

Duplicate *FLORICAULA/LEAFY* homologs *zfl1* and *zfl2* control inflorescence architecture and flower patterning in maize

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SUMMARY

The homologous transcription factors *FLORICAULA* of *Antirrhinum* and *LEAFY* of *Arabidopsis* share conserved roles in flower meristem identity and floral patterning. While roles for *FLORICAULA/LEAFY* homologs in flower development have been demonstrated in numerous dicots, little is known about the function of these meristem identity genes in the more distantly related flowering plants, the monocots. We used reverse genetics to investigate the role of two duplicate *FLORICAULA/LEAFY* homologs in maize (*Zea mays* L. ssp. *mays*) – a monocot species with dramatically different flower and inflorescence morphology from that of dicot species. Transposon insertions into the maize genes, *zfl1* and *zfl2*, led to a disruption of floral organ identity and patterning, as well as to defects in inflorescence architecture and in the vegetative to reproductive phase transition. Our results demonstrate that these genes share conserved roles with

their dicot counterparts in flower and inflorescence patterning. The phenotype of *zfl1; zfl2* double mutants suggests that these maize *FLORICAULA/LEAFY* homologs act as upstream regulators of the ABC floral organ identity genes, and this along with previously published work, indicates that the transcriptional network regulating flower development is at least partially conserved between monocots and dicots. Our data also suggest that the *zfl* genes may play a novel role in controlling quantitative aspects of inflorescence phyllotaxy in maize, consistent with their candidacy for quantitative trait loci that control differences in inflorescence structure between maize and its progenitor, teosinte.

Key words: Maize, Inflorescence architecture, *FLORICAULA*, *LEAFY*

INTRODUCTION

Comparative studies have begun to shed light on evolutionary conservation not just of individual gene functions, but of complex regulatory networks controlling morphological development. In insects for example, the germ band stage of development is patterned by a conserved network of genes (Patel, 1994). Similarly, studies in diverse angiosperms (flowering plants) have shown that expression and function of 'ABC' floral organ identity genes are widely conserved, suggesting that the determination of floral organ identity is achieved through similar genetic interactions in numerous species (Ambrose et al., 2000; Ma and dePamphilis, 2000; Ng and Yanofsky, 2001; Weigel and Meyerowitz, 1994).

Within the angiosperms, the divergence of monocots and dicots is estimated to have occurred over 150 million years ago (Wikstrom et al., 2001). While the basic organization of flowers is conserved between these groups, both monocots and dicots include some species with distinctive types of flowers.

Among monocots, the grasses in particular have highly divergent floral morphology when compared with typical dicots. For example, the grasses do not have clear homologs to the sepals and petals. Nevertheless, despite differences in floral morphology, at least some aspects of ABC gene function in flower development are conserved between maize (a grass) and dicots (Ambrose et al., 2000; Mena et al., 1996).

Currently, little is known about grass genes that act upstream of the conserved floral organ identity genes to regulate the transition from vegetative to reproductive growth and to control inflorescence architecture and floral meristem identity. Work with dicots, especially *Arabidopsis thaliana* and *Antirrhinum majus*, has begun to define a conserved transcriptional network upstream of the ABC genes (Araki, 2001; Bradley et al., 1997; Bradley et al., 1996; Carpenter et al., 1995; Ferrandiz et al., 2000). Central to this network is the meristem identity gene *FLORICAULA* (*FLO*) from *Antirrhinum* and its *Arabidopsis* homolog *LEAFY* (*LFY*) (Coen et al., 1990; Weigel et al., 1992). *FLO/LFY* plays an important role in the reproductive transition

and controls flower development by establishing the expression of the ABC floral organ identity genes (Coen and Meyerowitz, 1991; Huala and Sussex, 1992; Parcy et al., 1998; Weigel and Meyerowitz, 1994). Mutant phenotypes of *FLO/LFY* homologs in several other dicot species suggest that the function of *FLO/LFY* during reproductive development is largely conserved among the dicots, though its function during other stages of development may vary (Ahearn et al., 2001; Hofer et al., 1997; Molinero-Rosales et al., 1999; Souer et al., 1998).

To begin addressing whether the regulatory network involving *FLO/LFY*-like genes upstream of the ABC genes is conserved between maize and dicots, we analyzed loss-of-function mutants for the duplicate maize *FLO/LFY* homologs, *zfl1* and *zfl2*. The maize mutant phenotypes revealed that *zfl1* and *zfl2* play similar roles in the reproductive transition and in flower development as their dicot homologs. The mutant phenotype observed in flowers specifically suggests a conserved role for the *zfl* genes as upstream regulators of the maize counterparts of the dicot ABC floral organ identity genes. The mutant phenotype also suggests that the *zfl* genes play a novel quantitative role in inflorescence phyllotaxy, supporting *zfl2* as a candidate gene for a maize domestication quantitative trait locus (QTL) controlling differences in inflorescence architecture between maize and its wild ancestor, teosinte (Doebley, 1992).

MATERIALS AND METHODS

Cloning and sequencing of *zfl1* and *zfl2*

A *zfl1* sequence segment (a gift from Detlef Weigel) was used to design two primers (5'ACCAACCAGGTGTTCCGGTACGC3'; 5'CTGGCGCAGCCTGGTGGGCACGTA3') that amplified a 283 bp segment of *zfl1*. This segment was used to screen a cDNA library constructed in λ Zap II (Stratagene) using mRNA from ear primordia of the maize inbred line A632. We recovered and sequenced a 1323 bp *zfl1* cDNA clone (GenBank AY179882) that was then used to screen genomic libraries constructed in λ Dash II (Stratagene) with *Bam*HI digested genomic DNA of the maize inbred lines W22 and A632. From the W22 library, we isolated a 17 kb *Bam*HI clone that contained *zfl1* (GenBank AY179883) and a 10 kb *Bam*HI clone that contained *zfl2*. From A632, a 10 kb clone was isolated for *zfl1* and a 17 kb clone was isolated and sequenced for *zfl2* (GenBank AY179881). Because of a conserved *Bam*HI site just downstream of the ATG start codon in both genes, the genomic clones are missing the first three base pairs of the coding sequence.

Similar and identical amino acids in protein alignment were identified using Boxshade v.3.1.1 (<http://workbench.sdsc.edu>) with default settings. Percentage identity and similarity were calculated by 2-way BLAST (<http://www.ncbi.nlm.nih.gov>) using the BLOSUM62 amino acid similarity matrix with default settings except that 'filter' was turned off.

Isolation of *Mutator* insertions in *zfl1* and *zfl2*

Approximately 42,000 F₁ plants carrying active *Mutator* (*Mu*) transposable elements were screened at Pioneer Hi-Bred International by PCR for *Mu* insertions in *zfl1* and *zfl2* using a *Mu* terminal repeat specific primer (5'AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC3') in combination with either a *zfl1* (5'TGTGTGTTTTGCCTCTGCGAGCAATGTG3') or *zfl2* (5'GGATCTCGGAGCTCGGGTTCAC3') specific primer (Meeley and Briggs, 1995). PCR products were sequenced to verify insertions. Two *zfl1* insertion events (*zfl1-mum1*, *zfl1-mum2*) and six *zfl2* insertion events (of which three were analyzed; *zfl2-mum1*, *zfl2-mum2*, *zfl2-mum4*) were identified. To

generate families segregating for the *Mu* insertion alleles at both *zfl1* and *zfl2*, plants carrying the different insertion alleles were first crossed to the W22 inbred line for one or two generations to improve the vigor of the stocks. The progeny of these crosses were then intercrossed to create a set of plants heterozygous for an insertion and wild-type allele at both *zfl1* and *zfl2*. Doubly heterozygous plants were either selfed to create a family segregating for both *zfl1* and *zfl2* in W22 background, or crossed to a 'Mu-Killer' stock (*les28/+*; *a1-mum1*) that suppresses *Mu* activity to further improve plant vigor (Martienssen and Baron, 1994). The progeny of these latter crosses were selfed to obtain plants segregating for both *zfl1* and *zfl2* in a background in which the mutagenic effects of *Mu* had been quelled.

Throughout this breeding process, we used restriction fragment length polymorphism (RFLP) analysis to trace the insertion and wild-type alleles. Specific alleles were identified by RFLP analyses in which genomic DNAs were digested with *Hind*III (for *zfl1-mum2*; *zfl2-mum4*, and *zfl1-mum1*; *zfl2-mum2* segregants) or *Xba*I (for *zfl1-mum1*; *zfl2-mum1* segregants), Southern blotted, and probed as previously described (Doebley and Stec, 1991) with a *zfl1* cDNA probe. Novel RFLPs were associated with *zfl* *Mu* insertion alleles by PCR with *Mu*- and *zfl*-specific primers.

Phenotyping

Phenotypic characterization of the double mutants was performed using three families that segregated for different combinations of the insertion alleles: (i) MK family – *zfl1-mum1*; *zfl2-mum1* in *Mu*-Killer background (299 plants), (ii) W1 family – *zfl1-mum2*; *zfl2-mum4* in W22 background (87 plants) and (iii) W2 family – *zfl1-mum2*; *zfl2-mum2* in W22 background (55 plants). Association of quantitative phenotypes with *zfl* genes was tested in the MK family segregating for *zfl1-mum1*; *zfl2-mum1* using analysis of variance (ANOVA) in the JMP software program (SAS Institute). The traits analyzed included days to pollen shed, total leaf number, number of long tassel branches, and ear and tassel rank (inflorescence phyllotaxy). Inflorescence phyllotaxy was measured as the number of ranks of spikelet-pairs around the circumference of the ear or central tassel spike (Doebley, 1992).

RNA isolation and analysis

TRI reagent (Molecular Research Center) was used to isolate total cellular RNA. RNA for RT-PCR was isolated from several developmental stages and tissues of the W22 inbred line. Vegetative shoot apical meristems (Veg. SAM) were pooled from seven plants to obtain sufficient tissue for RNA isolation. 'Early ear' RNA was obtained from three ears collected from three plants, and 'older ear' RNA was from two ears. 'Young tassel' RNA was isolated from a mixture of tissue from five plants, and 'older male' RNA was obtained from the tassel of a single plant. Vegetative leaf RNA was also obtained from a single plant. RNA used for northern analysis was isolated from developing ears of wild-type and *zfl1-mum1*; *zfl2-mum1* double mutant plants segregating in the *Mu*-killer background.

For RT-PCR, 1 μ g of total RNA was reverse transcribed with Superscript II (Invitrogen) using a primer designed to anneal to both *zfl1* and *zfl2* (5'ACATCGACGACGCAGCTAGA3'). PCR reactions were performed across intron 2 with a primer pair designed to amplify both *zfl* genes (5'GAACGGGCTTGACTACCT3'; 5'GCGTAGCAGTGCACGTAG3'). Since *zfl2* possesses a *Pst*I site in this PCR product that is absent in *zfl1*, RT-PCR products were restricted with *Pst*I to differentiate between the transcripts of the two genes. The fragments were visualized on 3.5% MetaPhor agarose gels (BioWhittaker Molecular Applications). As a cDNA synthesis control, the same RNA samples were reverse transcribed with a mixture of two maize actin primers (5'TCATGGCAGTTCATGTATTG3'; 5'AACTCTGAGGCAACACGTTA). Actin PCR reactions were performed in parallel with *zfl* reactions, using a primer pair that spans an 883 bp intron (5'CATGAGCCACGTACAACCT3'; 5'TCATGGCAGTTCATGTATTG3') and gives a 415 bp product. Primers were designed based on GenBank sequences AY104628 and U60508.

For northern blots, 6 µg total RNA was electrophoresed in formaldehyde gels and transferred as previously described (Sambrook et al., 1989) to Hybond-XL nylon membranes (Amersham). Membranes were hybridized with a ³²P-labeled *zfl* exon 3 probe and washed as previously described (Doebley and Stec, 1991). The same blots were stripped and probed with a maize ubiquitin cDNA probe to verify equivalent loading and RNA quality (Christiansen and Quail, 1989).

Histology and in situ hybridization

Samples for histological analysis were fixed in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol), dehydrated in an ethanol series and infiltrated with HistoClear (National Diagnostics). Samples were then embedded in Paraplast Plus (Oxford Labware). 8-10 µm sections were mounted on ProbeOn Plus glass slides (Fisher Scientific), stained in 0.5% aqueous Safranin overnight, counterstained with 1% Fast Green FCF in 95% ethanol for 30-60 seconds, and cleared in HistoClear. Some sections were stained directly with aqueous 0.05% Toluidine Blue O for 10-30 minutes. The protocols were adapted from Berlyn and Miksche (Berlyn and Miksche, 1976).

Methods for preparing tissue from immature male inflorescences and in situ hybridization with digoxigenin-labeled RNA probes were described previously (Ambrose et al., 2000). The antisense RNA probe was generated by first subcloning a 426 bp *SacI-HincII* fragment of the *zfl1* cDNA into the *SacI HincII* restriction sites of pBluescript SK (Stratagene). This clone was then linearized by cutting at an internal *NotI* restriction site and transcribed with T7 RNA polymerase in the presence of digoxigenin-coupled UTP (DIG RNA labeling mix; Boehringer Mannheim) to produce an in situ hybridization probe containing 287 bases of *zfl1* sequence. The probe spans parts of exons one and two and is 89% identical between *zfl1* and *zfl2*.

Scanning electron microscopy (SEM)

Developing ears and tassels from wild-type and *zfl* double mutant plants in either the MK or W2 families were fixed in 2% glutaraldehyde in phosphate buffer (0.05 M KPO₄ pH 7.0) overnight at 4°C, then dehydrated in an ethanol series and critical point dried. Mounted samples were sputter coated with gold and viewed at 5 kV accelerating voltage in a Hitachi S570 SEM.

RESULTS

Cloning of the maize *FLORICAULA/LEAFY* homologs

We isolated a 1323 bp cDNA clone from an immature ear cDNA library. The clone contains an open reading frame encoding a putative protein of 392 amino acids (Fig. 1A). Based on high homology to the rice *FLORICAULA(FLO)/LEAFY(LFY)* gene, *RFL* (Kyojuka et al., 1998), and to other *FLO/LFY* genes, the gene represented by the cDNA was named *zfl1* (*Zea FLO/LFY 1*). Screening of genomic libraries constructed from inbred lines A632 and W22 yielded genomic clones of both *zfl1* and a second maize *FLO/LFY* gene, *zfl2*. Comparison of cDNA and genomic clones revealed that both *zfl1* and *zfl2* contain three exons and that their intron-exon structure is conserved with other *FLO/LFY* genes (Fig. 1B).

The putative ZFL1 and ZFL2 proteins are 91% identical and 92% similar to one another, and 80% and 83% identical (85% and 88% similar) to RFL, respectively. The ZFL1 and ZFL2 proteins are about 57% and 56% identical (67% and 65% similar) to FLO, respectively (Coen et al., 1990). ZFL1 and ZFL2 are 54% and 55% identical (65% and 66% similar) to

LFY, respectively (Weigel et al., 1992). The highest degree of conservation is in the C-terminal region (Fig. 1A), which has been reported to bind DNA in *Arabidopsis* (Gocal et al., 2001). A proline-rich region, a leucine repeat region and basic and acidic domains known from dicot *FLO/LFY* (Coen et al., 1990;

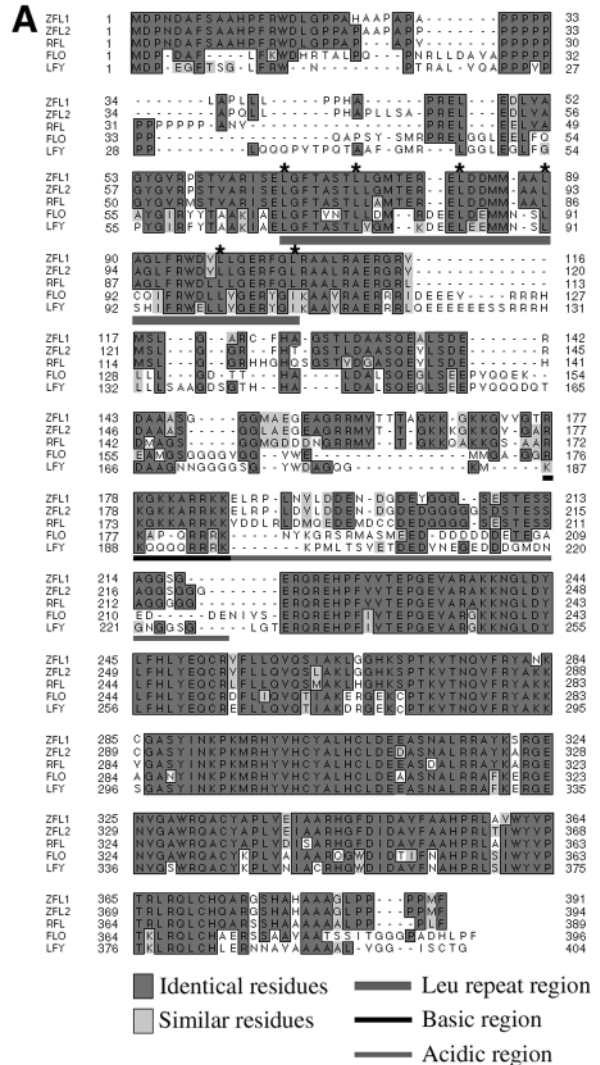


Fig. 1. *zfl1* and *zfl2* gene structures. (A) Alignment of *zfl1* and *zfl2* proteins deduced from cDNA sequences with *RFL* (Kyojuka et al., 1998), *FLO* (Coen et al., 1990) and *LFY* (Weigel et al., 1992). Repeated leucine residues are indicated by a star. Similar amino acids were identified using BoxShade version 3.1.1. (B) Exon/intron arrangement and sizes (in bp) of *zfl1* and *zfl2*. Five independently derived *Mu* insertion sites are indicated with triangles and labeled with corresponding *mum* allele names.

Weigel et al., 1992) are also present in the maize proteins (Fig. 1A). Although the functions of these regions are unknown, these similarities suggest conserved functions between the grass and dicot proteins.

Genomic Southern blots probed with *zfl1* cDNA consistently revealed two bands in maize inbreds W22 and A632, suggesting that *zfl1* and *zfl2* are the only *FLO/LFY* homologs in maize. These genes were previously mapped and are listed as *ucsd(lfya)* (= *zfl1*) and *ucsd(lfyb)* (= *zfl2*) in the Maize Database (www.agron.missouri.edu). *zfl1* maps near *umc44a* on chromosome 10, while *zfl2* maps near *umc6a* on chromosome 2. These chromosomal regions contain numerous other duplicate genes thought to have arisen via genome duplication (Berhan et al., 1993; Devos and Gale, 1997; Gale and Devos, 1998; Moore et al., 1995). We calculated synonymous nucleotide divergence (K_s) between *zfl1* and *zfl2* as described by Gaut and Doebley (Gaut and Doebley, 1997). The divergence ($K_s=0.1798$) is within the range observed for many other duplicated maize genes, suggesting that the *zfl* genes were duplicated in the tetraploidy event thought to have occurred approximately 11 million years ago in the lineage leading to maize and its relatives (Gaut and Doebley, 1997).

Expression of *zfl1* and *zfl2*

Expression studies in numerous species have shown that *FLO/LFY* homologs are transcribed both prior to and during reproductive development. To determine whether this is true of the *zfl* genes in maize, we used an RT-PCR approach that distinguishes the two transcripts (Fig. 2). We detected both *zfl1* and *zfl2* transcripts in 20-day old vegetative apices (including a few leaf primordia) and during male and female reproductive development (Fig. 2B). Expression was strongest relative to actin controls during early reproductive development. We did not detect *zfl* transcripts in samples from developing leaves (Fig. 2B).

We examined the *zfl* expression pattern during reproductive

development in more detail using mRNA in situ hybridization. The probe used is expected to hybridize to both *zfl1* and *zfl2*, and the pattern observed likely reflects the combined expression of both genes. *zfl* mRNA was initially detected at the flanks of both female and male inflorescence meristems in regions where spikelet-pair meristems initiate (Fig. 2C,D). The spikelet-pair primordia continue to strongly express *zfl* as they develop and branch to form two spikelets (small determinate branches that give rise to two florets each) (Fig. 2C-E). *zfl* mRNA continues to be expressed at high levels in spikelet meristems (Fig. 2E,F). Following the initiation of two florets

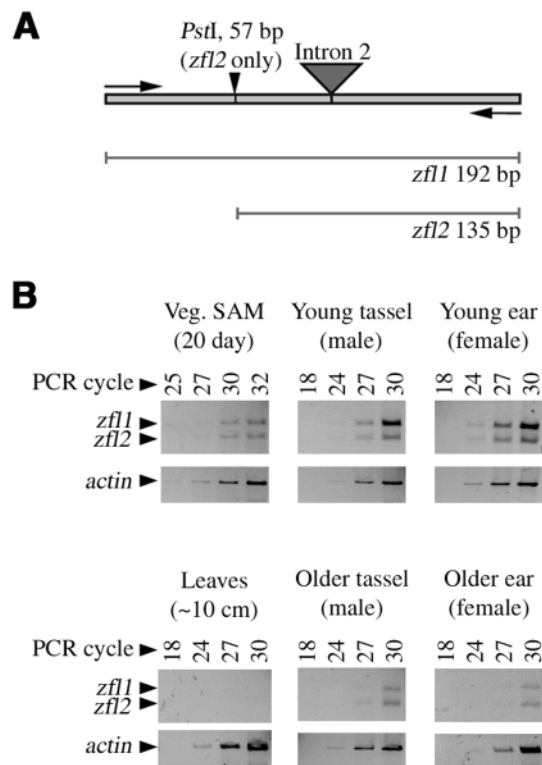
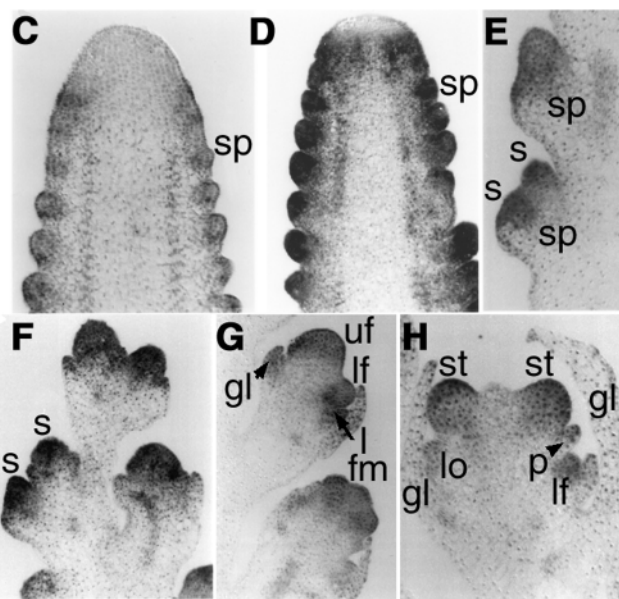


Fig. 2. Expression analysis of *zfl*. (A) 192 bp RT-PCR product and position of the *PstI* site used to discern *zfl1* and *zfl2* transcripts. (B) Inverted ethidium bromide-stained gel images of *zfl* RT-PCR products restricted with *PstI*, and actin cDNA synthesis and PCR controls. Developmental stages are indicated. Vegetative shoot apical meristem (Veg. SAM) RNA includes the youngest two to three leaf primordia. Higher cycle numbers were used for this tissue because of low actin amplification. Vegetative leaves were collected prior to emergence from the leaf whorl. 'Young tassel' RNA was collected at 34 days, just after reproductive transition, while the apex is producing branches and beginning to initiate spikelet pairs. 'Older tassel' RNA was collected from inflorescences with differentiated stamens evident in the florets, but prior to tassel emergence. 'Young ears' were 3-5 mm long and producing spikelet pairs and spikelets. 'Older ears' were 1-1.5 cm long and had differentiated organs visible in their florets. (C-H) *zfl* expression analysis by mRNA in situ hybridization. (C) Developing ear. (D) Developing tassel. Developing spikelet-pairs (sp) are visible. (E) Male spikelets (s) developing from the spikelet pair meristem (sp). (F) Spikelet meristems (s) and initiating subtending glume primordia. (G) Branching spikelets forming upper (uf) and lower (lf) florets. Arrows indicate glume (gl) and primordia lemma (l). A floral meristem (fm) with stamens and gynoecium apparent is also visible. (H) Later male floret with developing stamen primordia (st), palea (p), lemma, lower florets (lf), lodicules (lo) and glumes (gl).



in each spikelet, *zfl* expression becomes restricted to the upper half of the floret meristems (Fig. 2G,H) and is absent from the region between the developing florets (Fig. 2G). As florets develop, *zfl* mRNA is detected in developing floral organ primordia (Fig. 2H).

Transposon mutagenesis of *zfl1* and *zfl2*

Using a reverse genetics approach, we isolated and analyzed two independent *Mutator* (*Mu*) transposon insertion alleles of *zfl1* (*zfl1-mum1* and *zfl1-mum2*) and three independent insertion alleles of *zfl2* (*zfl2-mum1*, *zfl2-mum2* and *zfl2-mum4*) (Fig. 1B). All *Mu* alleles analyzed carry insertions in one of the first two exons of *zfl1* or *zfl2*.

To determine whether the insertion alleles are loss-of-function (RNA nulls), we used northern blot analysis with *zfl1-mum1* and *zfl2-mum1*. Since these *Mu* insertion alleles had novel restriction fragment length polymorphisms (RFLPs), we could identify plants homozygous for insertion alleles at both *zfl1* and *zfl2* (double mutants) by Southern hybridization (Fig. 3A). A northern blot with total RNA from immature ears of double mutant and wild-type plants produced a hybridization signal at about 1400 nt for wild-type plants, but RNA from plants doubly homozygous for *zfl1* and *zfl2* *Mu* alleles showed no signal (Fig. 3B). The failure to detect normal transcript suggests that *zfl1-mum1* and *zfl2-*

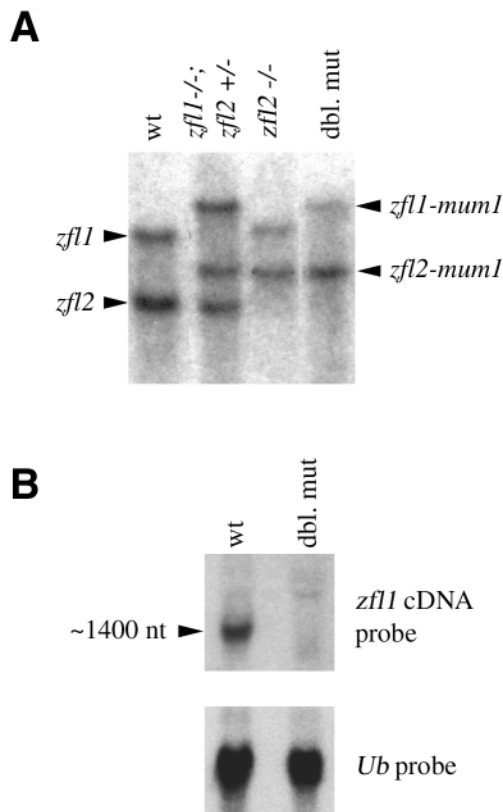


Fig. 3. (A) Autoradiograph of Southern blot with *Hind*III-digested genomic DNA showing novel bands corresponding to *Mu* alleles. (B) Autoradiograph of northern blot of total RNA from developing ears probed with a *zfl* exon 3 PCR product. Signal was detected only in wild type at about 1400 nt (left). The same membrane was stripped and re-probed with maize ubiquitin cDNA (*Ub*).

mum1 are likely null alleles, and is consistent with the anticipated consequence of *Mu* transposon insertions within the exons of these genes.

To determine the nature of the double mutant phenotype, two independent families (MK and W1) that segregate for different combinations of the insertion alleles were analyzed. Plants of both families were genotyped by RFLP analysis and the genotypic ratios for *zfl1* and *zfl2* RFLP alleles fit Mendelian expectations for two independently segregating loci ($\chi^2 \leq 12.9$; $P \geq 0.12$). A novel qualitative phenotype (loss of floral meristem identity, described below) was observed in both families at frequencies of 12/299 (MK family) and 5/87 (W1 family). This phenotype was found only in plants homozygous for insertion alleles at both *zfl1* and *zfl2*, and all such double homozygotes exhibited this phenotype. The frequency of the novel phenotype in both families fit a 15:1 ratio as expected for two redundant loci ($\chi^2 \leq 2.6$; $P \geq 0.10$).

Loss of *zfl* function affects the vegetative to reproductive transition

Vegetative development in *zfl1*; *zfl2* double mutant plants is normal (Fig. 4A), but morphological defects become apparent during the transition to reproductive development (Fig. 4A-D). Whereas wild-type maize plants switch abruptly from forming leaves to forming tassel branches, in *zfl1*; *zfl2* double mutants, this transition is severely compromised. The upper nodes of *zfl1*; *zfl2* double mutant plants regularly produce 'tassel ears,' branched reproductive structures with husk leaves surrounding a female inflorescence often with a terminal spike of male flowers (Fig. 4C,D). Toward the tip of the plant, these structures become progressively more like standard long tassel branches (Fig. 4D). We quantified the frequency of 'tassel ears' in a family (MK) of 299 plants segregating for *zfl1-mum1* and *zfl2-mum1*. In this family, the double mutant plants generated from zero to eight 'tassel ears' (avg. 3.4 ± 0.9 vs. 0 in wild-type siblings). In double mutant plants, internodes between the 'tassel ears' are frequently short and twisted, with leaves often partially fused to two adjacent nodes. These aberrant internodes are interspersed with normal internodes, resulting in a twisted stem and uneven leaf distribution in the upper part of the plant, a phenotype that is strikingly similar to the *terminal ear1* mutant in maize (Veit et al., 1998). Above the aberrant internodes, *zfl* double mutants form a reduced number of tassel branches (avg. 0.6 ± 0.9 vs. 9.7 ± 0.6 in wild-type siblings) and a normal central tassel spike with polystichous spikelet-pair phyllotaxy as in their wild-type siblings.

Ear shoots (lateral branches terminating with female inflorescences) in wild-type maize form in leaf axils about five nodes below the tassel. After producing a number of modified leaves (husks), ear shoots transition abruptly to the polystichous inflorescence (the ear) and do not develop long branches. Double mutant plants initiated ear shoots with husk leaves as in wild-type siblings. However, these often developed secondary ears in husk leaf axils at the base of the main ear. These secondary ears were not observed in wild-type siblings and may be equivalent to the 'tassel ears' produced during the transition to tassel development. Owing to defects in flower development (described below), ears on double mutant plants were largely sterile, but kernels sometimes formed late in development (Fig. 4E).

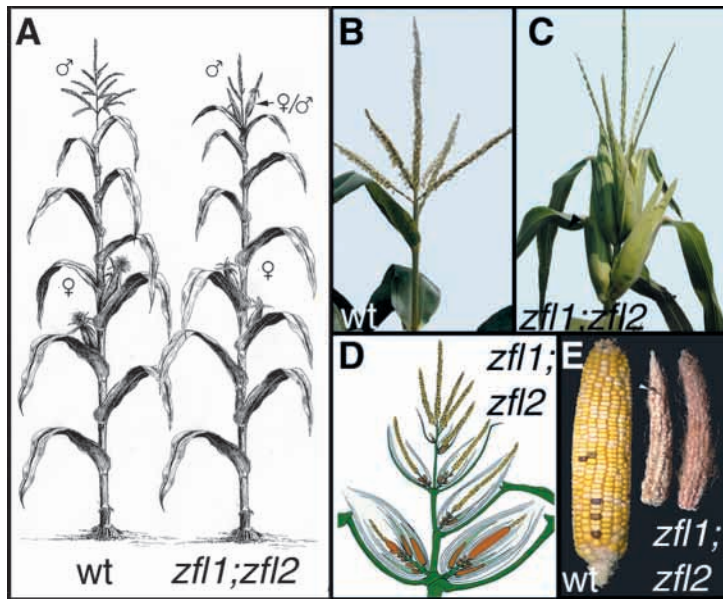


Fig. 4. Whole plant defects in *zfl* double mutant plants. (A) Illustration of wild type (left) and double mutant (right). (B) A wild-type tassel. (C) Apex of a *zfl* double mutant plant showing several 'tassel ears'. (D) Diagrammatic illustration of apical region of an individual double mutant plant showing complex axillary structures with husk leaves, multiple ears, and male (yellow) and female (brown) florets often subtended by husk leaves. (E) Wild type (left) and two double mutant (right) ears from sibling plants. Arrowhead indicates a single kernel on a double mutant ear.

Loss of *zfl* function disrupts floral development

The wild-type maize ear bears its flowers in spikelets, which are arranged in pairs along the axis of the ear (Fig. 5A). During spikelet development, a pair of glumes is initiated first, followed by the lower and upper florets. The lower floret aborts early in development such that each female spikelet has only one mature floret (Fig. 5A,C). Each female floret initiates a lemma, palea, three stamens and a gynoecium. The stamen primordia subsequently abort (Fig. 5C). The gynoecial primordium forms a ridge that expands to enclose the developing ovule and then part of the gynoecial ridge elongates to form the silk (Fig. 5E,G,I) (Cheng et al., 1983). Growth of the female floret meristem terminates with differentiation of a single ovule (Fig. 5I, Ov).

As in wild-type plants, spikelets are formed in pairs on the ears of *zfl1; zfl2* double mutant plants, each spikelet producing normal glumes, and initiating two florets with normal paleas and lemmas (Fig. 5B). Subsequent development deviates severely from wild type. Stamen primordia are rarely observed in double mutant female florets. Instead, the floret meristem frequently branches to give rise to additional meristem-like structures, sometimes associated with separate lemmas or paleas (Fig. 5D). Double mutants also fail to exhibit normal whorled organ phyllotaxy and a normal gynoecium rarely forms (Fig. 5F). Older double mutant female florets produce a proliferation of carpelloid organs with silks and other organs of unclear identity (Fig. 5F,H,J), suggesting a loss of determinacy and defects in organ identity. Ectopic vegetative outgrowths were occasionally observed on organs of double mutant florets (Fig. 5L),

suggesting defects in the maintenance of organ identity. Unlike the wild type, the center of the floral meristem of double mutant plants frequently continues to produce lateral structures of unclear identity, at times in a spiral arrangement (Fig. 5K, asterisk). Early-arising spikelet-pairs on double mutant ears often become highly branched and occasionally develop inflorescence or branch-like structures in place of flowers (Fig. 5M,N). Kernels can rarely form on double mutant ears (Fig. 4E).

Similar to the ear, the wild-type maize tassel bears its flowers in spikelets, which are arranged in pairs along the axis of the tassel branches and central spike (not shown). Tassel spikelet meristems initiate a pair of glumes, followed by the lower and upper florets, both of which develop fully (Fig. 6A,C). Male florets consist of a lemma and palea that subtend two lodicules and three stamens. Like female florets, wild-type male florets are initially bisexual, but the central gynoecium aborts while the three stamens develop to maturity (Cheng et al., 1983).

In *zfl1; zfl2* double mutant plants, early male spikelet development is similar to that of wild-type plants with the initiation of two glumes surrounding two florets, each with a lemma and palea. Subsequently, double mutant male florets proliferate organs with vegetative characters (palea?) in a spiral phyllotaxis (Fig. 6B,E,F,H), suggesting defects in floret determinacy, organ identity and phyllotaxy. In contrast to female development, branching of the male floral meristem was rarely observed in double mutants (Fig. 6B).

Second and third whorl organ development in male flowers is severely affected in double mutant plants. The second whorl lodicules of wild-type male florets swell at maturity to open the florets and facilitate pollen shed. Lodicules of double mutant plants are frequently chimeric with lemma/palea-like outgrowths or are missing entirely, and consequently, the florets rarely open at maturity. The third whorl of wild-type male florets contains three stamens. Double mutant florets develop few or no stamens (Fig. 6D-H), and those that do develop show defects including twisting (Fig. 6F,G), a decreased number and size of locules (Fig. 6D), and lemma or palea-like outgrowths (Fig. 6G, asterisk). Though pollen grains are sometimes present in the locules, these plants do not shed pollen, suggesting defects in stamen maturation or dehiscence.

Quantitative variation associated with *zfl* genotype

In addition to the qualitative morphological defects resulting from loss of *zfl* function, we found statistically significant associations ($P < 0.05$) between some quantitative traits and active *zfl* copy number. This was done using the MK family of 299 plants segregating for the *zfl1-mum1* and *zfl2-mum1* alleles. All plants were genotyped by RFLP analysis at both *zfl1* and *zfl2*, and classified for total number of active (wild-type) copies of *zfl* from zero (double mutant) to four (fully wild type). Associations between phenotypic traits and *zfl1* and *zfl2* genotype and total *zfl* copy number were assessed by ANOVA. Dominance/additivity (d/a) ratios were calculated to determine whether trends were additive ($|d/a| < 0.5$) or dominant ($|d/a| > 0.5$), where $a = wt/2 - mut/2$ and $d = Het - (mut/2 + wt/2)$. Data from the double mutant class were excluded (except where noted) to ensure that quantitative trends were not

influenced by the major morphological defects of the double mutant genotypic class.

Flowering time is significantly associated with genotype for both *zfl1* and *zfl2* whether measured in developmental (leaf number) or actual (days to pollen shed) time (Fig. 7A). An additive trend of increasing leaf number is associated with a decreasing number of active *zfl1* copies, while *zfl2* is associated with a dominant trend (Fig. 7A). Similarly, an additive trend of increasing time to pollen shed is significantly associated with a decreasing number of active *zfl1* copies, while *zfl2* is associated with a dominant trend (Fig. 7A).

Variation in several inflorescence architecture traits is also

associated with *zfl* copy number. A decrease in the number of long tassel branches is significantly associated with decreasing active *zfl2* copy number, while decreasing active *zfl1* copy number is significantly associated with an increase in the number of long tassel branches (Fig. 7B). In this case, both trends are dominant. Since *zfl1* genotype is associated with a trend opposite to that associated with *zfl2*, we asked whether *zfl1* is able to promote branch initiation in the absence of ZFL2 activity. To answer this, we analyzed a subset of 65 plants homozygous for *zfl2-mum1*. Decreasing *zfl1* copy number in the absence of active ZFL2 is associated with a statistically significant ($P < 0.0001$) decrease in tassel branch number from

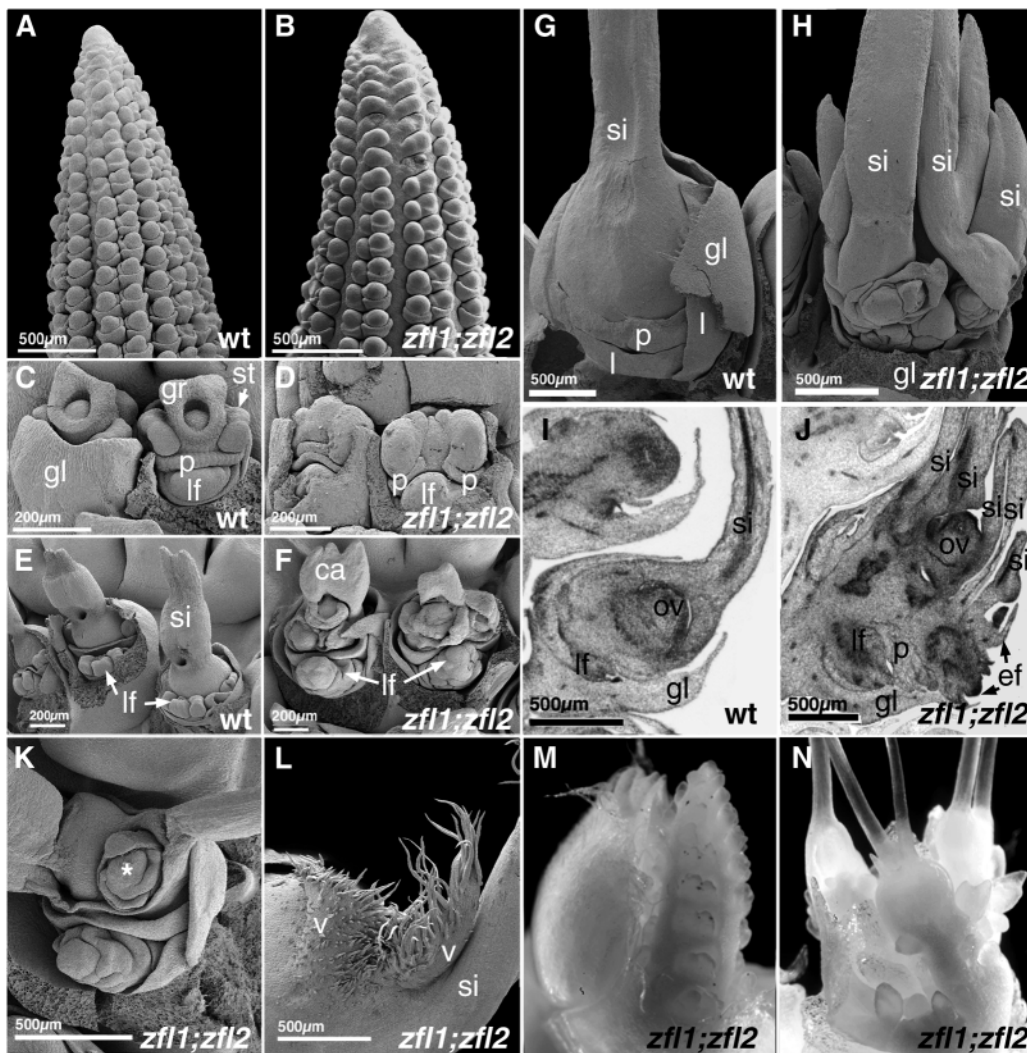


Fig. 5. Scanning electron microscopy and histology of developing female reproductive organs. (A) Wild-type ear showing developing spikelet pairs. (B) Double mutant ear with normal spikelet-pair initiation. (C) Wild-type spikelet pair showing two upper florets with glume (gl), palea (p), stamen primordia (arrow; st) and a gynoeceal ridge (gr) that surrounds the ovule. The lower floret (lf) is visible. (D) Double mutant spikelet pair with upper floret initiating multiple floret meristems subtended by separate paleas (p). (E) Wild-type female florets showing carpels forming silk (si). (F) Double mutant florets generating abnormal organs in aberrant arrangements; ca, a carpel-like organ in the left spikelet. (G) Wild-type floret with a single, fully formed silk (si). (H) Double mutant floret with many silks. (I) Longitudinal section of a wild-type spikelet showing fully formed silk and the carpel surrounding the ovule (ov). (J) Longitudinal section of a double mutant spikelet. Ectopic florets (ef) are visible. Multiple silks arise from multiple carpel layers surrounding an ovule. (K) Double mutant spikelet showing a spiral of organ primordia at the center of one floret (*). (L) A chimeric carpel on a double mutant floret with vegetative outgrowth (v). (M) An early-arising floret replaced by an inflorescence-like structure. (N) A portion of a highly branched early floret of a double mutant plant.

9.3±0.6 in plants with two copies of *zfl1* and 8.2±0.4 in plants with one copy of *zfl1* to 0.64±0.9 branches in double mutants. This suggests that despite the negative branching trend associated with *zfl1* when wild-type copies of *zfl2* are present, a single active copy of *zfl1* is sufficient to rescue the branching defect of double mutant plants.

We measured inflorescence phyllotaxy by scoring tassel and ear rank (the number of spikelet-pair ranks, or vertical rows, produced around the circumference of the male and female primary inflorescence axes). A statistically significant additive trend of decreasing tassel rank is associated with a decreasing number of active *zfl1* copies (Fig. 7B). The association of tassel

rank with *zfl2* shows the same trend, although the *P* value for statistical significance falls just above the usual *P*=0.05 cut off. A statistically significant additive decrease in ear rank is associated with decreasing active copy number of both *zfl1* and *zfl2* (Fig. 7B).

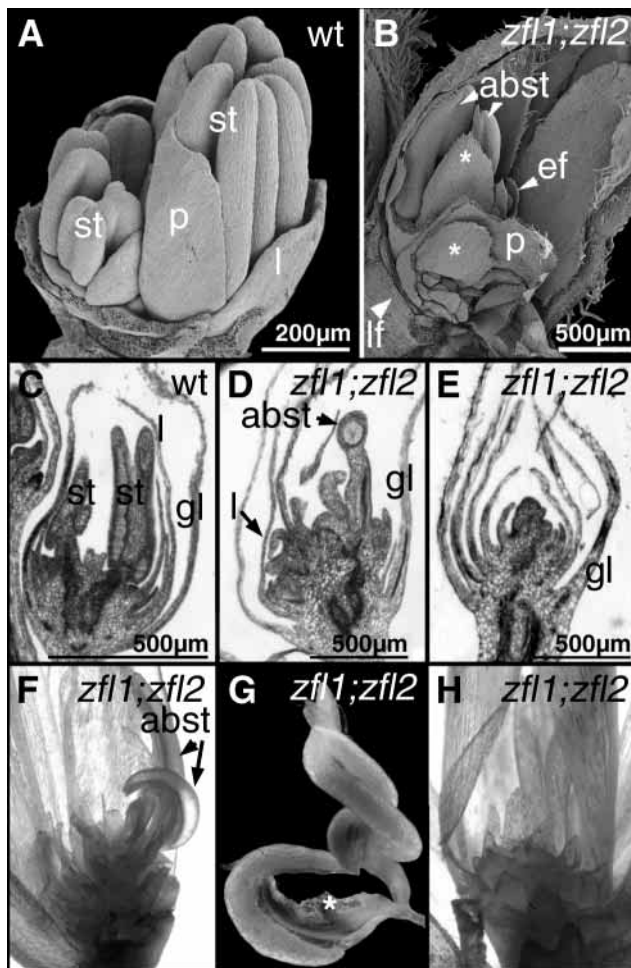


Fig. 6. Scanning electron microscopy and histology of developing male reproductive organs. (A) Developing wild-type male spikelet showing the upper (right) and lower floret with stamens (st), a palea (p) and lemma (l). (B) A double mutant male spikelet showing a broken palea (p), abnormal stamens (abst) in the upper floret, extra vegetative organs (*) in both florets, and a small ectopic floret (ef). (C) Longitudinal section of a wild-type male spikelet showing glumes (gl), lemma (l), and two normal stamens (st). (D) A double mutant spikelet showing glume, lemma and an abnormal stamen with a small locule. (E) An indeterminate double mutant spikelet. (F) Whole-mount image of a double mutant floret with two abnormal stamens and overproliferating vegetative organs. (G) Stamens from a double mutant floret showing twisting and vegetative outgrowths (*). (H) A double mutant floret with overproliferating vegetative organs and no stamens.

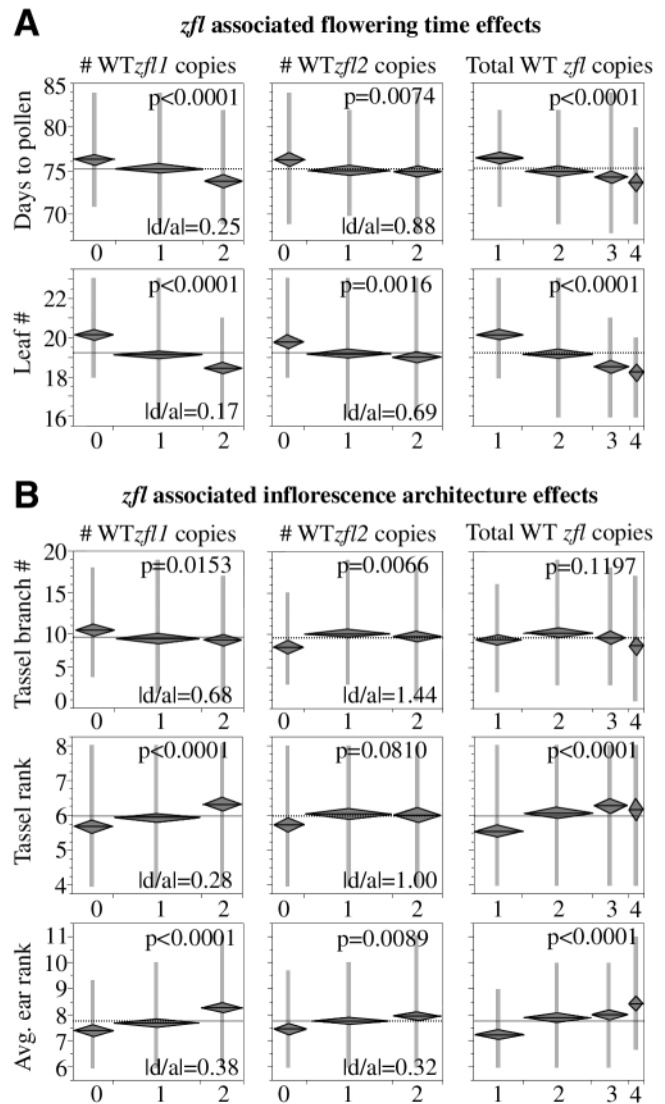


Fig. 7. Quantitative effects associated with the *zfl* genotype, excluding the double mutant class. Trait values are plotted on the Y axis of each graph, and active (wild-type) *zfl* copy number is plotted on the X axis. Grey bars represent the range for each genotype. Diamonds in each category are centered on the mean value for the trait within a genotypic class. The width of the diamond is proportional to the number of individuals in each class, and height represents the 95% confidence interval for each class. The left column of graphs shows associations with *zfl1*, the middle column shows associations with *zfl2*, and the right column shows effects associated with total active *zfl* copy number (numbers of active *zfl1* and *zfl2* in the plant combined). Each graph has the *P* value for the associated ANOVA indicated in the upper right corner, and a ld/al ratio in the lower right corner. (A) Flowering time effects were measured by leaf number and days to pollen production. (B) Inflorescence architecture effects, including tassel branch number, and tassel and ear rank.

DISCUSSION

zfl plays a conserved role in floral development

The phenotype of the *zfl1*; *zfl2* double mutant suggests that the function of ZFL in floral meristem identity, phyllotaxy and organ identity is largely conserved between maize and dicots. In *Arabidopsis*, *LFY* has been shown to control flower development largely via activation of downstream ABC floral organ identity genes (Bowman et al., 1991; Coen and Meyerowitz, 1991; Huala and Sussex, 1992; Parcy et al., 1998; Weigel et al., 1992; Weigel and Meyerowitz, 1994), and flowering defects in plants mutant for *FLO/LFY* homologs in other species suggest that this role is conserved in many dicots (Ahearn et al., 2001; Coen et al., 1990; Hofer et al., 1997; Molinero-Rosales et al., 1999; Souer et al., 1998). The applicability of the ABC model to flower development in the grasses has been tested in maize, and despite divergent flower morphology, maize homologs of dicot B and C genes were found to have largely conserved functions (Ambrose et al., 2000; Mena et al., 1996). Maize plants homozygous for B and C gene mutations have similar defects in floral organ identity and determinacy as those observed in *zfl* double mutant flowers (Ambrose et al., 2000). This suggests that *zfl* may establish expression of the ABC flower patterning genes in maize as *FLO/LFY* does in dicots, and by extension, that the transcriptional network that regulates flower development in dicots is at least partially conserved within the monocots.

Concordant with the similarity in mutant phenotype between *zfl* in maize and *FLO/LFY* in dicots, these genes share a similar expression pattern during floral development. *zfl* mRNA is expressed throughout early floral meristems and subsequently relegated to developing organ primordia (Fig. 2C-H). This pattern is similar to the floral expression patterns of *FLO/LFY*-like genes reported in several dicot species (Coen et al., 1990; Hofer et al., 1997; Kelly et al., 1995; Molinero-Rosales et al., 1999; Souer et al., 1998; Weigel et al., 1992). The conserved expression pattern and similar mutant effects on floral development, suggest that dicot *FLO/LFY* and *zfl* play a conserved role in floral development.

Divergent expression patterns of *FLO/LFY* homologs have been reported for two grasses, rice and *Lolium temulentum* (Gocal et al., 2001; Kyojuka et al., 1998). The rice homolog, *RFL*, was detected in developing panicle branches, but not in initiating branch or flower meristems. Kyojuka et al. proposed that *RFL* may be important for inflorescence architecture and is probably not essential for flower patterning (Kyojuka et al., 1998). The *Lolium temulentum* homolog, *LtLFY*, was initially detected in spikelet meristems, glumes and lemmas, but not during later stages of floret development (Gocal et al., 2001). It will be interesting as more data become available to determine whether diversity in *FLO/LFY* expression patterns is characteristic of monocots in general. However, since both *FLO* and *LFY* can act non cell-autonomously during flower development (Hantke et al., 1995; Sessions et al., 2000) and *LFY* protein has been detected in cells in *Arabidopsis* flowers where *LFY* mRNA was not observed (Parcy et al., 1998), mutants in additional monocot species are needed to address whether the diversity of expression patterns reflects a diversity of functions.

ZFL functions in the reproductive transition

FLO and *LFY* have been implicated in *Antirrhinum* and *Arabidopsis* in coordinating the abrupt transition from vegetative to inflorescence development by ensuring that independent aspects of inflorescence fate are adopted simultaneously (Bradley et al., 1996; Ferrandiz et al., 2000; Liljegren et al., 1999). This function appears to be shared by the *zfl* genes of maize. The transition from vegetative to reproductive state occurs more gradually in *zfl* double mutants than in wild-type plants with some vegetative characteristics being maintained after the onset of the reproductive phase.

Several other mutants in maize also have aberrant expression of vegetative traits in inflorescences. These mutants, including the dominant *Teopod* mutations, *Tp1-Tp3* (Poethig, 1988), *Lax-midrib1-O* (Schichnes and Freeling, 1998) and *liguleless2* (Walsh and Freeling, 1999), have been characterized as having defective phase transitions. All of these mutants show, to varying degrees, development of abnormal transition nodes expressing both vegetative and reproductive characteristics, suggesting defects in generating an abrupt boundary between the vegetative and reproductive phases (Freeling et al., 1992; Poethig, 1988). Given the similarity in phenotypes among these mutants and the *zfl* mutant, it would be of interest to test if they act in the same or different developmental pathways.

ZFL functions in inflorescence architecture

zfl mutants have a dramatically reduced number of tassel branches and there is a quantitative association between an increase in *zfl2* copy number and an increase in the number of long tassel branches. These observations suggest that in maize, *zfl* plays a direct role in promoting branch establishment in addition to its roles in flower development. A function for *zfl* in promoting inflorescence branching is somewhat difficult to reconcile with results in dicots. Most *FLO/LFY* single mutants in dicots show an increase in branching due to the conversion of flowers into shoots (Coen et al., 1990; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Weigel et al., 1992), suggesting *FLO/LFY* suppresses branching by promoting flower development. However, *LFY* has been implicated in promoting branch meristem establishment when combined with the *Arabidopsis wiggum* (*wig*) and *filamentous flower* (*fil*) mutations (Running et al., 1998; Sawa et al., 1999). Thus, it is plausible that ZFL promotes inflorescence branching, although in a genetic background-dependent manner.

We have also observed a quantitative decrease in inflorescence phyllotaxy that is associated with decreasing *zfl* activity in a family segregating for *zfl1-mum1*; *zfl2-mum1* (Fig. 7B). We confirmed this association in an independent family segregating for *zfl1-mum2*; *zfl2-mum4* (The W1 family; data not shown). An effect on inflorescence phyllotaxy has not been described for *FLO/LFY* homologs in other species. This suggests that ZFL may play a novel role in promoting higher orders of inflorescence phyllotaxy in maize, perhaps by influencing inflorescence meristem organization or size to promote formation of increased numbers of primordia around the circumference of the meristem. We caution, however, that at present our knowledge of the quantitative effects on inflorescence phyllotaxy only show them to be 'associated' with ZFL, and must await confirmation using transgenic methodologies.

Possible roles for FLO/LFY homologs in morphological evolution

Though *FLO/LFY* homologs have conserved functions in flower development in divergent species (Ahearn et al., 2001; Coen et al., 1990; Hofer et al., 1997; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Souer et al., 1998; Weigel et al., 1992), several species appear to have evolved additional functions for this gene. For example, the pea and tomato homologs promote compound leaf development (DeMason and Schmidt, 2001; Hofer et al., 1997; Molinero-Rosales et al., 1999), and the tobacco *NFL* genes are required for proper shoot apical meristem development (Ahearn et al., 2001). In violet cress, changes in *FLO/LFY* expression have also been associated with an accelerated reproductive transition and the concurrent change in inflorescence structure (Shu et al., 2000).

Defects associated with loss of *ZFL* in maize suggest that the role of the *zfl* genes in flower patterning does not differ dramatically between maize and dicots. However, variation in the number of active copies of *zfl* is associated with variation in inflorescence structure. This observation suggests a novel role for *zfl* in inflorescence architecture in maize and perhaps other grasses. Crucially, varying *zfl* copy number from one to four showed significant associated effects on inflorescence branching and phyllotaxy without compromising flower development. Therefore, natural or human selection might quantitatively alter grass inflorescence architecture by modulating *ZFL* activity. Since *FLO/LFY* homologs share conserved roles in flower development, but variable roles in other aspects of development, involvement of these genes in morphological evolution of flowering plants may reflect independent appropriations to roles outside flower development. Dramatic changes in protein function are likely to be limited by the constraint that these genes are essential for normal flower development in diverse species. Thus, we anticipate that these novel functions result principally from alterations in the pattern of *FLO/LFY* expression or from changes in downstream targets.

Finally, we have shown that an increase in the number of active copies of *zfl* is associated with an increase in the number of ranks of spikelet-pairs around the circumference of the maize ear. This is one of the key morphological changes involved in the evolution of maize from its progenitor, teosinte (*Zea mays* ssp. *parviglumis*). Domesticated maize produces decussate (four ranked) or polystichous (many ranked) ears and tassels, while teosinte invariably produces two-ranked (distichous) inflorescences (Beadle, 1939; Galinat, 1983). A number of QTL controlling ear rank differences between maize and teosinte have been identified. An ear rank QTL of large-effect that maps near *zfl2* on chromosome 2 was identified in multiple studies, while a smaller and more variable QTL that maps to chromosome 10 near *zfl1* was identified in a few studies (Doebley, 1992). The associated trends between *zfl* copy number and ear rank support the candidacy of *zfl1* and *zfl2* as the genes underlying these QTL and suggest that human selection for increasing ear rank (higher kernel row number) during maize domestication may have led to increased *ZFL2* activity in the inflorescence meristem.

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