**thick tassel dwarf1 encodes a putative maize ortholog of the Arabidopsis CLAVATA1 leucine-rich-repeat receptor-like kinase**

Peter Bommert1,*, China Lunde2, Judith Nardmann1, Erik Vollbrecht2,‡, Mark Running2,‡, David Jackson3, Sarah Hake2 and Wolfgang Werr1,§

1Institut für Entwicklungsbiologie, Universität zu Köln, Gyrhofstrasse 17, D-50923 Köln, Germany
2Plant Gene Expression Center, Agricultural Research Service – USDA, 800 Buchanan St, Albany, CA 94710, USA
3Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

*Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
‡Present address: Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA
†Present address: Donald Danforth Plant Science Center, 975 Warson Rd, St Louis, MO 63146, USA
§Author for correspondence (e-mail: werr@uni-koeln.de)

Accepted 17 December 2004

Development 132, 1235-1245
Published by The Company of Biologists 2005
doi:10.1242/dev.01671

**Summary**

Development in higher plants depends on the activity of meristems, formative regions that continuously initiate new organs at their flanks. Meristems must maintain a balance between stem cell renewal and organ initiation. In fasciated mutants, organ initiation fails to keep pace with meristem proliferation. The *thick tassel dwarf1* (*td1*) mutation of maize affects both male and female inflorescence development. The female inflorescence, which results in the ear, is fasciated, with extra rows of kernels. The male inflorescence, or tassel, shows an increase in spikelet density. Floral meristems are also affected in *td1* mutants; for example, male florets have an increase in stamen number. These results suggest that *td1* functions in the inflorescence to limit meristem size. In addition, *td1* mutants are slightly shorter than normal siblings, indicating that *td1* also plays a role in vegetative development. *td1* encodes a leucine-rich-repeat receptor-like kinase (LRR-RLK) that is a putative ortholog of the *Arabidopsis* CLAVATA1 protein. These results complement previous work showing that *fasciated ear2* (*fe2*) encodes a CLAVATA2-like protein, and suggest that the CLAVATA signaling pathway is conserved in monocots. *td1* maps in the vicinity of quantitative trait loci that affect seed row number, spikelet density and plant height. We discuss the possible selection pressures on *td1* during maize domestication.

Key words: Meristem, Stem cells, Inflorescence, *thick tassel dwarf1*, Maize

**Introduction**

Plant growth is characterized by reiterative developmental events that depend upon the activity of meristems. The aerial part of the plant body is elaborated from the shoot apical meristem (SAM), which produces leaves, stems and axillary meristems in a repetitive manner (Galinat and Naylor, 1951; Steves and Sussex, 1989). Established during embryogenesis, the SAM is self-maintained as a pool of undifferentiated cells during the plant life cycle. A small group of pluripotent stem cells is maintained apically, in the central zone (CZ) of the SAM. Surrounding the CZ is the peripheral zone (PZ), where cells are recruited for elaboration of lateral organs that will form the plant body (reviewed by Kerstetter and Hake, 1997).

Analysis of mutations has identified a few of the genes that control meristem function. One class of mutations is perturbed in meristem formation and/or maintenance. For example, in strong alleles of *shootmeristemless* (*stm*), seedling development stops after formation of the two cotyledons (Barton and Poethig, 1993), whereas analysis of weak *stm* alleles indicates a role for *STM* throughout all stages of shoot meristem development (Clark et al., 1996; Endrizzi et al., 1996). The maize homolog *knotted1* (*kn1*) is also required for shoot meristem establishment and maintenance (Kerstetter et al., 1997; Vollbrecht et al., 2000). The *kn1* and *STM* homeobox genes are expressed throughout the SAM, except in the cells that are destined to become the next leaf primordium (Smith et al., 1992; Jackson et al., 1994; Long et al., 1996). Plants carrying loss-of-function mutations at the *WUSCHEL* (*WUS*) locus also fail to maintain the SAM (Laux et al., 1996). *WUS* encodes a novel subtype of homeodomain protein that is nuclear localized and predicted to act as a transcription factor (Mayer et al., 1998). *WUS* mRNA is first detected when embryos reach the 16-cell stage and becomes localized to a group of cells that underlies the presumptive stem cells, suggesting that it may act in promoting stem cell fate non-autonomously.

A second class of mutations displays meristem enlargement, which can lead to fasciation in extreme cases. Fasciation, derived from the Latin word *fascis*, meaning ‘bundle’, is a reflection of increased proliferation. Fasciated variants are reported to have increased crop yields, for instance by increasing fruit size in tomato (Luckwill, 1943; Zielinski, 1945). Fasciation also has a long history of study in maize (Emerson, 1912): *Fascicled ear1* (*Fas1*), *fasciated ear1* (*fe1*), *fasciated ear2* (*fe2*) and *thick tassel dwarf1* (*td1*) are
Development characterization of the meristems during maize development (Taguchi-Shiobara et al., 1997; Jackson and Hake, 1999).

In Arabidopsis, mutations in the three CLAVATA loci (CLV1, CLV2 and CLV3) result in a phenotype opposite to that of wus and stm (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). The embryonic SAM of clv-mutants is slightly larger than that of wild-type embryos. During postembryonic growth, the SAM gradually increases in size, resulting in altered phyllotaxy and supernumerary floral organs. These mutant phenotypes indicate a role for the CLV genes in restricting the size of the stem cell population. All three CLV genes have been identified and their protein products probably constitute a single receptor-ligand complex, consistent with the three mutations having an almost identical phenotype (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999). The CLV1 protein is a receptor-like kinase composed of a leucine-rich repeat-containing extracellular domain with putative receptor function and a cytoplasmic Ser/Thr kinase domain linked through a transmembrane domain (Clark et al., 1997). CLV2 is structurally similar to CLV1 but lacks a cytoplasmic kinase domain (Jeong et al., 1999). CLV3 encodes a 96 amino acid polypeptide that contains a putative signal sequence and is secreted (Rojo et al., 2002). CLV3 transcripts are preferentially found in the outer L1 and L2 layers, whereas CLV1 is expressed specifically in the L2 and L3 layers of the SAM. CLV2 is expressed ubiquitously (Jeong et al., 1999). The WUS and CLV pathways are interdependent (Brand et al., 2000; Schoof et al., 2000); WUS promotes stem cell fate, whereas CLV signaling restricts the number of stem cells (reviewed by Sharma et al., 2003a).

The maize ear comprises a series of meristem types (reviewed by McSteen et al., 2000) and changes in meristem size and identity may underlie the yield increases brought about during the domestication of maize (Kellogg and Bircher, 1993) from teosinte inflorescences (Beadle, 1980; Doebley, 1992). Whereas seeds are arranged in single alternating rows in teosinte, present-day maize lines have a polystichous repeat-containing extracellular domain with putative receptor function and a cytoplasmic Ser/Thr kinase domain linked through a transmembrane domain (Clark et al., 1997). CLV2 is structurally similar to CLV1 but lacks a cytoplasmic kinase domain (Jeong et al., 1999). CLV3 encodes a 96 amino acid polypeptide that contains a putative signal sequence and is secreted (Rojo et al., 2002). CLV3 transcripts are preferentially found in the outer L1 and L2 layers, whereas CLV1 is expressed specifically in the L2 and L3 layers of the SAM. CLV2 is expressed ubiquitously (Jeong et al., 1999). The WUS and CLV pathways are interdependent (Brand et al., 2000; Schoof et al., 2000); WUS promotes stem cell fate, whereas CLV signaling restricts the number of stem cells (reviewed by Sharma et al., 2003a).

The maize ear comprises a series of meristem types (reviewed by McSteen et al., 2000) and changes in meristem size and identity may underlie the yield increases brought about during the domestication of maize (Kellogg and Bircher, 1993) from teosinte inflorescences (Beadle, 1980; Doebley, 1992). Whereas seeds are arranged in single alternating rows in teosinte, present-day maize lines have a polystichous arrangement of seeds consisting of 8 to 18 rows, and in some varieties up to 36 rows (USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network; http://www.ars-grin.gov/cgi-bin/npgs/). The increased number of axillary meristems in maize is thus a major acquired character, which has been instrumental in the domestication of maize as a crop plant. It would be of interest to understand how ear size relates to the stem cell concept and reflects modulations in the size of the stem cell population, since the increase in seed row number of the maize ear is reminiscent of supernumerary lateral organs that arise due to the increase in seed row number of the maize ear. The maize ear in the plant kingdom could reflect permanent modifications of specific signaling pathways, such as the CLV pathway, may have contributed to the domestication of crop plants. Here we describe a reverse genetics approach to functionally characterize CLV1 homologs from maize. The maize gene most similar to CLV1 encodes thick tassel dwarf1 (td1). The fasciation phenotype of td1 mutant alleles provides further evidence that meristem size in maize is controlled by the CLV signaling pathway. However, significant qualitative and quantitative differences exist between the td1 and CLV1 expression patterns. These differences emphasize that it is important to study developmental processes in agronomic crops such as maize, as well as in model species such as Arabidopsis.

Materials and methods

Genetics

The glassfingers (gfl) mutation (now called td1-gfl) was mapped using waxy translocations to chromosome 5S. Complementation crosses with td1-Ref (obtained from the Maize Genetics Cooperative Stock Center) confirmed allelism. Both td1-gfl and td1-Ref were introgressed into B73 and W22 for phenotypic observations. Additional alleles (see Table 1) were obtained by crossing td1-Ref or td1-gfl homozygotes as males onto Mutator stocks. Screens were carried out in Davis, California and in Illinois. td1-mum3 was identified in a F2 family of the Maize Gene Discovery Project (Lunde et al., 2003). A F2 family segregating for fea2, td1 double mutants was created by crossing individuals homozygous for fea2-0 and td1-gfl respectively, and selfing the resulting progeny. Both mutations were in the B73 background.

Isolation of the full-length ZmKin5 gene

By using the degenerate primers (5′-GG5 AAR GGM GGM GC5 GGR ATY GTS TA-3′; 5′-TTR GCR AGS ACR AAR TCR GCR AC-3′), and genomic DNA as a template, five CLV1 related kinase sequences, designated as ZmKin1 to 5, have been isolated. A cDNA library constructed in Zap II (Stratagene) using mRNA from leaf-stage 4 embryos [staged according to Abbe and Stein (Abbe and Stein, 1954)] was screened using individual ZmKin5 specific 5′-biotinylated forward and reverse primers (5′-GTTGGAGACAAAGCCGAGCAGC-3′, 5′-GCCGACACCCTTCTCCCCCACCAG-3′) in linear PCR. PCR products from these reactions were purified using Dynabeads according to the manufacturer’s instructions (DYNALT) and amplified exponentially with nested ZmKin5 primers and vector-specific primers. This cDNA-based strategy yielded partial sequence information extending from the last extracellular LRR-repeat through the kinase domain to the stop codon, plus sequences of the 3′ untranslated region (UTR). To obtain the full length ZmKin5 sequence, a genomic library of genotype HD5 × HD7 in vector EMBL3 SP6/T7 (Clontech) was screened with a 32P-labeled ZmKin5 fragment representing 450 bp of the less conserved carboxy terminal part of the kinase domain plus 170 bp of the 3′ UTR. Genomic maize DNA from positive plaques was isolated and cloned into pBlueScript II SK (Stratagene) and sequenced.

td1 expression analysis

Maize tissues were ground to a fine powder in liquid nitrogen and RNA was purified using the Trizol procedure (Invitrogen). PolyA+ RNA was isolated using oligo-DT cellulose columns or a Dynabeads mRNA purification kit (DYNALT). RNA electrophoresis, transfer to a...
Hybridization with the $^{32}$P-labeled 620 bp probe (450 bp of kinase coding sequence + 170 bp 3' UTR) was performed at 42°C overnight. Blots were washed in 0.1 x SSC, 0.1% SDS at 65°C and autoradiographed on Kodak X-ray film or by visualization on a PhosphorImager (Molecular Dynamics).

For real-time PCR analysis, tissue-specific cDNAs were synthesized using the SuperScriptIII™ reverse transcriptase according to the manufacturer's instructions (Invitrogen). Real-time RT-PCR analysis was performed on a GeneAmp® 5700 Light Cycler. The on-line detection of amplification rates was performed using SYBR® Green (Applied Biosystems). Normalization was performed against Ubiquitin with primers: UBI-5′-TAAGCTGCGATGCTGCT-GG-3′ and UBI-3′ (5′-CTGAAAGACAGAGCAATATGAGACGCA-CAG-3′). Knotted1-specific amplicons were generated with primers: kn1-3′ (5′-ACCCAGGCTCCTGAAGTTGATG-3′) and kn1-5′ (5′-CTAGGCCGTGGGGTGTGAAATGCT-3′). The td1-specific primers were: td1-Ref-5′ (5′-GCTGCTGCGAGCTTCA-CA-3′) and td1-Ref-3′ (5′-GAGCGGTGACGGCAAAAG-3′).

Female and male inflorescences for in-situ hybridizations were dissected and fixed at 4°C overnight in 4% formaldehyde with 0.1% TritonX100 (Sigma) in PBS, dehydrated in an ethanol series, cleared in Histoclear (Roth) and embedded in paraffin wax (Paraplast plus, Sigma). Sections 7-9 µm thick were mounted on coated slides (SuperFrost®Plus). The td1 probe (620 bp, see above) was cloned into pBluescript II SK (Stratagene). The resulting amplicons were cloned and Platinum Pfx proofreading polymerase (Invitrogen) or Fisher Taq polymerase. DNA sequence analysis followed cloning and Platinum Pfx proofreading polymerase (Invitrogen) or by visualization on a PhosphorImager (Molecular Dynamics).

Scanning electron microscopy

Scanning electron microscopy was performed according to the protocol outlined in Sommer et al. (Sommer et al., 1990) and images were processed digitally.

PCR on td1 alleles and double mutant families

Genomic DNAs of homozygous td1-Ref and td1-Nickerson plants were used as templates in PCR reactions with primers 5′-CATGCCACCTGGCCTTGAC-3′ and 5′-GAGCGGTGACGGCAAAAG-3′ and Platinum Pfx proofreading polymerase (Invitrogen) or Fisher Taq polymerase. DNA sequence analysis followed cloning into the pCR2.1-TOPO vector (Invitrogen) or pGemT-EZ vector (Promega).

The Mu-alleles were amplified using platinum Pfx proofreading polymerase (Invitrogen) using forward or reverse td1-primers ~500 bp apart and covering the entire coding region, and Mu-specific primers 5′-GCCCTCAATTTGCTGAATC-3′ or Pioneer’s 9242 Mu-specific primer 5′-AGAGAAGCCACGCAGCCTGCCTAATGCTAC-3′ (Blauth et al., 2001) located in the terminal inverted repeat sequences of the transposable element. The resulting amplicons were cloned and sequenced to determine the Mu-insertion site.

Genotypes of individuals in the segregating F2 families used for double mutant analysis were determined by using primers specific to fea2 (fea2-5′: 5′-GCTGCTGGCAGACCTCTACA-3′; fea2-3′: 5′-GACGATGCGACGGCAAAAG-3′) or td1 (td1-5′: 5′-TCCACGACACATGTCCTAC-3′; td1-3′: 5′-CAACACCTGTAGTCGCC-3′) and the 9242 primer. Wild-type chromosomes were detected by amplifying with gene-specific primers flanking the known Mu insertion sites in each allele.

Results

**td1 mutant phenotype**

Maize, a monocoeous plant, develops distinct male and female inflorescence structures. The terminal shoot apical meristem gives rise to the tassel, the male inflorescence, whereas axillary meristems, initiated in leaf axils, produce the female inflorescences, or ears. The recessive *thick tassel dwarf1* allele (*td1-ref*) was first reported by E. G. Anderson and has been donated to the MGC stock center (M. Sachs, personal communication). *td1* mutants are fertile, and the most pronounced phenotypic alterations are detected in the inflorescences, although plants are also noticeably shorter in some inbred backgrounds.

The mature tassel of maize consists of a central rachis and a few long branches at the base, with each axis lined with rows of spikelets. In the tassel of *td1* mutant plants, the main rachis was thicker than normal, and this was more obvious when spikelets were removed (Fig. 1A,B). This increase in rachis diameter was reflected in a higher spikelet density. Whereas normal sibling tassels on average produced around 14 spikelets/cm on the main rachis, *td1* mutant tassels could produce more than 20 spikelets/cm (Table 2). This increase in spikelet density was proportionally similar in tassel branches (Table 2). However, the total number of spikelets per tassel was not always increased, as thickening of the main axis in *td1* tassels was sometimes correlated with a reduction in length (Table 2). The spikelets are arranged in pairs, with a pedicellate and sessile spikelet (Fig. 1E). Male spikelets contain two glumes that surround two florets. Each floret contains three stamens, two lodicules and an aborted pistil (Cheng et al., 1983). A closer inspection of male spikelets in *td1* mutants showed a high frequency of single spikelets instead of paired spikelets (Fig. 1C; Table 2). The spikelets themselves were occasionally irregular, with extra glumes (Fig. 1C), and florets often contained more than three stamens (Fig. 1D, Table 2). These supernumerary floral organs may have resulted from an enlarged floral meristem.

Development of the ear was also significantly altered in *td1* mutants. During normal ear development, the inflorescence meristem (IM) initiated spikelet pair meristems (SPMs) in a...
regular phyllotaxy and the SPMs later branched to generate a pair of spikelet meristems (SMs) that were aligned in adjacent vertical rows, which corresponded to rows of seeds in the mature cob (Fig. 2A,B). Mutant td1 ears were fasciated and wider than corresponding wild-type ears (Fig. 2A). The consequences varied among siblings; in some cases, enlargement of the IM led to a ridge-like structure (Fig. 2C). In other cases, the IM was bifurcated, as shown in Fig. 2D, which resulted in branched ears at maturity, or showed ring fasciation (Fig. 7B).

In summary, loss of function in the tassel caused thickening of the rachis, a higher spikelet density and extra glumes and stamens. The phenotype was more pronounced in the ear, where fasciation and bifurcation of the IM was accompanied by supernumerary and larger SPMs that branched to produce clusters of SMs of abnormal size. Additionally, a weak vegetative phenotype could be observed in td1 mutant plants. These phenotypes are all consistent with the observation that td1 functions to limit meristem size.

Isolation of CLAVATA1 homologous genes from maize

Like most eukaryotic protein kinases, leucine-rich repeat receptor-like kinases (LRR-RLKs) share 12 highly conserved subdomains within their intracellular catalytic kinase domains. These subdomains have been designated I-XII (Hanks and Quinn, 1991) and invariant amino acid residues in individual subdomains provide excellent anchor points to design degenerated PCR primers. Based on invariant amino acids in subdomains I and VII and guided by flanking sequences in CLV1 (GI: 15222877), and its closest relatives in the
Arabidopsis or rice genomes (GI: 15239123 and GI: 18855025) respectively, PCR primers were designed (for sequences see Materials and methods). The relative position of these primers in the kinase domain of CLV1 are indicated in Fig. 3A.

These primers amplified five CLV1-like kinase sequences from genomic maize DNA, which we designated ZmKin1-5. To estimate phylogenetic relationships, we extracted highly related LRR-RLK sequences from the National Center for Biotechnology Information (NCBI; http://www3.ncbi.nlm.nih.gov) or DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) databases. The CLUSTALW program was used to align the kinase domain sequences and estimation of the phylogenetic relationships was performed with the Minimum Evolution algorithm provided by the MEGA2.1 software (Kumar et al., 2001). The resulting phylogenetic tree is depicted in Fig. 3B and shows two distinct CLV1-like subfamilies, which we call subfamily A and B, substantiated by high bootstrap values. CLV1 is in a distinct branch of subfamily A, separated from other dicot sequences. Two monocot genes, the maize Kin5 and the rice CLVI ortholog FON1 (Suzaki et al., 2004), are also found within subfamily A.

Three other maize kinases, ZmKin1-3, fall into subfamily B, which also splits into monocot and dicot clades. This splitting validates the phylogenetic reconstruction and suggests that a common ancestral gene may have duplicated to give rise to two separate lineages of LRR-RLK genes prior to the divergence of monocots and dicots. We used the more distantly related ZmKin4 to root the tree. This phylogeny

Table 3. Ear spikelet number and leaf number in segregating td1-glf/fea2-Ref double mutant families

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of spikelets</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>td1/td1; fea2/fea2</td>
<td>123.5 (23.3; 2)</td>
<td>14.8 (1.1; 5)</td>
</tr>
<tr>
<td>+/+; fea2/fea2</td>
<td>68.5 (22.4; 6)</td>
<td>17.8 (1.0; 6)</td>
</tr>
<tr>
<td>td1/td1; +/+</td>
<td>53.7 (16.7; 6)</td>
<td>17.8 (0.8; 6)</td>
</tr>
<tr>
<td>td1/+; fea2/+</td>
<td>15.4 (1.9; 15)</td>
<td>19.8 (0.7; 10)</td>
</tr>
<tr>
<td>+/+; +/+</td>
<td>15.5 (1.5; 14)</td>
<td>19.6 (1.0; 7)</td>
</tr>
</tbody>
</table>

 Numbers are mean values, with standard deviations in parentheses, followed by the number of individuals measured.

*Number of spikelets were counted from a cross-section of the ear at the widest point. Data are combined for plants grown in the field and greenhouse.

Values are from plants grown in the greenhouse.

Development

Arabidopsis or rice genomes (GI: 15239123 and GI: 18855025) respectively, PCR primers were designed (for sequences see Materials and methods). The relative position of these primers in the kinase domain of CLV1 are indicated in Fig. 3A.

These primers amplified five CLV1-like kinase sequences from genomic maize DNA, which we designated ZmKin1-5. To estimate phylogenetic relationships, we extracted highly related LRR-RLK sequences from the National Center for Biotechnology Information (NCBI; http://www3.ncbi.nlm.nih.gov) or DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) databases. The CLUSTALW program was used to align the kinase domain sequences and estimation of the phylogenetic relationships was performed with the Minimum Evolution algorithm provided by the MEGA2.1 software (Kumar et al., 2001). The resulting phylogenetic tree is depicted in Fig. 3B and shows two distinct CLV1-like subfamilies, which we call subfamily A and B, substantiated by high bootstrap values. CLV1 is in a distinct branch of subfamily A, separated from other dicot sequences. Two monocot genes, the maize Kin5 and the rice CLVI ortholog FON1 (Suzaki et al., 2004), are also found within subfamily A.

Three other maize kinases, ZmKin1-3, fall into subfamily B, which also splits into monocot and dicot clades. This splitting validates the phylogenetic reconstruction and suggests that a common ancestral gene may have duplicated to give rise to two separate lineages of LRR-RLK genes prior to the divergence of monocots and dicots. We used the more distantly related ZmKin4 to root the tree. This phylogeny

Development
identified ZmKin5 as the most likely maize ortholog of CLV1 from Arabidopsis.

ZmKin5 gene structure

To isolate the full-length ZmKin5 coding sequence, we screened a maize embryo cDNA library with PCR primers from the kinase domain and the vector. Although successful for the 3’ end of the gene, this strategy failed for the 5’ end. We therefore isolated a ZmKin5 genomic clone by screening with a ZmKin5-specific probe comprised of the carboxy terminus of the kinase domain and 3’ UTR sequences. Analysis of the genomic clone indicated that the predicted ZmKin5 open reading frame is 2991 bp in length. The predicted size of 3262 bp from the ATG translation start codon to the polyA tail was consistent with a single ~3.4 kb transcript detected in RNA gel blot experiments (Fig. 5A). A single intron in the kinase domain was conserved in position in ZmKin5 and CLV1. The amino terminal leucine-rich repeat coding sequences and transmembrane domains were devoid of intervening sequences in maize, as in Arabidopsis. The predicted protein sequences encoded by ZmKIN5 and CLV1 are compared in Fig. 4A.

Motif searching using the SMART algorithm (http://smart.embl-heidelberg.de/) predicted a hydrophobic 26 amino acid residue ER targeting signal peptide at the amino terminus, which according to the TargetP cleavage site prediction program (http://www.cbs.dtu.dk/services/TargetP/) may be processed. This signal peptide is followed by 21 tandem copies of 23-25 amino acid residue long leucine-rich repeats (LRRs) that, as in CLV1, are flanked by pairs of spaced cysteines. According to the classification of Shiu and Bleeker
(Shiu and Bleeker, 2001), both 21 LRR motifs of ZmKin5 and CLV1 belong to the LRR-XI subfamily of LRR motifs. The LRR domain is followed by a predicted transmembrane domain (ZmKin5 residues 648-665). The intracellular kinase domain comprises the characteristic 12 subdomains with all invariant amino acid residues conserved in comparison to other eukaryotic protein kinases (Hanks and Quinn, 1991) (Fig. 4A). Conserved motifs in subdomains VIb (HRDVKSNN) and VIII (GSGYGIAPEY) predict ZmKin5 functions as a serine/threonine protein kinase (Fig. 4A). Amino acid similarity and identity to CLV1 are 79 and 72%, respectively, within the kinase domain and 66 and 58%, respectively, over the entire predicted proteins.

**ZmKin5 is encoded by the td1 locus**

We mapped ZmKin5 using the BNL T232/CM37 recombinant inbred lines (Burr et al., 1988) to chromosome 5 (bin 5.03) between markers uaz77 and ici287, close to the td1 locus. To test the relationship between ZmKin5 and td1, we isolated and sequenced ZmKin5 genomic DNA from homozygous td1-Ref plants. The td1-Ref allele harbors a cluster of four base pair substitutions surrounding a 15 bp deletion in the predicted ER targeting signal sequence (Fig. 4A). This mutation is predicted to abolish ER targeting of the protein by the target algorithm. Altered localization of the ZmKin5 receptor kinase might explain the td1-Ref mutant phenotype. The same sequence differences within the putative ER targeting signal were found in the td1-Nickerson allele, although both td1 alleles have been maintained as distinct mutations in the Maize Stock Center, and are believed to have arisen independently (M. Sachs, personal communication). Our data indicate, however, that both mutations may share a common origin.

To determine whether or not ZmKin5 corresponded to the td1 locus, we generated additional td1 mutant alleles. td1-Ref and td1-glf were crossed as males onto Mutator (Robertson, 1978) plants and the resulting progeny were screened for the td1 mutant phenotype. Twelve putative mutant td1 alleles were found from screens of approximately 150,000 plants. DNA from putative new alleles was analyzed by PCR for the presence of Mu elements in the ZmKin5 sequence (see Materials and methods). Among 12 alleles examined, five Mu insertion alleles were identified, referred to as td1-mum1 to -mum5 (Table 1; Fig. 4B). Sequencing of the PCR amplicons revealed Mu insertions in the first exon, disrupting the extracellular LRR domain for td1-mum1, td1-mum2 and td1-mum3. The Mu element in td1-mum4 was located within the intracellular kinase domain, and in td1-mum5 was 29 bp downstream of the splice donor site in the single intron. We also found that the td1-glf allele had a Mu insertion in the first exon. This allele also carried the same polymorphisms as the td1-Ref allele within the putative ER targeting signal sequence. Southern hybridizations confirmed co-segregation of a ZmKin5-specific Mu insertion with the td1 mutant phenotype in over 40 chromosomes (data not shown). In conclusion, six independently isolated td1 alleles were shown to contain Mutator element insertions in the ZmKin5 coding region, demonstrating that ZmKin5 corresponds to the td1 gene.

We also compared td1 transcript levels in immature ears of normal siblings and td1-glf homozygous mutant plants by RNA gel blot experiments. As shown in Fig. 5A, a larger, aberrant transcript was detected using a td1-specific probe in RNA from the td1-glf mutant. Additionally, real-time RT-PCR showed that the level of td1 RNA in td1-glf was reduced to 20% that of wild type, whereas the level of the knl meristem marker was slightly increased (Fig. 5B). This increase in knl RNA may reflect an increase in IM size or the presence of supernumerary SPMs. Sequence analysis of an RT-PCR product from td1-glf RNA revealed the presence of 180 nucleotides of the Mu terminal inverted repeat, which would lead to a frame shift and premature translation stop in the extracellular LRR domain. These results demonstrate that the Mu element insertion in td1-glf is spliced out incompletely, and that td1-glf may represent a null allele. In summary, by identifying five independent td1 Mu-alleles, we have shown that td1 is ZmKin5, a maize ortholog of CLV1.

**Expression pattern of the td1 gene**

Organ specificity of td1 transcription was determined via RNA gel blot hybridizations (data not shown) and quantified by real-time RT-PCR analysis (Fig. 6A). Normalized to ubiquitin transcript levels, the highest td1 transcript level was detected in the apex of the vegetative seedling, and was arbitrarily set at 100%. The RNA level was lower in the ear (58%) and tassel (43%) relative to wild type, whereas the level of the knl meristem marker was slightly increased (Fig. 6C). In contrast to CLV1 (Clark et al., 1997), a significant td1 transcript level was detected in young leaves (63%). Primer specificity for td1 was verified by direct sequencing of the PCR amplicons.

To analyze the expression pattern of td1 RNA at a finer resolution, in-situ hybridization experiments were performed with a td1-specific probe corresponding to the carboxy terminus of the kinase domain and 170 bp of the 3′ UTR. td1 transcripts were detected in the vegetative shoot apex in leaf primordia and leaves. The shoot meristem itself was free of detectable td1 expression (Fig. 6B). During reproductive development, weak td1 expression was detected in the three outermost layers of the IM, and on its flanks at positions of prospective SPMs (Fig. 6C,D). The expression in the IM was always significantly lower than in the emerging SPMs. td1 transcript levels remained high as SPMs produce SMs (Fig. 6D) and in the apex of the floral meristems in the L1, L2 and L3 layers (Fig. 6E,F). By contrast to the CLV1 pattern (Clark et al., 1997), but similar to that of FON1 (Suzuki et al., 2004), td1 transcripts were detected in primordia of glumes, lemmas
and stamens (Fig. 6E,F). Thus, the td1 RNA was not confined to meristematic cells but was also found in lateral organs of the ear, as in the vegetative shoot. td1 expression during male inflorescence development was identical to that of the female (data not shown).

**td1/fea2 double mutant analysis**

CLV1 and 2 are reported to act in a common pathway in Arabidopsis (Kayes and Clark, 1998), and this is supported by biochemical analysis (Jeong et al., 1999). To ask whether td1 and fea2, the maize orthologs of CLV1 and 2, respectively, act in a common pathway, we analyzed td1/fea2 double mutants. At early stages of ear development td1/fea2 double mutants (Fig. 7A) were strikingly similar to strong td1 mutants (Fig. 7B), in showing a severe ring fasciation, whereas fea2 mutant siblings were less strongly fasciated (Fig. 7C). At maturity, td1/fea2 double mutants showed additive or synergistic phenotypes, as they initiated twice as many rows of spikelets as either td1 or fea2 single mutants (Table 3), and their ears were reduced in length (Fig. 7D). The decrease in leaf number was also more pronounced in td1/fea2 double mutants compared with either single mutant (Table 3), indicating that, like td1, fea2 also functions during vegetative development.

Following the assumption that ear spikelet number correlates with inflorescence meristem size, our data indicate that td1 and fea2 do not act exclusively in a single pathway to control meristem size.

**Discussion**

The td1 gene of maize regulated meristem size during inflorescence development. The ears showed the strongest effect, with massively fasciated tips and additional and irregular kernel rows. Tassels of td1 mutants were also thicker, and the plants were slightly shorter, with fewer leaves. Using a combination of reverse genetics and targeted transposon tagging, we have identified the td1 gene as an ortholog of CLV1. td1 mutants were similar to fea2 mutants, and given that fea2 encodes a CLV2-like protein one could assume that FEA2 and TD1 may function in a common pathway, like CLV1 and
CLV2 in Arabidopsis. Our analysis, however, suggests that td1 and fea2 do not function exclusively in a single pathway, as the double mutants between the putative null alleles show some additive and synergistic phenotypes. Comparisons of the phenotypes and expression patterns revealed further similarities and differences between maize, rice and Arabidopsis CLV1-orthologs.

**Phenotypic consequences of td1 mutants**

The most conspicuous aspect of td1 mutants was the dramatic fasciation of the ear tip, due to an enlarged inflorescence meristem. Ear tips showed line or ring fasciation and were often bifurcated. The broadening of the tip led to extra rows of SPMs and extra SMs, which sometimes produced abnormal single spikelets. By contrast to the dramatic defect in the ear, tassels of td1 mutants appeared less affected. However, a closer analysis showed that spikelet density was increased by about 40% in the main rachis of the tassel, but it did not fasciate. Interestingly, knl null mutants reduced meristem size, and while knl mutant ears and tassels shared a set of meristem defects, only in the ear did the inflorescence meristem sometimes abort, leading to plants without ears (Kerstetter et al., 1997; Vollbrecht et al., 2000). Thus, the ear appeared to be more sensitive than the tassel to mutations that alter inflorescence meristem size.

Given the similarity of ear and tassel primordia at early stages of development (Cheng et al., 1983), it is interesting to speculate on the reasons for the differences between the mature tassel and ear of td1 mutants. One possibility is that the ear is more sensitive to changes in CLV signaling, due to the selective pressure placed on the ear during agricultural selection. Maize was domesticated from teosinte, in which the inflorescence rachis contains only a single alternating row of spikelets. By contrast, a typical maize ear contained 16-18 spikelet rows (Doebely and Stel, 1991). Evidence for a general relaxed control of meristem size in the ear also comes from the observation that it is not uncommon to find spontaneously fasciated ears, whereas such abnormalities are rare for the tassel (Neuffer et al., 1997). Interestingly, td1 maps near the location of a quantitative trait locus (QTL) for seed row number between the markers BNL6.25 and BNL5.02 (Doebely et al., 1990) and a QTL for tassel spikelet density on tassel branches (T. Rocheford, personal communication). The rice FON1 gene also encodes a CLV1 ortholog (Suzaki et al., 2004), and additional branches are made in rice fon1 inflorescences to a similar magnitude as the formation of extra spikelets in male td1 inflorescences, suggesting that TD1 and FON1 bear equivalent functions. This finding again strengthens the view that the maize ear reflects a unique structure among grass species, with a distinct genetic program from the tassel, possibly as a result of the intense selective pressure during domestication.

Other meristems were also affected in td1 mutant inflorescences. SPMs were frequently enlarged and irregularly arranged, leading to extra spikelets that were often single instead of in pairs. Although we did not observe more than two florets per spikelet, we did observe extra glumes. As glumes are products of the SM (McSteen and Hake, 2001), the SMs were therefore also affected in td1 mutants. td1 mutant male flowers also showed an increase in the number of stamens, which may be a consequence of an increase in floral meristem size. Finally, td1 mutant plants were shorter and developed fewer leaves. Given that td1 maps near a QTL for plant height (Beavis et al., 1991), it could also be responsible for natural variation in height.

In summary, td1 functions to limit meristem size during inflorescence and flower development.

**td1 encodes a CLV1-like protein**

We have shown that td1 encodes a putative ortholog of CLV1. clv1 mutants affect all shoot meristem types, including those of the embryo and vegetative phase. With the exception of the ear inflorescence meristem, the effects of the td1 mutation were relatively mild, and this could be a result of genetic redundancy. Although td1 appears to be a single-copy gene by low stringency Southern hybridizations, sequence database searches indicate a plethora of LRR receptor-like genes in maize, and these could account for the genetic redundancy. From the Arabidopsis genome, 216 LRR-RLKs have been identified (Shiu and Bleeker, 2001), and recent studies of clv1 dominant negative alleles strongly suggest the presence of additional receptor kinases that function redundantly with CLV1 in the Arabidopsis SAM (Dievart et al., 2003). Our phylogenetic analysis indicates an ancestral gene duplication prior to the divergence of monocot and dicot species, giving rise to two discrete branches. CLV1, FON1 and TD1 fall into subfamily A, whereas the three maize kinases encoded by ZmKin1 to 3 fall into subfamily B, along with OsLRK1 from rice and the two Arabidopsis LRR-RLKs, A.t. RLK1 and A.t. RLK2. Interestingly, OsLRK1 antisense plants have an increased number of floral organs, implicating a function in control of floral meristem size (Kim et al., 2000). The presence of A.t. RLK1 and 2 in the same subfamily (B) indicates that redundancy presumably is ancient and these two genes still may act redundantly to CLV1 in Arabidopsis.

**TD1 and FEA2 do not interact in an exclusive receptor complex**

The td1 mutant phenotype was similar to that of fea2, which also has ear fasciation, an increased number of stamens, and a thicker tassel. FEA2 is predicted to be a CLV2 ortholog (Taguchi-Shiobara et al., 2001). In Arabidopsis, the CLV1 and CLV2 gene products interact to form a heterodimeric LRR-transmembrane receptor kinase complex [reviewed by Sharma et al. (Sharma et al., 2003a)], and this is supported by genetic analysis that shows that strong clv1 and clv3 alleles are epistatic to clv2 in regulating meristem size (Kayes and Clark, 1998). Given the similar phenotypes of fea2 and td1 mutants, it is intriguing to speculate whether they encode two subunits of a heterodimeric CLV-like LRR-receptor kinase complex in maize.

Our analysis of td1/fea2 double mutants, however, indicates that the situation in maize is more complex. The formation of twice as many kernel rows, as well as the enhanced reduction of leaf number, in td1/fea2 double mutants compared with either single mutant strongly suggests that td1 and fea2 do not simply function in a single pathway, for example as an exclusive receptor-co-receptor complex. One could speculate that td1 and fea2 are incorporated into different receptor complexes, thereby acting in independent pathways in regulating shoot and inflorescence meristem size. Alternatively, they may function in partially overlapping...
pathways, for example forming a co-receptor complex as well as dimerizing with other LRR partners, as has been also recently proposed for CLV1 function in Arabidopsis (Diévart et al., 2003). Our data suggest that several CLV1-CLV2-like complexes with partially redundant functions contribute to the regulation of inflorescence meristem size, and that the degree of redundancy may differ in the ear and tassel. As there are multiple CLV3-like genes in maize (Cock and McCormick, 2001; Sharma et al., 2003b), further specificity may be dictated by specific ligand-receptor interactions.

The CLV1 and TD1 protein structures are highly conserved. Like CLV1 (Clark et al., 1997), the extracellular receptor domain of TD1 is composed of 21 complete LRR motifs, each of 23-25 amino acids in length. These LRR motifs are commonly used for protein-protein interactions (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995) and all motifs in TD1, as in CLV1, belong to the LRR-XI subfamily (Shiu and Bleeker, 2001). Sequence analyses of strong clvl alleles in Arabidopsis reveal lesions within LRRs four, five or nine, implying these regions may be important for ligand specificity (Diévart et al., 2003). Interestingly, sequence similarity between TD1 and CLV1 is more pronounced within LRR-5 and LRR-9, supporting the hypothesis that these LRRs play a conserved role in the receptor-ligand interaction. This situation is reminiscent of the FEA2 and CLV2 proteins, which also show blocks of significant similarity in specific LRRs (Taguchi-Shiobara et al., 2001).

Another common feature of LRR-RLKs are paired cysteines flanking the extracellular LRR domain. These cysteines are implicated in receptor dimerization (Trotchaud et al., 1999). TD1, FON1 and CLV1 share both cysteine pairs, although the spacing of the carboxy terminal cysteines is only two amino acid residues in TD1 relative to six in CLV1 and seven in FON1. Interestingly, in FEA2 the carboxy terminal cysteine pairs are also closer together, spaced by only four amino acids, compared with seven in CLV2 (Taguchi-Shiobara et al., 2001; Jeong et al., 1999). Therefore, TD1 and FEA2 contain putative compensatory alterations that might stabilize heterodimers by intermolecular disulfide bridges, via the carboxy terminal pair of cysteines.

Expression analysis shows a potentially wider role for td1 compared with CLV1

Significant differences in expression pattern can be found between TD1 in maize and CLV1 in Arabidopsis. td1 expression was not restricted to the internal L2/L3 meristem layers, as reported for CLV1 (Clark et al., 1997). Furthermore, similar to the FON1 expression in rice, td1 was expressed in inflorescence organ primordia, such as glumes, lemma and stamens, whereas CLV1 is expressed only in meristems. More significantly, we did not observe td1 expression in the shoot apical meristem of embryos or seedlings using in-situ hybridizations. One explanation for the lack of detection at the cellular level, however, is a ubiquitous but low level of expression in embryonic or seedling shoot meristems.

An interpretation of the low level of td1 expression in the ear inflorescence meristem again takes the domestication of maize into consideration. The change in phyllotaxy and increase in spikelet row number during selection for maize ear size may have been accompanied by an increase in inflorescence meristem size, by modification of genetic pathways, such as the CLV pathway. In other words, selection for low td1 expression in the ear inflorescence meristem may be responsible for the large size of the ear inflorescence meristem.

An alternative hypothesis is that SPMs, which express high levels of td1 mRNA, regulate the size of the inflorescence meristem by a long-distance signaling mechanism. An interaction between inflorescence and axillary meristem is also seen with barren inflorescence2 (bif2) mutants, which do not develop axillary meristems and exhibit fasciated inflorescence meristems (McSteen and Hake, 2001). bif2 is also expressed in axillary meristems but not in the inflorescence meristem (McSteen and Hake, unpublished). The same scenario can also be postulated for the vegetative shoot meristem, as td1 is expressed in leaves.

Conclusion

In summary, the td1 fasciated and enlarged meristem phenotypes are explained by a deficiency in CLV-type signaling. The finding that td1 and FON1 encode orthologs of CLV1 supports the notion that the regulation of stem cell proliferation by CLV-type signaling is of general importance in plants. However, our data point out important differences that justify the study of such pathways in a variety of model systems. For example, td1 expression was not detected, or was very weak, in vegetative and inflorescence apical meristems, and analysis of td1/fea2 mutant plants indicated that td1 and fea2 do not exclusively act in a simple co-receptor complex. Moreover, the proximity between td1 and QTLs affecting seed row number, spikelet density and plant height suggests that CLV signaling may have been a target of selection during domestication of maize as a crop. Our results suggest that genes such as td1 could be manipulated to improve crop yields.

The authors thank R. Waites for inspiring discussions and technical assistance at the start of the project, and J. Chandler and S. Dass for critically reading the manuscript. Thanks to T. Rocheford for growing out the td1 transposon screens in Illinois and for aid in mapping td1 relative to his floral QTLs, and to J. Lam. M. Rezapour and B. Burr for plant measurements and statistical analysis. P.B., J.N. and W.W. were supported by QLKR-2000-00196 and QLRT-2000-00302. S.H., C.L., E.V., D.J. and M.R. were supported by NSF BDI-0110189 and the USDA-ARS. P.B. and D.J. were also supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number #2003-35304-02625.

References


Burr, B., Burr, F. A., Thompson, K. H., Albertson, M. C. and Stuber, C.


