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Protein kinases are an abundant class of enzymes which play important roles in numerous signal transduction systems. *Arabidopsis* TOUSLED kinase is a serine/threonine kinase which is essential for cell-cell communication within the shoot meristem. TOUSLED is encoded by a single gene in *Arabidopsis*. Recessive mutants in this gene show mild vegetative defects and severe floral abnormalities including a random reduction in the number of floral organs produced and defects in the formation of the gynoecium.

This thesis describes the cloning and characterization of three maize genes with homology to *TOUSLED*. These genes are known as the *TOUSLED-LIKE KINASE (TLK)* genes. Partial genomic and cDNA clones of the maize *TLK* genes have been sequenced and analyzed. These genes show remarkable similarity to each other and to *TOUSLED* over the region corresponding to the *TOUSLED* catalytic domain. The *TLK* genes fall into two distinct classes on the basis of nucleotide and amino acid sequence. Both classes appear to be expressed throughout the plant. In addition, database searches reveal that *TOUSLED*-like genes are present in a diverse array of other eukaryotes, indicating that the *TLK* genes are members of a widespread, evolutionarily conserved class.

Two approaches have been taken to find mutants in the *TLK* genes. This thesis describes the *tassel-less1 (tls1)* mutant, a possible mutant in one of the *TLK* genes. The *tls1* mutation maps to the same chromosomal location as one of the *TLK* genes, and may represent a lesion in this *TLK* gene. Characterization of the *tls1* mutant reveals that disruption of the *TLS1* gene results in variable, progressive vegetative defects and severe reduction of the reproductive structures. The *tls1* phenotype is consistent with the hypothesis that *TLS1* plays a role in regulating meristem activity. In addition, *TLK* sequences have been used in a reverse genetics screen to isolate families which contain *Mutator* transposable element insertions into the *TLK* genes. These families are currently being analyzed for phenotype and allelism to *tls1*.

Analysis of the Maize *TOUSLED-LIKE KINASE* Gene Family

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Elizabeth S. Yoon, Author

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1. Protein Kinases and Signal Transduction in Plants

In order for cells to grow, divide or differentiate appropriately, there must be some means of coordinating biochemical activity in response to internal and external conditions. One means of coordinating cellular activity is through the regulation of protein activity by reversible phosphorylation. Protein kinases are the class of enzymes which catalyze the addition of phosphate groups to other proteins, thereby activating or inactivating their function (Hunter, 1995; Ranjeva and Boudet, 1987). Protein kinases can act on and regulate the activity of metabolic enzymes. This provides a direct means of controlling cellular activity. Kinases can also phosphorylate transcription factors, resulting in changes in gene expression (Hill et al., 1993). Finally, kinases can phosphorylate other kinases, creating a signalling relay or cascade. This can be extremely effective in amplifying a weak signal as well as in relaying information from one part of the cell to another (Hunter, 1995).

Many protein kinases are expressed in eukaryotes. Roughly 1-3% of eukaryotic genes are likely to encode protein kinases (Stone and Walker, 1995). Most eukaryotic protein kinases fall into two general classes on the basis of their catalytic activity: serine/threonine kinases and tyrosine kinases. In addition, a few histidine kinases have been isolated from eukaryotes, although this class is more commonly found among prokaryotes (Chang et al., 1993; Stone and Walker, 1995). Protein kinases can be recognized and tentatively classified by examining conserved regions in their catalytic domains (Hanks and Quinn, 1991; Stone and Walker, 1995), although kinase activity needs to be confirmed biochemically.

Protein kinases play key regulatory roles in processes such as cell division and cell-cell communication. In eukaryotes, a number of conserved protein kinases have been identified which regulate the cell cycle. For example, entry into mitosis is regulated

by the activation of cyclin dependent kinases (CDKs) in complexes with specific cyclin proteins. This M-phase kinase complex is then thought to activate other proteins directly involved in mitotic events such as chromatin condensation and breakdown of the nuclear envelope (Nurse, 1990). Not only is the onset of mitosis controlled by a protein kinase, activity of the CDK itself is regulated through phosphorylation by other kinases (Lundgren et al., 1991; Nurse, 1990).

Other pathways are essential for relaying information from the cell surface to other parts of the cell. The MAP kinase pathways are well-characterized signal transduction cascades that appear to be conserved among animals and yeast. One of the best characterized MAP kinases pathways is the pheromone response pathway in yeast (reviewed in Herskowitz, 1995). Peptide mating factors are perceived by G-protein-coupled seven-transmembrane receptors at the cell surface. The signal is then relayed through activation of the G-protein and the activation of a module of three kinases, each activating the next signal transduction component. The output of the cascade is the phosphorylation of the FAR1 protein and transcription factor STE12 (Neiman, 1993). Interestingly, this pathway ultimately allows regulation of the cell cycle discussed above; FAR1 interacts with the cyclin/CDK complex, inactivating it and arresting the cell cycle in preparation for mating (Tyers and Futcher, 1993).

Both the cell-cycle components and the three component MAP kinase module are highly conserved throughout eukaryotes. Similar MAP kinase modules participate in a wide range of response pathways, although the triggering mechanism can vary. In animals, for example, the cascade is often triggered by the binding of ligands to specific receptor tyrosine kinases at the cell surface (Hill and Treisman, 1995; Hunter, 1995). Other types of receptors have been identified in yeast and plants, which do not appear to have receptor tyrosine kinases.

Signal Transduction in Plants

Plants provide an interesting context in which to examine signal transduction. Because of their sessile nature, plants must be able to perceive and respond to environmental changes exclusively on a developmental or biochemical level, rather than through behavioral responses. In general, higher plants cannot flee their environment or alter it to make it more favorable. On the other hand, plants are able to modify their developmental program according to environmental cues. For example, the physical pressure that a germinating seedling might encounter when blocked by soil particles has been shown to induce the production of the plant hormone ethylene (reviewed in Ecker, 1995). In response to ethylene, seedlings show increased thickening of the roots and hypocotyl, and an exaggeration of the apical hook, which may facilitate growth of seedlings through soil while minimizing damage to the shoot apex. Plants also respond to environmental cues for normal developmental processes. In *Arabidopsis* seedlings, for example, light stimulates morphological programs including the differentiation of chloroplasts in the shoot, expansion of cotyledons, and production of new leaves (Deng, 1994).

Signal transduction is also likely to be critical in the process of laying down the plant body. Because plant cells have rigid cell walls, all morphogenetic processes must take place by cell division, differentiation or death. New organs are initiated by meristems, pools of undifferentiated stem cells, which proliferate to give rise to the reiterated structures of the plant body. Clonal analysis has shown that cell lineage is not the primary determinant of cell fate in plants, as it is in animals (Poethig et al., 1990). Rather, cells rely on positional information for developmental cues, suggesting that cell-cell communication plays a large role in determining cell fate in plants. In addition, the continual process of organ initiation makes plants an ideal system in which to study the signal transduction systems which govern plant development. Plants grow and develop new structures throughout their lives. Therefore, cell division and differentiation, as well

as the regulatory mechanisms which control these processes, are ongoing and can be studied throughout the life cycle of a plant.

Protein Kinases in Plants

Complete signalling pathways have not yet been worked out in plants, but a great deal of evidence indicates that protein kinases play critical signal transduction roles in plants as well as in animals and yeast. Numerous genes encoding putative protein kinases have been isolated from plants, including some which have been been demonstrated to play important signalling roles (Stone and Walker, 1995). Three protein kinases from *Arabidopsis* have been implicated in mediating the response to the plant hormone ethylene (Ecker, 1995; Hua et al., 1996). ETR1 and ERS are putative histidine kinases which act early in ethylene perception (Chang et al., 1993; Hua et al., 1996). Dominant mutants in both the *ETR1* and *ERS* genes are defective in ethylene binding and have an ethylene insensitive phenotype (Bleecker et al., 1988). ETR1 and ERS may act as an ethylene receptors or components of an ethylene sensing complex and start the signalling cascade which ultimately results in changes in gene expression. Recessive mutants in the *CTR1* gene display the opposite phenotype; plants show a constitutive ethylene response when grown in the absence of ethylene (Kieber et al., 1993). *CTR1* encodes a putative serine/threonine kinase (Kieber et al., 1993). Epistasis studies suggest that CTR1 acts downstream of ETR1 and ERS to negatively regulate ethylene signal transduction (Ecker, 1995; Hua et al., 1996; Kieber et al., 1993). CTR1 shows homology to the mammalian Raf kinases which operate in MAP kinase pathways; CTR1 may play a similar role as an intermediate step in signal transduction (Kieber et al., 1993).

Although receptor tyrosine kinases have not been found in plants, plant receptor-like kinases are likely to play a similar role in cell-cell communication and the integration of extracellular signals. Plant receptor-like kinases feature diverse extracellular receptor

domains, a transmembrane domain, and conserved intracellular serine/threonine catalytic domains (Stone and Walker, 1995; Walker, 1994). The receptor-like kinase genes which have been identified include genes from *Brassica napus* (Goring and Rothstein, 1992) and *B. oleracea* (Stein and Nasrallah, 1993) which are tightly linked to self-incompatibility loci. Some self-compatible *Brassica* lines have been shown to contain mutations which result in non-functional S-Locus Receptor Kinase genes, suggesting that these kinases are necessary for the self-incompatibility response (Goring et al., 1993; Nasrallah et al., 1994). The *CLAVATA1* gene of *Arabidopsis* also encodes a putative receptor kinase (S. Clark, personal communication). Mutants in this gene show progressive enlargement of the meristem throughout development, suggesting that the CLAVATA1 receptor kinase is necessary for restricting meristem size (Clark et al., 1993).

Calcium fluxes in plant cells occur in response to a variety of stimuli and many protein kinases have been identified which are directly or indirectly activated by calcium (Stone and Walker, 1995). Unlike animals and fungi, plants contain a large class of kinases which can be activated directly by calcium. These calcium-dependent protein kinases feature a calmodulin-like domain fused to a serine/threonine catalytic domain (Hrabak et al., 1996). Although the specific actions of these kinases are not known, some have been shown to be functional kinases which are activated by calcium (Hrabak et al., 1996).

Many other putative protein kinases have been identified in plants. Several genes with homology to yeast cell-cycle components have been cloned. One *Arabidopsis* CDK homologue has been shown to play a role in cell division. A dominant negative mutant phenotype which suppresses cell division results when a mutated version of this CDK gene is over-expressed in tobacco (Hemerly et al., 1995). In addition, kinases with similarity to those regulated by second messengers in other systems have been identified in plants (Stone and Walker, 1995). Although the exact function of these kinases is not

yet known, it is clear that protein kinases are common and play important roles in signal transduction in plants.

Genetic Redundancy in Protein Kinase Families

Many protein kinases are members of families of closely related genes, often with overlapping function. Functional redundancy of genes is a common phenomenon. Thomas (1993) classified various scenarios under which genetic redundancy could be selectively maintained. Genes with identical function could serve simply to increase gene dosage, allowing greater production of an important gene product. Alternatively, having redundant genes for extremely important processes such as cell-cycle regulation ensures that the loss or mutation of one gene will not render an organism inviable. Finally, redundant genes may evolve new functions, either distinct from a shared function or in combination with each other, providing a new function.

There are many examples of genetic redundancy among protein kinases. For example, in the yeast pheromone response pathway, the MAP kinases FUS3 and KSS1 both can phosphorylate the transcription factor STE12, activating gene expression. Single mutants in either FUS3 or KSS1 show no defect in this pathway, but double mutants are unable to respond to pheromones. However, while these two kinases are completely redundant in this pathway, KSS1 cannot compensate for the loss of FUS3 in other pathways (reviewed in Herskowitz, 1995). FUS3 and KSS1 have overlapping but non-identical functions.

Redundant kinase families have also been documented in plants. The *Arabidopsis* ethylene response genes *ETR1* and *ERS* give translation products with 75% homology in the amino terminal domains (Hua et al., 1995). Dominant mutants in both genes display identical ethylene insensitive phenotypes, suggesting a similar role for the two kinases. No recessive mutants have been isolated in either gene, which may be due to functional redundancy. If one gene can compensate for the loss of the other, both

genes would need to be knocked out in order to completely disrupt ethylene perception (Hua et al., 1995).

Another family of closely related kinases is the *PTO* family in tomato. *PTO* encodes a serine/threonine kinase which confers resistance to the pathogen *Pseudomonas syringae* pv. *tomato*. Southern analysis indicates that there are at least six closely related genes in tomato (Martin et al., 1993). One of these related genes, *FEN*, has been cloned and shown to encode a serine/threonine kinase which is 80% identical to PTO at the amino acid level. However, the phenotype conferred by the *FEN* gene is quite different; *FEN* mediates sensitivity to the insecticide fenthion, but does not confer resistance to *Pseudomonas* (Martin et al., 1994). Although these two kinases are very closely related, they have different functions. Additional players in the pathway have also been identified. *PTI1* encodes a different serine/threonine kinase which interacts specifically with PTO but not FEN (Zhou et al., 1995). *PRF* has been shown to act upstream of both PTO and FEN (Salmeron et al., 1994). Clearly, PTO and FEN are components of closely related, but not completely overlapping signalling systems.

TOUSLED Kinase

TOUSLED kinase (TSL) is a putative serine/threonine kinase which is essential for many aspects of *Arabidopsis* development (Roe et al., 1993). *TSL* is a single gene in *Arabidopsis*. It contains 16 exons and encodes a 2.7 kb transcript. *TSL* is expressed throughout the plant, but is expressed most highly in developing floral buds. In addition to a C-terminal catalytic domain, the N-terminal portion of TSL has a glutamine rich region, three nuclear localization signals and two predicted coiled-coil regions (Roe et al., 1993). *TSL* has been expressed in yeast, and *in vitro* studies show that it encodes a functional serine/threonine kinase which can both autophosphorylate and transphosphorylate exogenous substrates (Roe et al., 1997a). TSL fusion proteins localize to the nucleus when expressed in tobacco protoplasts, indicating that the nuclear

localization signals are functional (Roe et al., 1997a). Studies of deletion mutants indicate that the N terminal coiled-coil region is essential for catalytic activity. Coiled-coils such as those found in TSL, are α-helical regions with a hydrophobic face. They are thought to be important in protein-protein interactions (Cohen and Parry, 1986). In TSL, oligomerization, perhaps through interactions of these coiled-coil regions, is necessary for catalytic activity (Roe et al., 1997a). Oligomerization with different protein partners may play a role in the regulation of TSL activity.

TOUSLED plays a critical role in *Arabidopsis* morphogenesis. Recessive mutants in the *TSL* gene display a variety of abnormal traits (Roe et al., 1993). Leaf shape is slightly altered and cauline leaves curl up around the main stem, rather than lying perpendicular to it. Flowers in *tsl* mutants consistently lack the normal complement of floral organs, although the particular organs which are missing vary from flower to flower. In addition, the gynoecium fails to fuse completely, and lacks a complete style and stigma. Microscopy of *tsl* mutants reveals that the floral meristem fails to initiate organs in the proper position or number. In the gynoecia, cell division appears to be poorly coordinated, resulting in uneven growth and failure of the carpels to fuse and form a closed style and stigma (Roe et al., 1997b). The phenotype of *tsl* mutants is consistent with the expression pattern and the possible role of TSL in cell-cell communication and signal transduction in the meristem. TSL clearly plays an important role in coordinating proper development in *Arabidopsis*.

The *TOUSLED-LIKE KINASE* Gene Family of Maize

The catalytic domain of TOUSLED is quite conserved and *TOUSLED*-like homologues have recently been found in a diverse array of organisms including maize, *Caenorhabditis* species, mice, and humans (Roe et al., 1997a). Mice and humans have two *TOUSLED*-like genes each. The mammalian genes fall into two distinct classes, with one gene of each class found each species. In maize, a small family of genes shares

homology to the TOUSLED catalytic domain. A partial cDNA of one maize *TOUSLED-LIKE KINASE,* or *TLK* gene was isolated and sequenced by Helentjaris et al. (1995). When used to probe genomic Southern blots, multiple bands are detected, indicating that this *TLK* gene is a member of a small family of related genes, rather than a single gene as in *Arabidopsis.* Helentjaris et al. (1995) mapped three *TLK* loci to chromosomes 1L, 4L , and 5S.

It is not surprising that the *TLK* genes form a small gene family in maize, while *TOUSLED* is a single gene in *Arabidopsis.* Mapping studies in maize have shown that there are large chromosomal blocks which are duplicated elsewhere in the genome (Helentjaris et al., 1988). The *TLK* loci on chromosomes 1L and 5S are on one such duplicated block, suggesting that they may have been duplicated as part of a large scale genome duplication. Comparisons between maize and other closely related grasses show that these blocks are conserved linkage groups among the cereals, although they are only represented once in the genome of species such as rice (Ahn and Tanksley, 1993). Maize is currently a true diploid with a haploid chromosome number of 10. However, cytogenetic studies suggest an allotetraploid origin for maize, with a base chromosome number of 5 (Molina and Naranjo, 1987). The genome duplication could have occurred when two related diploid species hybridized to form the tetraploid maize progenitor. Some duplications could have been subsequently lost. Alternatively, duplications of large chromosomal regions could have occurred internally (Helentjaris et al., 1988).

Duplicated loci include many known structural genes, some of which display functional overlap (Gottleib, 1982; Rhoades, 1951). Two genes, *C2* and *Whp*, both encode the anthocyanin biosynthesis enzyme chalcone synthase (Franken et al., 1991). Although they display functional overlap in mediating pollen coloration and viability (Coe et al., 1981), the two genes are regulated differently at both the transcriptional and translational levels and do not complement each other in other parts of the plant (Franken et al., 1991). These two closely related genes have evolved independent specificity

despite their functional similarity. There is genetic redundancy in the regulation of the anthocyanin pathway as well. The genes *B* and *R* are roughly 85% identical at the nucleotide level (Chandler et al., 1989), and both encode putative transcriptional activators (Chandler et al., 1989; Ludwig et al., 1989). There are numerous alleles of each gene, and the degree of functional overlap between the genes varies according to alleles and tissues examined. These genes map to chromosomal locations believed to be duplicated regions of each other, suggesting that this is an ancient duplication of genes which now have overlapping but non-identical functions (Chandler et al., 1989).

Whether or not the *TLK* genes currently display functional overlap, this gene family provides a convenient system in which to examine the evolution of multiple gene families. The presence of three mapped loci can be explained by either the loss of one gene following duplication of an entire genome containing two *TLK* genes, or the independent duplication of one of two *TLK* loci present in an ancestral genome. It is possible that *TLK* gene function has diverged since duplication, either through independent regulation of the expression of each individual gene, or from specification in the action of the gene products. The latter may occur through modifications in the non-catalytic protein-protein interaction domain, allowing for specific regulation through interactions with different protein partners. Molecular analysis of the *TLK* genes will yield information about the structural and functional relationship between the various members of the family.

Mutants in the *TLK* Genes

Mutant analysis is extremely useful in elucidating the role of particular genes in a whole organism. The *tassel-less1* morphological mutant (Albertsen et al., 1993) maps to the same location as the chromosome 1L *TLK* gene, and may represent a lesion in this gene. The *tassel-less1* phenotype has only been briefly characterized (Albertsen et al., 1993), but may be due to a mutation in the same gene as the *barren-sterile* mutant

(Woodworth, 1926), which has been lost. Both mutants display a highly variable phenotype resulting in severe reduction of both the male and female inflorescences and reduced stature. The phenotype is similar to that of *tousled* mutants, with major floral defects and a milder vegetative phenotype.

Analysis of *tassel-less1,* a potential *TLK* mutant, and isolation of other *TLK* mutants will be useful in determining the role of the various *TLK* genes. It will be particularly interesting to examine mutant phenotypes in maize, which has very different plant architecture from *Arabidopsis* and which has multiple related *TOUSLED-LIKE KINASE* genes. In addition to allowing analysis of the phenotypes resulting from disruption of each individual *TLK* gene, the construction of double and triple mutants will yield information on the interaction of the *TLK* genes with each other. Isolation of mutants will also permit studies of the interactions between the *TLK* genes and other genes known to play a role in maize development. The combination of molecular information on the *TLK* genes and gene products with the characterization of mutants will provide powerful tools for understanding one of the signal transduction pathways which is likely to be critical in coordinating plant development.

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2. Maize Contains a Small Family of *TOUSLED-LIKE KINASE* Genes With High Homology to *Arabidopsis TOUSLED*

Abstract

The *TOUSLED* gene of *Arabidopsis* encodes a member of a novel class of serine/threonine protein kinases. *TOUSLED* plays a role in shoot development and is essential for the correct partitioning of the floral meristem into organs (Roe et al., 1993). In maize, a small family of genes show homology to the *TOUSLED* catalytic domain (Helentjaris et al., 1995). Partial genomic and cDNA clones representing three distinct maize *TOUSLED-LIKE KINASE (TLK)* genes show extremely high homology to each other and to the catalytic domain of *TOUSLED.* The *TLK* genes are highly conserved, but appear to fall into two classes, on the basis of both nucleotide and predicted amino acid sequences. Preliminary expression studies indicate that both classes of *TLK* genes are expressed in maize. Database searches reveal the presence of *TOUSLED*-like genes in a diverse array of other eukaryotes, indicating that the maize *TLK* genes are part of a widespread, evolutionarily conserved class of kinases.

Introduction

Growth of higher plants occurs as cells in the meristems, pools of undifferentiated stem cells, proliferate and give rise to the various organs which make up the plant body. Development is an ongoing process in plants, and is a primary means by which plants respond to their environment. New organ primordia must be initiated correctly in time and space, and then must perceive and respond to developmental cues in order to undergo appropriate patterns of growth and differentiation. The coordination of cell division and differentiation is a complex process involving cell-cell communication and regulated gene expression. It is possible to gain some insight into these aspects of plant development by examining some of the genes involved in the process.

Recently, analysis of mutant phenotypes has led to the identification of many genes with critical roles in plant development, including some which coordinate shoot apical meristem activity in angiosperms. Some of these genes have been cloned, yielding information on the possible specific roles of these genes. These include a number of probable transcription factors such as those encoded by genes related to the maize homeobox gene *KNOTTED1* (Hake et al., 1995), and the various MADS box genes which play important roles in floral development (Okada and Shimura, 1994; Weigel and Meyerowitz, 1994). Another major class of developmentally important genes include signal transduction elements. Phosphorylation cascades are one important means of signal transduction (reviewed in Hill and Treisman, 1995), and numerous protein kinases have been identified in plants (Stone and Walker, 1995). Plant protein kinases have been implicated in the relay of a number of environmental signals, including the presence of the plant hormone ethylene (reviewed by Ecker, 1995), recognition of self pollen by self-incompatible plants (Goring et al., 1993; Stein and Nasrallah, 1993), and response to pathogen attack (Martin et al., 1993). CLAVATA1 is a receptor-like kinase which is necessary for the maintenance of meristem size in *Arabidopsis* (S. Clark, personal communication). Mutants in *Clavata1* display an abnormally large meristem and developmental defects including fasciation and the production of extra carpels (Clark et al., 1993).

The *Arabidopsis* gene *TOUSLED (TSL)* is another plant kinase with a probable role in meristem function. It was first identified as a floral mutant, and is likely to play a role in cell signaling in the meristem (Roe et al., 1993). Disruption of the *TSL* gene causes mild vegetative defects and severe defects in floral morphology. *tsl* mutant plants display a random reduction in the number of floral organs produced (Roe et al., 1993) and show incomplete development of the gynoecium, with significant defects in the stigma and style (Roe et al., 1997b). *TSL* encodes a functional serine/threonine kinase which is nuclearly localized and acts as an oligomer (Roe et al., 1997a). In addition to

the catalytic domain, the TSL protein contains an N-terminal coiled-coil region which is necessary for activity and likely is involved in oligomerization (Roe et al., 1997a).

Although *TOUSLED* is a single gene in *Arabidopsis,* there appear to be multiple *TOUSLED-LIKE KINASE (TLK)* genes in maize. A partial cDNA clone from maize with very high homology to the catalytic domain of *TSL* was isolated by Helentjaris et al. (1995) as part of a maize sequencing project. This clone identifies three loci in maize, which map to chromosomes 1L, 4L and 5S (Helentjaris et al., 1995).

Multiple gene families and genetic redundancy are common phenomena. Protein kinases may be duplicated to ensure a functional kinase for important processes or may have arisen through duplications but subsequently diverged, allowing functional diversity (Thomas, 1993). This study describes the isolation of partial genomic clones of the *TLK* genes from maize. Analysis of the structural relationships between the members of this small gene family and examination of gene expression should help reveal how the multiple *TLK* genes of maize are related to each other and to *Arabidopsis TOUSLED* both structurally and functionally.

Methods and Materials

TLK1 Partial cDNA Clone

A partial cDNA clone representing one *TLK* gene was obtained from T. Helentjaris (then at University of Arizona). This cDNA was isolated from a developing endosperm library. The ΔSph subclone of this cDNA corresponds to the translated portion of the catalytic domain and was used as a probe in the experiments described below.

Isolation of Genomic and cDNA Clones

The ΔSph probe was used to screen a B73 maize genomic library in EMBL3 (Clontech). Duplicate plaque lifts were made on Magnagraph 0.45 μm nylon filters

(MSI). Filters were baked for two hours in an 80° oven and hybridized with 1.5 x 10^6 dpm/ml ^{32}P-dCTP labeled random-primed DNA (Feinberg and Vogelstein, 1984) in 5 ml of 250 mM NaH_2PO_4, 7% SDS. Lifts were prehybridized for 30 minutes and hybridized overnight at 65° in a rotating hybridization oven (Robbins Scientific). Lifts were washed three times for 45 minutes each in approximately 100 ml of 0.2X SSC, 0.1% SDS. Positive clones were visualized by exposing lifts to X-omat AR X-ray film (Kodak) for 2-3 days. Positive clones were isolated and purified.

A cDNA library in λ-Zap II (Stratagene) made from B73 developing ear shoot RNA was provided by Sarah Hake (U. C. Berkeley). This library was also screened as described.

Analysis of DNA

Lambda DNA was extracted from positive clones using the Wizard Lambda DNA Purification kit according to manufacturer's directions (Promega). Clones were restriction mapped by running 250 ng DNA digested with various restriction endonucleases on 0.8% agarose gels in Tris-Borate buffer (Sambrook et al., 1989). Gels were capillary blotted onto 0.45μm Magnagraph nylon membranes (MSI) and baked and hybridized as described above. Hybridization was detected by exposing blots to a phosphorimager detection screen (Molecular Dynamics). Results were analyzed using the ImageQuant program (Molecular Dynamics).

Southern blots were performed in the same manner to analyze plasmid subclones and maize genomic DNA. Maize DNA was purified according to Dellaporta (1994), and 3 to 4 μg DNA was digested and run per lane.

Subcloning and Sequencing

The entire cDNA insert and hybridizing restriction fragments of genomic clones were gel purified using the Geneclean II kit according to manufacturer's protocol (Bio 101). These inserts were ligated into pBluescript II SK- (Stratagene) and transformed

into competent *E. coli* strain DH5α. DNA was prepared from cells carrying recombinant plasmids using the QIAprep-spin plasmid miniprep kit according to instructions (Qiagen, Inc.). Plasmids were sequenced by Oregon State University's Central Services Laboratory using dye primer chemistry on an automated sequencer (Applied Biosystems).

Sequences were analyzed and compared to each other using Genetics Computer Group Version 8 (1994) software. Sequences were compared to databases over the World Wide Web using FASTA (Pearson and Lipman, 1988).

RNA Analysis

Plant material was taken from B73 and W22 plants grown in the field nursery and greenhouse in Corvallis, OR. Tissue was immediately frozen in liquid nitrogen and stored at -80° until used. RNA was isolated from 7 day old W22 seedlings, 17 DAP W22 kernels, mature B73 leaves, and an immature 3 cm W22 ear. Large scale RNA preps were done according to Wessler (1994). RNA micropreps were done using the PUREScript R-5500A RNA Isolation Kit (Gentra Systems) according to the protocol for plant material. This RNA was further purified by precipitation with an equal volume of 4 M LiCl before use. For northern blots, poly(A) RNA was prepared using the Poly ATtract IV System (Promega). Total or poly(A) RNA was run on 1.2% agarose, 2.2 M formaldehyde gels in MOPS buffer (Sambrook et al., 1989). Gels were blotted and hybridized as described for DNA gels. Reverse transcription PCR (RT-PCR) was also done, as described below.

PCR Analysis

Genomic and cDNA sequences were used to develop PCR primers which amplify various portions of the genes (Figure 2.1). Primers A(5'GGTTCATCCAAACATTGT-CAGGCTATGGGA-3'), B (5'-AGCTGGCTTTGAAGGGAACTCCACTCT-3'), C (5'-CAATGCACGGAGAGTGGAGTTCCCTTCAA-3'), D (5'GGTGGTTTCTGATT-

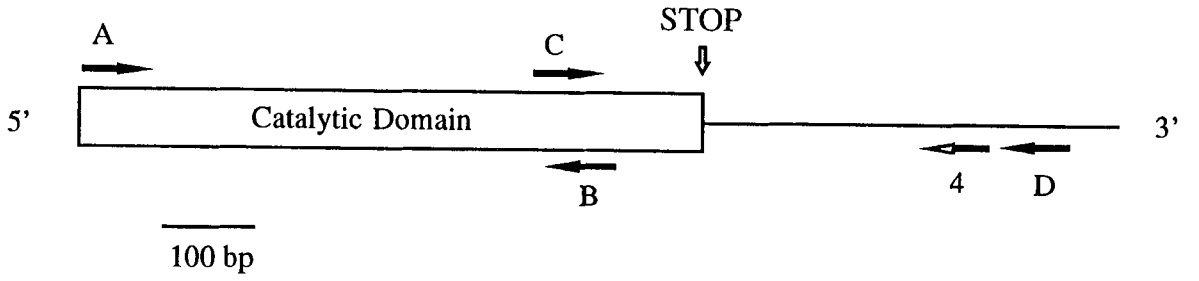

Figure 2.1 PCR primers and their positions relative to the *TLK1* cDNA clone. Primer 4 is specific to clones MTK3 and MTK4.

TGGCAGACCTTACC-3') and 4 (5'AAGGAGATCTCAGTCAAAGGGTAAATGG-3') were synthesized by the Central Services Laboratory (Oregon State). Primers C and D were used to amplify genomic DNA. 50µl PCR reactions were run in 0.25 mM dNTP mix, 10% sucrose, 1.5 mM $MgCl_2$ with 3 U Hot Tub DNA Polymerase (Amersham). 40 ng of B73 total genomic DNA was used as template. Amplification was conducted in a TwinBlock EasyCycler (Ericomp), with 10 min at 94° (once), 1 min at 94° and 62°, 2 min at 72° (40 times), and 5 min at 72° (once). The DNA polymerase was added after the initial 10 min incubation at 94°.

Primers pairs A-B, A-D and A-4 were used in RT-PCR. First strand cDNAs were prepared from total RNA using the SuperScript Preamplification System (Life Technologies). These cDNAs were then amplified using the PCR reaction mix described above with a reaction profile of 10 min at 94° (once), 1 min at 94° and 58°, 2 min at 72° (40 times), and 5 min at 72° (once). Negative controls included reactions with no template and with RNA treated with no reverse transcriptase.

PCR products were analyzed by running 10 µl of the reaction on 1.0% agarose gels and blotting and hybridizing as described above. Some fragments were gel purified, cloned and sequenced as described above. RT-PCR products from primers A to D were digested with SacI before being analyzed.

Results

Isolation of *TLK* Clones

860,000 plaques from a B73 random genomic library were screened with the ΔSph probe. Five positive clones were identified for further analysis. These clones are referred to as Maize Tousled Kinases (MTK) because they have not been assigned to specific *TLK* loci. All the MTK genes described here appear to fall into the *TLK* family. Restriction mapping indicates that two clones cover overlapping regions of the same gene (MTK2). The other three clones, MTK1, 3, and 4, represent distinct loci (Figure 2.2).

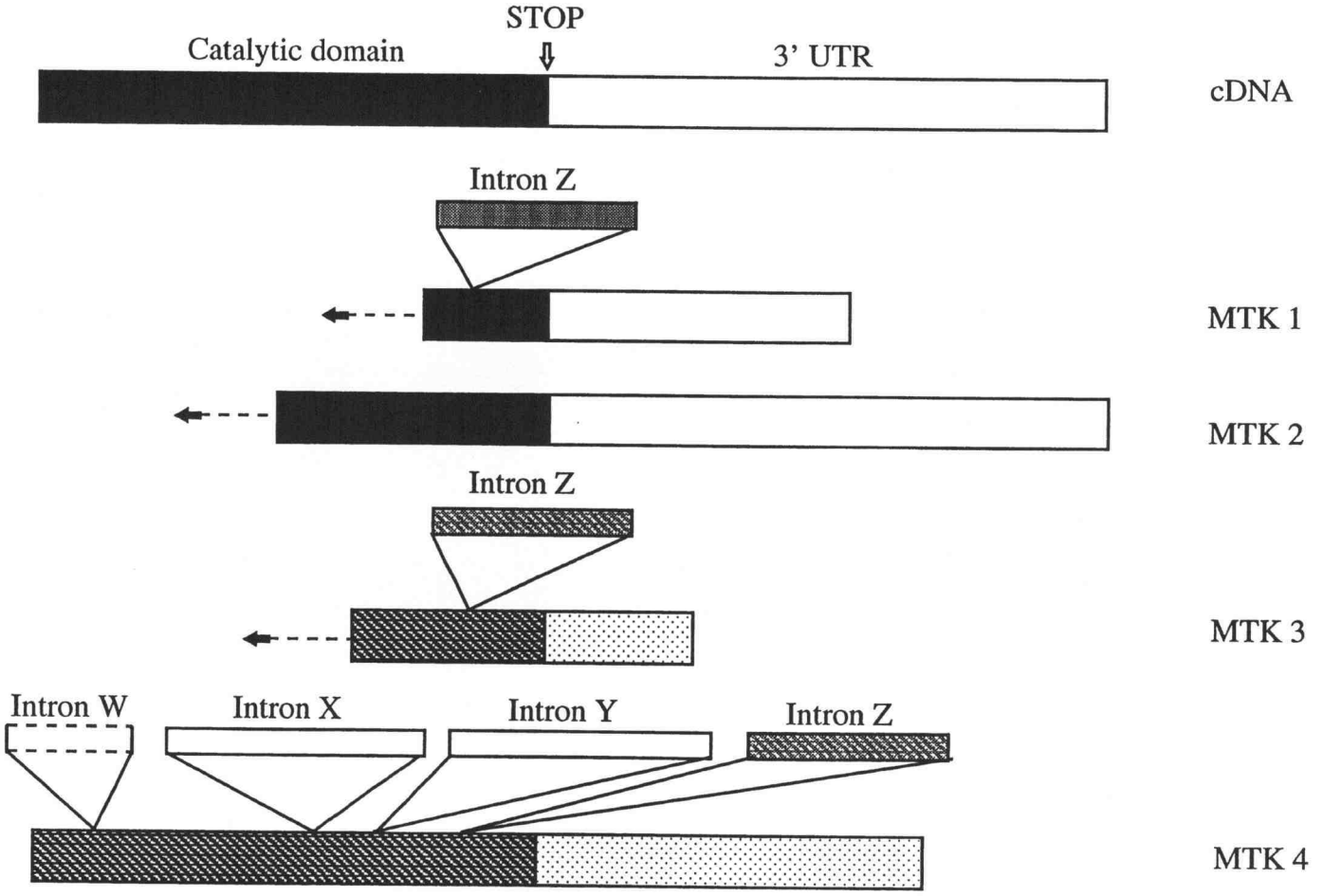

Figure 2.2 The MTK clones are highly homologous to the *TLK1* cDNA. The coding regions of MTK1 and MTK2 are identical to the cDNA, while MTK3 and 4 are only 91% identical to the cDNA. Intron Z is also highly conserved.

Restriction fragments which hybridized to the ΔSph catalytic domain probe were subcloned, mapped more thoroughly, and sequenced. All five clones contained sequences with high homology to the 3' end of the *TLK1* partial cDNA clone. After obtaining more information about the orientation of the gene sequence within the clones, it was possible to subclone flanking regions of MTK4. Unfortunately, none of the lambda clones contained complete copies of the genes; the most 5' sequence of each clone ended within the catalytic domain.

Additional MTK1 sequence was obtained through PCR amplification of B73 genomic DNA using primers C and D. These primers were expected to amplify a product of 508 bp from an intronless gene or cDNA and a product of roughly 790 bp in a gene containing introns. When used to amplify genomic DNA, the primary product was 790 bp. When cloned and sequenced, this fragment corresponded to MTK1. This PCR clone included 5' sequence which was not present in the lambda clone due to a rearrangement. Interestingly, I was not able to amplify the 508 bp product predicted to correspond to the intronless gene represented by the MTK2 clones. Genomic Southern blots also failed to give RFLPs predicted by MTK2, although other bands were consistent with the sequence information from the other clones.

160,000 plaques from a developing ear shoot cDNA library were also screened, yielding one positive clone, cEAR. This clone is identical in sequence to Helentjaris's *TLK1* partial cDNA, except that it extends 300 bp further upstream. In addition, the cEAR clone lacks the most 3' portion of the untranslated region. This clone ends in an A-rich region, suggesting that the oligo(T) primer used to initiate cDNA synthesis may have annealled to this site rather than to the poly(A) tail further downstream. The cEAR clone includes sequence corresponding to nearly the complete catalytic domain of *Arabidopsis TSL*, but lacks the extensive 5' non-catalytic portion of *TSL*. Both cDNAs also correspond exactly to the predicted transcripts of MTK1 and 2.

Primers A and 4 were used to amplify a seedling cDNA. The resulting fragment was cloned and sequenced. This RT-PCR product corresponded to MTK4 and also contained 5' sequence which was not present in the genomic clone.

Comparison of the Maize *TLK* Genes to Each Other

The maize *TLK* genes which we have cloned are extremely similar to each other, but appear to fall into two distinct classes on the basis of nucleotide sequence (Figure 2.2). The coding regions of MTK1 and 2 are 100% identical to each other, but MTK1 contains an intron, while MTK2 lacks introns over the entire region sequenced. The putative coding regions of MTK3 and 4 are 96% identical to each other, but only 91% identical to MTK1 and 2.

Interestingly, the 3' untranslated regions of all the genes are also conserved. MTK3 and 4 are 94% identical to each other over the sequenced portions of their 3' untranslated regions, and 75% identical to MTK1 and 2. The most 3' intron is also conserved. This intron is 84% identical in MTK3 and 4, which are both 75% identical to MTK1 in this region. Intron homology apparently does not continue to this degree in the more 5' regions of the genes. A portion of intron W of MTK4 (as shown in Figure 2.2) can be used as a gene-specific probe, identifying a single band on genomic Southern blots while the ΔSph probe recognizes multiple bands (Figure 2.3).

Homology to *Arabidopsis TSL* and Other *TSL* Homologues

All four genes cloned have extremely high homology to each other and to *TSL* (Figure 2.4). The predicted translation products of the maize genes are over 84% identical and 91% similar to *TSL* at the amino acid level. In addition, gene structure is conserved in three of the four genes. MTK1, MTK3 and MTK4 all contain introns in exactly the same positions as they occur in *TSL*. Although the MTK clones do not cover the complete catalytic domain, the last 8 of 11 conserved serine/threonine kinase subdomains are present and contain the conserved residues common to all

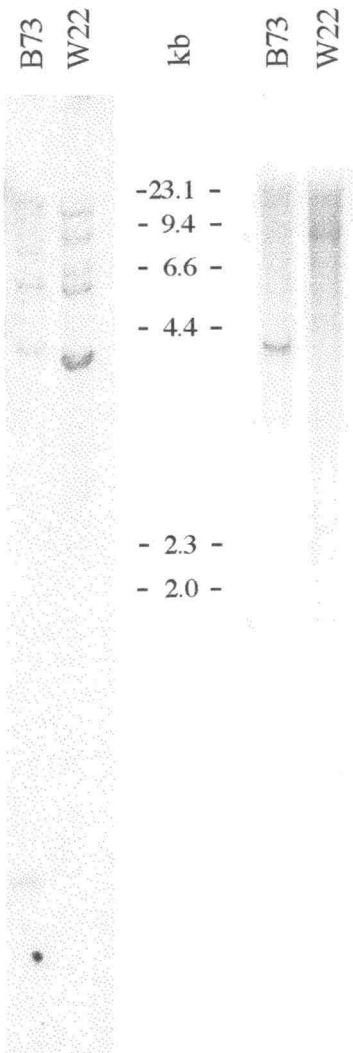

Figure 2.3 Genomic Southern blot probed with ΔSph (left) and MTK4 intron W (right). B73 and W22 maize DNA was digested with BglII. The position of size standards is indicated in the middle.

```
 TSL   EEKKQSYIRH ANRECEIHKS LVHHHIVRLW DKFHIDMHTF CTVLEYCSGK
cEAR   .......... .......... .VHPNIVRLW DIFEIDHNTF CTVLEYCSGK
MTK1   .......... .......... .......... .......... ..........
MTK2   .......... .......... .......... .......... ..........
MTK3   .......... .......... .......... .......... ..........
MTK4   .......... .......... .VHPNIVRLW DIFDIDHNTF CTVLEYCSGK

                                                    * *  *
 TSL   DLDAVLKATS NLPEKEARII IVQIVQGLVY LNKKSQKIIH YDLKPGNVLF
cEAR   DLDAVLKATP ILPEKEGRII IVQIFQGLVY LNKRGQKIIH YDLKPGNVLF
MTK1   .......... .......... .......... .......... ..........
MTK2   .......... .......... .......... .......... ..........
MTK3   .......... .......... .......... .......... ..........
MTK4   DLDAVLKATP ILPEKEARII IVQIFQGLVY LNKRGQKIIH YDLKPGNVLF

             *  *                                   **
 TSL   DEFGVAKVTD FGLSKIVEDN VGSQGMELTS QGAGTYWYLP PECFELNKTP
cEAR   DEVGVAKVTD FGLSKIVEND VGSQGMELTS QGAGTYWYLP PECFDLSKTP
MTK1   .......... .......... .......... .......... ..........
MTK2   .......... ......VRND VGSQGMELTS QGAGTYWYLP PECFDLSKTP
MTK3   .......... .......... .......... .......... ..........
MTK4   DEVGVAKVTD FGLSKIVEDD VGSQGMELTS QGAGTYWYLP PECFDLSKTP

          *     *
 TSL   MISSKVDVWS VGVLFYQMLF GKRPFGHDQS QERILREDTI IKAKKVEFPV
cEAR   FISSKVDVWS AGVMFYQMLF GKRPFGHDQT QERILREDTI INARRVEFP-
MTK1   .......... .......... .......... .......... .NARRVEFP-
MTK2   FISSKVDVWS AGVMFYQMLF GKRPFGHDQT QERILREDTI INARRVEFP-
MTK3   .......... ...FMYQMLY GRCPFGHDQT QERILWEDTI INARRVEFP-
MTK4   FISSKVDVWS AGVMFYQMLY GRRPFGHDQT QERILREDTI INARRVEFP-

                     *
 TSL   TRPAISNEAK DLIRRCLTYN QEDRPDVLTM AQDPYLAYSK K
cEAR   SKPAVSNEAK DLIRRCLTYN QSERPDVLTI AQDPYLSYAK R
MTK1   SKPAVSNEAK DLIRRCLTYN QSERPDVLTI AQDPYLSYAK R
MTK2   SKPAVSNEAK DLIRRCLTYN QSERPDVLTI AQDPYLSYAK R
MTK3   SKPAVSNEAK DLIRRCLTYN QSERPDVLTI TQDPYLSYAK K
MTK4   SKPAVSNEAK DLIRRCLTYN QSERPDVLTI TQDHYLSYAK K
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Figure 2.4 Comparison of translation products of *Arabidopsis Tousled* catalytic domain and partial maize *TOUSLED-LIKE KINASE* clones. Stars (*) indicate residues which are conserved among all serine/threonine kinases.

serine/threonine kinases (Hanks and Quinn, 1991), indicating that the *TLK* genes encode potentially functional kinases.

Database searches reveal the presence of homologous genes in such distantly related species as *Caenorabditis elegans*, mouse and human. Genes from these diverse species show extensive stretches of homology over the catalytic region and encode translation products with 44% identity in this area (Figure 2.5).

Expression of the *TLK* Genes

Cloning of partial cDNAs and an RT-PCR product indicate that the *TLK* genes corresponding to clones MTK1 or 2 and MTK4 are transcribed in maize. Northern blots show a transcript size of roughly 2.8 kb, which is similar to the size of the *TSL* transcript. The *TLK* genes are expressed at very low levels and are difficult to detect and impossible to distinguish from one another on northern blots. Therefore, RT-PCR was used to monitor expression of the *TLK* genes in different tissues. The translated region of MTK2 contains a SacI restriction site which is not present in MTK4. The corresponding regions of the MTK1 and MTK3 genes have not been sequenced, but MTK1 is likely to contain the SacI site and MTK3 is likely to lack it, based on their high degree of similarity to MTK2 and MTK4 respectively. By amplifying cDNAs using primers A and D, and then digesting the PCR products with SacI, it is possible to distinguish between MTK2-related and MTK4-related transcripts. Both classes of genes are expressed in all tissues examined (Figure 2.6).

Discussion

The Maize *TOUSLED-LIKE KINASE* Genes Form a Small Gene Family

Genomic and cDNA clones representing portions of four distinct genes with high homology to the catalytic domain of the *Arabidopsis* gene *Tousled* have been isolated from maize and sequenced. These maize *TOUSLED-LIKE KINASE* genes are likely to

```
   TSL  .VHHHIVRLW DKFHIDMHTF CTVLEYCSGK DLDAVLKATS NLPEKEARII
maize1  .VHPNIVRLW DIFEIDHNTF CTVLEYCSGK DLDAVLKATP ILPEKEGRII
 C.elg  .DHCRIVKQY DLLTIDNHSF CTVLEYVPGN DLDFYLKQNR SISEKEARSI
 mouse  .DHPRIVKLY DYFSLDTDSF CTVLEYCEGN DLDFYLKQHF LMSEKEARSI
 human  .DHPRIVKLY DYFSLDTDTF CTVLEYCEGN DLDFYLKQHK LMSEKEARSI

   TSL  IVQIVQGLVY LNKKSQKIIH YDLKPGNVLF ...DEFGVAK VTDFGLSKIV
maize1  IVQIFQGLVY LNKRGQKIIH YDLKPGNVLF ...DEVGVAK VTDFGLSKIV
 C.elg  IMQVVSALVY LNEKSTPIIH YDLKPANILL ESGNTSGAIK ITDFGLSKIM
 mouse  IMQIVNALKY LNEIKPPIIH YDLKPGNILL VNGTACGEIK ITDFGLSKIM
 human  VMQIVNALRY LNEIKPPIIH YDLKPGNILL VDGTACGEIK ITDFGLSKIM

   TSL  EDN..VGSQG MELTSQGAGT YWYLPPECFE LNKTP.MISS KVDVWSVGVL
maize1  END..VGSQG MELTSQGAGT YWYLPPECFD LSKTP.FISS KVDVWSAGVM
 C.elg  EGESDDHDLG IELTSQFAGT YWYLPPETFI V..PPPKITC KVDVWSIGVI
 mouse  DDDSYNSVDG MELTSQGAGT YWYLPPECFV VGKEPPKISN KVDVWSVGVI
 human  DDDY..GVDG MDLTSQGAGT YWYLPPECFV VGKEPPKISN KVDVWSVGVI

   TSL  FYQMLFGKRP FGHDQSQERI LREDTIIKAK KVEFPVTRPA ISNEAKDLIR
maize1  FYQMLFGKRP FGHDQTQERI LREDTIINAR RVEFP.SKPA VSNEAKDLIR
 C.elg  FYQCIYGKKP FGNDLTQQKI LEYNTIINQR EVSFP.SKPQ VSSAAQDFIR
 mouse  FYQCLYGRKP FGHNQSQQDI LQENTILKAT EVQFPP.KPV VTPEAKAFSR
 human  FYQCLYGRKP FGHNQSQQDI LQENTILKAT EVQFPV.KPV VSSEAKAFIR

   TSL  RCLTYNQEDR PDVLTMAQDP YLAYSKK*
maize1  RCLTYNQSER PDVLTIAQDP YLSYAKR*
 C.elg  RCLQYRKEDR ADVFELAKHE LFRPRGASV
 mouse  RCLAYRKEDR IDVQQLACDP YLLPHIRKS
 human  RCLAYRKEDR FDVHQLANDP YLLPHMRRS
```

Figure 2.5 Alignment of predicted translations of the catalytic domains of *TOUSLED*-like genes from five different organisms. Predicted translation products are based on cDNA sequences from *Arabidopsis TOUSLED* (Roe et al., 1993), maize (cEAR clone), *C. elegans* (Wilson et al., 1994), mouse (Shalom and Don, 1996) and human (Nomura, 1995). Boldface residues are conserved among all five species.

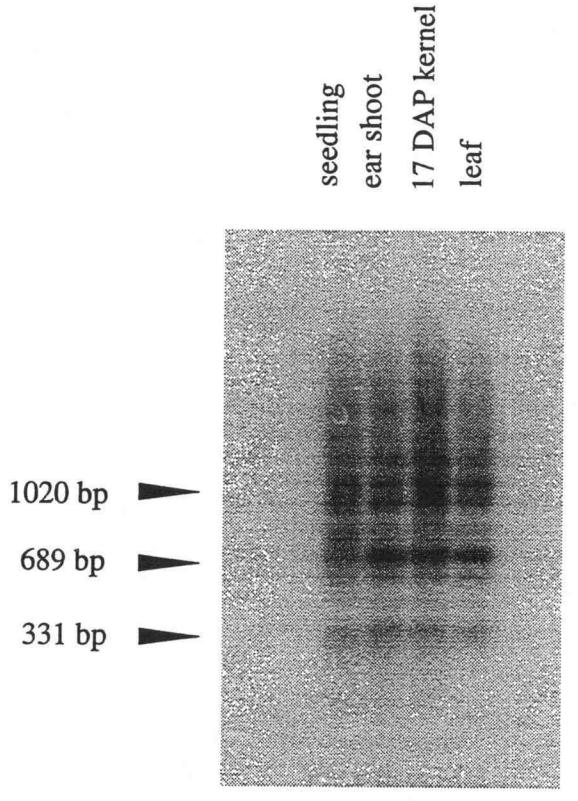

Figure 2.6 Southern blot of RT-PCR products digested with SacI and probed with the cEAR clone. PCR amplification using primers A and D was done on first strand cDNAs from maize seedlings, developing ears, kernels and mature leaves. The MTK1/MTK2 class transcripts are cleaved by SacI resulting in fragments of 689 and 331 bp. MTK3/MTK4 class transcripts remain uncleaved, at 1020 bp.

encode functional serine/threonine kinases, based on their predicted translation products. At least two of these genes are expressed throughout the plant. These clones are likely to represent all of the *TLK* genes in maize, based on the number of genomic clones screened, and previous mapping data (Helentjaris et al., 1995).

It is possible that the two intronless MTK2 clones do not represent an actual gene, but are instead some sort of artifact in the library we screened. I have been unable to confirm the existence of an intronless gene in B73 genomic DNA. In addition, the MTK1 and MTK2 sequences are 100% identical, even in the 3' untranslated region, except for the lack of introns in MTK2. The two MTK2 clones may actually be cDNAs of MTK1 transcripts. Although the MTK2 clones could also represent a pseudogene, such a locus should still be detectable by PCR and Southern blotting. The finding of three actual *TLK* genes is consistent with the mapping data of Helentjaris et al. (1995), although two closely linked loci would not necessarily be detectable as two separate genes in a mapping survey. It is unclear how cDNAs could have appeared in a genomic library. The development of gene-specific probes and subsequent mapping of the MTK sequences to previously established *TLK* loci may help clear up this uncertainty.

Mapping will also provide clues to the evolution of this gene family in maize. Comparative mapping studies between maize and other related grasses have shown that large portions of the maize genome have been duplicated in blocks. The 1L and 5S *TLK* loci reported by Helentjaris et al. (1995) fall within chromosomal regions which appear to be syntenous. Large blocks of the maize genome are duplicated, either as a result of an tetraploidization event, or through duplication of individual chromosome segments (Helentjaris et al., 1988). It will be interesting to see if the more closely related MTK3 and 4 map to these locations. If so, they may have arisen through a relatively recent duplication event, explaining the higher homology they share with each other than with MTK1/MTK2. Maize is presumed to have a tetraploid origin, and it is possible that the progenitors of maize each had two genes. Following hybridization, one of the MTK1-

like genes could have been lost from the new tetraploid resulting in the current three genes. Alternatively, there could have been two *TLK* genes following tetraploidization, perhaps due to the hybridization event. The MTK3/MTK4 progenitor gene could have then been duplicated to give the current number of three genes.

Regardless of the exact number of *TLK* genes in maize, at least two *TLK* genes are expressed. The presence of multiple, related genes may lead to functional overlap. For example, the *Arabidopsis* ethylene response genes *ETR1* and *ERS* share sequence homology, and are likely to share function too. Dominant mutations in either gene result in an identical ethylene insensitive phenotype (Hua et al., 1995). The failure to recover recessive mutants in these genes may be due to complete functional overlap; both genes would need to be eliminated in order to see a phenotype. In other cases, genetic redundancy may lead to a diversification of function. In tomato, a cluster of homologous genes have evolved distinct functions. Two of these genes, *PTO* and *FEN*, encode active protein kinases with 80% identity and 87% similarity at the amino acid level (Martin et al., 1994). Despite their structural similarity, they have non-overlapping functions: *PTO* confers resistance to *Pseudomonas syringae* pv *tomato*, while *FEN* leads to sensitivity to the insecticide fenthion (Martin et al., 1993; Martin et al., 1994). Functional copies of both genes are needed to give both phenotypes.

Expression data suggest that there may be differences in the relative levels of the two classes of *TLK* genes which are expressed in different tissues, but it is premature to conclude that there is differential regulation of the *TLK* genes at the transcriptional level. The development of gene-specific probes will facilitate quantitative comparisons of the expression level of each *TLK* gene in different tissues.

The structure of the TOUSLED protein suggests the potential for the evolution of distinct functions in the *TLK* gene family. TSL contains an extensive N-terminal domain which includes coiled-coil regions likely to be involved in protein-protein interactions (Roe et al., 1993). This portion of the *TSL* gene is not as highly conserved between

Arabidopsis and maize, based on Southern analysis (data not shown). It is possible that the various members of the maize *TLK* family each have unique non-catalytic domains, allowing their specific regulation at the protein level through interactions with specific protein partners. Alternatively, different TLK proteins could interact with each other, creating various combinations of homo- and heterodimers with varying activities. Cloning and analysis of the 5' portions of the *TLK* genes will allow comparison of the non-catalytic domains and permit biochemical studies on the action of and interaction between various members.

The *TLK* Genes Contain Highly Conserved Untranslated Sequences

The extremely high sequence homology of the 3' untranslated region (3' UTR) and the most 3' intron was surprising. Although untranslated, these sequences may play important roles in the regulation of these genes. Either region could act as a distal regulatory element such as an enhancer. Such an enhancer has been found in an intron in a mouse immunoglobin heavy chain gene. This intron contains three enhancer sites of 8 nucleotides each which can act as enhancers even when moved elsewhere in the gene (Gillies et al., 1983). In plants, transposable elements inserted into introns have been implicated in altering gene expression. Insertions into introns of both maize *KNOTTED1* and *Antirrhinum PLENA* cause dominant mutations marked by expression of the *KN1* and *PLENA* genes in tissues where they are not normally expressed. It has been suggested that either the transposable elements themselves act as enhancers, or they prevent binding or activity of inhibitors associated with sequences near the insertions (Bradley et al., 1993; Greene et al., 1994). Either way, these findings suggest that intron sequences are potentially important in regulating gene expression.

The 3' UTR could also play a role in transcript stability. Various proteins have been identified which bind mRNA and affect message stability (reviewed in Ross, 1996). A variety of motifs have been identified in 3' UTRs which are binding sites for proteins

which affect message stability. The poly(A)-binding protein confers stability to mRNAs with a poly(A) tail. AURE-binding proteins are believed to bind to AU-rich regions in the 3' UTRs of many mRNAs to destabilize the message (Ross, 1996). Other proteins have been found which bind specifically to the 3' UTRs of unique or closely related mRNAs to alter stability, sometimes under highly specific conditions. For example, transforming growth factor β_1 stimulates binding of various protein factors to a 6 nucleotide repeat in the 3' UTR of the hyaluronan receptor RHAMM mRNA, resulting in increased stability (Amara et al., 1996). Finally, 3' UTRs may contain sites for endoribonucleoytic cleavage, leading to degradation of the message. It has been shown that the transferrin receptor mRNA is cleaved at a specific site in the 3' UTR, likely beginning the degradation process (Binder et al., 1994).

The Maize *TLK* Genes are Members of an Evolutionarily Conserved Class of Kinases

Until recently, *TOUSLED* was classified as a novel type of serine/threonine kinase, with no homologues identified in other organisms (Stone and Walker, 1995; Roe et al., 1993). However, the cloning of a family of maize *TOUSLED-LIKE KINASES*, as well as the recent addition of *TSL* homologues from various species to sequence databases indicates that *TSL* is in fact a representative of an evolutionarily conserved class of kinases. The high degree of conservation in the predicted catalytic domains from such distantly related organisms as *C. elegans*, plants, and mammals may indicate that there has been strong selective pressure to minimize alterations in TOUSLED-like proteins. Interestingly, no *TSL* homologues have been found in yeast (Hunter and Plowman, 1997) so the conservation of *TOUSLED*-like genes specifically in higher eukaryotes suggests that this family of kinases plays a important role in multicellular organisms.

There are two classes of *TOUSLED*-like genes in mouse and human as well as in maize. The redundancy of apparent *TSL* homologues may indicate the need for fidelity

of *TSL* function. A second possibility is that gene duplication may have led to diversification of function in organisms other than *Arabidopsis*. Alternatively, multiple functions could have been taken over by a single gene following the loss of other family members in *Arabidopsis*. Although the mouse and human genes can be clearly assigned to two classes, with each species containing one member of each, these do not appear to correspond to the two classes of maize genes. Each of maize *TLK* genes is equally similar to both classes of mammalian genes. The existence of two *TOUSLED*-like genes appears to predate the radiation of mammals, but it is not known how many *TLK* genes may have been present in the common ancestor of plants and animals or of maize and *Arabidopsis*.

The specific biochemical function of the TOUSLED protein has not yet been determined, but it has been shown to be a nuclear-localized, active serine/threonine kinase with a probable role in cell-signalling events (Roe et al., 1993, 1997a). By continuing analysis of this gene family in maize, as well as of the single gene *TSL* in *Arabidopsis*, it should be possible to gain more insight into the role of *TOUSLED*-like kinases in general and the coordination of families of closely related *TSL* homologues within single organisms. Future studies of the maize *TOUSLED-LIKE KINASE* gene family can help address questions on a number of levels, from issues surrounding the evolution of multiple gene families to the specific role of *TOUSLED-LIKE KINASES* in the signalling pathways involved in plant development.

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3. Characterization of the *tassel-less1* Mutant of Maize

Abstract

Mutant analysis is a powerful tool in gaining understanding of developmental processes. The *tassel-less1 (tls1)* mutant of maize is a recessive mutation which shows a unique phenotype of variable vegetative and reproductive defects. Mutant plants consistently lack a well-developed, fertile tassel, and display a severe reduction in ear size and fertility. In addition, *tls1* plants can show reduction in stature, leaf size, and alterations in leaf morphology. The range and severity of defects is environmentally sensitive. The broad effects of the *tls1* mutation on organ formation suggest that the *TLS1* gene product may play a critical role in regulating the partitioning of the meristem and/or the initiation of organ primordia.

Introduction

Maize has a distinctive architecture and well-defined developmental program. A large body of descriptive and genetic information has been gathered on maize, making it a model system for the study of developmental processes. The shoot apical meristem produces vegetative nodes through the proliferation of a ring of cells around the base of the meristem to form a leaf primordium (Sylvester et al., 1990). The sheath of maize leaves wraps around the shoot axis. The blade extends outwards from the axis and has characteristic ligule and auricle structures at the blade-sheath boundary (Sylvester et al., 1990). After producing a fixed number of vegetative nodes, the shoot apical meristem elongates and becomes developmentally determined to from a terminal inflorescence, the tassel (Irish and Nelson, 1991). The transition from vegetative to a floral meristem identity occurs in a series of steps. Tassel branches are initiated and then spikelet pair primordia form. Each spikelet pair primordium forms two spikelets in which two florets each are initiated (Cheng et al., 1983). In tassels, the gynoecium of each floret is aborted, leading to staminate flowers. Axillary buds in the lower portion of the plant can

form tillers, or basal branches, in which the structure of the main axis is reiterated. In intermediate nodes, axillary branches form ear shoots, which are terminated by female inflorescences, or ears. Ears are unbranched, and one floret of each spikelet, as well as the stamens of the remaining floret, are aborted, giving rise to a pistillate inflorescence (Cheng et al., 1983).

Various mutants of maize have been described in which defects in meristem activity have been implicated in causing abnormal shoot development. Several mutants have been described in which the leaf-sheath boundary is disrupted, resulting in displacement of sheath tissue into the blade and displacement or absence of the ligule and auricles. These include dominant gain of function mutants in several homeobox genes of the *Knotted1* family, whose expression is normally limited to the shoot meristem (reviewed in Freeling, 1992).

Overall leaf shape can also be affected by mutations. The *narrow sheath* mutant has short internodes and narrow leaves (Scanlon et al., 1996). The leaf phenotype is most severe at the base of the plant, but upper leaves are still unusually narrow. Scanlon et al. (1996) propose that the *NARROW SHEATH* gene is necessary for the recruitment of meristematic cells to the lateral regions of new leaf primordia.

There are also many maize mutants with altered inflorescence development (reviewed in Veit et al., 1993), although the specific role of these genes in the inflorescence apex has not been determined. These include sex determination mutants. Mutations in *anther ear* and some *dwarf* genes result in stamen development in ears, while *tasselseed* mutants contain pistillate tassels (Irish, 1996). Other inflorescence mutants include the *barren stalk* mutants which fail to produce ear shoots, the *ramosa* mutants, in which some spikelet pairs are transformed into branches in both the ear and tassel, and *branched silkless* in which florets are converted to indeterminate structures (Veit et al., 1993).

The *tassel-less1* (*tls1*) mutant of maize displays a variety of defects and is likely to represent a lesion in a gene required for proper meristem function. This mutant was briefly described by Albertsen et al. (1993) and is likely to be defective in the same gene as the *barren-sterile* mutant described by Woodworth (1926) but subsequently lost. The *tls1* phenotype segregates as a simple recessive. The severity of the mutant phenotype is variable. However, the most striking defects are the lack of a tassel and the severe reduction of the ears, although ear shoots are produced. This study represents an attempt to characterize the *tls1* phenotype more fully, in order to gain a better understanding of the role of this gene in maize development.

Methods and Materials

Plant Material

Seeds from three families segregating for the *tassel-less1* (*tls1*) mutation were obtained from M. Albertsen (Pioneer). These lines had been introgressed previously into the A632 genetic background. Seeds were grown in the Corvallis, OR field nursery in the summer of 1995. Progeny of these families were grown in the field nursery in 1996, and in the greenhouse in 1996 and 1997. Stocks were maintained and increased by crossing phenotypically wild type heterozygous *tls1/+* plants with each other and onto *tls1/tls1* siblings.

Observations and Data Collection

Plants were observed throughout the growing season and observations were made from the time that *tls1* plants could reliably be detected as phenotypically distinct from their wild type siblings. Observations continued until the time that wild type siblings were fully mature, at anthesis, and with expanded silks.

In 1995, measurements were taken on mature *tls1* plants. Height was measured as the distance from the ground to either the top of the highest structure on the main axis,

or to the point of emergence of a terminal structure. Nodes were counted from the first node above the soil to the uppermost structure on the main axis. Similar measurements were made on heterozygous siblings at the same time. Height was measured as the distance between the ground and the base of the tassel. The number of above-ground nodes below the tassel was recorded. These measurements were made late in the season, 2 to 4 weeks after anthesis.

Field-grown *tls1* plants were dissected in order to more closely examine shoot apices and lateral branches in 1995 and 1996. Shoot apices were examined under 12X and 20X magnification in a Zeiss dissecting microscope. Observations were recorded on the contents of lateral branches and the presence of any apical structures or additional unexpanded leaves which could not be seen in the intact plant.

Results

tassel-less1 mutant plants displayed a wide range of defects including altered vegetative growth, abnormal leaf morphology, and a severe reduction of reproductive structures. The mutant phenotype was extremely variable and appears sensitive to environmental conditions.

Severe *tls1* Phenotype

Field-grown *tls1* plants were strikingly abnormal and could be positively identified as different from their wild type, heterozygous siblings by four to five weeks post germination. Mutant plants were shorter, with less-expanded internodes. Leaf morphology became progressively more abnormal toward the top of the plant, and upper leaves were shorter, narrower, rougher, and much stiffer than comparable leaves on wild type plants.

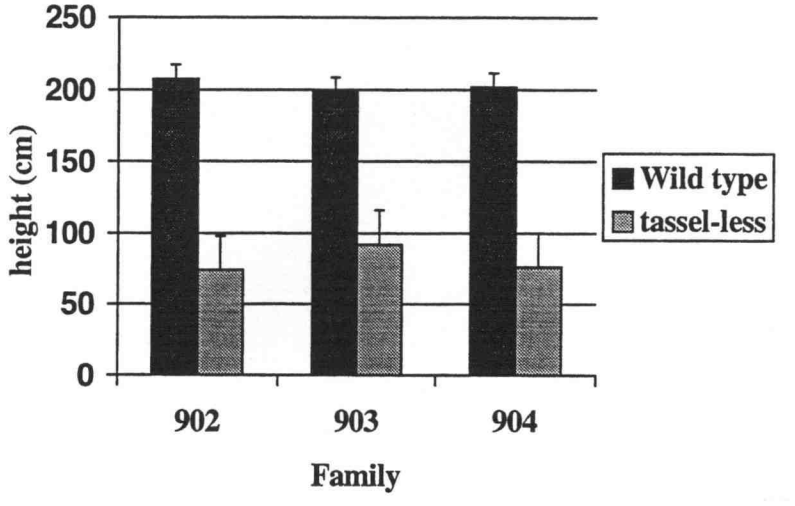
By approximately 10 weeks after germination, heterozygous plants were reproductively mature, having produced an average of 16 above-ground vegetative nodes, and a fertile tassel. In 1995, these wild type plants were approximately 2 meters

tall, and also produced one or more fertile ear shoots. In contrast, *tls1* plants were variable in height, but averaged under a meter, and produced an average of only 10 above-ground nodes (Figure 3.1). None of the mutants had a tassel of any sort, or produced fertile ear shoots. The upper portions of these plants were extremely abnormal. In many cases, the uppermost one or two nodes were not fully expanded, and could be found only by dissecting away outer, older leaves. Leaves in the upper third of the plant were much smaller than comparable wild type leaves, and were extremely stiff and somewhat thick. Many leaves displayed a reduction in the leaf blade, so that it was either asymmetrical, with one side of the leaf wider than the other, or virtually absent, leading to a spike-like appearance (Figure 3.2a). The ligule and auricles were missing in many of these upper leaves and the blade-sheath boundary was not clear, especially since there was no culm continuing up inside the leaf to define a functional sheath region.

In 1995, the main axis of all *tls1* plants terminated in an abnormal vegetative structure. In many plants, the most apical structure was leaf-like, but very narrow and reduced (Figure 3.2). These structures were often solid cylinders of tissue at the base, but flattened out to have more leaf-like bilateral symmetry at the tip. This is unlike the flat wild type leaves, which wrap around the main axis to form a hollow cylinder.

Mutant plants produced lateral branches at the appropriate nodes for ear shoots, but these lacked well-developed ears inside. Most ear shoots either appeared arrested or terminated in structures similar to those found at the top of the main axis. A few ear shoots contained apices which looked distinctly reproductive, having an elongated shape and a few spikelet or spikelet pair primordia. However, all apices examined were at a very early stage of development even though wild type plants of the same age had produced mature ears with silks. In many plants, the ear shoots expanded and elongated while the primary axis did not, so that the highest part of a plant was part of an axillary branch, rather than the main axis (Figure 3.2b).

a)



b)

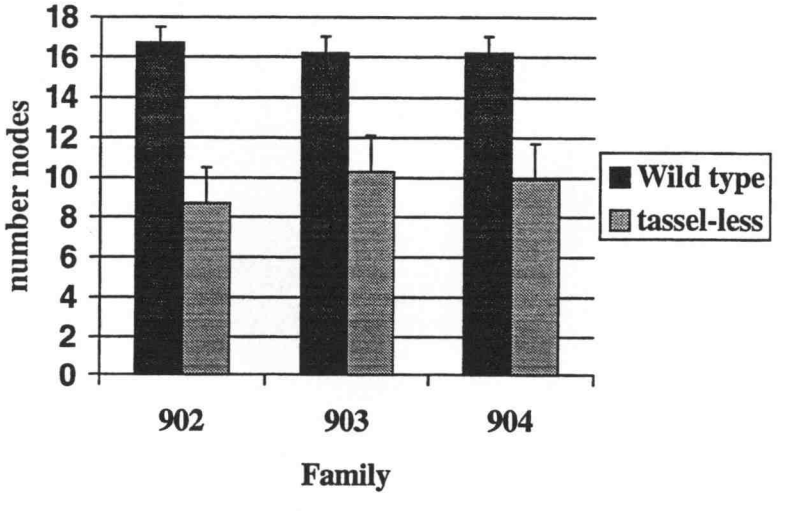


Figure 3.1 Comparison of wild type and *tassel-less* siblings from three families, grown in the summer of 1995 showing a) height from ground to base of tassel or other most apical structure and b) number of above ground nodes.

a)

b)

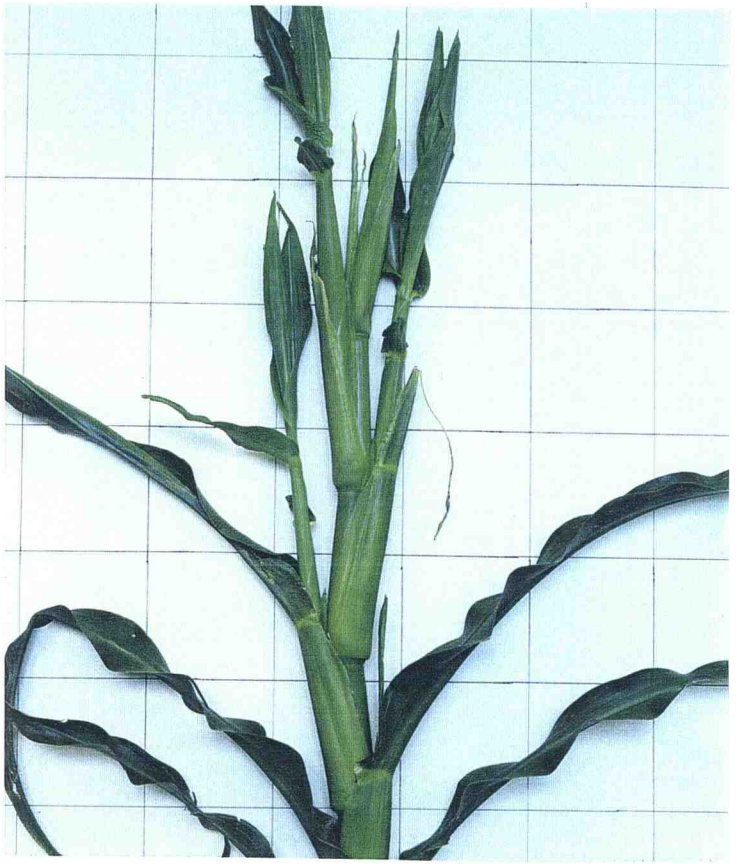


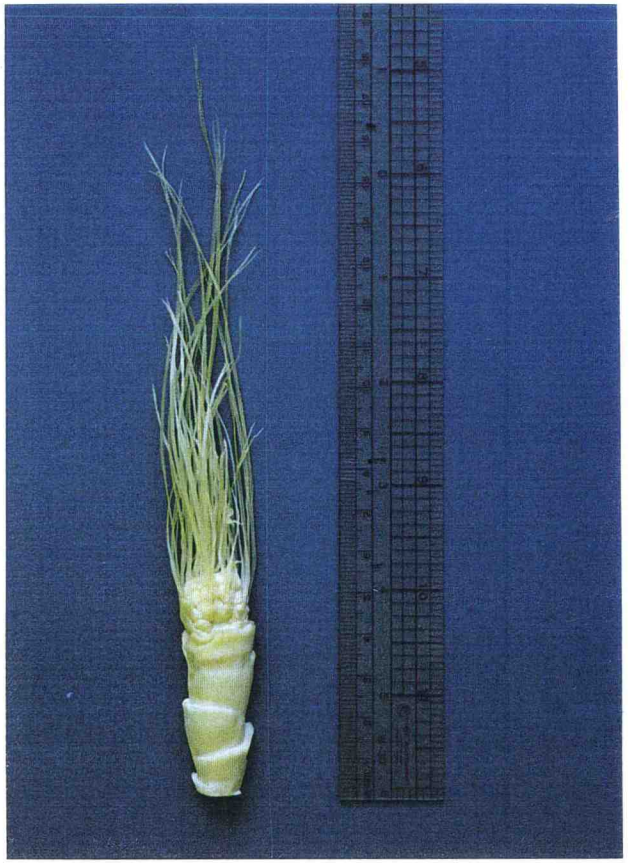
Figure 3.2 Field-grown *tls1* plants. a) Many plants produced abnormal vegetative structures at the apex. b) In some plants, elongated ear shoots were the highest point on the plant.

Plants grown in the 1996 field nursery showed similar defects, although most plants examined produced reproductive primordia in the ear shoots. These varied from small elongated apices with a few spikelet primordia to very reduced but otherwise normal ears (Figure 3.3a). Seed was obtained from 9 plants out of 60, with small ears producing 1 to 50 seeds each. A few plants produced a tassel structure, although tassels failed to expand and emerge out the top of the plant, and were completely sterile (Figure 3.3b).

Mild *tls1* Phenotype

Greenhouse-grown *tls1* plants showed a much milder phenotype, and could not be distinguished from their wild type siblings until about nine weeks after germination. Mutants were as tall as their heterozygous siblings, produced as many vegetative nodes, and had phenotypically normal leaves at all but the most uppermost nodes. Some plants displayed reduced, stiff upper leaves and a complete lack of tassel, but grew to roughly the same height as wild type siblings (Figure 3.4a). Other plants had a rudimentary, sterile tassel (Figure 3.4b). Over half the plants produced partially fertile ears, with seed set varying from 1 to 35 seeds per ear in 1996. Although ears were fertile, ear morphology was altered. Many ears were truncated and some looked spherical when harvested. Three ears out of 19 harvested were branched, with three, six, and eight branches respectively. All branches were clearly ear-like in nature, most with some seed set. Kernels were not all aligned properly on the ear. Normally, the kernel is positioned with the embryo on the upper, abaxial side of the seed. A few kernels were observed on *tls1* ears in which the embryo faced to the side, perpendicular to the normal position. Many kernels were mispositioned at an intermediate angle. This alteration in kernel alignment occurred on unbranched as well as branched ears.

a)



b)

Figure 3.3 *tls1* a) ear and b) tassel.

a)

b)

Figure 3.4 Greenhouse-grown *tls1* plants with a) no expanded tassel and b) a rudimentary tassel.

Discussion

Mutations in the *tassel-less1* gene cause a variable range of vegetative and reproductive defects. The phenotype appears to be highly sensitive to environmental conditions and was much milder in greenhouse-grown plants than in plants grown in the Corvallis, OR field nursery. In the field, *tls1* plants show striking vegetative defects. The plants become progressively more abnormal toward the top of the plant, suggesting that the *TLS1* gene product is critical for maintaining proper development. These plants generally terminate in unusual vegetative structures. In greenhouse-grown *tls1* plants, vegetative development is relatively normal, but mutants fail to produce functional tassels or complete ears.

Comparison to Similar Developmental Mutants

Mutants in *tls1* show a number of defects in organ production. Other organ formation mutants have been described, but do not display the pleiotropy found in *tls1* mutants. One type of defect found in *tls1* plants is the disruption of the blade-sheath boundary in upper leaves. Several *liguleless* mutants show a displacement or absence of ligules and auricles, with the displacement of sheath tissue into the blade (Freeling, 1992). These include the dominant mutants *Knotted1* and *Rough Sheath*, which encode homeodomain proteins normally expressed only in the meristem (Hake et al., 1995). Both recessive and dominant *liguleless* mutants are characterized by the presence of sheath tissue in the blade region.

Because maize leaves differentiate from tip to base, one interpretation of the *liguleless* phenotypes is that patches of blade tissue are delayed in becoming determined and do not assume an identity until the developmentally appropriate time to assume sheath fate (Freeling, 1992). It is possible that a similar delay in cell determination could be operating in *tls1* plants, if overall plant growth is slowed. If absolute growth is slowed in *tls1* plants, but developmental timing is not retarded to the same extent, it is

possible that very few cells will be competent to perceive and respond to a "make blade" signal when it is produced. This scenario could also explain the more severe reduction of blade than sheath.

In addition to lacking the blade-sheath boundary, *tls1* plants display abnormally narrow, and sometimes asymmetrical leaves. Mutations in the *narrow sheath* gene dramatically reduce leaf width and often lead to asymmetrical postitioning of the remaining blade tissue around the midrib (Scanlon et al., 1996). However, the blade-sheath boundary remains intact, and leaf narrowing is most severe at the base of the blade and in lower leaves. These aspects of the *narrow sheath* phenotype are unlike the characteristics of *tls1* mutants.

In severe cases, *tls1* plants produce abnormal vegetative structures which are cylindrical at the base and then flatten somewhat at the tip. Two *Arabidopsis* mutants, *arrested development 1* and *2*, produce two to three leaf primordia before arresting when grown under high temperature conditions. The leaves which are produced are "finger-like projections that lack dorsoventrality" (Pickett et al., 1996). Pickett et al. (1996) suggest that not only is a functional meristem necessary for leaf initiation, but it is also necessary for proper leaf morphogenesis and the establishment of dorsoventrality. The formation of abnormal spike-like structures in *tls1* plants may also result from the failure of leaf primordia to receive proper developmental signals from the meristem.

The reproductive defects in *tls1* plants are more consistent than the alterations in leaf morphology, but also differ from defects described in other well-characterized mutants. Ears are present, but poorly developed, while tassels are absent or extremely rudimentary and sterile. There does not appear to be any disruption of sex determination, as the occasional tassel floret is staminate, though sterile, and ear florets are consistently pistillate. The presence of branched ears is an interesting finding. In the *ramosa* mutant, spikelet pair primordia in both the tassel and ear develop into branches instead of spikelets (Gernert, 1912). However, in *tls1* plants, the tassel is very reduced and rarely

has any branches at all, while the ear branches appear to be all from the base, rather than all the way up the axis as in *ramosa*. The unusual positioning of kernels on some ears may be due to a disruption of positional information. One possibility is that the lack of a single strong apical-basal axis in severely truncated or branched ears interferes with important physical or chemical positional cues.

Many field-grown *tls1* plants show an unusual appearance due to the extensive growth of ear shoots, while the main axis remains small. Often an ear shoot is the highest structure on the plant. These ear shoots are much more elongated than normal, suggesting that they may have a partial tiller identity, or be otherwise released from inhibition of internode elongation. In addition, these ear shoots are often much larger in circumference than the main axis, suggesting an alteration in the relative numbers of cells or degree of cell growth and proliferation in the primary shoot as compared to the axillary meristems which give rise to the ear shoots.

The Role of the *TLS1* Gene Product

The *tls1* phenotype could be explained in part if the meristem initiates an appropriate number of nodes in *tls1* plants, but upper nodes then fail to develop, arresting or becoming necrotic. The observations of greenhouse plants are consistent with this hypothesis; all nodes appear to be initiated but the tassel fails to expand and appears arrested as do infertile ears. However, dissections of mature field-grown plants often reveal arrested vegetative apices or abnormal, terminal vegetative structures, suggesting that reproductive nodes are never initiated. Closer examination of apices at different developmental stages is necessary to eliminate the possibility that inflorescence primordia are initiated but become necrotic extremely early in development. However, this model fails to provide an explanation for the abnormal ear and leaf morphology observed.

An alternative model suggests that the *TLS1* gene product is necessary for the initiation of organs. *TLS1* may be necessary for meristem maintenance or partitioning. In mutants, the meristem may fail to partition cells correctly, or be defective in replenishing and maintaining itself, so that all meristematic cells are used up by organogenesis prematurely. A gradual depletion of the meristem may explain the progressive nature of the *tls1* phenotype. Allocation of an unusual number or distribution of cells to new primordia may lead to the increasingly defective morphology of upper leaves. Improper recruitment of cells to the lateral regions of new leaf primordia has been proposed in the *narrow sheath* mutant (Scanlon et al., 1996). Improper partitioning of the meristem could also explain the presence of large, robust axillary ear shoots while primary shoots are extremely small. Perhaps a disproportionate percentage of meristem cells are allocated to the axillary meristems, allowing the formation of large ear shoots, but depleting the cells available for the continuation of the main axis. In addition, ear branches could possibly arise if too many cells were allocated to some spikelet pair primordia, transforming them into indeterminate branch primordia. Alternatively, the primary inflorescence meristem itself may branch.

The *tls1* phenotype is clearly environmentally sensitive. The rate of meristem depletion in the model described above could be affected by environmental conditions. Greenhouse plants grown in pots are under different nutritional stresses than soil-grown plants. In addition, both light intensity and temperature are very different in the greenhouse than in the field. Light induces numerous developmental pathways which could be affected by disruption of *TLS1* gene function. Temperature can affect process such as protein folding and complex formation as well as enzyme activity. There is a large temperature difference at night. The average low temperature for western Oregon is only 12° C during the growing season, while the greenhouse is maintained at a minimum temperature of 20°. If the *tls1* mutation causes production of an abnormal protein, it may retain more activity under greenhouse conditions than field conditions. Alternatively,

loss of the *TLS1* gene product may be partially compensated for by other proteins, which have higher activity, or higher affinity for the TLS1 substrate(s) at higher temperatures. The effect of specific environmental conditions on the *tls1* phenotype can be assessed in future studies.

The observations in this study are consistent with *TLS1* playing a role in meristem partitioning and organ initiation. This is a particularly intriguing finding because *tls1* maps to the same location on chromosome 1 as a homologue of the *Arabidopsis* gene *Tousled* (Helentjaris et al., 1995). Mutants in *Tousled* have a similar phenotype, with mild vegetative defects and severe floral abnormalities. *Tousled* encodes a serine/threonine kinase which is essential for the appropriate initiation of floral organs (Roe et al., 1993). Recently, several families containing probable *Mu* insertions in the maize *TOUSLED-LIKE KINASE* genes have been identified. Future studies will focus on the analysis of these families for mutant phenotypes as well as testing for allelism to *tassel-less1*.

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4. Conclusions and Future Directions

The studies described in this thesis cover preliminary molecular analysis of a small family of *TOULSED-LIKE KINASE (TLK)* genes in maize as well as a descriptive study of the maize mutant *tassel-less1*, which may represent a lesion in one of the *TLK* genes. By approaching the question of *TLK* function in maize from both the molecular and organismal levels, this work should contribute to an understanding of the role of the *TLK* genes and *TASSEL-LESS1* in maize development. Through detailed analyses of specific genes such as these, it will ultimately be possible to construct models of how different genes interact to coordinate developmental processes in plants. The *TLK* gene family appears to be part of a highly conserved class of kinases which have been found in a diverse array of multicellular eukaryotes. By studying the *TLK* genes, it may be possible to address broad evolutionary issues as well as the details of plant development.

Molecular Analysis of the *TLK* Genes

I have cloned and sequenced portions of the three *TOUSLED-LIKE KINASE* genes from maize. The sequenced portions of all three genes show remarkable similarity to each other and to the catalytic domain of TOUSLED kinase from *Arabidopsis*. The maize *TLK* genes fall into two distinct classes; two genes are more similar to each other than either is to the third gene. Expression data indicate that both classes of genes are expressed throughout the plant. In addition to the extreme conservation of *TOUSLED*-like genes between maize and *Arabidopsis*, database searches have revealed the presence of homologues in much more distantly related eukaryotic species. Clearly, the *TLK* genes are members of a larger class of conserved kinases. Based on the high degree of conservation of *TOUSLED*-like genes from such distantly related species, it is likely that this class of kinases performs some essential function in multicellular organisms.

Members of the Rivin lab are continuing to screen for genomic clones which extend further upstream. Isolation of new clones will yield information on both the

complete catalytic domains and any non-catalytic regions which are present in the maize *TLK* genes. Tim Helentjaris and colleagues (Pioneer HiBred International) have recently provided some additional cDNA clones which they have isolated and sequenced. Preliminary sequence data indicate that two of these clones overlap the 5' portion of genomic clone MTK4, but extend further upstream. The region covered is within the highly conserved catalytic domain, so these new clones should be useful in isolating upstream regions of the other genes, as well as in providing additional information about the MTK4 gene.

Obtaining more sequence data will be valuable for several reasons. Additional sequence information will allow further comparisons of the individual *TLK* genes to each other and to *TOUSLED.* It will be particulary interesting to see whether the high degree of conservation extends into the non-catalytic region. It is possible that the *TLK* genes are divergent in this region, allowing individual regulation through interactions with specific protein partners. Ultimately, full-length clones will be useful in conducting biochemical studies of *TLK* function and in isolating interacting proteins.

Divergent regions will be useful in making gene specific probes and primers. In additions to the possibility of divergent non-catalytic domains, it is likely that upstream introns have diverged. Gene-specific probes will allow the cloned sequences to be assigned to specific chromosomal locations. This information will be valuable in the isolation and characterization of mutants in the *TLK* genes, and potentially could offer some insight into the origins of this duplicated gene family.

Gene-specific probes and primers will also be helpful in conducting further studies on the expression of the *TLK* genes. It remains to be determined whether all the *TLK* genes are expressed. The development of gene specific probes and primers will be useful in gaining more information about the specific expression patterns of each gene. This will be helpful in elucidating the functional relationship of the various *TLK* genes.

Mutant Analysis

Mutant analysis will be a very useful tool for studying the roles of the maize *TLK* genes individually and as a group. The *tassel-less1 (tls1)* mutant of maize, described in the previous chapter, is one possible *TLK* mutant. The *tls1* gene maps to the same chromosomal location as one of the *TLK* genes (Helentjaris et al., 1995) and may represent a lesion in this gene. Mutant plants display variable vegetative defects, ranging from abnormally stiff upper leaves, to the production of unusual, small spike-like leaves and a dramatic reduction in overall height and nodes produced. *tls1* plants consistently fail to produce a fertile tassel, and produce greatly reduced ears with limited fertility. The severity of the *tls1* phenotype is strongly affected by environmental conditions. The *tls1* phenotype may be due to the disruption of a gene which is necessary for proper meristem partitioning, maintenance, or organ initiation. The phenotype of severe reproductive abnormalities accompanied by some vegetative defects is similar to that of the *Arabidopsis tousled* mutant (Roe et al., 1993), and is consistent with the hypothesis that *tls1* represents a mutant in a *TLK* gene.

Despite these similarities, maize has a very different architecture than *Arabidopsis*, and the *TLK* genes may show some functional overlap. Therefore, accurately predicting a mutant phenotype is difficult. These problems would complicate a traditional mutant screen based on phenotype. However, a reverse genetics system has been established in maize. The Trait Utility System for Corn (TUSC) screen allows researchers to identify lines containing *Mutator (Mu)* transposon insertions into any gene with known sequence (Meeley and Briggs, 1995). Scientists at Pioneer HiBred International have developed a large collection of plants containing *Mu* insertions throughout the genome. DNA and self-pollinated seeds were collected from each plant. In an automated PCR-based screen, scientists at Pioneer use gene-specific primers in combination with primers to the *Mu* terminal repeats to detect insertional mutants in specific genes (Figure 4.1). Only DNA from plants containing an insertion in a particular

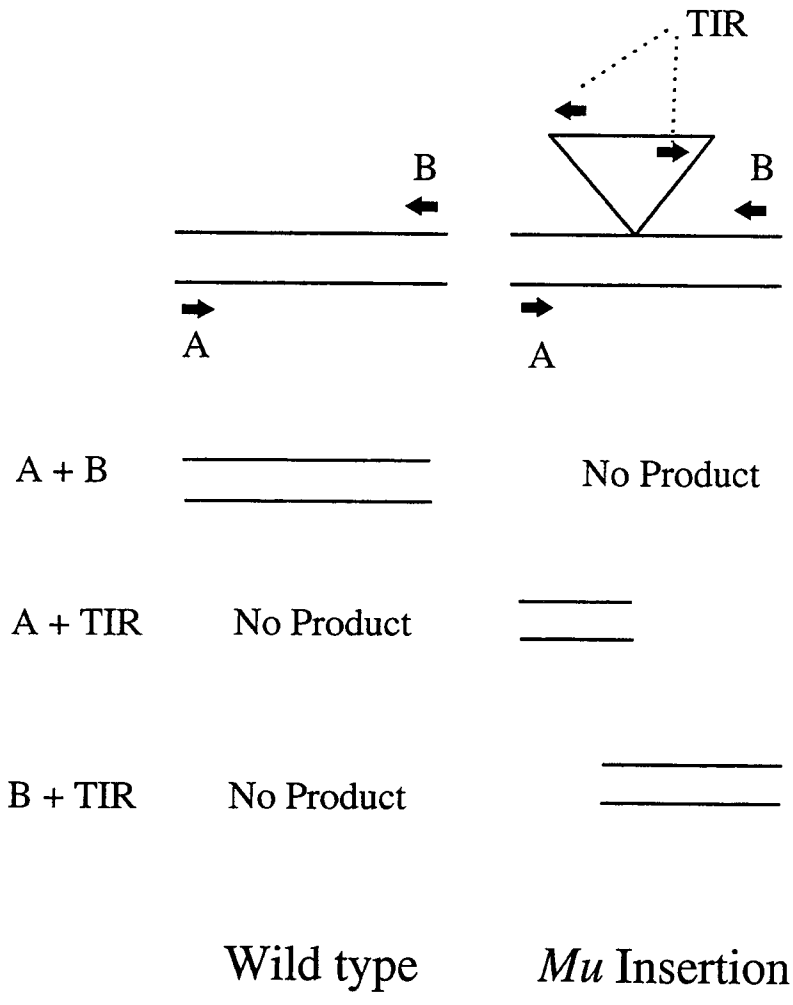

Figure 4.1 The TUSC reverse genetics screen uses gene-specific primers (indicated by A and B) in combination with *Mu* terminal inverted repeat primers (TIR). In this example, primers A and B amplify a specific fragment when used with DNA from a wild type plant. In plants which contain a *Mu* insertion between A and B, both A and B in combination with TIR amplify fragments, while primers A and B together amplify a very large product or no product.

gene will give amplification products when amplified with one gene specific primer and the *Mu* terminal repeat primer. In a collaboration with Pioneer, *TLK* primers B and D were used (described in Chapter 2) to identify nine families with putative insertions into a *TLK* gene.

Seeds from six TUSC families are currently growing in the greenhouse. There are numerous *Mu* elements throughout the genome in all the TUSC lines, so it is premature to propose a link between any of the wide array of abnormal phenotypes in these families and mutations in the *TLK* genes. However, these plants are being outcrossed and molecular analysis is underway to determine which individuals contain insertions into a *TLK* gene. Any positive PCR products will be cloned and sequenced, allowing determination of which gene contains the insertion as well as the position of the insert within the gene.

PCR screens will allow TUSC plants and their progeny to be accurately assigned a genotype at the *TLK* loci. This will permit the identification of individuals which are heterozygous or homozygous for an insertion, facilitating mutant analysis. In addition, it will permit positive identification of plants carrying insertions, even if mutant phenotypes are difficult to detect. If the *TLK* genes are functionally redundant, then mutations in a single *TLK* gene may have minimal effects on a plant. However, it will be possible to construct double or triple mutants by following the PCR genotypes. Construction of double and triple mutants will also offer insight into the interactions between the different *TLK* genes. In addition, *TLK* mutants will be crossed to *tls1* plants to test for allelism.

Future Directions

Ultimately, future studies involving the *TLK* genes could explore a wide variety of biological questions. Because the *TLK* genes are so highly conserved, they may be useful for phylogenetic and evolutionary studies. Comparisons of maize *TLK* sequences with those of closely related species may offer insight into the origins of this redundant

gene family in maize. If the gene duplication was in part due to a polyploid event, comparison of *TLK* genes in members of the genus *Zea* and in closely related taxa may shed some light on the origins of maize and its congeners.

The redundancy of this gene family also opens up various genetic and developmental questions. How are the individual members related functionally, and do they play independent or overlapping roles in maize development? One possibility is that the various TLK proteins interact with each other to regulate overall TLK activity. The combination of molecular sequence information and available mutants will be ideal for addressing these issues. Biochemical studies of TLK proteins can address the details of the interactions of these kinases with each other and with other proteins. The universality of *TLK* function could be tested by transforming *Arabidopsis tousled* mutants with the *TLK* genes. If the TLK proteins are as highly conserved as partial gene sequences suggest, one or all of the maize genes may be able to rescue *tousled* mutants. In maize, analysis of double and triple mutants in the *TLK* genes, as well as in combinations with other developmental mutants, can yield insight into the role of the *TLK* genes in the whole organism. While some studies of this nature have been done in *Arabidopsis* (Roe et al., 1997b), both the unique architecture of maize and the availability of mutants in different types of genes make mutant analysis a worthwhile pursuit in maize as well.

The *TLK* gene family of maize provides an excellent context in which to study plant development. The *TLK* genes are part of a family of highly conserved kinases in eukaryotes and may play a role in some fundamental processes common to all multicellular organisms. Molecular data will permit studies of the mechanisms of *TLK* gene regulation and protein function. It is possible that the findings of these studies can be extended to other organisms. Mutant analysis provides a means for studying the role of these kinases and their interactions with each other and with other gene products in an

intact organism. In addition, overall questions of maize genome evolution and the evolution of multiple gene families can be addressed by studying the maize *TOUSLED-LIKE KINASES.*

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