

Evidence for a common sex determination mechanism for pistil abortion in maize and in its wild relative *Tripsacum*

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ABSTRACT Cultivated maize (*Zea mays*) and several other members of the Tribe Andropogoneae produce unisexual florets. In maize, the formation of two staminate florets in each spikelet on the tassel and a single pistillate floret in each spikelet on the ear includes a pistil abortion process that requires the action of the *TASSELSEED2* gene. In Eastern gamagrass (*Tripsacum dactyloides*) the *GYNOMONOECIOUS SEX FORM1* gene appears to perform a similar role in pistil abortion. These genes were shown to be homeologs by restriction fragment length polymorphism mapping and by the failure of the *gsf1* and *ts2* alleles to complement one another in intergeneric hybrids. Molecular analysis of the *gsf1* allele shows that it is caused by a 1.4-kb deletion mutation. Both *TASSELSEED2* and *GYNOMONOECIOUS SEX FORM1* show similar expression patterns in subepidermal cells of pistils just before abortion. These results suggest that the formation of staminate florets in the Andropogoneae represents a monophyletic trait.

Maize and Eastern gamagrass are two monoecious species in the tribe Andropogoneae of the family Poaceae. Several members of this tribe, including maize and gamagrass, produce unisexual flowers, called “florets” in grasses. In both maize and Eastern gamagrass, two florets, the upper and lower florets, enclosed by a pair of leaf-like glumes, form a spikelet (Fig. 1C), the unit of grass inflorescences. In maize, the male and female florets are segregated in separate inflorescences. Two staminate florets are formed in each spikelet of the terminal inflorescence, the tassel, and a solitary pistillate floret is formed in each spikelet of the axillary inflorescence, the ear. Both tassel and ear spikelets are paired (Fig. 1B; ref. 1). Gamagrass forms terminal and axillary inflorescences of mixed sexuality with the apical two-thirds to three-quarters comprised of paired staminate spikelets, each containing two staminate florets, and the basal portion bearing solitary pistillate spikelets containing only one functional pistillate floret (2). Pistillate spikelets are unpaired because one of each pair of spikelets arrests and aborts in the pistillate region of the inflorescence (Figs. 1A and 2A). There is usually an abrupt transition between the zone of solitary pistillate spikelets and that of paired staminate spikelets, but occasionally a solitary staminate spikelet or a pair of pistillate spikelets is found at the boundary between these two zones (3).

In both maize and Eastern gamagrass, the unisexuality of florets is established through a process of selective elimination or maturation of preformed floral organs in an initially bisexual immature floret (1, 3). In staminate spikelets, preformed pistil initials degenerate while stamen initials mature. In pistillate spikelets, the development of all stamen initials

and the pistil initials of the lower florets is blocked, with only the pistils of the upper florets reaching maturity. The process of pistil abortion in maize requires the action of the *tasselseed1* and *tasselseed2* genes (4, 5). Mutations in these genes result in the failure to abort pistils, including the pistils in tassel spikelets and the lower floret pistils of ear spikelets (Fig. 1B; ref. 6). *Ts2* has been cloned and shown to encode a short chain dehydrogenase expressed in subepidermal cells of pistils just before abortion (8). Little is known about the genetics of unisexuality in gamagrass except for a spontaneous recessive mutation called *gynomonoecious sex form1* (*gsf1*) found in Kansas (2). The *gsf1* mutation blocks pistil abortion in the apical staminate spikelets as well as the lower florets of the basal pistillate spikelets. Pistil development in these spikelets is associated with arrest of stamen initials. The stamens are arrested at different developmental stages, from rudimentary structures at the base to fully developed stamens at the upper portion of the inflorescence, as shown in Figs. 1A and 3. The *gsf1* mutation does not affect the pattern of spikelet abortion; the basal portion of the inflorescence still contains solitary spikelets. As a result of feminization by *gsf1*, seed production in this variant is increased 10- to 25-fold (9), an important agronomic trait for the utility of Eastern gamagrass as a foraging crop.

MATERIALS AND METHODS

Plants and Intergeneric Crosses. Maize *ts2* stock was originally obtained from the Maize Genetics Cooperative, Champaign–Urbana, IL, and was grown in Hamden, CT for two generations. *Tripsacum* wild-type (WW1218), *gsf1* mutant (WW1582), and heterozygous (WW1748) plants were obtained from the United States Department of Agriculture/Agricultural Research Service, Southern Plains Range Research Station, Woodward, OK. The intergeneric crosses were performed as described by Mangelsdorf and Reeves (10). The diploid ($2n = 20$), heterozygous (*Ts2/ts2*) maize plants were crossed as females by pollen from diploid ($2n = 36$), heterozygous (*Gsf1/gsf1*) *Tripsacum* plants (WW1748) (2). Hybrid seeds were harvested and kept at 4°C for more than 4 weeks. Afterward, hybrid seeds were surface-sterilized with 50% commercial bleach (Clorox, Oakland, CA) for 10 minutes and washed in distilled water, then germinated on Murashige minimal organics medium with 0.8% phytagar (GIBCO/BRL, Oakland, CA) at 28°C with a 16-h photoperiod. Two weeks after germination, seedlings were transferred to soil and grown in a greenhouse.

Scanning Electron Microscopy. Samples for scanning electron microscopic analysis were prepared as described (8).

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Abbreviations: *Ts2* and *ts2*, the wild-type and mutant alleles of the maize *tasselseed2* gene; *Gsf1* and *gsf1*, the wild-type and mutant alleles of the *Tripsacum gynomonoecious sex form1* gene.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. U89270 and U89271).

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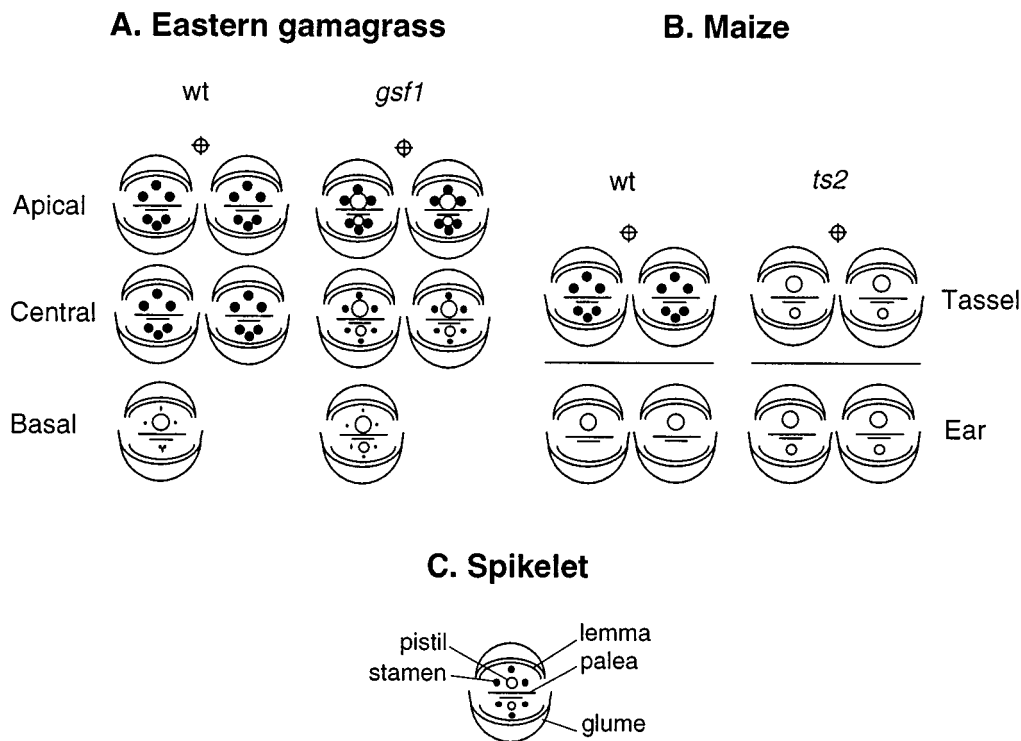


FIG. 1. Diagrams of spikelet and floral structures of Eastern gamagrass and maize. (A) Floral diagrams of *Gsf1* and *gsf1* spikelets. Eastern gamagrass produces inflorescences with both staminate and pistillate spikelets. Staminate spikelets are paired and located on the upper two-thirds of the inflorescence. Each staminate spikelet contains two functional male florets. Pistillate spikelets are solitary, contain one functional pistil, and are located on the basal one-third of the inflorescence. In the *gsf1* mutant, there is no pistil abortion. As a result, every floret contains a functional pistil. Stamens develop to different degrees, from rudimentary structures in the basal region of the inflorescence to fully developed ones at the apex. The ratio of solitary-to-paired spikelets is unaffected by the *gsf1* mutation. (B) Floral diagrams of *Ts2* and *ts2* spikelets. Maize forms separate male and female inflorescences: tassel and ear. Staminate spikelets are paired, and each spikelet has two male florets. Pistillate spikelets also are paired, and only the upper floret in each spikelet develops. In the *ts2* mutant, every spikelet contains two female florets, and each floret has a well developed pistil and no stamens. (C) Keys to floral structures. An immature bisexual spikelet of maize or Eastern gamagrass is comprised of two florets, the upper and the secondary lower florets, subtended by a pair of glumes. Each floret contains a lemma, a palea, three stamens, and a pistil. Lodicules are not shown.

Immature *Tripsacum* inflorescences were fixed in 50% ethanol/10% formaldehyde/5% acetic acid overnight, then dehydrated by passage through an ethanol series. Samples were then subjected to critical point dry and coated with gold/palladium. Coated inflorescences were examined in an International Scientific Instruments model SS40 scanning electron microscope.

Southern Blots and Sequence Analysis. Southern analysis was carried out under stringent conditions as described (11). The *gsf1* allele was cloned from mutant plants (WW1582), and the wild-type allele was cloned from a phenotypically normal plant found near the site that the first mutant plant was found

in Ottawa County, Kansas (12). Both strands of wild-type and mutant alleles were sequenced.

RNA *in Situ* Hybridization. *Tripsacum* and maize immature inflorescences (1–2 cm) were fixed in 4% formaldehyde in PBS solution. *In situ* hybridization was performed according to published methods (7). Sense and antisense riboprobes were derived from the maize *Ts2* gene (8).

RESULTS

Mapping the *gsf1* Trait. Eastern gamagrass *Gsf1* and maize *Ts2* genes have been suggested to be related genes based on



FIG. 2. The inflorescences of *Tripsacum*. The wild-type (A) inflorescence consists of solitary pistillate spikelets at the base and paired staminate spikelets above. The *gsf1* inflorescence (B) is feminized; all florets except those in the most apical region contain well developed pistils.

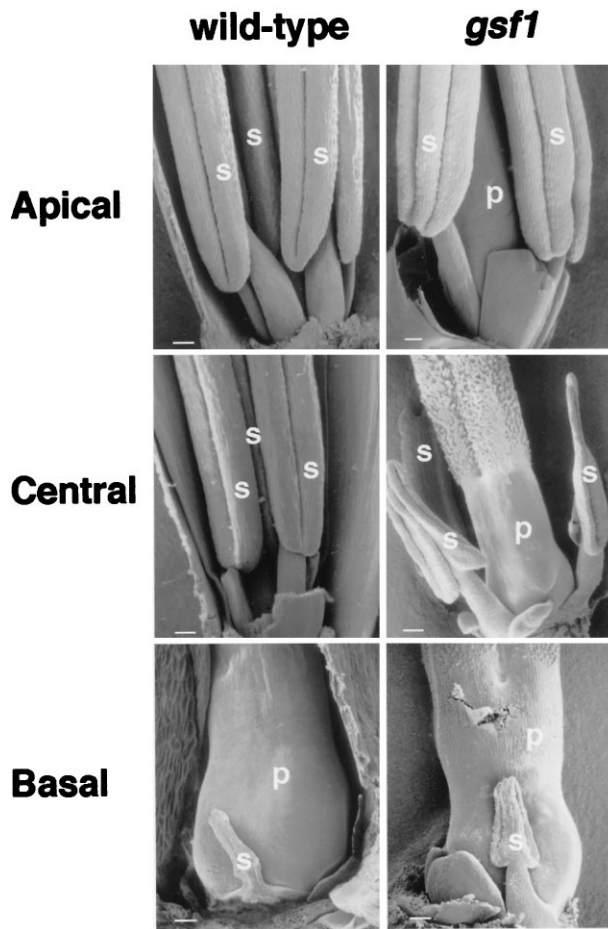


FIG. 3. Scanning electron microscopy analysis of wild-type and *gsf1* mutant florets. Staminate florets in the wild-type inflorescence (*Left*) contain three functional stamens and no remnants of pistils regardless of the location of florets; pistillate florets at the base each contain a fully developed pistil and three rudimentary stamens. In the *gsf1* mutant (*Right*), all florets contain fully developed pistils. Stamens arrest at different developmental stages, from fully developed in the apical region, to immature in the central region, to rudimentary at the base. Wild-type (WW1218) and *gsf1* mutant (WW1582) plants (2) were greenhouse grown. s, stamen; p, pistil. (Bar = 100 μm .)

phenotypic similarity (Fig. 2) and on linkage of each trait to molecular markers (13, 14). To investigate the possibility that *Gsf1* and *Ts2* are homologous genes, we used *Ts2* cDNA probes for Southern analysis of gamagrass F2 populations segregating for the *gsf1* trait. Results (summarized in Fig. 4) indicated that gamagrass contained a single *Ts2*-hybridizing band that mapped to linkage group I, 5.9 cM from the maize-derived molecular marker *npi286* and 9.2 cM from the gamagrass-derived molecular marker *tda48*. This position was closely linked (no recombinants detected in 113 individuals) to the *gsf1* trait. The restriction fragment length polymorphism data also revealed that the *Ts2* gene and *gsf1* trait are located in the homeologous segments of maize chromosome 1 and gamagrass linkage group I, respectively (Fig. 4).

Cloning and Sequencing *Gsf1* and *gsf1* Alleles. Further indication that the *Gsf1* and *Ts2* are homeologs came from molecular cloning of the gamagrass homology, which revealed that *Gsf1* has a gene structure (two exons and one intron) similar to the maize *Ts2* gene and that these two genes share greater than 90% identity in coding sequences (Fig. 5A). The predicted gamagrass protein shows 95% identity with the maize TS2 protein and contains three conserved motifs found in other short chain dehydrogenases: an N-terminal cofactor binding motif, a central catalytic motif, and a C-terminal

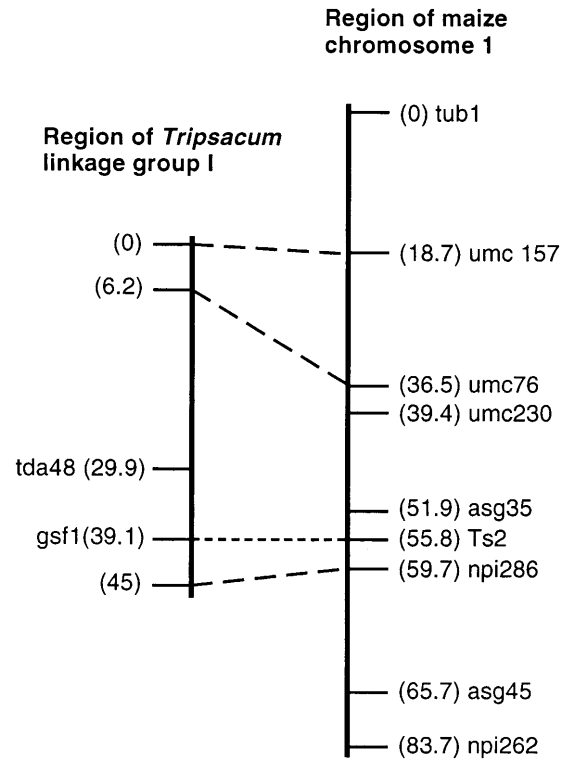


FIG. 4. The restriction fragment length polymorphism linkage maps of gamagrass linkage group I and maize chromosome 1. No recombination was observed between the *Ts2* gene and the *gsf1* phenotype in the Eastern gamagrass F2 mapping population (113 individuals) segregating 3:1 for the *gsf1* trait (13, 14). *Ts2* was mapped 3.9 cM distal to *npi286* and 3.9 cM proximal to *asg35* on chromosome 1 in the maize UMC IF2 population (54 individuals) (15). Comparative map analysis also revealed that two chromosomal regions (41 cM on maize chromosome 1 and 45 cM in gamagrass Group I) are homeologous segments. The order of the three "anchor loci," *npi286*, *umc76*, and *umc157*, was conserved. Data were analyzed using MAPMAKER 2.0 (E. S. Lander, Whitehead Institute for Biomedical Research, Cambridge, MA). The molecular marker *tda48* is derived from Eastern gamagrass; all others are derived from maize.

domain of unknown significance (8, 16). Additional evidence that the gamagrass *Ts2* homology represents the *Gsf1* locus was obtained by comparing the *Ts2* homologies in *gsf1* and *Gsf1* DNA. This analysis indicated that a major structural rearrangement occurred in the 3' end of the coding region in *gsf1* DNA. A restriction map generated by Southern data shows that the rearrangement was caused by a deletion of 1.4 kb (Fig. 5B). Sequence analysis of *gsf1* DNA indicated that 25 bp of DNA of unknown origin was added between the breakpoints during deletion formation (Fig. 5B), a common phenomenon associated with deletions in maize (17). The deletion found in the *gsf1* allele results in a predicted product containing a substitution of 53 amino acids for the last 163 nucleotides found in the *Gsf1* allele. There were also minor sequence polymorphisms detected between the *Ts2*-homologous regions in *Gsf1* and *gsf1* DNA located in the noncoding regions (data not shown).

We examined the expression of the gamagrass *Ts2* homeolog in young staminate florets by RNA *in situ* hybridization. As seen with *Ts2* expression in maize (8), the gamagrass gene also is expressed in the subepidermal cells of the pistil primordia before their abortion (Fig. 6).

Complementation Test. Complementation studies between *Ts2* and *Gsf1* were performed, and all four possible genotypic combinations of *Gsf1* and *Ts2* alleles among intergeneric F1 hybrids were identified by PCR analysis. Maize *Ts2/ts2* plants were fertilized with pollen from gamagrass *Gsf1/gsf1* plants,

A

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Gsf1 tcGagACaCa ctacgACATA CATGCACGCT AGCCTCGCCT CCTACGCCGC 50
Ts2 ca·ca···ac aacat····· .....

Gsf1 GGCAGCTATG CCGGCGCTGG ACCTCCGCC CGAGATAGCG CACGCGCACC 100
Ts2 .....

Gsf1 AGCCCGTCAT GTCGCCCTCg CACCACGGCT GGGACGGCAA TGGCCCGcCA 150
Ts2 .....t .....a..

Gsf1 GCCGTGCCCA CACctATGCC CAAGAGGCTG GACGGGAAGG TGGCCATTGT 200
Ts2 .....g.....

Gsf1 GACGGCGCGC GCGCGgGGGA TCGGCGAGGC CATCGTGCgt CTGTTCGCCA 250
Ts2 .....c.....g.....

Gsf1 AG^ACGGGGC CCGGGTGGTG ATCGCGGACA TCGACGACGC CGcGGGGAG 300
Ts2 .....g.....

Gsf1 GCGGTGGCGg CGGCGCTGGG CCCGCAGGTC AGCTTCGTGC GCTGCGACGT 350
Ts2 .....t .....

Gsf1 GTCgGTGGAG GAgGACGTCC GCGCGCGCGT GGACTGGGCG CTGTCCGCGCC 400
Ts2 .....c.....c.....

Gsf1 ACGGCGGCCG gCTCGACGTg TACTGCAACA ACGCCGGGGT GCTGGGCCGg 450
Ts2 .....c.....c.....

Gsf1 CAGACGCGCG CCGCCAaGAG CATCCtGTCC TTCGACGCGG gCGAGTTCGA 500
Ts2 .....g.....c.....

Gsf1 CCGCGTGTCT CCGCTCAACG CGTGGGGCGC CGCGCTCGGG ATGAAGCACG 550
Ts2 .....

Gsf1 CGGCGCGgGC CATGGCCCGC CGCCGCGCGG GGAGCATCGT CTCCGTCGCC 600
Ts2 .....c.....

Gsf1 AGCGTCGCGG ggGTGCTcGG CGGCTCGGC CCGCACGCT ACACCGCCTC 650
Ts2 .....cc.....g.....

Gsf1 CAAGCACGCC ATCGTgGGG TCACCAAGAA CGCCGCCTGC GAGTcgGCG 700
Ts2 .....c.....gc...

Gsf1 CGCACGGcGT CCGGGTCAAC TGCGTCTCGC CCTTCGGCGT CGCCACGCC 750
Ts2 .....g.....

Gsf1 ATGCTCATCA ACGCCTGGCG CCAGGGCCAC GACGgCGCCg CC----- 800
Ts2 .....a.....a ..gccgacgc

Gsf1 -----GAC gcgGAaCTCG ACCTCGAcAt CAaCGTCCCG AGCGACCAGG 850
Ts2 cgaccga... ctc·c.....g...c.....

Gsf1 AGGTGGAGAA GATGGAGGAG GTGGTCAGGG GCCTGGCCAC GCTCAAGGGC 900
Ts2 .....

Gsf1 CCCACGTgA GGCCAGGGA CATCGCCGAG GCGGTGCTgT TCCTGGCCAG 950
Ts2 .....c.....c.....

Gsf1 CGACGAGGCC AGGTATATAT CcGGCCACAA CCTcGTCTGT GACGCGCGCG 1000
Ts2 .....g.....t.....

Gsf1 TCACCACcTC CAGGAACCTC ATCGGCTTGT G^AATgAATgc CA--tCcaTc 1050
Ts2 .....a... ..c...t ..atc·gt·t

Gsf1 CcAATATCCC ATTCCCATGG CTAGGCTAAT TAGAGAAGGA GAGAGA-AAA 1100
Ts2 .a.....g...
    
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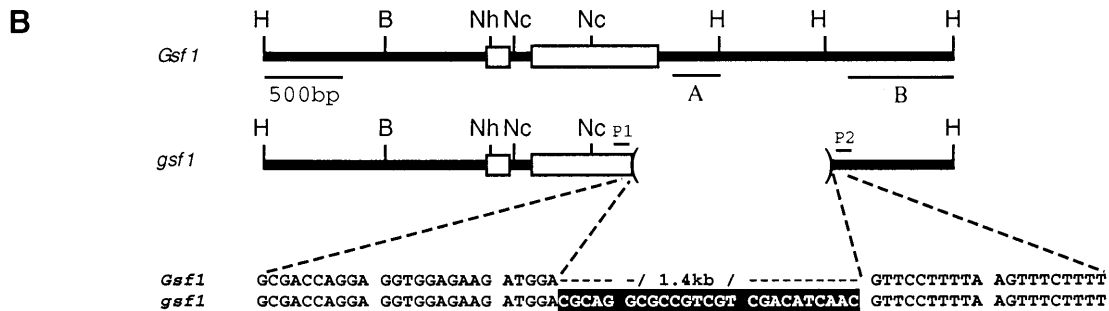


FIG. 5. *Gsf1* and *Ts2* genes. (A) Shown are the *Gsf1* coding sequence (top line) with the start and stop codons underlined and the mismatches in the *Ts2* gene sequence indicated below. The coding sequences from *Gsf1* and *Ts2* show more than 90% identity. (B) Results from Southern analysis and DNA sequencing of the *gsfl* mutation are summarized. Probe A hybridized to *Gsf1* DNA but not to *gsfl* DNA. Probe B hybridized to both genomic DNAs, but the *gsfl* fragment was 1.4 kb smaller than in wild type (data not shown). PCR analysis using P1 and P2 as primers also indicated that the *gsfl* amplification product was 1.4 kb smaller than the product found in wild type. Sequence analysis of both alleles indicated that, between the deletion breakpoints, 25 bp of filler DNA is inserted (highlighted nucleotides). In addition to the 1.4-kb deletion, several minor nucleotide polymorphisms in intron and 5' noncoding sequences were detected between wild-type and mutant alleles (not shown). H, *HindIII*; B, *BamHI*; Nh, *NheI*; Nc, *NcoI*.

and 17 recovered hybrids flowered. Regardless of the genotype, all hybrids were highly tillered, and most shoots produced

branched terminal inflorescences with a few solitary spikelets at the base and paired spikelets above. These phenotypes

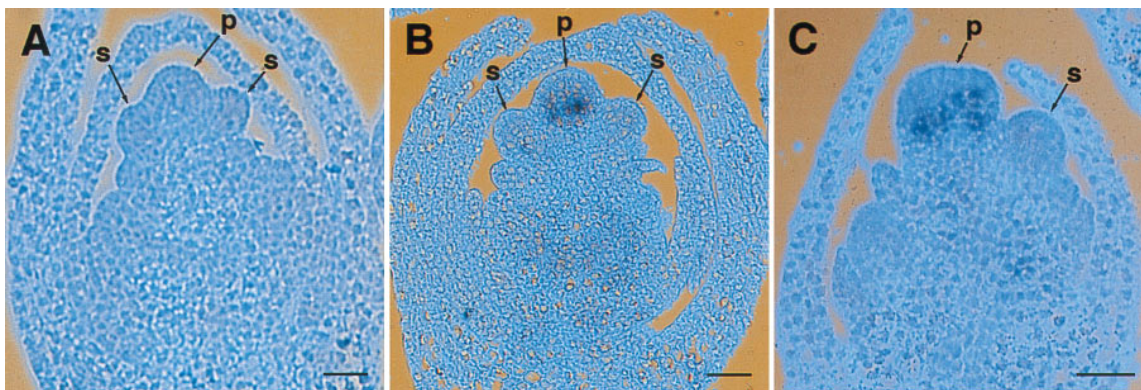


FIG. 6. The expression of *Gsf1* gene detected by RNA *in situ* hybridization. Thin sections of Eastern gamagrass (A and B) and maize (C) immature staminate florets hybridized with maize *Ts2* sense (A) and antisense (B and C) probes. The pistil (p) and stamen (s) primordia are indicated by arrows. (Bar = 50 μ m.)

resembled those observed in other maize–gamagrass hybrids (18). Thirteen hybrids produced the expected wild-type pattern of solitary spikelets containing a single pistillate floret and paired spikelets containing two staminate florets (Fig. 7A). All 13 of these hybrids contained at least one copy of the functional gene *Gsf1* or *Ts2*. Their genotypes were *Gsf1/Ts2*, *Gsf1/ts2*, or *gsf1/Ts2* (Table 1). Four hybrids produced atypical inflorescences that were almost completely feminized except for 5–10 spikelets in the most apical region (Fig. 7B). The genotypes of these hybrids were determined to be *gsf1/ts2* (Table 1). All spikelets on these atypical inflorescences contained a pair of florets with well developed pistils, but the stamens were arrested at different developmental stages (not shown), similar to what is seen in the gamagrass *gsf1* mutant inflorescence. Stamen arrest occurred when initials were rudimentary in basal spikelets, but with increasing degrees of development seen in more apical regions. These results clearly

showed that mutations in *Ts2* and *Gsf1* failed to complement each other genetically in intergeneric F1 hybrids.

DISCUSSION

We conclude that *Gsf1* and *Ts2* are homeologous genes based on restriction fragment length polymorphism linkage between *Ts2* homology and the *Gsf1* trait, the detection of a structural rearrangement associated with the mutant *gsf1* allele, and noncomplementation of the maize *ts2* and gamagrass *gsf1* mutant alleles in intergeneric hybrids. Both genes perform similar roles in these species in that they are responsible for the abortion of pistils in staminate spikelets and the abortion of the lower floret pistils in pistillate spikelets. The *Gsf1* gene does not appear to have a role in the process of spikelet abortion; the ratio of solitary to paired spikelets is unaffected by the *gsf1* mutation, even though sexual fate is profoundly affected, indicating that spikelet abortion and pistil abortion pathways are distinct.

There are major differences in spatial organization of the sexes in these two species. In Eastern gamagrass, sexes are contained within a single inflorescence whereas in maize the sexes are separated into different inflorescences: tassels and ears. Yet despite these major morphological differences, the sex determination process appears to function through a common mechanism of TASSELSEED2-induced pistil abortion. Even though the maize tassel and ear have been modified by continual artificial selection and cultivation, the same genetic pathway formed by natural selection appears to



FIG. 7. The inflorescences of maize–gamagrass intergeneric hybrids. (A) Representative inflorescence from a plant carrying at least one wild-type gene (*Ts2* or *Gsf1*). (B) The feminized inflorescence from a plant with the genotype *gsf1/ts2*.

Table 1. The phenotypic characterization of maize–*Tripsacum* intergeneric hybrids

Genotype	<i>Gsf1/Ts2</i>	<i>Gsf1/ts2</i>	<i>gsf1/Ts2</i>	<i>gsf1/ts2</i>
Plants flowered, <i>n</i>	6	2	5	4
Branches per inflorescence	6–12	4–6	6–12	6–10
Paired spikelets on the main branch (X2)	21–32	27	25–40	30–39
Solitary spikelets on main axis	0–4	0	0–5	0–2
Pistillate spikelets on the main axis	0–4	0	0–3	(27–34) \times 2

Intergenic hybrid plants were genotyped by PCR analysis; *Ts2* and *ts2* alleles were identified by a microsatellite repeat located 3 kb downstream of the *Ts2* gene in maize (19). *Gsf1* and *gsf1* alleles were distinguished using primers P1 and P2 (shown in Fig. 5B) amplifying a 300-bp fragment from *gsf1* mutant DNA and a 1.6-kb product from wild-type DNA. Thirteen plants (genotyped as *Gsf1/Ts2*, *Gsf1/ts2*, or *gsf1/Ts2*) produced normal inflorescences (Fig. 7A). Four plants (genotyped as *gsf1/ts2*) formed feminized inflorescences (Fig. 7B).

be in use. This suggests that the *Ts2/Gsf1*-based sex determination mechanism for pistil abortion is ancestral to both *Zea* and *Tripsacum*. This pathway may have an even more ancient origin; based on phylogenetic analysis, the formation of staminate florets appears to be a monophyletic trait in the Andropogoneae (20, 21), and our results support the view that a common genetic mechanism for pistil abortion exists in at least two genera in the tribe. This shared trait indicates that sex determination studies in maize will have important evolutionary and mechanistic implications for understanding the forces of natural selection that resulted in unisexual flowers.

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