Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 308 (2007) 462–473
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Maize sex determination and abaxial leaf fates are canalized by a factor that maintains repressed epigenetic states

Susan E. Parkinson, Stephen M. Gross, Jay B. Hollick *

Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA

Received for publication 16 April 2007; revised 31 May 2007; accepted 5 June 2007
Available online 12 June 2007

Abstract

In maize (*Zea mays* ssp. *mays*), the meiotically heritable maintenance of specific transcriptionally repressed epigenetic states is facilitated by a putative RNA-dependent RNA polymerase encoded by *mediator of paramutation1* (*mop1*) and an unknown factor encoded by the *required to maintain repression6* (*rmr6*) locus. These so-called "paramutant" states occur at certain alleles of loci encoding regulators of anthocyanin pigment biosynthesis. Here we show *Rmr6* acts to canalize leaf and inflorescence development by prohibiting the ectopic action of key developmental regulators. Phenotypic and genetic analyses suggest that *Rmr6* ensures proper adaxial–abaxial polarity of the leaf sheath by limiting the expression domain of a putative adaxializing factor. Similar tests indicate that *Rmr6* maintains maize's monoecious pattern of sex determination by restricting the function of the pistil-protecting factor, *silkless1*, from the apical inflorescence. Phenotypic similarities with *mop1* mutant plants together with current models of heterochromatin maintenance and leaf polarity imply *Rmr6* functions to maintain epigenetic repression established by non-coding small RNA molecules.

Keywords: *Rmr6*; Epigenetics; Sex determination; *Sk1*; Leaf polarity; Paramutation; *Zea mays*

Introduction

The observation that largely uniform development occurs despite genotypic or environmental variations implies that the regulation of genes controlling developmental pathways is to some extent canalized or buffered (Waddington, 1942). Brink (1964) proposed a two-tiered system in which developmental patterns of gene expression are established by the action of developmental signals on specific DNA regulatory elements, while more generalized epigenetic chromosome-level alterations maintain these expression patterns independent of the original developmental stimuli. Consistent with Brink's hypothesis, patterns of activity and repression of *Drosophila* homeotic genes are initiated by a series of transcriptional regulators and subsequently maintained by specific chromatin structures (reviewed in Bantignies and Cavalli, 2006). In plants and many other animals, a similar relationship between specific chromatin structures and reversible DNA modifications is suggested by observations that the maintenance of cytosine methylation patterns is often important for ensuring proper development (reviewed in Attwood et al., 2002; Grant-Downton and Dickinson, 2006). It seems reasonable to presume that the degree to which a particular gene expression pattern is canalized, in a sense, reflects the fidelity of maintaining these chromosome-level epigenetic marks.

Recent studies suggest the establishment and maintenance of repressive chromatin structures in eukaryotes involves non-coding small RNA molecules (Bernstein and Allis, 2005). In *Arabidopsis*, heterochromatin appears to be maintained through the action of short interfering RNAs (siRNAs) produced by a process requiring a double-stranded RNase (DICER-LIKE3), RNA-DEPENDENT RNA POLYMERASE2 (RDR2) (Xie et al., 2004), and a form of RNA polymerase IV (Pontier et al., 2005). These siRNAs appear to target cytosine methylation to transposons and repetitive sequences through interaction with a small RNA binding protein, ARGONAUTE4 (Chan et al., 2006;

* Corresponding author. Fax: +1 510 642 0355.
E-mail address: hollick@nature.berkeley.edu (J.B. Hollick).

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.06.004

Zilberman et al., 2004), and an alternate form of RNA Polymerase IV (Pontier et al., 2005).

MicroRNAs (miRNAs) represent another class of small non-coding RNA produced using similar cellular machinery, but these appear to act primarily at a post-transcriptional level to effect gene repression. Through binding complementary sequences, miRNAs can target RNA transcripts for degradation (Llave et al., 2002) or inhibit translation (Olsen and Ambros, 1999). By either of these two modes of action, miRNAs ensure developmentally directed expression patterns of specific genes. Recent results from Bao et al. (2004) suggest that miRNAs also function to direct cytosine methylation at genomic targets. They found miRNA-dependent cytosine methylation 3′ of the miR-165/166 complementary site of the *Arabidopsis PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) genes, which each encodes a class III homeodomain-leucine zipper (HD-ZIP III) transcription factor. The miR-165/166 complementarities in both *PHB* and *PHV* span introns, and dominant mutations that disrupt splicing of these introns (and therefore the miR-165/166 contiguous complementarity) are associated with hypomethylation of the *PHB* and *PHV* coding regions. Hypomethylation associated with the dominant *phb-1d* mutation occurred in *cis*, which suggests that the miRNA interacts with the nascent RNA transcript to direct methylation to the *PHB* gene. These findings suggest a small RNA-based link between the gene-specific and chromosome-level tiers of control that Brink hypothesized are required for proper development.

Small-RNA-directed chromatin modifications are also implicated in paramutation, a meiotically heritable alteration in regulatory states influenced by certain allelic interactions (Alleman et al., 2006; Chandler et al., 2000). The maize (*Zea mays* ssp. mays) *mediator of paramutation1* (mop1; Dorweiler et al., 2000) locus, which encodes a protein similar to RDR2, is required for maintenance of epigenetic repression on alleles subject to paramutation (Alleman et al., 2006; Woodhouse et al., 2006). In *Arabidopsis*, *RDR2* is required for *de novo* methylation of tandemly repeated promoter sequences of a *FWA* transgene (Chan et al., 2004), for maintenance of cytosine methylation found in CpNpG and CpNpN contexts, and for histone 3 lysine 9 methylation (H3mK9) at *AtSN1* retroelement sequences (Xie et al., 2004). Because a putative maize RDR2 ortholog is required for maintaining repressed expression states of complex transgenes (McGinnis et al., 2006), RNAi-silenced transposons (Woodhouse et al., 2006), and paramutant alleles (Dorweiler et al., 2000), it has been suggested that paramutation involves the establishment of repressive chromatin structures (Alleman et al., 2006).

Plants deficient for *mop1* display stochastic occurrences of delayed flowering, decreased stature and tassel feminization (Dorweiler et al., 2000), implying a mechanistic connection between paramutation and developmental gene control. Developmental phenotypes associated with mutations in *required to maintain repression6* (*rmr6*), another *trans*-acting factor involved in maintaining paramutation-induced repression, strengthens this connection. The particular defects seen in *rmr6* mutant plants suggest a relationship between the epigenetic marks maintained on alleles subject to paramutation and those used to canalize the developmental programs responsible for domesticated maize leaf and inflorescence architecture. Using a genetic approach, we find *Rmr6* maintains tissue-specific repression of developmental regulators controlling flowering time, internode elongation, developmental phase transition, leaf adaxial/abaxial (dorsal/ventral) polarity, and sex determination. The phenotype of double mutants with the adaxializing *Rolled1-O* (*Rld1-O*) mutation leads to the hypothesis that *Rmr6* ensures proximal leaf adaxial–abaxial polarity by maintaining regional expression domains of an adaxializing factor. Epistasis data show that *Rmr6* restricts the *silkless1* (*sk1*)-encoded pistil protection activity from the terminal inflorescence of the primary axis (apical inflorescence or tassel). These results imply that the expression patterns of key developmental regulators are maintained, at least in part, by the same cellular machinery that restricts transcription of specific alleles of the *purple plant1* (*pl1*), *booster1* (*b1*), and *red color1* (*r1*) loci subject to paramutation (Hollick et al., 2005).

Materials and methods

Nomenclature

Nomenclature designations follow species-specific guidelines (Lawrence et al., 2007; http://www.arabidopsis.org/portals/nomenclature/namerule.jsp). Maize chromosomes, loci, alleles, and allelic states are designated by italic type. Recessive alleles and loci are designated in lower case, while the first letter of dominant alleles, including non-mutant alleles, is capitalized. In the presentation of double mutant analysis between *rmr6* and *sk1* mutants, non-mutant alleles are followed by a "+" and mutant alleles (including both *rmr6-1* and *rmr6-2* alleles) are followed by a "−". Chromosome translocation breakpoints are designated with a "*T*". Regulatory states of the *Pl1-Rhoades* allele are written as *Pl-Rh* for the fully expressed state, and *Pl′* for the repressed paramutant state (Hollick et al., 2005). Diploid genotypes are written with the pistillate (maternal)-derived allele first, followed by the staminate (paternal)-derived allele. For *Arabidopsis*, wild-type alleles are written in upper case italics while mutant alleles are written in lower case italics. Gene products for both species are written in upper case unitalicized type.

Germplasm and genetic crosses

Hand pollinations were used for all genetic crosses and detailed pedigree information is available upon request. For morphometric measurements, lines were constructed as detailed in Hollick et al. (2005) and the mean values were compared using 2-sample *z* tests. For comparisons of flowering time and plant height, 4 and 7 individual progenies segregating 1:1 for +/*rmr6-1* and *rmr6-1*/*rmr6-1* sibling plants were used, respectively. Internode length and leaf wax distribution were measured in *Pl′*/*Pl′* lines homozygous for the *T6-9 (043-1)* (*T*) interchange chromosome (Longley, 1961) that were segregating 1:1 for either +/*rmr6-1* and *rmr6-1*/*rmr6-1* or +/*rmr6-2* and *rmr6-2*/*rmr6-2* siblings. Stocks containing recessive *tassel seed* mutations (*ts1*, *ts2*, *ts4*, *ts*-PI200203*, *ts*-PI251881*, *ts*-PI267209*, *ts*-N2490*) and *sk1* were obtained from the Maize Genetics Cooperation Stock Center (USDA-ARS, University of Illinois, Urbana, IL). The *Rld1-O* allele was provided by Dr. M. Timmermans (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

For *sk1* epistasis analysis, F2 families segregating for mutations in both *rmr6* and *sk1* were derived from +/*rmr6-1*; *T Pl′*/*T Pl′* and *pl1*/*pl1*; *sk1*/*sk1* parents. To ensure proper identification of *rmr6* mutant homozygotes using anther pigment phenotypes, *Pl′*/*Pl′* individuals were enriched among F2 kernels. With the exception of rare recombination events, kernels with waxy endosperm are homozygous for both the *T6-9* interchange and *Pl′* as both *wx1* and *pl1* loci are linked (2.3 and 1.4 cM, respectively) to the interchange breakpoint (Hollick et al., 2005). To increase the likelihood of obtaining *rmr6-1*/*rmr6-1*; *sk1*/*sk1*

individuals in subsequent generations, F2 plants displaying mutant phenotypes were intercrossed or self-pollinated. F3 families were then evaluated for anther color, silkless ears, and feminized tassels. F3 families segregating *rmr6-2*/*rmr6-2*; *sk1*/*sk1* plants were generated in a similar fashion, with the exception that a *rmr6-2*/*rmr6-2*; *T Pl′*/*T Pl′* individual was used as the initial female parent.

Double mutant *rmr6-2*/*rmr6-2*; +/*Rld1-O* plants were generated by initially crossing *rmr6-2*/*rmr6-2*; *wx T Pl′*/*wx T Pl′* and *Rld1-O*/+; *pl1*/*pl1* parents. F1 siblings were intercrossed and F2 kernels with waxy endosperms were planted. Double mutants were identified by dark anther pigmentation and rolled leaf phenotypes.

Microscopy

For scanning electron microscopy, sheath tissue was collected from mature plants and fixed for at least 2 h at 4 °C in 2% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.2, then rinsed in 0.1 M sodium cacodylate buffer. Post fixation was performed by immersion in 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer, followed by dehydration in an ethanol series. Material was then critical point dried, mounted onto stubs using carbon dots, and sputter coated with gold to a thickness of 16 nm. Samples were analyzed on a Hitachi S-5000 electron microscope; images were collected digitally and adjusted for brightness and contrast using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Molecular analyses

Cytosine methylation at centromeric and 45S rDNA repeats and at the *pl1* locus was assayed by digestion of genomic DNA from +/*rmr6-1* and *rmr6-1*/*rmr6-1* siblings of families displaying tassel seed phenotypes. For the centromeric and *pl1* assays, DNA was isolated from flag leaf tissue as described (Dellaporta et al., 1983), and digested according to the manufacturer's instructions with *Bst*NI or *Psp*GI (New England Biolabs, Ipswich, MA). For the 45S rDNA assays, DNA was isolated as described (Voelker et al., 1997) from second seedling leaves. Size fractionation, membrane transfer, probe labeling, hybridization, and detection were as described (Dorweiler et al., 2000). The 45S rDNA (pZmRI) and centromeric clones (pSau3a9) have been described (Jiang et al., 1996; McMullen et al., 1986) and the 1.1 kb *Xho*I probe was derived from a 3′ portion of *pl1-Tx303* (Cone et al., 1993).

Results

Rmr6 affects timing of flowering and developmental phase transition and is required for adult internode elongation

Normal *Rmr6* function maintains weak seedling and anther pigmentation in plants homozygous for the *Pl1-Rhoades* allele found in a transcriptionally repressed expression state referred to as *Pl′* (Hollick et al., 2005). Thus *Pl′*/*Pl′* plants homozygous for *rmr6* mutant alleles are readily identified by dark anther pigmentation. In families segregating 1:1 for plants heterozygous or homozygous for ethyl methanesulfonate (EMS)-induced *rmr6* mutant alleles, we found common defects associated with both *rmr6-1* and *rmr6-2* recessive mutations. Plants homozygous for *rmr6-1* are significantly delayed in flowering (72.6 vs. 68.4 days; $n=98$ and 111, respectively; $z=12.6$, $p \ll 0.001$ for H_0 there is no difference in flowering time), and shorter (142 vs. 191.8 cm; $n=45$ and 54, respectively; $z=13.2$, $p \ll 0.001$ for H_0 there is no difference in plant height) than heterozygous siblings (Figs. 1A and B). To better understand the reduced stature phenotype, we compared internode lengths between +/*rmr6-1* and *rmr6-1*/*rmr6-1* siblings. While there are no significant differences in basal internode lengths, *rmr6-1*/*rmr6-1* plants have shorter vegetative internodes starting with the 7th internode from the base and continuing acropetally (Fig. 2). Measurements made in families segregating *rmr6-2*/*rmr6-2* individuals showed the same trend, with significant differences appearing in the 8th internode and increasingly diverging in more apical internodes (data not shown).

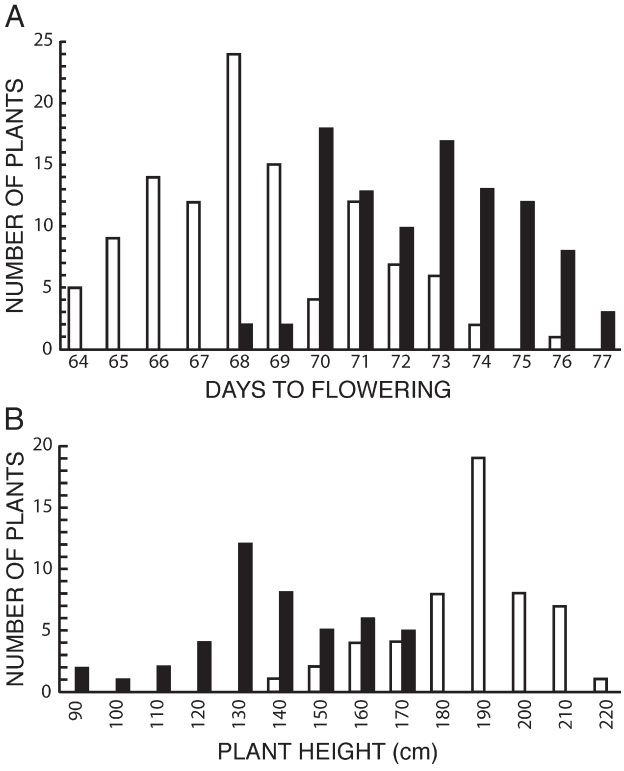

Fig. 1. Morphometric analysis of plants with specific *rmr6* genotypes. Histograms represent individual measurements for (A) days to flowering among 4 individual progenies and (B) binned plant height from 7 progenies segregating *rmr6-1*/*rmr6-1* (solid bars) and +/*rmr6-1* (open bars) siblings.

Interestingly, the divergence in internode length in *rmr6* mutants approximately corresponds to the developmental transition from juvenile to adult vegetative growth. In maize this transition is visually evident by phase specific leaf phenotypes (Poethig, 1988). Juvenile leaves are covered with wax, which gives them a dull appearance. In contrast, adult leaves, which lack this wax, are glossy in appearance. The developmental timing of the juvenile-to-adult transition can therefore be estimated by the appearance of the first partially glossy leaf. By this measurement, this transition is delayed in homozygous *rmr6* mutants compared to non-mutant heterozygous siblings [*rmr6-1*: first leaf with partial glossy character=6.91 vs. 6.17 for mutants and non-mutants, respectively ($n=22$ and 23; $z=7.2$, $p \ll 0.001$ for H_0 there is no difference; *rmr6-2*: first leaf with partial glossy character=7.00 vs. 6.48 for mutants and non-mutants, respectively ($n=21$ and 21; $z=3.5$, $p=0.0004$ for H_0 there is no difference)]. In addition to having reduced vegetative internodes, *rmr6* mutants also have compressed apical inflorescence architecture due primarily to decreased tassel internode length. The average distance between tassel branches is significantly shorter in *rmr6-1*/*rmr6-1* and *rmr6-2*/*rmr6-2* individuals compared to heterozygous siblings (*rmr6-1*: 0.38 vs. 0.63 cm; $n=31$ and 41; $z=8.2$, $p \ll 0.001$ for

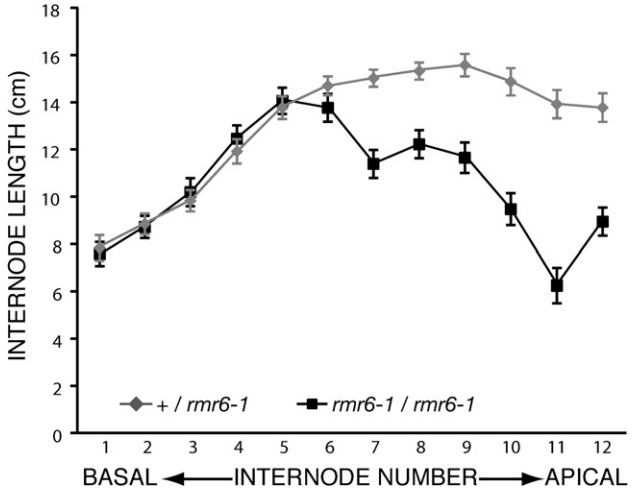

Fig. 2. Internode length in plants with specific *rmr6* genotypes. Average individual leaf internode length (±SE) for *rmr6-1*/*rmr6-1* ($n=31$) and +/*rmr6-1* ($n=41$) siblings.

H_0 there is no difference; *rmr6-2*: 0.21 vs. 0.82 cm; $n=14$ and 17; $z=3.54$, $p \ll 0.001$ for H_0 there is no difference). Thus *Rmr6* function is required for adult phase internode elongation in both vegetative and reproductive portions of the plant.

Rmr6 maintains abaxial leaf fates and lateral meristem repression

Approximately 52% of *rmr6-2* homozygotes display patches of smooth epidermis extending distally from the base of the abaxial sheath (Fig. 3A, Table 1). Scanning electron microscopy shows that these regions contain cellular markers typical of adaxial epidermis including straight cell walls and absence of macrohairs (Fig. 3D), while adjacent normal abaxial sheath epidermis has interdigitated cell walls and is rich in macrohairs (Fig. 3E). These atypical sheath regions do not appear to represent polarity reversals, as adaxial leaf surfaces show no abaxial characters. Although the patches of adaxial character typically terminate before the blade-sheath boundary; distal extensions into the blade are accompanied by macrohair-dense blade tissue and an ectopic ligule located at the proper abaxial blade/sheath boundary. The smooth epidermis patches are frequently bordered by pronounced laminar flaps, similar to *Antirrhinum* plants with mutations in the *PHANTASTICA* locus, which form laminar tissue at junctions of adaxial and abaxial leaf tissue (Waites and Hudson, 1995). Thus, a variety of cell markers and associated features indicate that the smooth abaxial epidermal regions seen in *rmr6* mutant plants represent ectopic sheath adaxializations.

Perhaps related to the observed adaxialized leaf fates, *rmr6* mutant plants can also produce lateral outgrowths. These outgrowths occur at primarily medial positions of the proximal abaxial sheath and most often initiate from the sheath base. A diverse set of tissues appear to be represented in these outgrowths, including leaflets, inflorescence primordia, and culm-like tissue. Interestingly, the apparent sheath adaxializations and lateral outgrowths were not manifest in initial M2 *rmr6-1* or

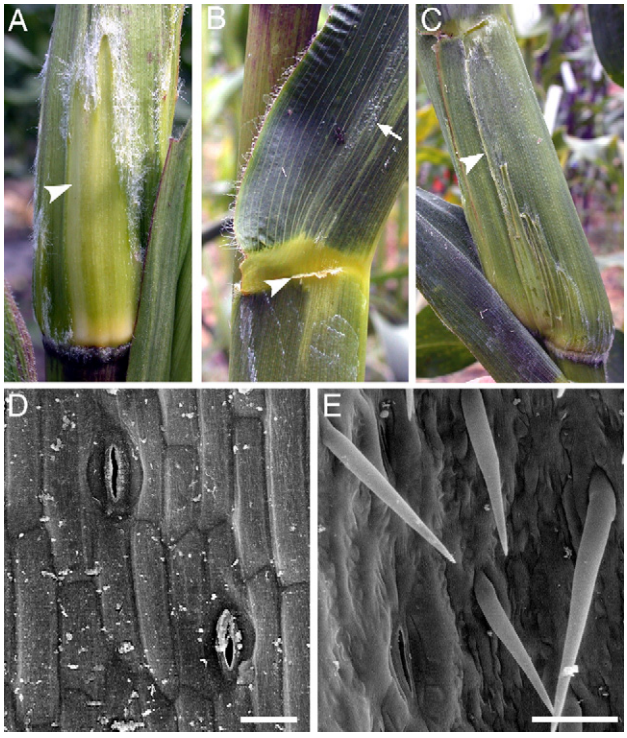

Fig. 3. Adaxialized tissue phenotypes in *rld1* and *rmr6* mutant plants. (A) Proximal sheath from an *rmr6-2*/*rmr6-2* plant displaying adaxial character (arrowhead). (B) *Rld1-O*/+ plant displaying ectopic ligule (arrowhead) and attendant adaxialized sectors on the abaxial blade (arrow) and sheath (below ligule). (C) Sheath of a *rmr6-2*/*rmr6-2*; *Rld1-O*/+ individual showing contiguous adaxialization of the sheath (arrowhead). (D) Scanning electron micrograph (SEM) of adaxialized sheath tissue from an *rmr6-2*/*rmr6-2* plant (scale bar=50 μm). (E) SEM of normal abaxial sheath tissue (scale bar=50 μm).

rmr6-2 mutant plants. These unusual phenotypes were first seen among homozygous *rmr6-1* and *rmr6-2* individuals following one or two generations of sibling crosses, respectively. Subsequently, however, both phenotypes show exclusive cosegregation with homozygous mutant progeny of crosses between +/*rmr6-2* and *rmr6-2*/*rmr6-2* siblings. Among 5 generations of sibling cross progenies, 0/159 +/*rmr6-2* individuals displayed sheath adaxialization or lateral outgrowths while 103/197 *rmr6-2*/*rmr6-2* individuals displayed evidence of

Table 1
Segregation of individuals with adaxialization and lateral outgrowth phenotypes among progeny of successive filial generations

Generation	Number of ear progenies examined	Number of individuals with specific *rmr6* genotypes and adaxialized sheaths/lateral outgrowths	
		Rmr6/rmr6-2	*rmr6-2/rmr6-2*
F2	5	22 (0,0)	37 (12,9)
F3	4	50 (0,0)	69 (29,21)
F4	5	23 (0,0)	35 (16,8)
F5	3	46 (0,0)	45 (36,15)
F6	2	18 (0,0)	11 (10,10)
Totals	19	159 (0,0)	197 (103,63)

Numbers in parentheses refer to individuals having sheath adaxialization and, among those, the number with lateral outgrowths, respectively.

sheath adaxializations; 63 of these 103 individuals displayed both phenotypes (Table 1). Thirty of 197 *rmr6-2/rmr6-2* individuals displayed lateral outgrowths with no apparent sheath adaxializations. Similar sheath patches of adaxial character and lateral outgrowths occur less frequently among *rmr6-1/rmr6-1* individuals (3 and 6 respective instances among 360 mutant individuals observed), consistent with previous genetic studies showing that *rmr6-2* is a more severe mutant allele than *rmr6-1* (Hollick et al., 2005).

The *rmr6*-associated proximal sheath phenotype bears striking resemblance to blade adaxialization associated with dominant gain-of-function mutations in *rolled leaf1* (*rld1*; Nelson et al., 2002), which encodes a HD-ZIP III transcription factor specifying adaxial cell fates (Juarez et al., 2004; Nelson et al., 2002). To directly compare leaf phenotypes conferred by *rmr6-2* and *Rld1-O* mutations, we synthesized and examined 23 independent ear progenies segregating *rmr6-2/rmr6-2*; +/*Rld1-O* double mutants. We identified *rmr6-2/rmr6-2* individuals by their compact tassel phenotype and anther pigmentation, and +/*Rld1-O* individuals by their rolled blade phenotype. The *Rld1-O* phenotype was identical to that previously described (Juarez et al., 2004), except that additional patches of adaxialized sheath extend short distances basipetally from the ectopic abaxial ligule found at the blade-sheath boundary (Fig. 3B). These distal regions of adaxialized sheath seen in +/*Rld1-O* individuals are distinct from the proximal adaxialized patches extending acropetally from the base of the sheath in *rmr6* mutant plants. In total, 43/455 F2 individuals were scored as *rmr6-2/rmr6-2*; +/*Rld1-O* double mutants. All but 2 of these showed a simple additive phenotype in that both basipetal and acropetal sheath adaxializations were present. Interestingly, in the 2 double mutant individuals in which sheath phenotypes appeared more severe than in single mutants, the *rmr6*-associated (acropetal) and *Rld1-O*-associated (basipetal) regions occurred in the same medial–lateral positions, forming contiguous adaxialized features along the entire proximal–distal length of the abaxial leaf surface (Fig. 3C). These results indicate both *Rld1* and *Rmr6* function to ensure proper dorso-ventral leaf polarity, albeit in different proximal–distal domains.

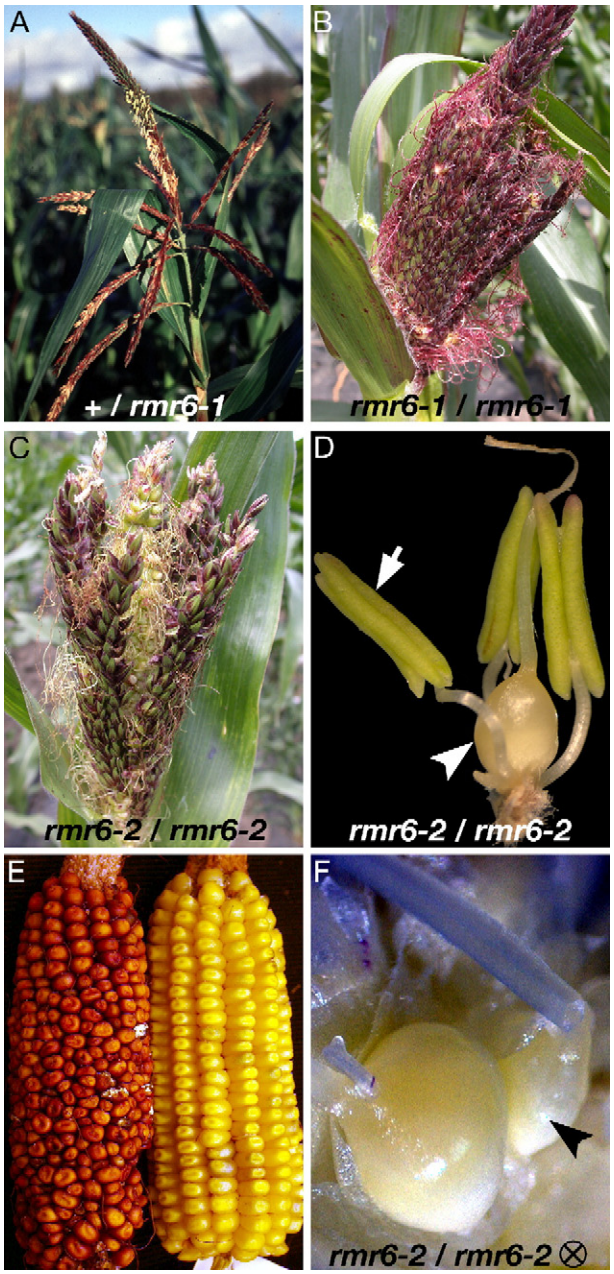

Fig. 4. Inflorescence phenotypes of plants with specific *rmr6* genotypes. (A) Normal tassel architecture displayed by +/*rmr6-1* individuals. (B, C) Compact, feminized tassels of *rmr6-1/rmr6-1* and *rmr6-2/rmr6-2* genotypes, respectively. (D) Dissected floret from an *rmr6-2/rmr6-2* tassel, displaying an unaborted ovule (arrowhead) and stamens (arrow). (E) Self-pollinated ears of *rmr6-1/rmr6-1* (left) and +/*rmr6-1* (right) siblings. (F) Ear spikelet from a self-pollinated *rmr6-2/rmr6-2* individual displaying an unaborted ovule in the secondary floret (arrowhead).

Rmr6 facilitates pistil abortion

As a monoecious plant, maize produces a male inflorescence (tassel) at the apex of the plant and lateral inflorescences (ears) in the leaf axils. During normal development (reviewed in Veit et al., 1993), both the ear and the tassel produce reiterated files of secondary branches known as spikelet pairs. Each spikelet contains an upper and lower floret, which are initially bisexual. Sexual differentiation of maize inflorescences occurs by independent organ abortion events: pistils abort in tassel florets and stamens arrest in ear florets. The pistil in the lower floret of the ear spikelets also aborts, such that only the upper floret develops. In *rmr6-1* and *rmr6-2* homozygotes, pistil abortion fails to occur in some tassel florets (Figs. 4A–C) and bisexual flowers are produced (Fig. 4D). This is most strikingly represented by the appearance of elongated pistils (silks) emanating from tassel florets. Open pollinated flowers from these florets develop kernels giving a clear tassel seed phenotype that suggests these pistils are functional. Consistent with this, we planted a set of these kernels and found 18/20 germinated and gave rise to fertile plants. Both silks growing out of the tassel, or, later, seeds forming in the tassel, are usually referred to as a "tassel seed" phenotype. Expressivity of this tassel feminization ranges from ~5 individual silks on the entire tassel to a mass of silks, in which all florets appear to have unaborted pistils;

regardless of the severity of tassel feminization, however, all mutants have a compact appearance due to the decreased internode lengths mentioned above. While silks emerge from florets harboring bisexual flowers, the anthers appear not to be extruded. However, complete tassel feminization is atypical; therefore, male florets from mutant individuals extrude anthers that release >95% normal pollen as viewed at 50× magnification (not shown). Although there is no evidence of bisexual flowers in lateral inflorescences, ears from *rmr6* mutants display full seed set with a crowded and irregular kernel arrangement (Fig. 4E). Dissection of developing ear spikelets from *rmr6-2/rmr6-2* plants revealed unaborted pistils in the secondary florets (Fig. 4F), indicating that *Rmr6* function is also required for normal pistil abortion from secondary ear florets. As with other *tassel seed* mutations, this aberrant pistil protection likely results in disorganized kernel arrangement on the mature ear (Irish et al., 1994). These phenotypes indicate that *rmr6* mutants are deficient in the pistil abortion aspect of sex determination, but that stamen arrest proceeds normally.

Rmr6 defines a novel tassel seed locus

Similar to the mutant leaf phenotypes noted above, tassel silks were not observed in *rmr6-1* nor *rmr6-2* M2 mutant plants. Tassel silks were first noted among *rmr6-1/rmr6-1* F2 plants derived from M2 *Pl′/Pl′*; *rmr6-1/rmr6-1* and standard *Pl′/Pl′*; +/+ parents (Fig. 5A). For *rmr6-2*, tassel silks were first noted among progeny of two intercrossed *rmr6-2/rmr6-2* M2 parents (Fig. 5B). Although the feminized tassel phenotypes appeared after at least one generation in the absence of normal *Rmr6* function, the developmental phenotype again cosegregated exclusively with the dark pigment phenotypes conferred by both *rmr6* mutations, indicating that the loss of *rmr6* function does not promote the formation of stable unlinked epialleles. Among *rmr6-1* mutants, the feminized tassel phenotype was 92% penetrant in subsequent filial generations (Table 2). Similar

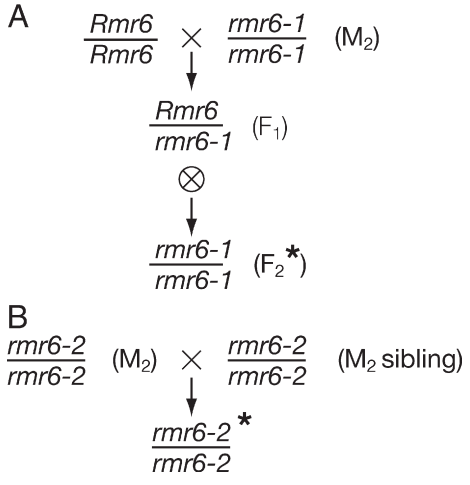

Fig. 5. Emergence of tassel silks in pedigrees containing *rmr6* mutant alleles. Tassel silks were initially observed in filial generations marked with an asterisk (*) and are exclusively associated with plants homozygous for *rmr6-1* (A) or *rmr6-2* (B) mutant alleles.

Table 2
Segregation of individuals with tassel silks among progeny of successive filial generations

Generation	Number of ear progenies examined	Number of individuals with specific *rmr6* genotypes and tassel silks	
		Rmr6/rmr6-1	*rmr6-1/rmr6-1*
F2	1	6 (0)	13 (12)
F3	2	33 (0)	20 (19)
F4	1	13 (0)	13 (13)
F5	1	21 (0)	15 (14)
F6	2	26 (0)	36 (33)
F7	2	28 (0)	34 (29)
F8	2	29 (0)	28 (27)
Totals	11	156 (0)	159 (147)

Numbers in parentheses refer to individuals having tassel silks.

penetrance is seen following back crosses of *rmr6* mutant alleles into standard inbred lines; for example, 7/10 *rmr6-1/rmr6-1* individuals in a 75% A632 background (BC1F2) and 7/7 *rmr6-2/rmr6-2* individuals in a 87.5% A619 background (BC2F2) display tassel feminization. For the *rmr6-2/rmr6-2* individuals included in Table 1, there was a slightly higher frequency of tassel seed phenotypes among those displaying sheath adaxializations than those without (85.4% vs. 70.2%; $n=103$ and 94 respectively; $\chi^2=6.68$, $p<0.01$ for H_0 that the two phenotypes are independent).

Mutations defining six mapped *tassel seed* loci also confer tassel feminizations (*ts1*, *ts2*, *Ts5*, Emerson et al., 1935; *Ts3*, *Ts6*, Nickerson and Dale, 1955; *ts4*, Phipps, 1928) so we questioned whether the tassel seed phenotypes seen in *rmr6* mutants were due to heritable defects at these loci. Genetic crosses and progeny evaluations show that *rmr6* mutations complement all known recessive *tassel seed* mutations (Table 3). Four unmapped recessive *tassel seed* mutations, *ts*-PI200203*, *ts*-PI251881*, *ts*-PI267209*, and *ts*-N2490* likewise complement *rmr6-1* (Table 3). Mapping experiments place *rmr6* in bin 1.06 (Hollick et al., 2005), a location distinct from those identified by the *Ts3*, *Ts5*, and *Ts6* dominant mutations. These genetic tests and mapping data indicate that *rmr6* is distinct from previously identified *tassel seed* loci but they do

Table 3
Genetic complementation between mutations in *rmr6* and other *tassel seed* loci

Parental genotype		Number of ear progenies examined	Total number of progeny individuals [a]
Female	Male		
ts1/ts1 [b]	*+/rmr6-1*	3	56 (0)
ts2/ts2	*rmr6-1/rmr6-1*	1	5 (0)
+/ts4	*rmr6-2/rmr6-2*	1	12 (0)
ts-PI200203/ts*-PI200203*	*+/rmr6-1*	1	14 (0)
ts-PI251881/ts*-PI251881*	*rmr6-1/rmr6-1*	2	37 (0)
ts-PI267209/ts*-PI267209*	*+/rmr6-1*	1	19 (0)
ts-N2490/ts*-N2490*	*+/rmr6-1*	1	13 (0)

[a] Numbers in parentheses refer to the number of individuals having tassel silks.

[b] Three different *ts1* alleles were tested: *ts*0714*, *ts* Anderson*, *ts*69-Alex-Mo17*.

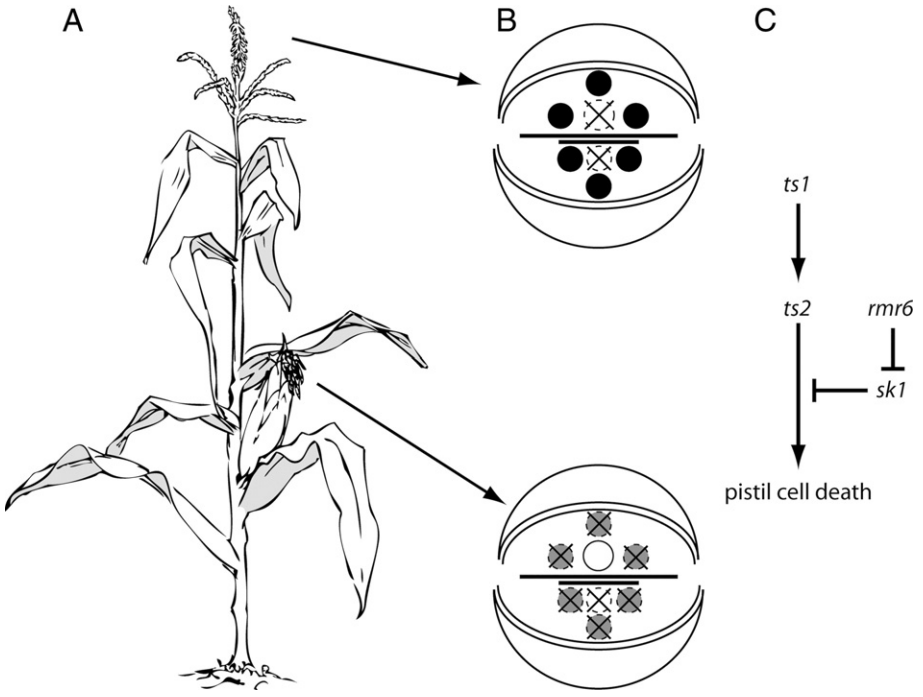

Fig. 6. Model for maize monoecious flower development (A) Monoecious sex determination in maize with male flowers borne apically and female flowers borne laterally. (B) In tassels, pistils (open circle) are aborted while stamens (filled circles) develop. In ears, pistils in primary florets develop while stamen growth is arrested and secondary florets are aborted. Aborted or arrested organs are indicated with an "×". (C) Pistil abortion occurs through a *ts1*, *ts2*-induced cell death pathway; however, *ts2* is expressed in all florets of the ear and tassel. Thus, the pistil protection activity of *Sk1* is required to counteract the effects of *ts2*. *Rmr6* acts to restrict the domain of *Sk1* activity from all floral regions except primary ear florets, thus ensuring pistil abortion in the tassel and secondary ear florets.

not exclude the possibility that the pistil protection seen in *rmr6* mutant tassel florets reflects a *trans*-acting defect in the regulation of known *tassel seed* loci.

silkless1 is epistatic to rmr6

The proposed role of *sk1* as encoding a pistil protector of a cell death pathway dependent on *ts1* and *ts2* action (Calderon-Urrea and Dellaporta, 1999; Fig. 6), suggests that the *sk1* gene may be inappropriately expressed in tassels of *rmr6* mutant plants. If so, *Sk1* function would be required for the observed tassel seed phenotype and thus *rmr6*$^{-}$/*rmr6*$^{-}$; *sk1*$^{-}$/*sk1*$^{-}$ double mutant plants would have no feminized tassel florets. Alternatively, if *Rmr6* affects pistil abortion through an independent pathway, then double mutant plants would retain the tassel feminization characteristic of *rmr6* single mutants. To test these alternatives, we synthesized families segregating *rmr6* and *sk1* double mutant plants – identified by their dark anther pigment (*rmr6*$^{-}$/*rmr6*$^{-}$) and silkless ears (*sk1*$^{-}$/*sk1*$^{-}$) – and examined these for tassel silks. For all seven *rmr6*$^{-}$/*rmr6*$^{-}$; *sk1*$^{-}$/*sk1*$^{-}$ putative double mutants that we crossed to both *rmr6*$^{-}$/*Rmr6*$^{+}$ and *Sk1*$^{+}$/*sk1*$^{-}$ testers, all progenies showed segregation ratios expected from double mutant parents (data not shown), indicating our genotype assignments were accurate. We examined six independent ear progenies segregating double mutants between *sk1* and either *rmr6-1* or *rmr6-2* in which the tassel feminization phenotype was highly penetrant among the *rmr6*$^{-}$/*rmr6*$^{-}$ single mutant plants (Table 4). Of the *rmr6* single mutants, 62/98 (63%) developed silks in their tassels. In contrast, 0 of 62 double mutants (*rmr6*$^{-}$/*rmr6*$^{-}$; *sk1*$^{-}$/*sk1*$^{-}$) displayed a tassel seed phenotype. Thus, *sk1* is epistatic to *rmr6* and ectopic *Sk1* function in the tassel florets of *rmr6* mutant plants appears responsible for pistil protection (Fig. 6C; $\chi^2 = 64.04$, $p \ll 0.001$ for H_0 there is no difference in the frequency of plants having silks in their tassels among *rmr6*$^{-}$/*rmr6*$^{-}$; *Sk1*$^{+}$/– and *rmr6*$^{-}$/*rmr6*$^{-}$; *sk1*$^{-}$/*sk1*$^{-}$ genotypes).

Rmr6 does not maintain genome-wide cytosine methylation patterns

Combined with prior findings that *Rmr6* functions to maintain transcriptional repression of paramutant *pl1* and *b1* alleles

Table 4
Epistasis analysis of *rmr6*; *sk1* double mutants

Ear progeny	*rmr6* allele	Number of individuals with specific genotypes			
		Rmr6^{+}/–		*rmr6*$^{-}$/*rmr6*$^{-}$	
		Sk1^{+}/–	*sk1*$^{-}$/*sk1*$^{-}$	*Sk1*$^{+}$/–	*sk1*$^{-}$/*sk1*$^{-}$
34459	6-1	13 (0)	8 (0)	12 (8)	4 (0)
41827	6-1	5 (0)	5 (0)	25 (11)	33 (0)
41834	6-1	0 (0)	0 (0)	19 (9)	17 (0)
42571	6-2	6 (0)	4 (0)	6 (5)	2 (0)
42574	6-2	8 (0)	2 (0)	15 (12)	3 (0)
44837	6-2	29 (0)	8 (0)	21 (17)	3 (0)
Totals		61 (0)	27 (0)	98 (62)	62 (0)

Rmr6^{+}/– and *Sk1*$^{+}$/– represent both *Rmr6*$^{+}$/*Rmr6*$^{+}$ and *Rmr6*$^{+}$/*rmr6*$^{-}$ genotypes or both *Sk1*$^{+}$/*Sk1*$^{+}$ and *Sk1*$^{+}$/*sk1*$^{-}$ genotypes, respectively. Numbers in parentheses refer to the number of individuals having tassel silks.

(Hollick et al., 2005), the similar phenotypic effects of *rmr6* mutations with those documented for the *Arabidopsis* cytosine methylation mutants *decrease in DNA methylation1* (*ddm1*) and *DNA methyltransferase1* (*met1*) (Finnegan et al., 1996; Kakutani et al., 1996) suggest that *Rmr6* function might broadly maintain genomic cytosine methylation patterns. To test this, we compared genomic DNA from +/*rmr6-1* and *rmr6-1*/*rmr6-1* sibling plants following digestion with *Bst*NI or *Psp*GI (restriction endonucleases with the same recognition sequences but insensitive or sensitive to cytosine methylation respectively) using Southern blot hybridizations with radiolabeled probes corresponding to centromere repeats, 45S rDNA, and the 3′ portion of the *pl1* coding region. If *Rmr6* functions to maintain cytosine methylation patterns found in CpNpG contexts throughout the genome, we would expect a greater abundance of digested *Psp*GI products in *rmr6-1*/*rmr6-1* individuals compared to their +/*rmr6-1* siblings. Our results show no differences in cytosine methylation at centromeric or 45S rDNA repeats (Figs. 7A, B), suggesting that *Rmr6* is not required for general maintenance of cytosine methylation in symmetrical CpNpG contexts. Similar results were reported for *mop1* mutant plants (Dorweiler et al., 2000). We also detected no substantial differences at the 3′ portion of the *pl1* coding region between +/*rmr6-1* and *rmr6-1*/*rmr6-1* individuals (Fig. 7C). Thus, *Rmr6*-dependent transcriptional repression of *Pl′* is not correlated with general cytosine methylation differences in this region.

Discussion

A locus that maintains meiotically heritable epigenetic marks associated with transcriptional repression of paramutant alleles, *rmr6* (Hollick et al., 2005), is also necessary for key developmental processes including flowering time, juvenile–adult phase change, adult internode elongation, abaxial sheath fate, and sex determination. The cosegregation of these phenotypes with independently isolated mutant alleles indicates that all these defects are caused by loss of *Rmr6* function and not by linked lesions introduced during EMS mutagenesis. Furthermore, EMS-based mutations in *rmr1* and *rmr2*, isolated from identical genetic stocks as those used to find *rmr6* mutations, do not display feminized tassel florets following similar breeding schemes (Hollick and Chandler, 2001), showing that tassel feminizations are not due to background modifiers. The lack of apparent developmental defects among M2 *rmr6* mutant individuals illustrates that even saturation M2 screens for mutant phenotypes may not reveal all possible genetic components governing a particular trait. Because the effects on development appeared among *rmr6* mutant plants only after the genome had been exposed to a meiotic division in the absence of *Rmr6* function, it is possible that *Rmr6* acts to maintain epigenetic marks at its target loci through meiosis. However, the failure to create heritable epialleles affecting leaf fate or floral development that persists after *Rmr6* function is restored by outcrossing suggests *Rmr6* functions somatically to reinforce epigenetic regulatory states that are established and maintained during typical development.

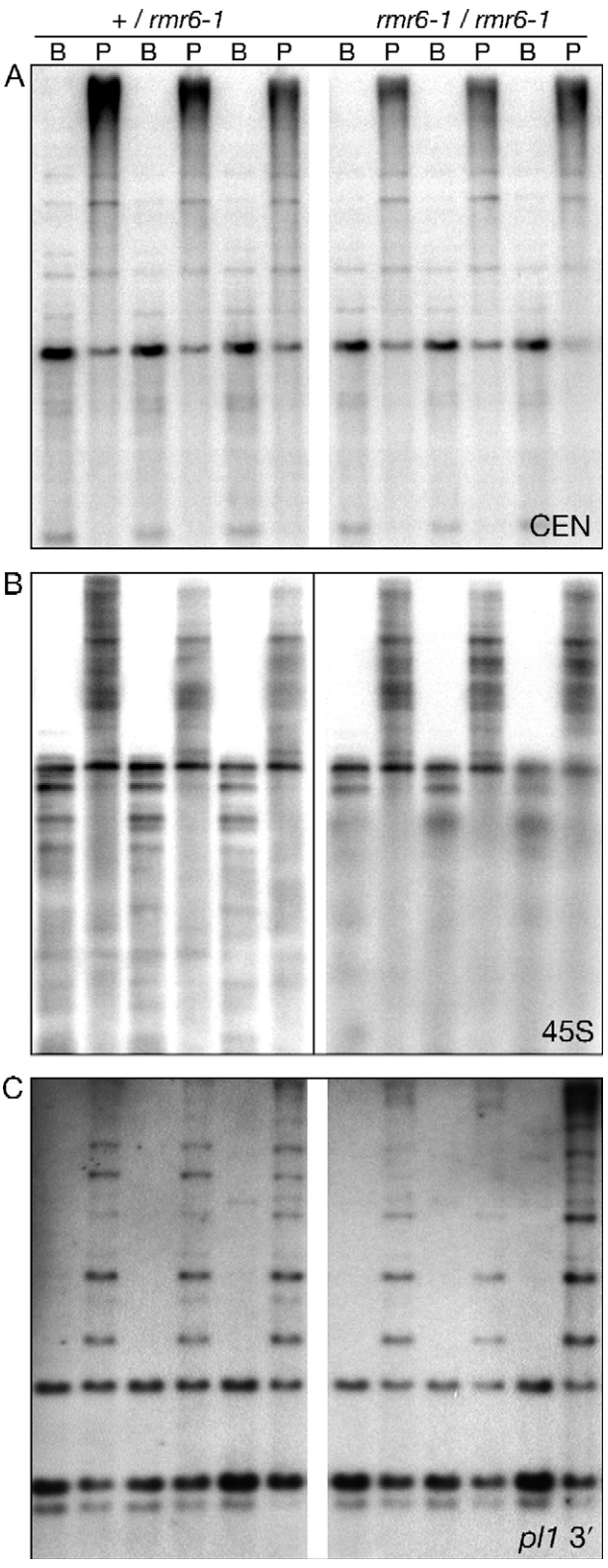

Fig. 7. Cytosine methylation patterns of specific *rmr6* genotypes. Genomic DNA from three +/*rmr6-1* plants and three *rmr6-1*/*rmr6-1* siblings was digested with either *Bst*NI (B, methylation insensitive) or *Psp*GI (P, methylation sensitive). Gel blots were hybridized with radiolabeled probes for (A) centromeric sequences, (B) 45S rDNA repeats, and (C) a 1.1 kb *Xho*I fragment that recognizes the 3′ portion of all known *pl1* alleles (Cone et al., 1993).

A model for Rmr6 function

Given the mechanistic similarities of *Rmr6* and *Mop1* in maintaining repression of paramutant *pl1*, *b1*, and *r1* alleles (Dorweiler et al., 2000; Hollick et al., 2005) and the stochastic developmental defects reported in *mop1* (Dorweiler et al., 2000) and *rmr6* mutant plants, the molecules encoded by these two loci appear to work in a common pathway at similar target genes. This pathway likely involves small RNA-mediated heterochromatin maintenance, as tandem repeats upstream of the *B1-Intense* allele are required for paramutation at that locus (Stam et al., 2002) and *mop1* encodes a putative RDR2 ortholog (Alleman et al., 2006; Woodhouse et al., 2006). Current models suggest that the RDR2-dependent 24 nt siRNAs help guide DNA methylation machinery including DRM (DOMAINS REARRANGED METHYLTRANSFERASE)-class methyltransferases to matching target DNA sequence (Pontes et al., 2006). This process appears to comprise a self-reinforcing loop, as DRM function is required for siRNA accumulation (Pontes et al., 2006; Zilberman et al., 2004). A similar mechanism may help ensure transcriptional repression at loci that are targets of *Rmr6* action and *Rmr6* may help maintain chromatin states in concert with MOP1. Loss of function for either of these molecules would presumably disrupt the maintenance of heterochromatin-type modifications, and thus allow atypical expression patterns of the target locus. While the chromatin modifications presumably maintained by MOP1 and *Rmr6* do not affect global cytosine methylation levels, we have documented CpG, CpNpG, and CpNpN hypomethylation in *rmr6* mutants at certain residues of a CACTA-like transposon fragment located 129 bp upstream of the *Pl1-Rhoades* translational start site (JBH and SMG manuscript in revision). While methylation of these upstream sequences is not correlated with different epigenetic regulatory states of the *Pl1-Rhoades* allele, these results show that *Rmr6* acts to maintain cytosine methylation patterns in all known sequence contexts at specific genomic features.

Although many *Arabidopsis* genes involved in establishing and maintaining small non-coding RNA production and cytosine methylation have been identified, there are no obvious orthologs in the syntenic rice interval to which *rmr6* maps (Hollick et al., 2005; SEP and JBH unpublished data). This suggests that *rmr6* encodes an as yet unknown component of epigenetic repression in higher eukaryotes. Although *rmr6* mutations were discovered by screening for genetic factors required to maintain repressed paramutant states of the *Pl1-Rhoades* allele, the *Rmr6*-mediated maintenance of epigenetic repression appears to affect a broad assortment of alleles beyond those that undergo paramutation.

Maize leaf polarity and lateral meristem repression

HD-ZIP III transcription factors are required to establish proper adaxial–abaxial leaf polarity in both monocots and dicots (Juarez et al., 2004; McConnell et al., 2001). In *Arabidopsis*, gain-of-function mutations in *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) lead to adaxialization of abaxial cell types in lateral organs (Emery et al., 2003; McConnell and Barton, 1998; McConnell et al., 2001). Similarly, gain-of-function mutations in *rld1*, the maize *REV* ortholog, lead to leaf blade adaxialization (Juarez et al., 2004). In addition to their role in leaf development, the *Arabidopsis* mutants suggest that HD-ZIP III factors promote shoot meristem formation. Gain-of-function *PHB* mutants display ectopic meristems at the base of adaxialized leaves (McConnell and Barton, 1998) and loss-of-function *REV* mutants have reduced branching and floral organ formation (Otsuga et al., 2001). All gain-of-function mutations in *Arabidopsis* and maize HD-ZIP III-encoding genes result from base pair changes in the predicted binding site of miR-165 and miR-166 (Emery et al., 2003; Juarez et al., 2004; McConnell et al., 2001) that presumably block the microRNA-dependent silencing pathway of these genes.

The adaxialized abaxial sheath and lateral outgrowths in *rmr6* mutant plants are reminiscent of gain-of-function mutations in *Arabidopsis* HD-ZIP III genes, and the former phenotype is also very similar to that conferred by *Rld1-O*; however, mutations in these two genes (*rld1* and *rmr6*) affect distinct proximal–distal portions of the leaf. Taken together, these findings suggest that *Rmr6* maintains canalized expression patterns of a factor affecting sheath abaxial epidermal fates, possibly an HD-ZIP III protein, in the proximal region of the leaf. Although HD-ZIP III proteins affecting proximal leaf polarity and lateral meristems have yet to be identified in maize, the presence of multiple HD-ZIP III factors with partially independent roles in *Arabidopsis* (Prigge et al., 2005) leaf development suggests that similar diversity exists in maize.

Maize monoecious sex determination pattern

Monoecy, defined by complete separation of male and female inflorescences on the same plant, distinguishes maize from most angiosperms (Richards, 1997). Our results suggest that maize monoecy is dependent on *Rmr6* action to repress pathways promoting female sexual determination. These pathways could be genetic, hormonal, or a combination thereof. Although our epistasis analysis implicates *sk1* regulation as integral to this pathway, both tassel feminization and decreased apical internode length phenotypes seen in *rmr6* mutant plants are consistent with aberrant hormone synthesis, regulation, or perception. Gibberellic acid (GA), for example, affects plant height and proper sex determination in maize. Mutations in maize *dwarf1*, *2*, *3*, *5*, and *8* and *anther ear1* loci, which are required for either biosynthesis or perception of GA, lead to decreased internode elongation and masculinization of lateral inflorescences (Neuffer et al., 1997; Phinney, 1961, 1984). However, defects in GA signaling are not entirely consistent with *rmr6* mutant phenotypes. While decreased apical internode length suggests a GA deficiency, tassel feminization suggests GA overabundance. In fact, exogenous application of GA causes tassel feminization (Hansen et al., 1976; Nickerson, 1959; Nickerson and Dale, 1955). Similarly, growth of plants under low light or in cool temperatures leads to increased GA levels and consequent tassel feminization (Richey and Sprague, 1932; Rood et al., 1980; Schaffner, 1930). Therefore, abnormal levels of GA do not

appear to be the cause of observed *rmr6* mutant phenotypes. Similarly, other *tassel seed* loci appear to act independently of GA action, as double mutants with *dwarf* mutations show additive phenotypes rather than restorative phenotypes (Irish et al., 1994).

There is a general correlation between the sex of the inflorescence and the internode lengths supporting it. Normal maize architecture features an apical male inflorescence subtended by elongated internodes and lateral female inflorescences subtended by compressed internodes demarcated by husk leaves. A functional relationship between these features is suggested by mutations in other *tassel seed* loci that are associated with reduced stature (Irish, 1996). Furthermore, husk internode lengths are increased in the masculinizing mutant *teosinte branched1* (*tba1*); however, *ts2*; *tba1* double mutants have elongated axillary branches that terminate in a female inflorescence (Hubbard et al., 2002). Our observations that *rmr6* mutants without a tassel seed phenotype still have reduced apical internodes suggest that additional factors, beyond the sexual status of the inflorescence, influence adult phase internode elongation.

An alternative explanation for the decreased apical internode length in *rmr6* mutant plants may relate to defects affecting the juvenile-to-adult phase transition. In maize, internode length varies with growth phase: internode length increases as plants proceed from juvenile to adult vegetative growth (Poethig, 1988). Interestingly, the maize juvenile-to-adult phase transition may be controlled by relative levels of miR-156, which promotes juvenile growth through down regulation of *teosinte glume architecture1* (Chuck et al., 2007), and miR-172, which promotes adult phase growth through repression of the *glossy15* gene (Lauter et al., 2005). Possibly, the lack of internode elongation in *rmr6* mutants associated with a delayed transition to the adult phases of vegetative development reflects a defect in canalizing the genetic program responsible for adult phase-specific internode elongation.

Regarding the tassel feminization of *rmr6* mutants, the genetic interaction of *sk1* with *rmr6* differs from the relationship between *sk1* and *ts1* or *ts2*, as mutations in these *ts* loci do not require *Sk1* function for tassel feminization (Irish et al., 1994; Jones, 1932). Data from our epistasis experiments, along with the role of *Rmr6* in maintaining transcriptional repression lead us to propose that an *Rmr6*-dependent mechanism is also responsible for maintaining tissue-specific regulation of *Sk1* expression either directly or through control of an unidentified upstream regulator. A prediction of this hypothesis is that *Sk1* RNA expression patterns in *rmr6* mutant plants are expanded relative to non-mutants, such that *Sk1* RNA would be found in tassel and secondary ear florets. Malcomber and Kellogg (2006) recently speculated that *sk1* encodes an miRNA that blocks translation of *ts2* mRNA, which would be consistent with the apparent link between *Rmr6* function and small RNA biology.

While hermaphroditism is an ancestral trait in the plant kingdom (Richards, 1997), unisexuality (either monoecy or dioecy) has arisen and been lost within angiosperm lineages generally and even within the grasses (Malcomber and Kellogg, 2006). Evidence for epigenetic control of sex determination has been demonstrated in the dioecious plant *Melandrium album*, in which flowers, like in maize, initiate as hermaphrodites but differentiate through selective abortion of pistils or stamens (Ye et al., 1991). Janousek et al. (1996) were able to induce heritable hermaphroditism on genetically male *M. album* plants through treatment with 5-azacytidine, which reduced cytosine methylation at CpG and CpNpG motifs. Their findings indicate that cytosine methylation can be used for the establishment or maintenance of unisexuality. We propose that monoecy in maize is epigenetically controlled, as evidenced by a requirement for *Rmr6* function in its maintenance.

Evolutionary significance of Rmr6 action

Epigenetic-based variation may help solve a conundrum of neo-Darwinian evolutionary theory: the improbability of accumulating the precise combination of adaptive traits without passing through a state of decreased fitness. Waddington's canalization hypothesis (1942) provides a step-wise scenario in which natural selection favors individuals that respond to environmental stimuli to an optimal degree regardless of the magnitude of the stimulus. The response to the stimulus is buffered – or canalized – in these individuals; therefore, they can accumulate genotypic variation that mimics the canalized response independent of the environmental stimulus. Because the genotypic fixation of these traits occurs after their phenotypic expression, they can arise without compromising fitness. The protein chaperone Heat-shock protein 90 (Hsp90) provides a protein-based phenotypic stabilizing mechanism that supports Waddington's hypothesis (Flatt, 2005). Loss of Hsp90 function – which leads to a reduced ability to refold functional protein conformations – reveals morphological variation that is presumably caused by unstable and dysfunctional protein conformations stemming from amino acid polymorphisms arising in highly homogenous populations of *Drosophila* and *Arabidopsis* (Queitsch et al., 2002; Rutherford and Lindquist, 1998). Because of its phenotypic buffering capacity, Hsp90 can relieve the selective pressure that would otherwise prevent accumulation of deleterious yet potentially adaptive morphological variation. The developmental abnormalities uncovered in *rmr6* mutants suggest that it also fulfills a phenotypic buffering role; however, *Rmr6* appears to affect stability of DNA or chromatin-based regulatory variation.

Because *Mop1* and *Rmr6* are required to maintain both epigenetic-based transcription states of paramutant alleles and the fidelity of proper developmental programs, it is likely that the epigenetic mechanism(s) determining patterns of transcriptional repression for many genes controlling maize development involve RDR2-dependent small RNAs. With the seminal observations that HD-ZIPIII-targeted miRNAs can effect epigenetic changes (Bao et al., 2004), it is possible that the fidelity of miRNA-initiated repression similarly requires *Rmr6*-type functions. Regardless of this specific possibility, *Rmr6* would provide developmental canalization by stabilizing transient or persistent small-RNA-directed expression patterns, and loss of *Rmr6* function would uncover this variation and lead to the abnormalities we observe in *rmr6* mutants. Alterations in

the developmental timing or spatial action of small RNAs, or in the maintenance of these induced chromatin states, could provide important epigenetic sources of phenotypic variation upon which select may act. Consistent with Waddington's thesis of genetic assimilation, these variations could persist within a population at levels needed for the accumulation of DNA sequence-based changes that help reinforce these novel expression patterns. Identification of the *rmr6* gene product will undoubtedly enhance our understanding of mechanisms underlying certain types of evolutionary change, as will identification of specific target sequences affected by *Rmr6* action. Efforts to address both of these issues are currently underway.

Acknowledgments

We thank Dr. Jiming Jiang (University of Wisconsin, Madison) and Dr. Michael McMullen (University of Missouri, Columbia), respectively, for the pSau3a9 and pZmRI clones. *Rld1-O* seed was kindly provided by Dr. Marja Timmermans (Cold Spring Harbor Laboratory). Thanks to Alison Jeffs for artwork and to Dr. Sarah Hake (USDA/ARS Plant Gene Expression Center) and members of the Hollick lab for helpful comments on the manuscript. This work was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (99-35301-7753, 2001-35301-10641, and 2006-35304-17399). The views expressed are solely those of the authors and are not endorsed by the sponsors of this work.

References

Alleman, M., Sidorenko, L., McGinnis, K., Seshadri, V., Dorweiler, J.E., White, J., Sikkink, K., Chandler, V.L., 2006. An RNA-dependent RNA polymerase is required for paramutation in maize. Nature 442, 295–298.

Attwood, J.T., Yung, R.L., Richardson, B.C., 2002. DNA methylation and the regulation of gene transcription. Cell. Mol. Life Sci. 59, 241–257.

Bantignies, F., Cavalli, G., 2006. Cellular memory and dynamic regulation of polycomb group proteins. Curr. Opin. Cell Biol. 18, 275–283.

Bao, N., Lye, K.W., Barton, M.K., 2004. MicroRNA binding sites in *Arabidopsis* class III HD-ZIP mRNAs are required for methylation of the template chromosome. Dev. Cell. 7, 653–662.

Bernstein, E., Allis, C.D., 2005. RNA meets chromatin. Genes Dev. 19, 1635–1655.

Brink, R.A., 1964. Genetic repression in multicellular organisms. Am. Nat. 98, 193–211.

Calderon-Urrea, A., Dellaporta, S.L., 1999. Cell death and cell protection genes determine the fate of pistils in maize. Development 126, 435–441.

Chan, S.W.L., Zilberman, D., Xie, Z.X., Johansen, L.K., Carrington, J.C., Jacobsen, S.E., 2004. RNA silencing genes control de novo DNA methylation. Science 303, 1336.

Chan, S.W.L., Zhang, X.Y., Bernatavichute, Y.V., Jacobsen, S.E., 2006. Two-step recruitment of RNA-directed DNA methylation to tandem repeats. PLoS. Biol. 4, 1923–1933.

Chandler, V.L., Eggleston, W.B., Dorweiler, J.E., 2000. Paramutation in maize. Plant Mol. Biol. 43, 121–145.

Chuck, G., Cigan, A.M., Saeteurn, K., Hake, S., 2007. The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. Nat. Genet. 39, 544–549.

Cone, K.C., Cocciolone, S.M., Burr, F.A., Burr, B., 1993. Maize anthocyanin regulatory gene *pl* is a duplicate of *c1* that functions in the plant. Plant Cell 5, 1795–1805.

Dellaporta, S.L., Wood, J., Hicks, J.B., 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1, 19–21.

Dorweiler, J.E., Carey, C.C., Kubo, K.M., Hollick, J.B., Kermicle, J.L., Chandler, V.L., 2000. *mediator of paramutation1* is required for establishment and maintenance of paramutation at multiple maize loci. Plant Cell 12, 2101–2118.

Emerson, R.A., Beadle, G.W., Fraser, A.C., 1935. A summary of linkage studies in maize. Cornell Univ. Agr. Exp. Sta. Mem. 180, 1–83.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F., Bowman, J.L., 2003. Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes. Curr. Biol. 13, 1768–1774.

Finnegan, E.J., Peacock, W.J., Dennis, E.S., 1996. Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. Proc. Natl. Acad. Sci. U. S. A. 93, 8449–8454.

Flatt, T., 2005. The evolutionary genetics of canalization. Q. Rev. Biol. 80, 287–316.

Grant-Downton, R.T., Dickinson, H.G., 2006. Epigenetics and its implications for plant biology 2. The 'epigenetic epiphany': epigenetics, evolution and beyond. Ann. Bot. 97, 11–27.

Hansen, D.J., Bellman, S.K., Sacher, R.M., 1976. Gibberellic acid-controlled sex expression of corn tassels. Crop. Sci. 16, 371–374.

Hollick, J.B., Chandler, V.L., 2001. Genetic factors required to maintain repression of a paramutagenic maize *pl1* allele. Genetics 157, 369–378.

Hollick, J.B., Kermicle, J.L., Parkinson, S.E., 2005. *Rmr6* maintains meiotic inheritance of paramutant states in *Zea mays*. Genetics 171, 725–740.

Hubbard, L., McSteen, P., Doebley, J., Hake, S., 2002. Expression patterns and mutant phenotype of *teosinte branched1* correlate with growth suppression in maize and teosinte. Genetics 162, 1927–1935.

Irish, E.E., 1996. Regulation of sex determination in maize. BioEssays 18, 363–369.

Irish, E.E., Langdale, J.A., Nelson, T.M., 1994. Interactions between *tassel* seed genes and other sex-determining genes in maize. Dev. Genet. 15, 155–171.

Janousek, B., Siroky, J., Vyskot, B., 1996. Epigenetic control of sexual phenotype in a dioecious plant, *Melandrium album*. Mol. Gen. Genet. 250, 483–490.

Jiang, J.M., Nasuda, S., Dong, F.G., Scherrer, C.W., Woo, S.S., Wing, R.A., Gill, B.S., Ward, D.C., 1996. A conserved repetitive DNA element located in the centromeres of cereal chromosomes. Proc. Natl. Acad. Sci. U. S. A. 93, 14210–14213.

Jones, D.F., 1932. The interaction of specific genes determining sex in dioecious maize. Proc. Sixth Intl. Cong. Genet. 2, 104–107.

Juarez, M.T., Kui, J.S., Thomas, J., Heller, B.A., Timmermans, M.C.P., 2004. microRNA-mediated repression of *rolled leaf1* specifies maize leaf polarity. Nature 428, 84–88.

Kakutani, T., Jeddeloh, J.A., Flowers, S.K., Munakata, K., Richards, E.J., 1996. Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proc. Natl. Acad. Sci. U. S. A. 93, 12406–12411.

Lauter, N., Kampani, A., Carlson, S., Goebel, M., Moose, S.P., 2005. *microRNA172* down-regulates *glossy15* to promote vegetative phase change in maize. Proc. Natl. Acad. Sci. U. S. A. 102, 9412–9417.

Lawrence, C.J., Schaeffer, M.L., Seigfried, T.E., Campbell, D.A., Harper, L.C., 2007. MaizeGDB's new data types, resources and activities. Nucleic Acids Res. 35, D895–D900.

Llave, C., Xie, Z.X., Kasschau, K.D., Carrington, J.C., 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. Science 297, 2053–2056.

Longley, A.E., 1961. Breakage points for four corn translocation series and other corn chromosome aberrations. USDA-ARS Crops Res. Bull. 34-16, 1–40.

Malcomber, S.T., Kellogg, E.A., 2006. Evolution of unisexual flowers in grasses (Poaceae) and the putative sex-determination gene, *TASSELSEED2* (*TS2*). New Phytol. 170, 885–899.

McConnell, J.R., Barton, M.K., 1998. Leaf polarity and meristem formation in *Arabidopsis*. Development 125, 2935–2942.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., Barton, M.K., 2001. Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots. Nature 411, 709–713.

McGinnis, K.M., Springer, C., Lin, Y., Carey, C.C., Chandler, V., 2006. Transcriptionally silenced transgenes in maize are activated by three mutations defective in paramutation. Genetics 173, 1637–1647.

McMullen, M.D., Hunter, B., Phillips, R.L., Rubenstein, I., 1986. The structure of the maize ribosomal DNA spacer region. Nucleic Acids Res. 14, 4953–4968.

Nelson, J.M., Lane, B., Freeling, M., 2002. Expression of a mutant maize gene in the ventral leaf epidermis is sufficient to signal a switch of the leaf's dorsoventral axis. Development 129, 4581–4589.

Neuffer, M.G., Coe, E.H., Wessler, S.R., 1997. Mutants of Maize. Cold Spring Harbor Laboratory Press, Plainview, NY.

Nickerson, N.H., 1959. Sustained treatment with gibberellic acid of five different kinds of maize. Ann. Mo. Bot. Gard. 46, 19–37.

Nickerson, N.H., Dale, E.E., 1955. Tassel modification in *Zea mays*. Ann. Mo. Bot. Gard. 42, 195–212.

Olsen, P.H., Ambros, V., 1999. The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. 216, 671–680.

Otsuga, D., DeGuzman, B., Prigge, M.J., Drews, G.N., Clark, S.E., 2001. *REVOLUTA* regulates meristem initiation at lateral positions. Plant J. 25, 223–236.

Phinney, B.O., 1961. Dwarfing genes in *Zea mays* and their relation to the gibberellins. In: Klein, R.M. (Ed.), Plant Growth Regulation. Iowa State University Press, Ames, IA, pp. 489–501.

Phinney, B.O., 1984. Gibberellin A1, dwarfism and the control of shoot elongation in higher plants. In: Crozier, A., Hillman, J.R. (Eds.), S.E.B. Seminar Series, Vol. 3. Cambridge University Press, Cambridge, pp. 17–41.

Phipps, I.F., 1928. Heritable characters in maize XXXI—Tassel seed-4. J. Hered. 19, 399–404.

Poethig, R.S., 1988. Heterochronic mutations affecting shoot development in maize. Genetics 119, 959–973.

Pontes, O., Li, C.F., Nunes, P.C., Haag, J., Ream, T., Vitins, A., Jacobsen, S.E., Pikaard, C.S., 2006. The *Arabidopsis* chromatin-modifying nuclear siRNA pathway involves a nucleolar RNA processing center. Cell 126, 79–92.

Pontier, D., Yahubyan, G., Vega, D., Bulski, A., Saez-Vasquez, J., Hakimi, M.A., Lerbs-Mache, S., Colot, V., Lagrange, T., 2005. Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA polymerases IV in *Arabidopsis*. Genes Dev. 19, 2030–2040.

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N., Clark, S.E., 2005. Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in *Arabidopsis* development. Plant Cell 17, 61–76.

Queitsch, C., Sangster, T.A., Lindquist, S., 2002. Hsp90 as a capacitor of phenotypic variation. Nature 417, 618–624.

Richards, A.J., 1997. Plant Breeding Systems. Chapman and Hall, New York.

Richey, F.D., Sprague, G.F., 1932. Some factors affecting the reversal of sex expression in the tassels of maize. Am. Nat. 66, 433–443.

Rood, S.B., Pharis, R.P., Major, D.J., 1980. Changes of endogenous gibberellin-like substances with sex reversal of the apical inflorescence of corn. Plant Physiol. 66, 793–796.

Rutherford, S.L., Lindquist, S., 1998. Hsp90 as a capacitor for morphological evolution. Nature 396, 336–342.

Schaffner, J.H., 1930. Sex reversal and the experimental production of neutral tassels in *Zea mays*. Bot. Gaz. 90, 279–298.

Stam, M., Belele, C., Dorweiler, J.E., Chandler, V.L., 2002. Differential chromatin structure within a tandem array 100 kb upstream of the maize *b1* locus is associated with paramutation. Genes Dev. 16, 1906–1918.

Veit, B., Schmidt, R.J., Hake, S., Yanofsky, M.F., 1993. Maize floral development—new genes and old mutants. Plant Cell 5, 1205–1215.

Voelker, R., Mendel-Hartvig, J., Barkan, A., 1997. Transposon-disruption of a maize nuclear gene, *tha1*, encoding a chloroplast SecA homologue: in vivo role of cp-SecA in thylakoid protein targeting. Genetics 145, 467–478.

Waddington, C.H., 1942. Canalization of development and the inheritance of acquired characters. Nature 150, 563–565.

Waites, R., Hudson, A., 1995. *phantastica*: a gene required for dorsoventrality of leaves in *Antirrhinum majus*. Development 121, 2143–2154.

Woodhouse, M.R., Freeling, M., Lisch, D., 2006. Initiation, establishment, and maintenance of heritable *MuDR* transposon silencing in maize are mediated by distinct factors. PLoS. Biol. 4, 1678–1688.

Xie, Z.X., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., Carrington, J.C., 2004. Genetic and functional diversification of small RNA pathways in plants. PLoS. Biol. 2, 642–652.

Ye, D., Oliveira, M., Veuskens, J., Wu, Y., Installe, P., Hinnisdaels, S., Truong, A.T., Brown, S., Mouras, A., Negrutiu, I., 1991. Sex determination in the dioecious *Melandrium*. The X/Y chromosome system allows complementary cloning strategies. Plant Sci. 80, 93–106.

Zilberman, D., Cao, X.F., Johansen, L.K., Xie, Z.X., Carrington, J.C., Jacobsen, S.E., 2004. Role of *Arabidopsis* ARGONAUTE4 in RNA-directed DNA methylation triggered by inverted repeats. Curr. Biol. 14, 1214–1220.