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

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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.

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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;...
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 paramutation (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 (skl)-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 (rl1) 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 skl 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 Pl-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 siblings were used, respectively. Internode length and leaf wax distribution were measured in Pl′/Pl′ lines homozygous for the T6-9 (043-1) (T) interexchange 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*P1200203, ts*P1251881, ts*P1267290, ts*N2490) and skl 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 skl epistasis analysis, F2 families segregating for mutations in both rmr6 and skl were derived from +/rmr6-1; T Pl′/Pl′ and pl1/pl1; skl/skl 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 interexchange and P1′ as both w1 and pl1 loci are linked (2.3 and 1.4 cM, respectively) to the interexchange breakpoint (Hollick et al., 2005). To increase the likelihood of obtaining rmr6-1/rmr6-1; skl/skl
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 P' T P' individual was used as the initial female parent.

Double mutant rmr6-2/rmr6-2; +/Bdl1-O plants were generated by initially crossing rmr6-2/rmr6-2; wx T P'/wx T P' and Bdl1-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 tetroxide 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 BstNI or PspGI (New England Biolabs, Ipswich, MA). For the 45S rDNA assays, DNA was isolated as described (Voelker et al., 1997) from 2nd 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 XhoI 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 darkened 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<0.001 for H0 there is no difference in flowering time), and shorter (142 vs. 191.8 cm; n=45 and 54, respectively; z=13.2, p<0.001 for H0 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).

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<0.001 for H0 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 H0 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<0.001 for
$H_0$ 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

$rmr6-2$ 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

<table>
<thead>
<tr>
<th>Generation</th>
<th>Number of ear progenies examined</th>
<th>Number of individuals with specific $rmr6$ genotypes and adaxialized sheaths/lateral outgrowths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$Rmr6/rmr6-2$</td>
</tr>
<tr>
<td>F2</td>
<td>5</td>
<td>22 (0.0)</td>
</tr>
<tr>
<td>F3</td>
<td>4</td>
<td>50 (0.0)</td>
</tr>
<tr>
<td>F4</td>
<td>5</td>
<td>23 (0.0)</td>
</tr>
<tr>
<td>F5</td>
<td>3</td>
<td>46 (0.0)</td>
</tr>
<tr>
<td>F6</td>
<td>2</td>
<td>18 (0.0)</td>
</tr>
<tr>
<td>Totals</td>
<td>19</td>
<td>159 (0.0)</td>
</tr>
</tbody>
</table>

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 \textit{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 \textit{rmr6-1/rmr6-1} individuals (3 and 6 respective instances among 360 mutant individuals observed), consistent with previous genetic studies showing that \textit{rmr6-2} is a more severe mutant allele than \textit{rmr6-1} (Hollick et al., 2005).

The \textit{rmr6-2}-associated proximal sheath phenotype bears striking resemblance to blade adaxialization associated with dominant gain-of-function mutations in \textit{rolled leaf1} (\textit{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 \textit{rmr6-2} and \textit{Rld1-O} mutations, we synthesized and examined 23 independent ear progenies segregating \textit{rmr6-2/rmr6-2};+/\textit{Rld1-O} double mutants. We identified \textit{rmr6-2/rmr6-2} individuals by their compact tassel phenotype and anther pigmentation, and +/\textit{Rld1-O} individuals by their rolled blade phenotype. The \textit{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 +/\textit{Rld1-O} individuals are distinct from the proximal adaxialized patches extending acropetally from the base of the sheath in \textit{rmr6} mutant plants. In total, 43/455 F2 individuals were scored as \textit{rmr6-2/rmr6-2};+/\textit{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 \textit{rmr6}-associated (acropetal) and \textit{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 \textit{Rld1} and \textit{Rmr6} function to ensure proper dorso-ventral leaf polarity, albeit in different proximal–distal domains.

\textit{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 \textit{rmr6-1} and \textit{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 un aborted 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 penetration 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%; χ² = 6.68, p < 0.01 for H₀ that the two phenotypes are independent).

Table 2

<table>
<thead>
<tr>
<th>Generation</th>
<th>Number of ear progenies examined</th>
<th>Number of individuals with specific rmr6 genotypes and tassel silks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rmr6/rmr6-1</td>
</tr>
<tr>
<td>F2</td>
<td>1</td>
<td>6 (0)</td>
</tr>
<tr>
<td>F3</td>
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</tr>
<tr>
<td>Totals</td>
<td>11</td>
<td>156 (0)</td>
</tr>
</tbody>
</table>

Numbers in parentheses refer to individuals having tassel silks.

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

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Number of ear progenies examined</th>
<th>Total number of progeny individualsa</th>
</tr>
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<tbody>
<tr>
<td>ts1/ts1b</td>
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<td></td>
</tr>
<tr>
<td>ts2/ts2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
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</tr>
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<tr>
<td>+/rmr6-1/rmr6-1</td>
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<td>37 (0)</td>
</tr>
<tr>
<td>+/PI251881</td>
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<td>+/PI267209</td>
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<td>13 (0)</td>
</tr>
<tr>
<td>+/N2490</td>
<td>1</td>
<td>13 (0)</td>
</tr>
</tbody>
</table>

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. 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.
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.

sklls1 is epistatic to rmr6

The proposed role of skl 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 skl gene may be inapparently expressed in tassels of rmr6 mutant plants. If so, Skl function would be required for the observed tassel seed phenotype and thus rmr6-1/skl-1 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 skl double mutant plants—identified by their dark anther pigment (rmr6-1/skl-1) and silkless ears (skl-1/skl-1)—and examined these for tassel silks. For all seven rmr6-1/skl-1; skl-1/skl-1 putative double mutants that we crossed to both rmr6-1/Rmr6+ and Skl+/skl-1 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 skl and either rmr6-1 or rmr6-2 in which the tassel feminization phenotype was highly penetrant among the rmr6-1/skl-1 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 (rmr6-1/skl-1; skl-1/skl-1) displayed a tassel seed phenotype. Thus, skl is epistatic to rmr6 and ectopic Skl function in the tassel florets of rmr6 mutant plants appears responsible for pistil protection (Fig. 6C; \( \chi^2 = 64.04, p < 0.001 \) for \( H_0 \) there is no difference in the frequency of plants having silks in their tassels among rmr6-1/skl-1; Skl+/− and rmr6-1/skl-1; skl-1/skl-1 genotypes).

Rmr6 does not maintain genome-wide cytosine methylation patterns

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

Table 4

<table>
<thead>
<tr>
<th>Ear progeny</th>
<th>rmr6 allele</th>
<th>Number of individuals with specific genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rmr6+/−</td>
<td>Rmr6−/−</td>
</tr>
<tr>
<td></td>
<td>Skl+/−</td>
<td>skl−/skl−</td>
</tr>
<tr>
<td>Totals</td>
<td>61 (0)</td>
<td>27 (0)</td>
</tr>
</tbody>
</table>

Rmr6+/− and Skl+/− represent both Rmr6+/Rmr6− and Rmr6−/Rmr6− genotypes or both Skl+/Skl− and Skl−/skl− 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 BstNI or PspGI (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 PspGI 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 BstNI (B, methylation insensitive) or PspGI (P, methylation sensitive). Gel blots were hybridized with radiolabeled probes for (A) centromeric sequences, (B) 45S rDNA repeats, and (C) a 1.1 kb XhoI 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, CpnG, and CpnPN hypomethylation in rmr6 mutants at certain residues of a CACTA-like transposon fragment located 129 bp upstream of the Pl1-Rhoades transcriptional 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 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 femization 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 dwarfl, 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 (Neuf-fer 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 femization suggests GA overabundance. In fact, exogenous application of GA causes tassel femization (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 femization (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 skl with rmr6 differs from the relationship between skl and ts1 or ts2, as mutations in these ts loci do not require Skl 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 Skl expression either directly or through control of an unidentified upstream regulator. A prediction of this hypothesis is that Skl RNA expression patterns in rmr6 mutant plants are expanded relative to non-mutants, such that Skl RNA would be found in tassel and secondary ear florets. Malcomber and Kellogg (2006) recently speculated that skl 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 (Quitsch 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 rnr6 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.

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