RESEARCH ARTICLES

Pod Corn Is Caused by Rearrangement at the Tunicate1 Locus

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Pod corn (Zea mays var tunicata) was once regarded as ancestral to cultivated maize, and was prized by pre-Columbian cultures for its magical properties. Tunicate1 (Tu1) is a dominant pod corn mutation in which kernels are completely enclosed in leaflike glumes. Here we show that Tu1 encodes a MADS box transcription factor expressed in leaves whose 5’ regulatory region is fused by a 1.8-Mb chromosomal inversion to the 3’ region of a gene expressed in the inflorescence. Both genes are further duplicated, accounting for classical derivative alleles isolated by recombination, and Tu1 transgenes interact with these derivative alleles in a dose-dependent manner. In young ear primordia, Tu1 proteins are nuclearly localized in specific cells at the base of spikelet pair meristems. Tu1 branch determination defects resemble those in ramosa mutants, which encode regulatory proteins expressed in these same cells, accounting for synergism in double mutants discovered almost 100 years ago. The Tu1 rearrangement is not found in ancestral teosinte and arose after domestication of maize.

INTRODUCTION

Modern cultivated maize (Zea mays), unlike other grasses in the Poaceae, has a unique feature of severe reduction in glume size that results in almost naked grains. By contrast, pod corn (Zea mays var tunicata) kernels are entirely enclosed in long glumes, resembling most grasses, and for this reason, pod corn was widely regarded as a primitive form of cultivated maize (Mangelsdorf, 1947). Pod corn was first described in the 19th century as an ancient variety of maize that had been preserved by pre-Columbian cultures in Brazil (Saint-Hilaire, 1829), Mexico, and Peru. Later, prehistoric cobs excavated from San Marcos Cave, dated 5200 to 3400 B.C., were found to have relatively long glumes and were believed to possibly be a weak form of pod corn (Mangelsdorf et al., 1964). Genetic mapping revealed that pod corn was attributable to a single dominant gene, Tunicate1 (Tu1) (Mangelsdorf and Galinat, 1964). Tu1 behaved as a compound locus, such that rare recombinants gave rise to weak (Tu1-l) and intermediate (Tu1-md) alleles (Mangelsdorf and Galinat, 1964). A third derivative, Tu1-d, was phenotypically similar to Tu1-l, which led to the idea that weak alleles had one or two recombilable components, whereas Tu1 had three components; in this way, doses could vary from one to six, and recombination could first dissect and then regenerate the full Tu1 phenotype (Mangelsdorf and Galinat, 1964; Langdale et al., 1994). Derivatives were rare; therefore, the duplication of a recombilable component was considered as an explanation for the compound Tu1 (Mangelsdorf and Galinat, 1964). The role of Tu1 in the origin of maize was fraught with controversy (Mangelsdorf, 1947, 1984).

The Tu1 phenotype is pleiotropic (Eyster, 1921; Nickerson and Dale, 1955; Langdale et al., 1994) and results in the conspicuous elongation of outer glumes, as well as sex reversal in the tassel and branching in the ear (Figure 1). Tu1 genetically interacts with various morphological mutants that relate to juvenile-to-adult transition, such as Corngrass1 (Cg1), Teosond1 (Ts1), and Ts2, branching, such as ramosa1 (ra1), and sex determination, such as the tassel seed mutants ts1, ts2, ts4, ts5, and ts6 (Collins, 1917; Langdale et al., 1994). Based on these genetic studies, Tu1 has been proposed to play roles in phase transition, branch meristem formation, spikelet initiation, and sex determination. Despite robust genetic and morphological studies, the molecular function of Tu1 in a diverse range of floral developmental processes is largely unknown, as is the nature of genetic modification that leads to the Tu1 phenotype.

MADS box genes in plants are well known for regulating floral organ identity as well as developmental phase transition (Hartmann et al., 2000; Ng and Yanofsky, 2001; Yu et al., 2002; Whipple et al., 2004). A MADS box gene in maize, Z. mays MADS19 (Zmm19), lies in the same genetic interval as Tu1 (Münster et al., 2002; He et al., 2004). Furthermore, ectopic expression of Zmm19 results in leaflike sepals in Arabidopsis thaliana, resembling the elongated glumes in Tu1 mutants (He et al., 2004; Wingen et al., 2012). Zmm19 is misexpressed in the inflorescence of Tu1 mutants, and two duplicated copies of the gene were found in Tu1, but only one copy was found in Tu1-l, Tu1-d, and Tu1-md (He et al., 2004; Wingen et al., 2012). Despite these promising indications, genome sequencing has
revealed several other candidate genes in the same interval (Schnable et al., 2009). For example, miR172c is found in this same genetic interval on chromosome 4 (Wei et al., 2009), and double mutants between Tu1 and miR172e/its4 (Chuck et al., 2007b) are strongly synergistic (Langdale et al., 1994) with respect to both branching and sex determination. Also, Tu1 has a synergistic interaction with Cg1, which encodes two miR156 genes, miR156b and miR156c (Langdale et al., 1994; Chuck et al., 2007a). miR156 shows antagonistic interactions with miR172, which, similar to MADS box genes, has regulatory roles.
Figure 2. Rearrangement of Tu1.

(A) Genetic map of the Tu1 locus with genetic markers. Recombination frequency is indicated by number of recombinants over total population size, with a recombination cold spot proximal to Tu1.

(B) Schematic representation of Tu1-A and Tu1-B loci that are distinct from wild-type Zmm19, which is GRMZM2G370777. An unknown gene (GRMZM2G006297, red line), is located 1.8 Mb from Zmm19 in the wild type but adjacent to both Tu1-A and Tu1-B in Tu1. A 3.5-kb insertion (yellow triangle) specific to Tu1-A, a 9-bp insertion (green triangle) specific to Tu1-B, and a novel Mutator-like insertion found in both Tu1-A and Tu1-B were absent from B73. Zmm19 is represented by a box, and internal lines denote exons.
in the transition from vegetative to reproductive growth and in specifying floral organ identity by targeting genes involved in floral determinacy (Aukerman and Sakai, 2003; Chen, 2004; Chuck et al., 2007a; Zhu and Hellwell, 2011). The extent of the duplication in Tu1 was not known; therefore, the possibility that one of these other candidates was responsible for the phenotype could not be eliminated.

Here, we describe phenotypic characteristics of Tu1 and demonstrate that the Tu1 locus is interrupted by a chromosomal rearrangement in the 5′ regulatory region of Zmm19. Our results indicate that the duplicate copies of Zmm19 in Tu1 are ~30 kb apart and that they can be recombined to result in a single copy with reduced phenotypic effects. At least one other gene is included in the duplication, but Tu1 phenotypes are reconstituted in Zmm19 transgenic plants when the rearranged locus is used to drive expression. Tu1 protein fusions show a discrete expression pattern comparable to that of ramosa genes during an early stage of inflorescence development that reflects fusion of the promoter via a large chromosomal inversion to a gene expressed in the inflorescence. Based on its expression pattern and on these dose-dependent phenotypes, we suggest that Tu1 is involved in inflorescence architecture by promoting indeterminate cell fate.

RESULTS

Tu1 Mutant Phenotypes Are Dose-Dependent

Tu1 was introgressed into the B73 background to examine its phenotype. Normal monoecious maize bears a distinct terminal male inflorescence, the tassel (Figures 1A and 1E), and a lateral female inflorescence, the ear (Figures 1D and 1H). Tu1 is a dominant mutation; therefore, plants heterozygous for Tu1 displayed pleiotropic defects in reproductive development, and plants homozygous for Tu1 became even more severe in a dose-dependent manner. Both tassels and ears of Tu1 mutants are associated with a spikelet formation defect in which outer glumes enclosing inner whorls are highly elongated in comparison with the wild type (Figures 1A to 1I). In heterozygous Tu1, elongated glumes were conspicuous at the base of the central rachis and lateral branches and became less prominent toward the inflorescence apex (Figures 1B and 1F), whereas homozygous Tu1 tassels produced very large glumes (Figures 1C and 1G). In the wild type, bisexual floral meristems (FMs) of maize convert into unisexual flowers by a process of selective abortion of pistil primordia within the tassel and stamen primordia within the ear (Le Roux and Kellogg, 1999). In heterozygous Tu1 tassels, pistils failed to abort. Male flowers were partially converted into female flowers near the base, and some bare kernels after fertilization (Figures 1B and 1F). This feminization was more prominent in homozygous Tu1 (Figures 1C and 1G).

Unlike the naked kernels found in the wild type (Figure 1H), kernels in heterozygous Tu1 were fully enclosed by glumes (Figure 1I). After removing leaflike glumes, several long branches, which are normally found only in the tassel, were found in homozygous Tu1 ears (Figure 1D, arrow). Using scanning electron microscopy of 2-mm developing ears, we observed the inflorescence meristem (IM), spikelet pair meristem (SPM), and spikelet meristem (SM) with bract growth in the wild type (Figure 1J). Figure 1L shows the irregular rows of the SM from homozygous Tu1 inflorescences, which are very different from the regular pattern of the organized SM on the flanks of the wild-type ear tip (Figure 1J). When ears become ~4 mm long, the two SMs, generated from each SPM, convert into FMs that initiate floral organs, including anthers and pistil primordia (Figure 1M). Heterozygous Tu1 ears mostly succeeded in FM conversion from SM, only producing an indeterminate branch rarely (Figure 1N, arrow). However, despite the obvious glume elongation in homozygous Tu1, we could observe numerous indeterminate long branches emerging as a consequence of failure in FM transition (Figure 1O), suggesting that the Tu1 ear adopts features of the male inflorescence (Bortiri and Hake, 2007). These phenotypes suggest that Tu1 mutants have defects in suppressing glume formation and in meristem fate and sex determinacy.

Fine Mapping of Tu1

The Tu1 mutation was mapped to the long arm of chromosome 4 (Mangelsdorf and Galinat, 1964). We set out to fine map the Tu1 locus by backcrossing Tu1 heterozygotes (Tu1+/+) to wild-type plants twice. We screened 738 F2 plants using markers from the region, and Tu1 was subsequently delimited between one cleaved-amplified polymorphic sequence (CAPS) marker for GRMZM2G386088 and one Insertion/Deletion (InDel) marker for GRMZM2G006297.

Figure 2. (continued).

(C) Schematic representation of wild-type Zmm19 with 293-bp insertion (blue triangle) in first intron, which is absent in B73 but present in unknown wild-type (WT) tu1 background. Allele-specific PCR showing that 5′ upstream region of wild-type Zmm19 is absent from Tu1. The positions of primers are depicted as arrows. Red arrow specific to 5′ upstream region of wild-type Zmm19.

(D) PCR verification of Tu1 locus. Although duplicated Tu1-A and Tu1-B loci are found in Tu1, half-tunicate mutants, which are Tu1-1 and Tu1-md, respectively, possess one copy of duplicated Tu1 loci. New Tu1 derivative allele, Tu1-rec, was detected in our mapping population (one out of 738) and bears only the Tu1-A but not Tu1-B. The position of primers is depicted as arrows. Green arrow specific to Mutator-like insertion, which is present only in Tu1 alleles.

(E) Both duplicated Tu1-A and Tu1-B loci have an unknown gene (GRMZM2G006297) as their 5′ upstream gene. Two amplicons are the product of two recombined genes between Zmm19 and the unknown gene. Blue arrow specific to GRMZM2G006297. Although forward primers were uniquely designed for allele-specific PCR, the same reverse primer (black arrow) was used as in (C).

(F) Duplicated Tu1-A and Tu1-B loci neighbor each other. GRMZM2G006297 is located at the 3′ downstream region of Tu1-B and at the 5′ upstream region of Tu1-A (Figure 2E; see Supplemental Figure 2 online). Purple arrow specific to GRMZM2G006297. Gel images were inverted for better contrast, and New England BioLabs 1 kb DNA ladder was used [(C) to (F)].
analyses between the Zmm19 Sequencing of BAC clones in a 22-Mb interval surrounding markers, and one simple sequence repeat (SSR) marker. In- two InDel markers, two insertion-deletion polymorphism (IDP) GRMZM2G081318, with only one recombinant each, covering Figure 3.

The immature tassels of the endogenous promoter region. novel (green box) and YFP showed one-half the expression level of Ubiquitin of the 3 zygous Tu1 showed one-half the expression level of Tu1 wild-type level of Supplemental Figure 3B online. Results are plotted as the ratio to the ears (Tu1/+, black bar). qRT-PCR of three biological replicates. See also Supplemental Figure 3A online. WT, wild type.

GRMZM2G081318, with only one recombinant each, covering a 2.5-Mb interval. To further narrow down the interval, we used two InDel markers, two insertion-deletion polymorphism (IDP) markers, and one simple sequence repeat (SSR) marker. Interestingly, these five genetic markers showed no recombination within a 1.8-Mb interval, revealing a recombination cold spot proximal to Zmm19 that did not recombine with Tu1 (Figure 2A). Sequencing of BAC clones in a 22-Mb interval surrounding Zmm19 (Wei et al., 2009) allowed comparative DNA sequence analyses between the Zmm19 gene copies found in Tu1, known as Tu1-A (GenBank number AJ850302) and Tu1-B (GenBank number AJ850303), and the Zmm19 copy found in the reference sequence of the B73 genome. This analysis revealed that both Tu1-A and Tu1-B are structurally rearranged by insertion of a novel 2-kb Mu-like element in the 5’ cis-regulatory region of Zmm19, which is fused with the 3’ flanking region of an unknown gene (GRMZM2G006297) located on the other side of the 1.8-Mb interval from Zmm19 in the opposite orientation (Figure 2B).

This 1.8-Mb chromosomal inversion would be expected to inhibit recombination and likely accounts for the cold spot (Figure 2A) that prohibited previous attempts to precisely map Zmm19 relative to Tu1 (Münster et al., 2002; He et al., 2004; Wingen et al., 2012). The analyses also revealed that Tu1-A is distinguished from Tu1-B by the presence of a 3.5-kb insertion in the first intron, a non-long terminal repeat retrotransposon (Figure 2B, yellow triangle). Allele-specific PCR confirmed that the wild type has a single copy of Zmm19 (Figure 2C), whereas Tu1 has both Tu1-A and Tu1-B (Figures 2D and 2E). Additional comparative sequence analyses and PCR showed that half-tunicate mutants, Tu1-A and Tu1-md, have only one copy of Tu1-B or Tu1-A, respectively (Figure 2D). Finally, in one rare recombinant out of 738, we observed de novo crossover between Tu1-A and Tu1-B (Tu1-rec, Figure 2D), reconstructing the half-tunicate phenotype (see Supplemental Figure 2 online) as previously reported (Mangelsdorf and Galignat, 1964). The primer pairs used in Figure 2E are separated by 1.8 Mb; therefore, only recombinant Tu1 alleles can be amplified but not the wild type. Long-range PCR and sequencing revealed that Tu1-B is positioned upstream of Tu1-A, and the intervening 30-kb DNA sequence includes gypsy-like and copia-like retrotransposons, dSpm-like and hAT-type DNA transposons, and another copy of GRMZM2G006297 fused with the promoter region of Tu1-A, indicating that GRMZM2G006297 is contained within the duplication (Figures 2E and 2F; see Supplemental Figure 2 online). We conclude that inversion preceded duplication at the Tu1 locus and that GRMZM2G006297 and Zmm19 are both strong candidate genes for Tu1 and cannot be further distinguished by recombination.

Zmm19 Transgenic Lines Phenocopy Tu1

We used transgenic plants to determine whether the Tu1 phenotype was caused by the rearrangement at Zmm19. We fused Tu1-A with yellow fluorescent protein (YFP) and Tu1-B with red fluorescent protein (RFP) at their C termini (Figure 3A) and generated several independent transgenic maize plants with each transgene (see Methods). Each transgene was driven by its own promoter, which comprised 3 kb of upstream sequence, including the 3’ flanking region of GRMZM2G006297 and the 2-kb Mu-like transposon. Maize plants were transformed individually with these two constructs (Tu1-A:YFP and Tu1-B:RFP) and were backcrossed to B73. We performed quantitative RT-PCR (qRT-PCR) analysis with RNA from immature tassel and ear. Each single transformant showed a significant increase in the Zmm19 transcript abundance in both tissue types, but the relative expression level was about one-half of the transcript level of heterozygous Tu1 (Tu1/+), which possesses both Tu1-A and Tu1-B (Figures 3B and 3C). Homozygous Tu1 (Tu1/Tu1)
further doubled Zmm19 transcript abundance compared with heterozygous Tu1 (Tu1/+). Expression of the transgene in transgenic immature tassels and ears was weaker than that observed in Tu1/+ heterozygotes, but was much higher than in wild-type inbreds. These data suggest that Zmm19 ectopic expression levels in inflorescences are correlated with Tu1 copy number.

Tu1-A:YFP and Tu1-B:RFP T1 transgenic plants produced elongated glumes in the tassel (Figures 4F and 4G) and were stable after backcrossing to B73 (Figures 4B and 4C). We tested the additive effect of the two transgenes by crossing Tu1-A:YFP and Tu1-B:RFP had further elongated glumes (Figure 4D) and occasional feminization that enables spikelets to bear seed in the tassel (Figure 4E), resembling Tu1/+ heterozygotes (Figure 1F). Elongated glumes in plants with both transgenes led the main rachis and lateral branches to seem thicker than those in single transgenic and nontransgenic plants, suggesting that both transgenes are functionally involved in the Tu1 tassel phenotype (Figure 4D; see Supplemental Figures 4A to 4F online). Similarly, tassel phenotypes were more conspicuous when transgenic lines were combined with Tu1-l, suggesting that both transgenes interact with Tu1-l in a dose-dependent manner (Figures 5A to 5G). We observed that Tu1-A:YFP and Tu1-B:RFP transgenic plants exhibited weak half-tunicate ear phenotypes, resembling those of Tu1-l single-copy derivatives (Figures 6A and 6B). Plants with both transgenes produced glumes that fully covered every kernel (Figure 6A) and were comparable to Tu1/+ heterozygotes (Figure 1I). Furthermore, crosses between Tu1-A: YFP or Tu1-B:RFP and Tu1-l resulted in a dramatic enhancement of the half-tunicate phenotype in the ear (Figure 6B), indicating that the transgenes complemented half-tunicate derivatives and provided Tu1 function (Mangelsdorf and Galinat, 1964). Thus, our data demonstrate that ectopic expression of Zmm19 derived from the Tu1 locus causes the dose-dependent Tu1 phenotype.

**Nuclear Localization of TU1-A:YFP and TU1-B:RFP Proteins**

Zmm19 contains a highly conserved DNA binding MADS box domain and is therefore expected to be nuclear-localized (Ng and Yanofsky, 2001; Münster et al., 2002). Confocal imaging...
revealed that TU1-A:YFP and TU1-B:RFP accumulated in nuclei of mature leaf and glume epidermis, as expected (Figures 7A to 7D). We further examined Tu1- A:YFP lines to investigate the expression of TU1 in different cell types. Nuclear localization was detected not only in vegetative tissues, mature leaves, and husks, but also in glumes, trichomes, and FMs (Figure 7; see Supplemental Figure 5 online). Colocalization of the two fusion proteins was consistent with their overlapping function (see Supplemental Figures 6A and 6B online). Expression of the fluorescent protein–tagged proteins in vegetative tissues was expected based on the transcription pattern of Zmm19 (GRMZM2G370777) in wild-type plants (see Supplemental Figure 7A online). Colocalization of the two fusion proteins was consistent with their overlapping function (see Supplemental Figures 6A and 6B online). Expression of the fluorescent protein–tagged proteins in vegetative tissues was expected based on the transcription pattern of Zmm19 (GRMZM2G370777) in wild-type plants (see Supplemental Figure 7A online). However, Zmm19 was ectopically expressed in the early inflorescence of Tu1 (Figure 3; see Supplemental Figure 3 online), and the fusion proteins persisted in even later developmental stages, when tassel glumes were fully developed with trichomes (Figures 7B and 7D; and Supplemental Figure 5B online).

In the maize inflorescence, meristem determinacy is progressively restricted, such that tertiary SMs arise from the secondary SPMs, which in turn arise from the primary IM (Vollbrecht et al., 2005). Whereas the spikelet pair is considered a short branch, long branches resemble the primary inflorescence and are normally found only at the base of the tassel. In homozygous Tu1/Tu1 mutants, long branches also arose at the base of the ear (Figures 1D and 1O), resembling ramosa mutants in this respect (Vollbrecht et al., 2005; Bortiri et al., 2006; Satoh-Nagasawa et al., 2006). Both functional TU1 fusion proteins were expressed in a small cup-shaped subset of cells at the base of the SPM in young ear primordia (Figures 8A and 8B). Remarkably, this expression pattern is similar to that of the short-branch determination genes, Ramosa1 (Ra1) and Ra3, in wild-type plants (Vollbrecht et al., 2005; Bortiri et al., 2006; Satoh-Nagasawa et al., 2006). Imaging of a double transgenic plant revealed that the YFP and RFP fusion proteins were colocalized to the nuclei in this domain (Figure 8C). These images suggest that ectopic expression of TU1/ ZMM19 at the base of developing SMs promotes their abnormal indeterminacy.

**DISCUSSION**

We have found that the pod corn mutant Tu1 is caused by the ectopic expression of the MADS box gene Zmm19 in the developing maize inflorescence. Zmm19 is normally expressed.
in husk and leaf tissues in the wild type (see Supplemental Figure 7A online) but is ectopically expressed in the inflorescence in Tu1 because of a chromosomal rearrangement (see Supplemental Figure 3 online), most likely a large inversion associated with the transposition of a Mutator-like transposon. This rearrangement led to a mild half-tunicate phenotype, in which glumes extend but fail to enclose the kernel. Subsequently, duplication of the two genes at the breakpoint of this rearrangement enhanced the phenotype so that glumes completely covered the kernels. Zmm19 has previously been proposed as a strong candidate gene to account for the Tu1 phenotype, but definitive proof was lacking because of the nature of the chromosomal rearrangement, which prohibited fine mapping. We have shown that half-tunicate phenotypes can be phenocopied by Tu1 transgenes and that these transgenes can interact with half-tunicate derivative alleles to reconstitute the full Tu1/pod corn phenotype.

The half-tunicate derivative alleles, Tu1-l, Tu1-d, and Tu1-md, were recovered by Mangelsdorf and Galinat after rare crossovers within the Tu1 locus, and Mangelsdorf and Galinat reported that they could reconstitute Tu1 by recombining Tu1-l and Tu1-d, suggesting that Tu1 was a compound locus (Mangelsdorf and Galinat, 1964). They continued to characterize the half-tunicate phenotypes of Tu1-l and Tu1-d through repeated backcrossing to an inbred line and observed that Tu1-d consistently had longer glumes than Tu1-l in tassels and ears. Although the origin of Tu1-md is unclear, the phenotype of Tu1-md (see Supplemental Figure 1A online) is more severe than that of Tu1-l (Figure 6B), and this may be the stronger half-tunicate allele reported by Mangelsdorf and Galinat (1964) (Langdale et al., 1994).

Previously, genomic cloning recovered two Zmm19 genes in Tu1, known as Tu1-A and Tu1-B, and one gene each in Tu1-d, Tu1-l, and Tu1-md (Munster et al., 2004; Wingen et al., 2012). Sequence analysis indicated that both Tu1-l and Tu1-d were analogous to Tu1-B, whereas Tu1-md was analogous to Tu1-A. We used PCR assays to confirm this organization and to reveal that the Tu1-A and Tu1-B genes were part of a larger 30-kb tandem duplication in Tu1 that included at least one other gene (Figures 2D to 2F; see Supplemental Figure 2 online). It is plausible that the derivative alleles were caused by unequal crossover within this duplication, and indeed we recovered a derivative allele Tu1-rec among our Tu1 mapping population at a frequency of one in 738, which is comparable to the one in 1300 frequency reported previously (Mangelsdorf and Galinat, 1964). The phenotype of Tu1-rec is comparable to that of Tu1-md (see Supplemental Figure 1 online) and is stronger than Tu1-l (Figure 6B), consistent with Tu1-md and Tu1-rec retaining Tu1-A, whereas Tu1-l retains Tu1-B (Figure 2D). We did not attempt to reconstitute the full tunicate phenotype from these

Figure 6. Tu1-A-YFP and Tu1-B-RFP Transgenic Ears Phenocopy Tu1.
(A) The half-tunicate phenotype was observed in transgenic ears carrying each single transgene (Tu1-A-YFP/+ or Tu1-B-RFP/+), whereas the wild-type (WT) kernels were naked. Two transgenes present in one transgenic line (Tu1-A-YFP/+; Tu1-B-RFP/+; Tu1-l-l-l/) caused glumes to be further elongated to fully enclose kernels, suggesting the additive genetic effect of the two transgenes.
(B) A single transgenic line (Tu1-A-YFP/+ or Tu1-B-RFP/) produced fully elongated glumes with the presence of Tu1-l, whereas plants heterozygous for a single copy of Tu1-l (Tu1-l-l/) represented the half-tunicate phenotype that was comparable to either the Tu1-A-YFP/+ or Tu1-B-RFP/+ phenotype (A).
derivative alleles (Mangelsdorf and Galinat, 1964), given that they only have one and not two components as previously proposed (Langdale et al., 1994) and that the mechanism of reconstitution therefore remains unclear.

By comparison with the finished sequence of 22 Mb surrounding Zmm19 in the B73 inbred line, we detected a large chromosomal inversion in Tu1 whose breakpoint lies in the promoter region of Zmm19. This breakpoint results in fusion with the 3’ flanking region of GRMZM2G006297, which is fused with the Zmm19 promoter by the Tu1 rearrangement. KN1 targets genes in the gibberellin and brassinosteroid pathways, which are involved in sex determination in maize (Bolduc and Hake, 2009; Hartwig et al., 2011; Bolduc et al., 2012). Thus, it is possible that the ectopic expression of Zmm19 may be associated with the Kn1 gene network.

SHORT VEGETATIVE PHASE (SVP) is the closest homolog of Zmm19 in Arabidopsis and regulates flowering repression during early tassel development (comparing samples taken at a tassel length of 1 to 2 mm with those at 5 to 7 mm), whereas GRMZM2G006297 expression increases. During the developmental progression from IM to FM, GRMZM2G006297 expression is upregulated in young ear primodia, where Zmm19 is not normally expressed (A. Eveland, A. Goldshmidt, and D. Jackson, unpublished data). Thus, this upstream gene may be the cause of the cup-shaped expression pattern of Tu1 fusion proteins, which typically resembles that of ramosa genes in young ear primodia. Interestingly, KNOTTED-1 (KN1) chromatin immunoprecipitation sequencing data (Bolduc et al., 2012) revealed that KN1 strongly binds this 3’ flanking region of GRMZM2G006297, which is fused with the Zmm19 promoter by the Tu1 rearrangement. KN1 targets genes in the gibberellin and brassinosteroid pathways, which are involved in sex determination in maize (Bolduc and Hake, 2009; Hartwig et al., 2011; Bolduc et al., 2012). Thus, it is possible that the ectopic expression of Zmm19 may be associated with the Kn1 gene network.

Figure 7. TU1-A:YFP and TU1-B:RFP Are Nuclear-Localized.

(A) and (B) TU1-A:YFP fusion proteins are nuclear-localized in leaf (A) and glume (B) epidermis.

(C) and (D) TU1-B:RFP fusion proteins are nuclear-localized in leaf (C) and glume (D) epidermis. Autofluorescence of YFP and RFP was detected from guard cells in the leaf epidermis (A) and (C). Bar in (A) = 20 μm; bars in (B) to (D) = 10 μm.

In the wild type, RNA sequencing expression data revealed that Zmm19 expression decreases during early tassel development (comparing samples taken at a tassel length of 1 to 2 mm with those at 5 to 7 mm), whereas GRMZM2G006297 expression increases. During the developmental progression from IM to FM, GRMZM2G006297 expression is upregulated in young ear primodia, where Zmm19 is not normally expressed (A. Eveland, A. Goldshmidt, and D. Jackson, unpublished data). Thus, this upstream gene may be the cause of the cup-shaped expression pattern of Tu1 fusion proteins, which typically resembles that of ramosa genes in young ear primodia. Interestingly, KNOTTED-1 (KN1) chromatin immunoprecipitation sequencing data (Bolduc et al., 2012) revealed that KN1 strongly binds this 3’ flanking region of GRMZM2G006297, which is fused with the Zmm19 promoter by the Tu1 rearrangement. KN1 targets genes in the gibberellin and brassinosteroid pathways, which are involved in sex determination in maize (Bolduc and Hake, 2009; Hartwig et al., 2011; Bolduc et al., 2012). Thus, it is possible that the ectopic expression of Zmm19 may be associated with the Kn1 gene network.

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(A) and (B) TU1-A:YFP fusion proteins are nuclear-localized in leaf (A) and glume (B) epidermis.

(C) and (D) TU1-B:RFP fusion proteins are nuclear-localized in leaf (C) and glume (D) epidermis. Autofluorescence of YFP and RFP was detected from guard cells in the leaf epidermis (A) and (C). Bar in (A) = 20 μm; bars in (B) to (D) = 10 μm.

By comparison with the finished sequence of 22 Mb surrounding Zmm19 in the B73 inbred line, we detected a large chromosomal inversion in Tu1 whose breakpoint lies in the promoter region of Zmm19. This breakpoint results in fusion with the 3’ flanking region of an unknown gene, GRMZM2G006297. This unknown gene is duplicated along with Zmm19 and is expressed in both husk leaves and inflorescences, unlike Zmm19, which is normally expressed in husk leaves (see Supplemental Figures 7A and 7B online). We considered three possibilities to explain why Zmm19 expression is drastically altered in Tu1. First, a novel Mu-like element located at the breakpoint of the inversion may enhance Zmm19 expression. However, we did not detect transcription start sites within the Mu-like element by 5’ RACE. Second, chromosomal inversion in the promoter of Zmm19 may cause removal of enhancer-blocking insulators or silencers to upregulate Tu1. However, transgenic fusion proteins that contained these sequences were expressed in a specific subset of cells, making this possibility unlikely. Finally, Zmm19 may adopt the expression pattern of the upstream gene GRMZM2G006297, accounting for ectopic expression in the male and female inflorescence.

In the wild type, RNA sequencing expression data revealed that Zmm19 expression decreases during early tassel development (comparing samples taken at a tassel length of 1 to 2 mm with those at 5 to 7 mm), whereas GRMZM2G006297 expression increases. During the developmental progression from IM to FM, GRMZM2G006297 expression is upregulated in young ear primodia, where Zmm19 is not normally expressed (A. Eveland, A. Goldshmidt, and D. Jackson, unpublished data). Thus, this upstream gene may be the cause of the cup-shaped expression pattern of Tu1 fusion proteins, which typically resembles that of ramosa genes in young ear primodia. Interestingly, KNOTTED-1 (KN1) chromatin immunoprecipitation sequencing data (Bolduc et al., 2012) revealed that KN1 strongly binds this 3’ flanking region of GRMZM2G006297, which is fused with the Zmm19 promoter by the Tu1 rearrangement. KN1 targets genes in the gibberellin and brassinosteroid pathways, which are involved in sex determination in maize (Bolduc and Hake, 2009; Hartwig et al., 2011; Bolduc et al., 2012). Thus, it is possible that the ectopic expression of Zmm19 may be associated with the Kn1 gene network.

SHORT VEGETATIVE PHASE (SVP) is the closest homolog of Zmm19 in Arabidopsis and regulates flowering repression during early tassel development (comparing samples taken at a tassel length of 1 to 2 mm with those at 5 to 7 mm), whereas GRMZM2G006297 expression increases. During the developmental progression from IM to FM, GRMZM2G006297 expression is upregulated in young ear primodia, where Zmm19 is not normally expressed (A. Eveland, A. Goldshmidt, and D. Jackson, unpublished data). Thus, this upstream gene may be the cause of the cup-shaped expression pattern of Tu1 fusion proteins, which typically resembles that of ramosa genes in young ear primodia. Interestingly, KNOTTED-1 (KN1) chromatin immunoprecipitation sequencing data (Bolduc et al., 2012) revealed that KN1 strongly binds this 3’ flanking region of GRMZM2G006297, which is fused with the Zmm19 promoter by the Tu1 rearrangement. KN1 targets genes in the gibberellin and brassinosteroid pathways, which are involved in sex determination in maize (Bolduc and Hake, 2009; Hartwig et al., 2011; Bolduc et al., 2012). Thus, it is possible that the ectopic expression of Zmm19 may be associated with the Kn1 gene network.
(Hartmann et al., 2000), whereas the closely related AGAMOUS-LIKE24 (AGL24) regulates FM identity and causes floral reversion with bract-like sepals and no petals when overexpressed (Yu et al., 2004). In rice (Oryza sativa), the ectopic expression of Os-MADS22, the Zmm19 ortholog, causes abnormal floral morphology, including loss of the palea from spikelets, elongated glumes, and a two-floret spikelet, a mild form of 5M indeterminacy (Sentoku et al., 2005). In barley (Hordeum vulgare), the Zmm19 homolog MADS1 (BM1) is expressed in vegetative tissues and repressed during floral development and also induces floral reversion by repressing spike development when ectopically expressed (Trevasakis et al., 2007). Thus, the molecular role of Zmm19 orthologs is at least partially conserved in other species. MADS domain proteins bind CAAT box cis-regulatory elements (Pollock and Treisman, 1991; Shore and Sharrocks, 1995), and these are present in 5’ upstream regions of Ra1, Ra2, Ra3, Ts1, Ts2, and Ts4, all of which interact with Tu1. It is plausible that ZMM19 may recognize these binding sites and contribute to pleiotropic alterations in inflorescence architecture that are exacerbated in double mutants. In addition to dramatically enhanced glume length, homozygous plants with four copies of the gene fusion have branch determination defects resembling ramosa mutants. These defects are consistent with the idea that Ramosa1 and Tu1 activity may be mutually repressive, via the coincidence of the expression patterns of Ra1, Ra3, and Tu1. Both ra1 and Tu1 mutants were first considered to be distinct subpecies, and Collins (1917) described hybrids of Zea ramosa and Zea tunicata (r1/r1 Tu1+/+) as sterile highly branched Califlower-like inflorescences (Langdale et al., 1994). This “monstrous” phenotype (Collins, 1917) presumably reflects the coexpression described here.

Mangelsdorf famously proposed that a half-tunicate form of Tu1 was present in the teosinte ancestors of maize but was only revealed phenotypically in crosses to the Mexican popcorn, Palomero tolucuero (Mangelsdorf, 1974). Several inbred accessions of teosinte have recently been sequenced, as well as the Mexican popcorn (Chia et al., 2012). We searched for the junctions of the 1.8-Mb inversion in these genomic sequences and found no evidence for the existence of this gene fusion, although the sequences on either side were intact. Although we cannot exclude the possibility that additional accessions of teosinte may have the rearrangement, our results are consistent with a single late origin for Tu1, after the domestication of maize.

METHODS

Plant Material

The Tu1 (number 412G), Tu1-l (number 416B), and Tu1-md (number 416E) alleles in an unknown genetic background were obtained from the Maize Genetics Coop Stock Center. Tu1, Tu1-l, and Tu1-md plants were introgressed into B73 two times. B73 was used as the wild-type line. Plants were grown in the field in the green house under standard conditions.

Fine Mapping

Heterozygous Tu1 plants were crossed to the B73 inbred line, and then F1 heterozygous Tu1 plants were backcrossed to B73 to generate mapping populations segregating equally for wild-type and mutant at a 1:1 ratio. Phenotypes of ~750 F2 plants were scored by visual inspection of mature tassels and ears. DNA preps were done on 738 plants for positional cloning. SSR and IDP markers were used on chromosome 4L, where the Tu1 locus is located, as previously described (Mangelsdorf and Galinat, 1964). The F2 population was screened with markers IDPB954 and umc2009 on chromosome 4L to identify recombinants. To narrow the mapping interval, CAPS (Konieczny and Ausubel, 1993) and InDel markers were designed for maize (Zea mays) genes by sequencing and identifying sequence polymorphisms between B73 and the Tu1 progenitor. Genetic markers were amplified with 20 to 60 ng of DNA and Phusion High-Fidelity DNA polymerase (Finnzymes) by PCR using primers listed in Supplemental Table 1 online. SSR and IDP markers were tested according to the recommended PCR conditions (http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php and magi.plantgenomics.iastate.edu/browseMarker.do, respectively). As a CAPS marker, GRMZM2G386088 PCR product was subsequently treated with the KpnI restriction enzyme at 37°C for 1 h and analyzed via 1% agarose gel electrophoresis.

Scanning Electron Microscopy

As described in Whipple et al. (2010), fresh samples of maize immature ears were dissected and mounted on disks with silver adhesive (Electron Microscopy Sciences) and placed on ice before imaging. A Hitachi S-3500N scanning electron microscope was used to capture images of the live sample by the electron beam using an accelerating voltage of 5.0 kV under high vacuum mode and a distance of 15 to 30 mm within 15 min.

Tu1-A:YFP and Tu1-B:RFP Transgenic Maize Lines

Tu1-A:YFP and Tu1-B:RFP transgenese were constructed by cloning Tu1-A and Tu1-B alleles, respectively, using homozygous Tu1 DNA. Tu1-A::YFP construct was generated by fusing YFP in-frame to the C terminus before the stop codon of the genomic sequence of Tu1-A, including 700 bp of the 3’ downstream region of GRMZM2G006297, 2 kb of the Mu-like element, 300 bp of the 5’ promoter, a complete Tu1-A coding sequence with a 3.5-kb insertion in the first intron, and 450 bp of the 3’ untranslated region (UTR). The MultiSite Gateway Four Fragment System (Invitrogen) was used, by modifying the method described in Mohanty et al. (2009). All fragments were amplified using Phusion Gateway polymerase (Finnzymes). The first fragment from the 5’ upstream region to the 3.5-kb insertion within the first intron was amplified with primers Tu1AB_attB1 and Tu1A_attB5(R) and cloned into the pDONR221 P1-P5 vector using BP recombinase (Invitrogen). The second fragment from the remaining first intron to the coding sequence before the stop codon was amplified with primers Tu1A_attB5 and Tu1AB_attB4 and cloned into the pDONR221 P5-P4 vector. The citrus YFP fragment was amplified with primers YFP_attB4(R) and YFP_attB3(R) and cloned into the pDONR221 P4r-P3r vector using BP recombinase (Invitrogen). The third fragment from the stop codon to the 3’ UTR was amplified with primers Tu1AB_attB3 and Tu1AB_attB2 and cloned into the pDONR221 P3-P2 vector. The pDONR221 P1-P5r, pDONR221 P5-P4, pDONR221 P4r-P3r, and pDONR221 P3-P2 vector fragments were combined and transferred to the pTf101 Gateway-compatible maize transformation vector by a multistep LR recombination reaction (Invitrogen). Confirmed clones were transferred to Agrobacterium tumefaciens and transformed into maize (Mohanty et al., 2009). The same protocol was applied for the Tu1-B::RFP construction. For the Tu1-B::RFP construct, Tu1B_attB5(R) and Tu1-B_attB5 primers were used to amplify the first and second fragment specific to Tu1-B. The mRFP1 fragment was amplified with primer RFP_attB4(R) and RFP_attB3(R) and cloned into the pDONR221 P4r-P3r vector. The same third fragment was used, because the 3’ UTR is identical between Tu1-A and Tu1-B. Likewise, four vector fragments were combined and transferred to the pTf101 vector. Confirmed clones were
transferred to *Agrobacterium* and were transformed into maize. The sequences of the primers are shown in Supplemental Table 2 online.

**Allele-Specific Long-Range PCRs and Sequencing**

For all PCR reactions, genomic DNA was extracted from wild-type inbred lines and plants homozygous for *Tu1* mutations. PCR amplification of *Tu1* alleles was performed with Phusion High-Fidelity DNA polymerase (Finnzymes) and allele-specific primers (see Supplemental Table 3 online) under optimal PCR conditions, according to Finnzymes’s recommendations. For the long-range PCRs, Takács LA Taq polymerase was used with primers (see Supplemental Table 3 online) under two-step PCR conditions following the provided protocol. The PCR product was subsequently prepared for Illumina DNA sequencing according to the manufacturer’s recommendations. Sequencing was performed on an Illumina Genome Analyzer “GAI” for PE50 cycles.

**qRT-PCR**

Total RNA was extracted from immature tassels (1 to 2 cm long) and immature ears (0.5 to 0.9 cm long) using TRIzol reagent (Invitrogen). Total RNA was treated with DNaseI and reverse transcribed using an oligo-dT primer and SuperScript III Reverse Transcriptase (Invitrogen). qRT-PCR was performed with IQ SYBR Green Supermix (Bio-Rad) using two technical replicates each of two or three biological replicates. qRT-PCR primers are listed in Supplemental Table 1 online. qRT-PCR reactions were performed with iQ SYBR Green Supermix (Bio-Rad) using two technical replicates each of two or three biological replicates. qRT-PCR reactions were annealed at 57°C. The relative expression values for all experiments were calculated based on the expression of the experimentally validated control gene *Ubiquitin* as previously described (Satoh-Nagasawa et al., 2006). qRT-PCR was performed on a CFX96 thermocycler, and the results were analyzed on the CFX Manager Software package (Bio-Rad). Relative expression was calculated using the delta-delta method equation $2^{-\Delta\Delta CT_{\text{sample}} - \Delta\Delta CT_{\text{control}}}$, where 2 represents perfect PCR efficiency.

**Confocal Microscopy**

Confocal images in different developmental stages were hand-sectioned and visualized in water. Immature ears were counterstained with Calcofluor for 2 min and washed with a buffer (50% glycerol, 1× PBS, and 0.1% NaAzide). Fluorescent proteins were detected on an LSM 710 confocal microscope (Carl Zeiss). Bright field images were collected together with fluorescence images using the transmitted light detector and were processed into a blue background using Zeiss ZEN software and Adobe Photoshop CS4.

**Accession Numbers**

Sequence data from this article can be found in the GenBank and MaizeSequence (MaizeSequence.org) databases under the following accession numbers: *Tu1-A* (AJ850302), *Tu1-B* (AJ850303), *Tu1-d* (AJ850299), *Tu1-l* (AJ850300), and *Tu1-md* (AJ850301), GRMZM2G370777, and GRMZM2G006297.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Half-Tunicate Phenotype of Single Copy *Tu1-md* and *Tu1-rec* Heterozygous Mutants.

**Supplemental Figure 2.** A 30-kb Tandem Duplication at *Tu1*.

**Supplemental Figure 3.** Upregulation of *Zmm19* Expression in *Tu1* Reproductive Tissues.

**Supplemental Figure 4.** *Tu1-A:YFP* and *Tu1-B:RFP* Transgenic Tassels Phenocopy *Tu1* in a Dose-Dependent Manner.

**Supplemental Figure 5.** *Tu1-A:YFP* Is Expressed in Vegetative and Reproductive Tissues.

**Supplemental Figure 6.** *Tu1-A:YFP* and *Tu1-B:RFP* Proteins Are Colocalized in Nuclei.

**Supplemental Figure 7.** Gene Expression Data of GRMZM2G370777 and GRMZM2G006297.

**Supplemental Table 1.** Primers Used in Fine-Mapping and qRT-PCR.

**Supplemental Table 2.** Primers Used in Transgene Construction.

**Supplemental Table 3.** Primers Used in Allele-Specific and Long-Range PCRs.

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**AUTHOR CONTRIBUTIONS**

J.-J.H. performed the experiments, D.J. provided advice, materials, and help with microscopy, and R.M., D.J., and J.-J.H. designed the experiments and wrote the article.

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**REFERENCES**


SUPPLEMENTAL MATERIAL

**SUPPLEMENTAL FIGURE 5.** *Tu1-A:YFP* Is Expressed in Vegetative and Reproductive Tissues.

**SUPPLEMENTAL FIGURE 6.** *Tu1-A:YFP* and *Tu1-B:RFP* Proteins Are Colocalized in Nuclei.

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