | chr3 | 121613610 | GGTGTAACT |
| **B73** | Mu12 | chr3 | 156342516 | GTCCCCAGC |
| **B73** | Mu12 | chr3 | 181510576 | CTCCCGAAC |
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| **B73** | Mu12 | chr3 | 216632366 | TACTGCAGT |
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| **B73** | Mu12 | chr4 | 58603310 | TGCGCGTGC |
| **B73** | Mu12 | chr4 | 73211942 | CAATGCCGC |

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| **B73** | Mu12 | chr4 | 211398529 | GCGAGCGAG |
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| **B73** | Mu12 | chr4 | 231573451 | GGTGTTTGA |
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| **B73** | Mu12 | chr5 | 5197195 | GCGTGGGCG |
| **B73** | Mu12 | chr5 | 5199460 | GCGTGGGCG |
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| **B73** | Mu12 | chr5 | 186969978 | AGAGGGGGG |
| **B73** | Mu12 | chr5 | 190223449 | TAAACCACC |
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| **B73** | Mu12 | chr6 | 2917476 | TCCATGATG |
| **B73** | Mu12 | chr6 | 14213991 | GTGTTCATG |
| **B73** | Mu12 | chr6 | 28306795 | GCTGCAGGA |
| **B73** | Mu12 | chr6 | 92047273 | ATGCAGATG |
| **B73** | Mu12 | chr6 | 92197928 | CCTTGCAAG |
| **B73** | Mu12 | chr6 | 129097781 | GGAACGAAT |
| **B73** | Mu12 | chr7 | 16535034 | AATGGTCCA |
| **B73** | Mu12 | chr7 | 20735964 | CCAATTCGG |

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| **B73** | Mu12 | chr7 | 50182115 | GCCCGTGAG |
| **B73** | Mu12 | chr7 | 121684992 | CCAAAAATG |
| **B73** | Mu12 | chr7 | 147322983 | GGCGTGCTA |
| **B73** | Mu12 | chr7 | 161229569 | ATCTGTCAG |
| **B73** | Mu12 | chr7 | 169729474 | CCATTTCCA |
| **B73** | Mu12 | chr8 | 5414131 | GCGCGCGAG |
| **B73** | Mu12 | chr8 | 5414433 | CTCGCGCGC |
| **B73** | Mu12 | chr8 | 42932444 | GACCTCAAA |
| **B73** | Mu12 | chr8 | 100614801 | CATTGTAGG |
| **B73** | Mu12 | chr8 | 106152637 | TCATTGCAA |
| **B73** | Mu12 | chr8 | 130925131 | GGCTGCGGA |
| **B73** | Mu12 | chr8 | 141273174 | GTAGATTGG |
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| **B73** | Mu12 | chr8 | 148887211 | CTGTTTGAA |
| **B73** | Mu12 | chr9 | 4231266 | GCACGCAAC |
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| **B73** | Mu12 | chr9 | 15061435 | GCGAGCGTC |
| **B73** | Mu12 | chr9 | 25697019 | CCTAATTTT |
| **B73** | Mu12 | chrUNKNOWN | 12783521 | AGTGGCCGG |
| **MO17** | Mu1-9 | chr1 | 152954540 | ACCAATTGG |
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| **MO17** | Mu1-9 | chr1 | 264263006 | TTAGGTCGG |
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| **MO17** | Mu1-9 | chr1 | 273709444 | ATCTCTGAG |
| **MO17** | Mu1-9 | chr10 | 88102930 | CTTGTGAAG |
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| **MO17** | Mu1-9 | chr10 | 129970873 | CTGGTAATA |
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| **MO17** | Mu1-9 | chr10 | 146912555 | CTCGATTTG |
| **MO17** | Mu1-9 | chr2 | 25300688 | GAGGCTCTC |
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| **MO17** | Mu1-9 | chr2 | 135007660 | GGCTGGCGG |
| **MO17** | Mu1-9 | chr2 | 135009766 | GGCTGGCGG |
| **MO17** | Mu1-9 | chr2 | 142272069 | CATCCAAAC |
| **MO17** | Mu1-9 | chr2 | 142324924 | CCATCCAAA |
| **MO17** | Mu1-9 | chr2 | 158559629 | CTCTCTTTC |
| **MO17** | Mu1-9 | chr2 | 214951873 | TTAGGTCGG |
| **MO17** | Mu1-9 | chr2 | 214951881 | TTAGATCGG |
| **MO17** | Mu1-9 | chr2 | 220443225 | CCAGCAGCC |

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| **MO17** | Mu1-9 | chr3 | 219993784 | CCCTTGATT |
| **MO17** | Mu1-9 | chr3 | 219993791 | CCCTTGATT |
| **MO17** | Mu1-9 | chr3 | 230139824 | CCCATGCAG |
| **MO17** | Mu1-9 | chr3 | 230143403 | CCCCTGCAG |
| **MO17** | Mu1-9 | chr4 | 118954521 | CCTTGTGAA |
| **MO17** | Mu1-9 | chr4 | 159505930 | GCCGTGCGA |
| **MO17** | Mu1-9 | chr4 | 159506805 | GCCGTGCGA |
| **MO17** | Mu1-9 | chr4 | 165969433 | GGGCCTAGG |
| **MO17** | Mu1-9 | chr5 | 17111268 | CAAGGTGGG |
| **MO17** | Mu1-9 | chr5 | 17197656 | CAAGGTGGG |
| **MO17** | Mu1-9 | chr5 | 116267118 | TCACCCAAG |
| **MO17** | Mu1-9 | chr5 | 116268617 | TCACCCGAG |
| **MO17** | Mu1-9 | chr5 | 178759106 | GTTTGGAGT |
| **MO17** | Mu1-9 | chr5 | 178759114 | GTTTGCAGT |
| **MO17** | Mu1-9 | chr5 | 201460195 | GTCGGTCTA |
| **6_8** | Mu1-9 | chr5 | 201460203 | GTCGGTCTA |
| **MO17** | Mu1-9 | chr6 | 47652355 | CCACAAATA |
| **MO17** | Mu1-9 | chr6 | 47652364 | CACAAATAG |
| **MO17** | Mu1-9 | chr6 | 107992299 | CACAAAAAA |
| **MO17** | Mu1-9 | chr6 | 167002619 | CTTCACAAG |
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| **MO17** | Mu1-9 | chr6 | 167011925 | CTTGTGAAG |
| **MO17** | Mu1-9 | chr8 | 85028236 | CAAGGTGGG |
| **MO17** | Mu1-9 | chr8 | 139599679 | ACAACTAAC |
| **MO17** | Mu1-9 | chr8 | 151461730 | CTCTCTACC |
| **MO17** | Mu1-9 | chr8 | 151463296 | CTCTCTACC |
| **MO17** | Mu1-9 | chr8 | 152812782 | ATTGAAAGG |
| **MO17** | Mu1-9 | chr8 | 152814820 | ATTGAAAAG |
| **MO17** | Mu1-9 | chr8 | 153725735 | GTTAGTTGT |
| **MO17** | Mu1-9 | chr8 | 153727357 | GTTAGTTGT |
| **MO17** | Mu1-9 | chr8 | 169907702 | CTCACACAC |
| **MO17** | Mu1-9 | chr8 | 169907706 | CTCACACAC |
| **MO17** | Mu1-9 | chr9 | 12106581 | TCTCCCGAG |
| **MO17** | Mu1-9 | chr9 | 26352347 | CTTGTGAAG |
| **MO17** | Mu1-9 | chr9 | 26352355 | CTTGTGAAG |
| **MO17** | Mu1-9 | chr9 | 106850972 | CACCGGGAG |
| **MO17** | Mu1-9 | chr9 | 106850980 | CACCGGGAG |
| **MO17** | Mu1-9 | chr9 | 129919432 | GGCGTGCGT |
| **MO17** | Mu1-9 | chr9 | 145681991 | GAAAGAGAG |
| **MO17** | Mu1-9 | chrUNKNOWN | 8109034 | CACCAGAAA |
| **MO17** | Mu1-9 | chrUNKNOWN | 13826404 | TGCAGTACA |
| **MO17** | Mu1-9 | chrUNKNOWN | 13829283 | TGCAGTACA |
| **MO17** | Mu10 | chr1 | 17669069 | GTTTGTCGC |

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| **MO17** | Mu10 | chr1 | 17669186 | GTTTGCCGC |
| **MO17** | Mu10 | chr1 | 127026395 | GCCCCAGAT |
| **MO17** | Mu10 | chr2 | 76218121 | TCGAGAGGG |
| **MO17** | Mu10 | chr2 | 76222892 | TCGAGAGGG |
| **MO17** | Mu10 | chr2 | 79137485 | GGTGGCAAC |
| **MO17** | Mu10 | chr2 | 187496817 | CTCAGCGCC |
| **MO17** | Mu10 | chr2 | 187496825 | CTCAGCGCC |
| **MO17** | Mu10 | chr2 | 220443225 | CCAGCAGCC |
| **MO17** | Mu10 | chr2 | 220448990 | CCTAGCAGC |
| **MO17** | Mu10 | chr3 | 32827320 | CCCGCTGCC |
| **MO17** | Mu10 | chr3 | 32832334 | CTCGCTGCC |
| **MO17** | Mu10 | chr4 | 40176738 | TCGAGCGCG |
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| **MO17** | Mu10 | chr8 | 162447325 | CAAATAAAC |
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| **MO17** | Mu10 | chr9 | 83025615 | CGCGCTCGA |
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| **MO17** | Mu12 | chr1 | 114596663 | CCATTTCCA |
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| **MO17** | Mu12 | chr10 | 45432395 | CATCATATT |

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| **MO17** | Mu12 | chr10 | 49804591 | TGACATCTC |
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| **MO17** | Mu12 | chr2 | 1208535 | CTTTGGATA |
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| **MO17** | Mu12 | chr2 | 6579102 | ACTGACGGG |
| **MO17** | Mu12 | chr2 | 24982189 | GACCTCAAA |
| **MO17** | Mu12 | chr2 | 25000049 | TTTGAGGTC |
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| **MO17** | Mu12 | chr2 | 37869180 | GGGCACGAG |
| **MO17** | Mu12 | chr2 | 90728755 | GGGTTCTGG |
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| **MO17** | Mu12 | chr2 | 207958804 | GTACAGAAC |
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| **MO17** | Mu12 | chr3 | 3839648 | GCTTGCCAA |
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| **MO17** | Mu12 | chr3 | 121611818 | GGCGTAACT |
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| **MO17** | Mu12 | chr3 | 156342516 | GTCCCCAGC |
| **MO17** | Mu12 | chr3 | 165906824 | TTGACAGGT |
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| **MO17** | Mu12 | chr4 | 68421868 | CCCACCGCC |

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| **MO17** | Mu12 | chr6 | 152474926 | GATTCAAAT |

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| **MO17** | Mu12 | chr6 | 157068221 | CCTCAAGAA |
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| **MO17** | Mu12 | chr7 | 29692026 | AGTTACGCC |
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| **MO17** | Mu12 | chr7 | 50179197 | GCCCGCGAG |
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| **MO17** | Mu12 | chr7 | 144119874 | GTGGGGGCC |
| **MO17** | Mu12 | chr7 | 161229569 | ATCTGTCAG |
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| **MO17** | Mu12 | chr8 | 141273174 | ATAGATTGG |
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| **MO17** | Mu12 | chrUNKNOWN | 10938872 | ATACATGCC |
| **MO17** | Mu12 | chrUNKNOWN | 12783520 | CAGTGGCCG |
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| **W22** | Mu1-9 | chr1 | 19746443 | TTTGAAATG |
| **W22** | Mu1-9 | chr1 | 101026009 | CTGTGCGAG |
| **W22** | Mu1-9 | chr1 | 101060278 | CTCGCACAG |
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| **W22** | Mu1-9 | chr1 | 264263006 | TTAGGTCGG |
| **W22** | Mu1-9 | chr1 | 264267812 | TTAGGTCGG |
| **W22** | Mu1-9 | chr10 | 10267286 | GAAAGAGAG |
| **W22** | Mu1-9 | chr10 | 129970865 | CTGGTAATA |
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| **W22** | Mu1-9 | chr10 | 132671325 | CCCCTACGG |
| **W22** | Mu1-9 | chr10 | 132671330 | CCCCTACGG |
| **W22** | Mu1-9 | chr10 | 146912555 | CTCGATTTG |
| **W22** | Mu1-9 | chr2 | 1696519 | GTCTCTGTG |
| **W22** | Mu1-9 | chr2 | 1696527 | GTCTCTGTG |
| **W22** | Mu1-9 | chr2 | 25300688 | GAGGCTCTC |
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| **W22** | Mu1-9 | chr2 | 71709108 | CTCTCTGAT |
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| **W22** | Mu1-9 | chr2 | 135007660 | GGCTGGCGG |
| **W22** | Mu1-9 | chr2 | 142272069 | CATCCAAAC |
| **W22** | Mu1-9 | chr2 | 158559629 | CTCTCTTTC |
| **W22** | Mu1-9 | chr2 | 194078115 | GCCGGGGGC |
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| **W22** | Mu1-9 | chr2 | 214951873 | TTAGGTCGG |
| **W22** | Mu1-9 | chr3 | 170233622 | GTCGCCAGC |
| **W22** | Mu1-9 | chr3 | 170235173 | GTCGCCAGC |
| **W22** | Mu1-9 | chr3 | 230143403 | CCCCTGCAG |
| **W22** | Mu1-9 | chr4 | 159505930 | GCCGTGCGA |
| **W22** | Mu1-9 | chr4 | 209048525 | GCTGGCGTC |
| **W22** | Mu1-9 | chr4 | 209048533 | GCTGGCGTC |
| **W22** | Mu1-9 | chr5 | 13208513 | GTCGCCAGC |
| **W22** | Mu1-9 | chr5 | 13215095 | GTCGCCAGC |
| **W22** | Mu1-9 | chr5 | 116267118 | TCACCCAAG |
| **W22** | Mu1-9 | chr5 | 116268617 | TCACCCGAG |
| **W22** | Mu1-9 | chr5 | 175353387 | TTTGACGGT |
| **W22** | Mu1-9 | chr5 | 175353388 | TTGACGGTG |
| **W22** | Mu1-9 | chr5 | 201460195 | GTCGGTCTA |
| **W22** | Mu1-9 | chr5 | 201460203 | GTCGGTCTA |
| **W22** | Mu1-9 | chr6 | 107992299 | CACAAAAAA |
| **W22** | Mu1-9 | chr6 | 112507608 | GTTCAGAAA |

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| **W22** | Mu1-9 | chr6 | 112507616 | GTTCAGAAA |
| **W22** | Mu1-9 | chr8 | 138633675 | GTTGTATTC |
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| **W22** | Mu1-9 | chr9 | 8145105 | CAAGACGTG |
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| **W22** | Mu1-9 | chr9 | 86968542 | ATCAAATCT |
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| **W22** | Mu1-9 | chr9 | 140075456 | TTCTCAGCC |
| **W22** | Mu1-9 | chr9 | 140075465 | TTCTCAGCC |
| **W22** | Mu1-9 | chrUNKNOWN | 13826404 | TGCAGTACA |
| **W22** | Mu1-9 | chrUNKNOWN | 13829283 | TGCAGTACA |
| **W22** | Mu10 | chr2 | 76222892 | TCGAGAGGG |
| **W22** | Mu10 | chr2 | 199398880 | GTTGTGTGG |
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| **W22** | Mu10 | chr4 | 40201165 | TCGAGCGCG |
| **W22** | Mu10 | chr4 | 40201169 | TCGGGCGCG |
| **W22** | Mu10 | chr4 | 133716929 | TCCCCTGCG |
| **W22** | Mu10 | chr4 | 133716937 | TCCCCTGCG |
| **W22** | Mu10 | chr4 | 170887654 | GCGGGCGGA |
| **W22** | Mu10 | chr4 | 204351539 | ATGATGTTG |
| **W22** | Mu10 | chr5 | 73323804 | GTCAAAATC |
| **W22** | Mu10 | chr5 | 73327895 | GTCAAAATC |
| **W22** | Mu10 | chr8 | 138633677 | GCTGTATTC |
| **W22** | Mu10 | chr9 | 10256387 | GATGGGGAG |
| **W22** | Mu12 | chr1 | 5855090 | CCCGTCAGT |
| **W22** | Mu12 | chr1 | 9045993 | CTAGATTTG |
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| **W22** | Mu12 | chr10 | 106665024 | CCCCATGGC |

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| **W22** | Mu12 | chr10 | 134125091 | CGCCACCGG |
| **W22** | Mu12 | chr10 | 138397252 | TCCCAGGAT |
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| **W22** | Mu12 | chr2 | 1207318 | CTTTGGATA |
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| **W22** | Mu12 | chr2 | 221528287 | GGTATACAA |
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| **W22** | Mu12 | chr3 | 3839648 | GCTTGCCGA |
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| **W22** | Mu12 | chr3 | 9282491 | ATTTCCCGT |
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| **W22** | Mu12 | chr3 | 54825892 | GGACTTGCG |
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| **W22** | Mu12 | chr3 | 192256105 | CGCCGCGAC |
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| **W22** | Mu12 | chr3 | 216632366 | TACTGCAGT |
| **W22** | Mu12 | chr3 | 217655664 | CCGTTTTTT |
| **W22** | Mu12 | chr3 | 220339229 | CTCGTGGAG |
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| **W22** | Mu12 | chr4 | 96634756 | GGGCTCAAT |
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| **W22** | Mu12 | chr4 | 152475445 | TTCTGCGTT |
| **W22** | Mu12 | chr4 | 166495091 | GTACATATG |
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| **W22** | Mu12 | chr4 | 199244475 | GGTTCGGAC |
| **W22** | Mu12 | chr4 | 210943904 | GAGAGAGAT |

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| **W22** | Mu12 | chr4 | 210945539 | GAGATGTCA |
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| **W22** | Mu12 | chr7 | 159353900 | AAAAAATGG |
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| **W22** | Mu12 | chr7 | 169729474 | CCATTTCCA |
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| **W22** | Mu12 | chr8 | 5414433 | CTCGCGCGC |
| **W22** | Mu12 | chr8 | 8045837 | CCGTTTTTT |
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| **W22** | Mu12 | chr8 | 100614801 | CATTGTAGG |
| **W22** | Mu12 | chr8 | 116461829 | GCTTGCCGA |
| **W22** | Mu12 | chr8 | 141273174 | GTAGATTGG |
| **W22** | Mu12 | chr8 | 141274171 | CTGATTGGG |

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| **W22** | Mu12 | chr8 | 142983538 | CCCGTCCCC |
| **W22** | Mu12 | chr8 | 142983546 | CCCGTCCCC |
| **W22** | Mu12 | chr8 | 174147997 | GCAAGCTTG |
| **W22** | Mu12 | chr9 | 14402800 | CCCGTGTTG |
| **W22** | Mu12 | chr9 | 14402808 | CCCGTGTTG |
| **W22** | Mu12 | chr9 | 25697019 | CTAATTTTG |
| **W22** | Mu12 | chr9 | 37587573 | CGTAGTGCA |
| **W22** | Mu12 | chr9 | 49227827 | CCATTTTTT |
| **W22** | Mu12 | chr9 | 89417958 | CCGTTTTTT |
| **W22** | Mu12 | chr9 | 107612912 | AAAAAAACG |
| **W22** | Mu12 | chr9 | 130763403 | GCAAACCAA |
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| **TIL-1** | Mu1-9 | chr1 | 273709444 | ATCTCTGAG |
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| **TIL-1** | Mu1-9 | chr1 | 293153277 | TTGGATCTG |
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| **TIL-1** | Mu10 | chr4 | 158906485 | AGAAGAGGG |
| **TIL-1** | Mu10 | chr4 | 158906493 | AGAAGAGGG |
| **TIL-1** | Mu10 | chr5 | 14699797 | CTCCCCAGC |
| **TIL-1** | Mu10 | chr5 | 14699805 | CTCCCCAGC |
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| **TIL-1** | Mu12 | chr2 | 5681848 | CTCAAAGCC |

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| **TIL-1** | Mu12 | chr2 | 9947891 | CTTGGACGA |
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| **TIL-1** | Mu12 | chr2 | 25001836 | CAAAGGGTC |
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| **TIL-1** | Mu12 | chr2 | 92129907 | TCTGCGCGG |
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| **TIL-1** | Mu12 | chr2 | 197215139 | CGCGGTGGC |
| **TIL-1** | Mu12 | chr2 | 229531941 | TAGAGGGGG |
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| **TIL-1** | Mu12 | chr3 | 3839648 | GCTTGCCGA |
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| **TIL-1** | Mu12 | chr3 | 227274385 | AAAAAATGG |
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| **TIL-14** | Mu1-9 | chr6 | 159357866 | CCGGCCCAC |
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| **TIL-14** | Mu1-9 | chr8 | 4819545 | CCGGCCCAC |
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| **TIL-14** | Mu12 | chr1 | 10975143 | CTCTCCTCG |
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| **TIL-14** | Mu12 | chr5 | 26466383 | AGCCTAGGA |
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| **TIL-15** | Mu10 | chr1 | 24057152 | ACCAGACAC |
| **TIL-15** | Mu10 | chr1 | 103305287 | GCGGGGCGG |
| **TIL-15** | Mu10 | chr2 | 21965511 | GTTCGCTTT |
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| **TIL-15** | Mu12 | chr3 | 137939804 | CACTATGCC |
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| **TIL-17** | Mu1-9 | chr2 | 170371447 | TCATTTCGG |
| **TIL-17** | Mu1-9 | chr2 | 222626068 | AACTGCATA |
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| **TIL-17** | Mu1-9 | chrUNKNOWN | 1099618 | AGACGGGAA |
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| **TIL-17** | Mu10 | chr10 | 24764948 | CTTTTTTCT |
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| **TIL-17** | Mu12 | chr1 | 10975143 | CTCTCCTCG |
| **TIL-17** | Mu12 | chr1 | 37644610 | ACCAAATGG |

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| **TIL-17** | Mu12 | chr4 | 67680404 | GTTTAGACT |
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| **TIL-17** | Mu12 | chr5 | 191610797 | AATGCAAGG |
| **TIL-17** | Mu12 | chr5 | 207526903 | CTATGCGGA |
| **TIL-17** | Mu12 | chr5 | 214442521 | GCTCGCGGG |
| **TIL-17** | Mu12 | chr5 | 214578190 | CTCCGCCGG |
| **TIL-17** | Mu12 | chr6 | 2575151 | GTTTTCCAA |
| **TIL-17** | Mu12 | chr6 | 2917476 | TCCATGATG |
| **TIL-17** | Mu12 | chr6 | 13851597 | GGCTACGGG |
| **TIL-17** | Mu12 | chr6 | 13851605 | GGATACGGG |
| **TIL-17** | Mu12 | chr6 | 38435508 | CCCGAGGCC |
| **TIL-17** | Mu12 | chr6 | 53962988 | ATGAAGACC |
| **TIL-17** | Mu12 | chr6 | 55872875 | ATCCCCGCC |

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| **TIL-17** | Mu12 | chr6 | 78114239 | CCCGTAGCC |
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| **TIL-17** | Mu12 | chr6 | 99963634 | GTGGATCTG |
| **TIL-17** | Mu12 | chr6 | 100137700 | CAGCTCAAT |
| **TIL-17** | Mu12 | chr6 | 100137708 | CAGCTCAAT |
| **TIL-17** | Mu12 | chr6 | 106449118 | CCCGTAGCC |
| **TIL-17** | Mu12 | chr6 | 107182865 | CCCGTAGCC |
| **TIL-17** | Mu12 | chr6 | 109240325 | GTAATGTGT |
| **TIL-17** | Mu12 | chr6 | 109240327 | ATCTAATGT |
| **TIL-17** | Mu12 | chr6 | 110826082 | GGCTACGGG |
| **TIL-17** | Mu12 | chr6 | 143020500 | GGAAGGGAT |
| **TIL-17** | Mu12 | chr6 | 149531955 | GAGTAGCAG |
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| **TIL-17** | Mu12 | chr6 | 158541306 | ATCTGCTCA |
| **TIL-17** | Mu12 | chr6 | 166433210 | CTTGCCATC |
| **TIL-17** | Mu12 | chr6 | 169129268 | CCCCCACCC |
| **TIL-17** | Mu12 | chr6 | 169241025 | CCCCCACCC |
| **TIL-17** | Mu12 | chr7 | 15575142 | TGAACAGGA |
| **TIL-17** | Mu12 | chr7 | 15575481 | TGAACAGGA |
| **TIL-17** | Mu12 | chr7 | 29508617 | CTCGGCATG |
| **TIL-17** | Mu12 | chr7 | 29913742 | GTCAAAGGT |
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| **TIL-17** | Mu12 | chr7 | 151005031 | ACGGCAAGA |
| **TIL-17** | Mu12 | chr7 | 161229569 | ATCTGTCAG |
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| **TIL-17** | Mu12 | chr7 | 169668703 | ACTAAAAGA |
| **TIL-17** | Mu12 | chr7 | 169729474 | CCATTTCCA |
| **TIL-17** | Mu12 | chr8 | 5414433 | CTCGCGCGC |
| **TIL-17** | Mu12 | chr8 | 8995040 | TCCATTTCA |
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| **TIL-17** | Mu12 | chr8 | 44535993 | CCCGTAGCC |

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| **TIL-17** | Mu12 | chr8 | 120021601 | CGAGACGGC |
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| **TIL-17** | Mu12 | chr9 | 22666174 | TCTCTCTCT |
| **TIL-17** | Mu12 | chr9 | 30376224 | GACGCCACG |
| **TIL-17** | Mu12 | chr9 | 37204160 | GGCTACGGG |
| **TIL-17** | Mu12 | chr9 | 37587573 | CGTAGTGCA |
| **TIL-17** | Mu12 | chr9 | 40524602 | CATCCCTGC |
| **TIL-17** | Mu12 | chr9 | 52945708 | CTCGGCACG |
| **TIL-17** | Mu12 | chr9 | 62651198 | ACTAAAAGA |
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| **TIL-17** | Mu12 | chr9 | 74188523 | CCGTAGCCT |
| **TIL-17** | Mu12 | chr9 | 78735749 | CTCGGCATG |
| **TIL-17** | Mu12 | chr9 | 78735757 | CTCGGCATG |
| **TIL-17** | Mu12 | chr9 | 80678772 | TCGGGGAGA |
| **TIL-17** | Mu12 | chr9 | 85510106 | CCCGTAGCC |
| **TIL-17** | Mu12 | chr9 | 95994917 | CCCGTAGCC |
| **TIL-17** | Mu12 | chr9 | 98334525 | GGCTACGGG |
| **TIL-17** | Mu12 | chr9 | 115623561 | GGCTACGGG |
| **TIL-17** | Mu12 | chr9 | 120688928 | CCCGTAGCC |
| **TIL-17** | Mu12 | chr9 | 130377493 | CTCTCTCTT |
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| **TIL-17** | Mu12 | chr9 | 140112790 | GGCTACGGG |
| **TIL-17** | Mu12 | chr9 | 146113969 | TCCTGTTCA |
| **TIL-17** | Mu12 | chr9 | 147125385 | TCTCTCTCT |
| **TIL-17** | Mu12 | chr9 | 147125393 | TCTCTCTCT |
| **TIL-17** | Mu12 | chrUNKNOWN | 756223 | GGCTATGGG |
| **TIL-17** | Mu12 | chrUNKNOWN | 833180 | GGCTACGGG |
| **TIL-17** | Mu12 | chrUNKNOWN | 1239802 | GGCTACGGG |
| **TIL-17** | Mu12 | chrUNKNOWN | 3164384 | GGCTACGGG |
| **TIL-17** | Mu12 | chrUNKNOWN | 6262680 | CTCGTAGCC |
| **TIL-17** | Mu12 | chrUNKNOWN | 6656498 | ATTGAGCTC |
| **TIL-17** | Mu12 | chrUNKNOWN | 8287600 | GGCTACGGG |
| **TIL-17** | Mu12 | chrUNKNOWN | 12184565 | CCCGTAGCC |
| **TIL-17** | Mu12 | chrUNKNOWN | 12184573 | CCCGTAGCC |
| **TIL-17** | Mu12 | chrUNKNOWN | 12783520 | CAGTGGCCG |

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# BIOGRAPHICAL SKETCH

Charles (Chip) Hunter grew up in Panama City, Florida before moving to Gainesville to attend the University of Florida, as both an undergraduate and graduate student. He majored in microbiology and cell science, and minored in plant molecular and cellular biology in the College of Agriculture and Life Sciences. He graduated Cum Laude, and joined the Plant Molecular and Cellular Biology program as a doctoral student in 2004. He was awarded the prestigious American Society of Plant Biology-Pioneer Hi-Bred International Graduate Student Prize in 2008 for conducting the most agriculturally-relevant graduate research in the United States. He has conducted research under the guidance of Dr. Karen Koch in the Department of Horticultural Sciences, where his experiments have focused on establishing roles for cell wall biosynthetic enzymes in maize, identifying related maize mutants, and in testing contributions by Mu transposable elements to diversity in maize and its wild ancestor teosinte.