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Paramutation: The Chromatin Connection

Paramutation has been studied extensively in maize since R.A. Brink described a heritable alteration of the *r* locus that defied principles of Mendelian inheritance (Brink, 1956). A recent publication on paramutation in maize now provides convincing evidence that chromatin-level regulation underlies the phenotypic differences between alleles that participate in paramutation (Stam et al., 2002a). Two additional articles—one detailing paramutation-like effects associated with mammalian imprinting and the other describing paramutation after a change in ploidy in Arabidopsis—challenge us to understand how these cases fit into the developing paradigm explaining the molecular basis for paramutation (Herman et al., 2003; Mittelsten Scheid et al., 2003). Together, these studies suggest a mechanistic link between paramutation and other types of chromatin-level epigenetic regulation of gene expression.

Paramutation is an allelic interaction in which one allele, referred to as paramutagenic, causes a heritable change in the expression of a homologous paramutable allele. Alleles unaffected by exposure to a paramutagenic allele are called neutral alleles. Crossing a plant carrying a paramutable allele with a plant carrying a paramutagenic allele results in reduced gene expression of the susceptible allele. When these hybrids are outcrossed to plants carrying paramutable alleles, most of the progeny exhibit the low-expression phenotype in this and the following generations, demonstrating a heritable change in expression state for the paramutable allele. In some cases, the formerly paramutable allele becomes paramutagenic and can now silence other paramutable alleles; this is called secondary paramutation. Thus, there are two distinct steps in paramutation: establishment and maintenance. Establishment occurs when the paramutable allele is exposed to the paramutagenic allele and the paramutable allele takes on an altered expression state. Maintenance refers to the mitotic or meiotic heritability of the new expression state, for example, its ability to be maintained stably in subsequent generations.

PARAMUTATION IN MAIZE

In maize, paramutation has been best studied for two genes: *booster* (*b*) and *red* (*r*). Both genes encode transcription factors that regulate synthesis of anthocyanin pigments. At both loci, paramutable alleles have higher expression than paramutagenic alleles, but several observations suggest that different mechanisms control paramutation for these two loci. They differ in genetic behavior, molecular organization of the loci, and the imposition of epigenetic marks, such as DNA methylation and altered chromatin structure.

At *b*, two alleles participate in paramutation (Figure 1A). When the paramutable *B-I* allele (dark pigmentation) is crossed with paramutagenic *B'* (light pigmentation), the F1 progeny exhibit the *B'* phenotype. This phenotype is stable, and the new *B'* allele is paramutagenic, such that crossing the F1 to new (naive) *B-I* leads to production of only the *B'* phenotype. At the molecular level, the *b* locus contains a single coding region. Reduced pigmentation of *B'* is because of reduced transcription. There are no differences in DNA methylation or DNA sequence in the coding regions of paramutagenic *B'* and paramutable *B-I* alleles (Patterson et al., 1993; Stam et al., 2002b).

To identify *cis*-linked elements regulating paramutation, recombination mapping between *B'* and neutral *b* alleles was used (Stam et al., 2002a). A regulatory element, termed an enhancer, was located ~100 kb upstream of the *b* coding region. In both *B-I* and *B'*, this enhancer includes seven tandem repeats of an 853-bp sequence. Strength of paramutagenicity is determined in part by the number of repeats, as recombinant alleles with a reduced number of repeats are only weakly paramutagenic. Analysis of DNA methylation and chromatin structure in this repeat region revealed that in the transcriptionally active *B-I* allele, the repeats are hypermethylated and have an open chromatin structure, as assessed by DNaseI sensitivity assay. Conversely, the transcriptionally repressed *B'* allele has lower levels of methylation and more closed chromatin (Stam et al., 2002a). In newly formed *B'* alleles, chromatin structure was intermediate between *B-I* and *B'*, and DNA methylation changes lagged behind the reduction in expression associated with the new paramutation event. Although the precise nature of the molecular trigger for establishing paramutation remains unclear, these results show that establishment of paramutation is associated with changes in chromatin structure. DNA methylation differences reflect altered chromatin states and may serve as a mechanism to ensure stable transmission through subsequent generations.

Paramutation at the *r* locus differs from paramutation at *b* in many ways. When the paramutable *R-r*:*standard* (darkly pigmented) is crossed to paramutagenic *R-stippled* (lightly pigmented), *trans*-silencing does not occur in the first-generation heterozygote (Figure 1B). Rather, meiosis appears to be required to change the epigenetic state; when the F1 is crossed to plants homozygous for a recessive neutral *r* allele, *R-r*:*standard* is silenced and none of the progeny express the *R-r*:*standard* phenotype. The paramutant phenotype of the newly silenced allele is heritable but not stable. When crossed to recessive *r*, it reverts to normal expression over subsequent generations.

The *r* locus is complex, frequently containing multiple copies of *r* coding sequences. A thorough study of paramutagenic, paramutable, and neutral alleles revealed that the paramutable ones often

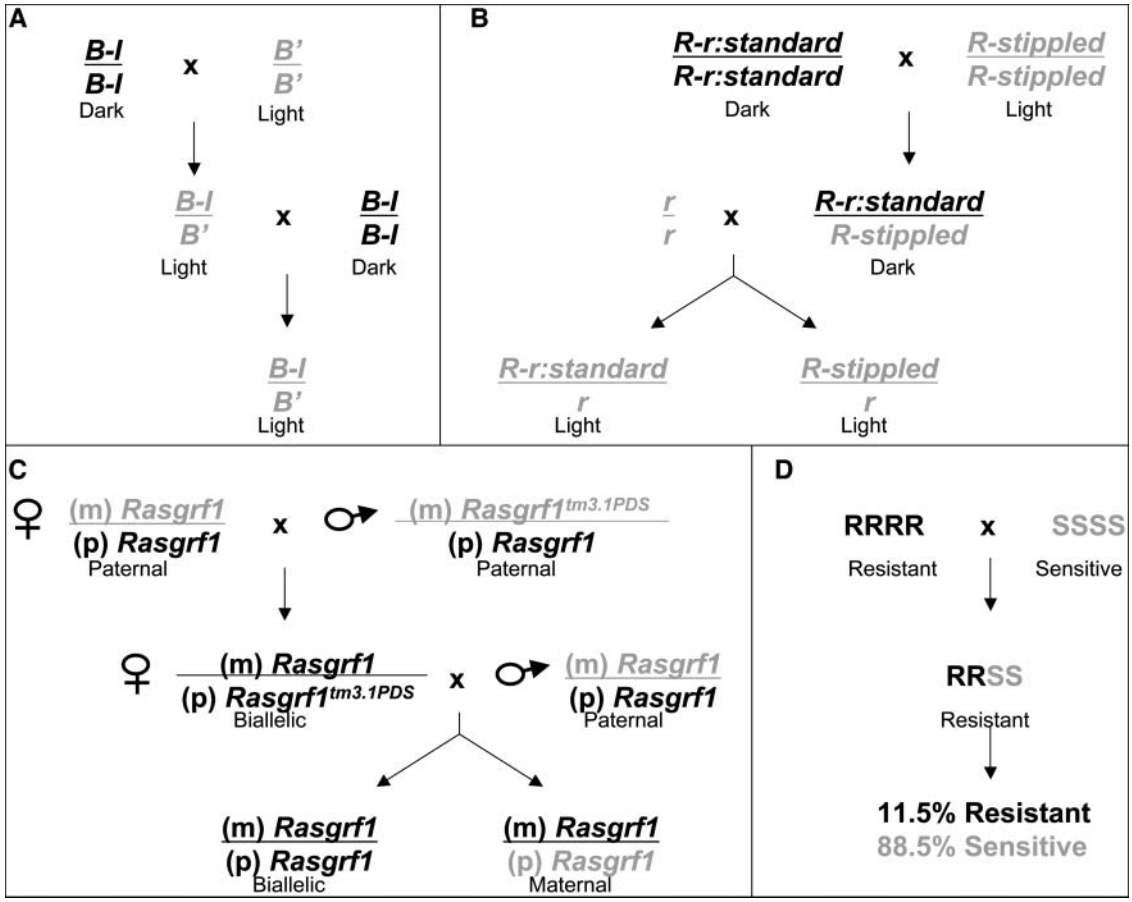

Figure 1. Four Examples of Paramutation.

In each panel, actively expressed alleles are shown in black, and silenced alleles are shown in gray. Phenotypes are listed below each genotype.

(A) In maize, the paramutable *B-I* allele is silenced by the paramutagenic *B′* allele in the first generation. In the next generation, the newly silenced *B-I* allele is paramutagenic and silences a naive *B-I* allele.

(B) In maize, the paramutable *R-r:standard* allele is expressed in the first generation when heterozygous with the paramutagenic *R-stippled*. When the heterozygote is crossed to a plant homozygous for a neutral *r* allele, *R-r:standard* is silenced in the second generation progeny.

(C) In mouse, the chimeric *Rasgrf1*$^{tm3.1Pds}$ allele, when crossed as male, activates maternal expression of the normally paternally expressed *Rasgrf1* allele, resulting in biallelic expression in the first generation. Maternal expression of *Rasgrf1* is maintained when the chimeric allele is segregated away in the second generation.

(D) In Arabidopsis autotetraploids, cross of hygromycin-resistant plants (RRRR) with hygromycin-sensitive plants (SSSS) carrying a silent transgene produces first generation progeny that are hygromycin resistant. Self-fertilization of the hybrids produces mainly hygromycin-sensitive (silenced) progeny.

have a similar allelic structure with multiple coding segments, some arranged in inverted repeat orientation (Walker and Panavas, 2001). Paramutability assays on several deletion derivatives of the *R-r:standard* revealed that sequences necessary for paramutability lie in the promoter shared by two divergently transcribed *r* coding segments (Kermicle et al., 1995).

However, paramutagenic alleles of *r* are not similar in structure to one another. Some paramutagenic alleles have the same organization as some neutral alleles (Walker and Panavas, 2001). For the paramutagenic *R-stippled* allele, paramutagenic strength correlates with the number of *r*-gene copies. The normal allele—with four *r*-homologous genes—has the strongest paramutagenicity, and derivatives with three, two, or one *r* genes have proportionately less paramutagenic activity (Kermicle et al., 1995). In terms of epigenetic marks, paramutagenic alleles consistently have a higher level of cytosine methylation in coding regions than paramutable alleles (Panavas et al., 1999; Walker and Panavas, 2001). Although chromatin structure of *r*

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alleles has not been examined directly, the wealth of data linking DNA methylation to changes in chromatin structure (Geiman and Robertson, 2002) is consistent with the notion that *r* paramutation is regulated at the level of chromatin.

Expression and paramutation of some *r* alleles is influenced by parent-of-origin genomic imprinting. When the paramutable *R-r:standard* allele is crossed as female by a recessive *r* tester, the gene is transcriptionally active and the kernel is fully pigmented. However, when transmitted through the male (pollen) parent, *R-r:standard* has a reduced level of transcription and the kernel is mottled or nearly colorless. Maternally transmitted *R-r:standard* is hypomethylated relative to the paternally transmitted allele, implicating a chromatin-based repression of the paternal allele (Alleman and Doctor, 2000). Imprinting also affects paramutation; only when *R-r:standard*/*R-stippled* heterozygotes are crossed as males to *r*/*r* females is paramutation evident. It is not clear whether this link is coincidental or whether there is a mechanistic relationship between imprinting and paramutation. However, rare *cis*-acting mutations that perturb imprinting of the paramutable *R-r:standard* allele have no effect on paramutability of that allele (Alleman and Doctor, 2000), implying that there are unique sequence elements for each process.

To identify *trans*-acting factors that regulate paramutation, genetic screens have been used to recover mutants that disrupt establishment or maintenance of paramutation. One recessive mutant, *mediator of paramutation1* (*mop1*), was isolated in a screen for modifiers of paramutation at *b*. The functional, dominant *Mop1* allele is required for establishment of paramutation at *b*. However, *Mop1* is not required for maintenance of the paramutant *B'* state. In the absence of *Mop1*, *B'* is expressed at high levels, but this change in expression is transient; when *Mop1* is added through crossing, *B'* returns to its silenced state. *Mop1* is also required for establishing paramutation at *r*, but its effect on maintenance of *r* paramutation was not reported (Dorweiler et al., 2000). This indicates that establishment and maintenance of paramutation are regulated by distinguishable processes.

PARAMUTATION AND GENOMIC IMPRINTING IN MAMMALS

Genomic imprinting refers to differential expression of maternally versus paternally inherited alleles. Imprinting has been reported for ~70 mammalian genes, and several mechanisms have been proposed for how imprinting occurs (Murphy and Jirtle, 2003). Recent work in mouse suggests a connection between genomic imprinting and paramutation and may provide some clues about how maternally imprinted genes are regulated.

Herman and colleagues (2003) studied imprinting of two loci, *Igf2r* (encoding insulin-like growth factor 2 receptor) and *Rasgrf1* (encoding RAS protein-specific guanine nucleotide-releasing factor 1). *Igf2r* is normally expressed from the maternal allele and is paternally silenced. Its expression is controlled by a sequence known as Region 2, in the second intron of the gene, which is hypermethylated in the expressed allele. *Rasgrf1* is normally expressed from the paternal allele and is silent on the maternal allele. Imprinted expression depends on a 1.6-kb repeated element located immediately downstream of a differentially methylated domain consisting of 40 direct repeats of 41 bp.

Homologous recombination was used to introduce a chimeric transgenic allele in which Region 2 of the *Igf2r* locus was substituted for the repeated DNA regulatory element in the *Rasgrf1* locus. The chimeric allele, *Rasgrf1*$^{tm3.1Pds}$, was analyzed for the ability to control imprinting of the wild-type *Rasgrf1* allele. When the chimeric allele was transmitted maternally, *Rasgrf1* was only expressed from the paternal allele—the normal state. However, when the chimeric allele was transmitted paternally (Figure 1C), expression was biallelic with low expression from the paternal chimeric allele and derepressed high-level expression from the maternal allele. This expression pattern was accompanied by methylation of both the paternal chimeric allele and the maternal wild-type allele. Thus, by substituting a maternal-specific regulatory sequence for the normal paternal-specific regulator, expression of *Rasgrf1* was switched from paternal to maternal. Moreover, the chimeric allele could heritably alter the methylation and expression pattern of a wild-type allele; this behavior is reminiscent of paramutation.

To test the heritability of the maternal derepression induced by *Rasgrf1*$^{tm3.1Pds}$, female mice carrying and expressing both a paternally derived *Rasgrf1*$^{tm3.1Pds}$ allele and a derepressed wild-type maternal *Rasgrf1* allele were mated with wild-type males (Figure 1C). Expression patterns then were analyzed for progeny carrying a wild-type *Rasgrf1* allele from both parents (e.g., progeny in which the chimeric allele had segregated away). There were two classes of progeny in approximately equal proportions. The first showed biallelic expression, namely, expression from both the paternally derived active allele and the maternally derived derepressed allele. This indicates that the active expression state of the derepressed maternal allele was transmitted through meiosis. The second class expressed only the derepressed allele from their mothers, suggesting that expression from this allele is able to silence expression from the paternally derived allele. In both cases, expression was correlated with DNA methylation of the expressed alleles.

These results imply that the *Igf2r* Region 2 is sufficient to direct maternal imprinting in *cis*. If imprinting involves alterations in chromatin structure as well as the documented changes in DNA methylation, it is possible that more than one generation might be required to fully convert a normally paternally expressed allele to maternal expression. This might explain why paternal expression persists in the first generation of progeny and is seen in some second generation progeny.

PARAMUTATION AND POLYPLOIDY

Another recent study points to paramutation-like regulation as an important determinant in controlling gene expression during changes in ploidy level (Mittelsten Scheid et al., 2003). Polyploidy can be divided into two categories. Allopolyploidy

arises when two or more genomes from distinct species are combined, for example, by interspecific hybridization followed by chromosome doubling. Autopolyploidy occurs when a single genome is multiplied. Polyploidy is an important mechanism for evolution in plants (reviewed in Wendel, 2000).

Altered phenotypes and novel patterns of gene expression often arise when plants undergo a change in ploidy. In an autopolyploid series of maize, gene expression for a number of loci increased as ploidy increased (Guo and Birchler, 1994), suggesting that gene expression and copy number are directly related in autopolyploids. There is accumulating evidence that the interaction between two different genomes triggers epigenetic changes in allopolyploids (Wendel et al., 2002; Osborn et al., 2003; Riddle and Birchler, 2003). In allopolyploid wheat, both upregulation and downregulation in gene expression were found. Some changes were specific to one of the parental genomes (He et al., 2003), and gene silencing was correlated with increased DNA methylation at the affected loci (Shaked et al., 2001). Changes in DNA methylation and gene expression are also seen in synthetic Arabidopsis allopolyploids (Madlung et al., 2002); in one study, 0.4% of 700 genes analyzed were transcriptionally silenced (Comai et al., 2000). These changes in gene expression may explain atypical phenotypes that arise when plants undergo a change in ploidy (Wendel et al., 2002; Osborn et al., 2003).

Although most studies of epigenetic changes in gene expression in polyploids have focused on allopolyploids, epigenetic silencing can also occur in Arabidopsis autopolyploids (Mittelsten Scheid et al., 1996). This group now provides evidence for a link between autopolyploidy and paramutation (Mittelsten Scheid et al., 2003). The new study focuses on derivatives of a transgenic Arabidopsis line called C^{14}. This diploid line is homozygous for a hygromycin phosphotransferase transgene, which confers resistance to the antibiotic hygromycin. When C^{14} was used to make autotetraploids, six lines were produced. One line (RRRR) had resistant progeny only, and this phenotype was stably maintained in subsequent self-crossed generations. Five lines produced progeny that were sensitive to hygromycin, indicating that the transgene had been silenced. Molecular analysis indicated that silencing was transcriptional and the silenced transgene was heavily methylated. When tetraploid sensitive, silenced plants (SSSS) were crossed to tetraploid resistant plants (RRRR), the progeny were all hygromycin resistant, as expected if the resistance phenotype is dominant; thus, silencing did not occur in the F1. Self-fertilization of hybrid RRSS plants should have generated only a small proportion of sensitive progeny. However, among 13 independent F2 populations (3530 plants), 88.5% were sensitive to hygromycin; for three of these families (742 plants), there were no hygromycin resistant plants (Figure 1D). This indicates that exposure of the R allele to the S allele resulted in a heritable silencing such that when the R allele was segregated away from the S allele (in RRRR progeny), the R allele remained silent. This behavior is typical of paramutation. The ability of the altered R allele to induce silencing of a naive allele was not tested. However, the silenced state was maintained even after several generations of self-fertilization.

The methylation state of the transgenes in sensitive and resistant plants was assayed by methylation-sensitive restriction enzyme digests. The silenced transgenes in the SSSS plants were hypermethylated relative to the active transgenes in RRRR plants. This hypermethylation was specific to the S loci, as flanking DNA was unmethylated. In F1 RRSS plants, hypomethylated DNA (from the R alleles) was observed, suggesting that the epigenetic changes in methylation of the R alleles occurs later, perhaps during meiosis. In F2 progeny, the R alleles were partially methylated, indicating that one of the changes that accompanies silencing is hypermethylation.

In an effort to probe the mechanism for this polyploid-induced silencing, diploid (SS) derivatives of the silenced tetraploids were crossed to mutants known to relieve transcriptional silencing of other transgenes. One of the mutants, *decrease in DNA methylation1* (*ddm1*), is deficient in a SWI/SNF-like protein that functions in chromatin remodeling (Brzeski and Jerzmanowski, 2003). Homozygous recessive *ddm1* mutant plants exhibit a reduction in DNA methylation, and after multiple generations of self-crossing, they show novel and heritable pleiotropic developmental phenotypes (Jeddeloh et al., 1998). When the diploid silenced (SS) plants were placed in a homozygous *ddm1* mutant background, initially there was no relief of silencing. However, in the F3 and F4, a small percentage of plants displayed a resistant phenotype. This result suggests that a functional chromatin remodeling activity is required for maintaining the silenced state of the S alleles.

CHROMATIN: A COMMON MECHANISM

The examples of paramutation-like behaviors discussed have similar genetic properties and probably share some of the same molecular mechanisms. In each case, one allele (paramutagenic; *B'*, *R-stippled*, *Rasgrf1*$^{tm3.1Pds}$, and SSSS) causes a heritable change in expression of a second allele (paramutable; *B-I*, *R-r:standard*, *Rasgrf1*, and RRRR). The underlying mechanism appears to involve, first, the imposition of a stable chromatin and expression state on the paramutagenic allele and, second, allele-specific transmission of that chromatin and expression state in *trans* to the paramutable allele. The stability of the altered expression states is maintained by epigenetic regulatory mechanisms, including DNA methylation, that serve to reinforce the chromatin structure of the altered allele.

A key question is how the allele-specific communication is accomplished. We propose a model invoking DNA methylation, changes in chromatin structure, and noncoding RNA as regulatory elements important for the establishment and maintenance of paramutation. In our view, DNA methylation and chromatin structure are key determinants for regulating expression, and noncoding RNA is the trigger for initiating changes in chromatin state. In the following discussion, we group *b* paramutation with imprinting of *Igf2r*/*Rasgrf1* because of similarities in the role played by

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cis-acting regulatory sequences in establishing the paramutagenic state. Similarly, we group *r* paramutation and autopolyploid-induced silencing based on similarities in genetic behavior and in organization of the genetic elements that appear to induce paramutation.

b PARAMUTATION AND IMPRINTING OF *Igf2r*/*Rasgrf1*

In *b* paramutation and in imprinting of *Igf2r* and *Rasgrf1*, DNA methylation is important in establishing the expression state. Deletion of Region 2 of the *Igf2r* locus or replacement of the repeated differentially methylated domain at *Rasgrf1* results in biallelic expression (Wutz et al., 1997; Yoon et al., 2002). When methylation of Region 2 is inhibited by introducing mutant alleles of DNA methyltransferase genes, imprinting is also lost. This demonstrates that methylation of Region 2, not just its presence, is required for imprinting (Verona et al., 2003). At the *b* locus, expression levels of paramutable and paramutagenic alleles of *b* are correlated with the DNA methylation status of the upstream repeat elements, but it is not clear if methylation is absolutely required for that expression state. This could be tested by introducing mutant alleles of genes known to be responsible for DNA methylation. Multiple copies of this 853-bp repeat are required for participation in paramutation, and it is possible that the number of repeats is correlated with the ability to induce methylation. An informative experiment would be to assay the methylation state of derivative lines with fewer repeats.

Accumulating evidence suggests a role for noncoding RNA in remodeling of chromatin structure. Imprinting at the *Igf2r* locus is dependent on an antisense transcript known as *Air* RNA (Lyle et al., 2000). *Air* is transcribed specifically from the silent paternal allele from a promoter that overlaps the paternally hypomethylated Region 2. *Air* is not transcribed from the maternal chromosome where Region 2 is hypermethylated. This observation suggests that the methylation state of Region 2 governs *Air* transcription, although how transcription of *Air* from the paternal allele leads to silencing is not yet clear. Homology with the *Igf2r* promoter is not required for imprinting because deletion or replacement of the homologous regions does not interfere with imprinting (Sleutels et al., 2002). A possibility is that transcription of *Air* RNA recruits chromatin-remodeling proteins that cause a change in chromatin state at flanking regions resulting in silencing of *Igf2r* (Verona et al., 2003). Such RNA-mediated chromatin remodeling is seen in X-inactivation in mammals and in *Caenorhabditis elegans*, whereby a noncoding RNA seems to recruit a protein complex that initiates chromatin remodeling of flanking DNA regions (Andersen and Panning, 2003).

Noncoding RNA may also regulate paramutation at *b*. Methylation status of the repeats in the upstream enhancer is associated with transcriptional activity of downstream genes (Stam et al., 2002a). It is possible that, as at *Igf2r*, hypomethylation of the repeats in the *B′* allele may allow transcription of a noncoding RNA from the enhancer that could then guide chromatin-remodeling factors to flanking DNA sites, preventing transcription of nearby genes, including *b*.

The production of noncoding RNA could also explain the *trans*-acting allelic interactions that define the ability of a paramutagenic allele to transfer its chromatin state to a paramutable allele. Several lines of evidence from plants and *Schizosaccharomyces pombe* indicate that RNA can mediate chromatin changes in *trans* (Chan et al., 2004; Verdel et al., 2004). While this is typically associated with RNA interference (see below), the machinery exists to use RNA as a guide for chromatin remodeling. This model explains the change in expression at the chimeric *Rasgrf1*$^{tm3.1Pds}$ locus to that of *Igf2r*. In this case, *Air* RNA transcribed from the intact *Igf2r* locus would, through homology with the Region 2 in the chimeric transgene, cause chromatin remodeling at that locus. This hypothesis can be tested directly by analysis of chromatin structure of the chimeric *Rasgrf1*$^{tm3.1Pds}$ allele. In maize, the role of noncoding RNA in mediating the *trans*-acting interactions between paramutagenic and paramutable alleles could be probed by crossing paramutagenic lines to lines containing knockout mutations in genes important for RNA-mediated chromatin remodeling.

r PARAMUTATION AND AUTOPOLYPLOID-INDUCED SILENCING

One similarity between *r* paramutation and autopolyploid-induced silencing is the genetic behavior of the allelic interactions. In the F1 generation, when the paramutagenic alleles (*R-stippled* and SSSS) are first together with the paramutable alleles (*R-r*:*standard* and RRRR), there is little if any change in expression of the paramutable allele. In the F2, however, the paramutant state is evident such that few second generation progeny have the normal phenotype. It appears that meiosis is required for establishment of the epigenetic state in these cases, but what role meiosis plays is not clear. In Drosophila and the fungus Ascobolus, the epigenetic state of one chromosome can be transferred to its homolog during chromosome pairing (Colot et al., 1996; Henikoff and Comai, 1998). However, pairing can be ruled out in the case of *r* paramutation. In trisomic stocks carrying one dose of a paramutagenic *R′* allele together with either one dose or two doses of the paramutable *R-r*:*standard* allele (e.g., *R′*/*R-r*:*standard*/*r* or *R′*/*R-r*:*standard*/*R-r*:*standard*), the number of doses of the paramutable allele did not affect the paramutant phenotype (Styles, 1970). In trisomics, only two of the three homologous regions of the chromosome are paired at any one time. If pairing is required for paramutation, then some of the progeny of *R′*/*R-r*:*standard*/*R-r*:*standard* plants should have had the active *R-r*:*standard* phenotype. This was not the case.

Several lines of evidence are consistent with the idea that *r* paramutation and polyploid-induced silencing occur by a mechanism similar to homology-dependent gene silencing (HDGS). HDGS is an epigenetic mechanism for controlling gene expression by targeted inactivation of tandem repeats, *trans*-inactivation of allelic or ectopic gene copies, or the coordinated silencing of a transgene and its endogenous homolog (Meyer and Saedler, 1996).

Loci with a repetitive structure—for example, multiple copies of promoter elements, inverted repeats of transcribed regions, or tandem repetitive elements—are potent triggers for HDGS (Vance and Vaucheret, 2001; Martienssen, 2003). HDGS can be classified as transcriptional or posttranscriptional. Transcriptional silencing refers to the heritable transcriptional repression of homologous loci. Posttranscriptional silencing, also known as RNA interference, occurs through the targeted degradation of aberrant or double-stranded RNA and subsequent degradation of homologous transcripts. In both types of HDGS, the silenced state is associated with increased DNA methylation of homologous sequences and the acquisition of a more tightly compacted chromatin structure. In plants, these modifications can be established by an RNA-directed DNA methylation. Recent evidence indicates that both transcriptional and posttranscriptional silencing may involve an RNA component. For example, in a transgenic tobacco system, when a promoter sequence is ectopically transcribed, genes driven by that promoter are transcriptionally silenced. This silencing is established by degradation of promoter transcripts and an RNA-directed methylation of homologous DNA sequences (Mette et al., 2000).

Key features of the *r* locus of maize and the hygromycin resistance transgene in Arabidopsis polyploids are consistent with regulation through HDGS. In terms of gene organization, both loci contain repeated elements. At *r*, both paramutagenic and paramutable alleles have multiple *r* gene copies (Kermicle et al., 1995; Walker and Panavas, 2001), and the Arabidopsis transgene contains a second copy of the 35S promoter downstream of the polyadenylation site (Mittelsten Scheid et al., 2003). In both *r* and the polyploid silencing, DNA methylation is an important determinant for maintaining (and perhaps establishing) a silenced state. The only consistent difference between poorly expressed paramutagenic and actively expressed paramutable *r* alleles is that paramutagenic alleles are more heavily methylated; in the F1, *r* alleles are transcriptionally active and hypomethylated, but when the *r* alleles are silenced in the F2, they become hypermethylated. In the case of autopolyploid-induced silencing, methylation state also corresponds to level of expression. In both *r* and the hygromycin transgene, methylation is restricted to the gene or transgene and does not spread to flanking regions. These results imply that DNA methylation is responsible for maintenance of the paramutant state in both *r* paramutation and polyploid induced silencing. The idea that methylation is correlated with changes in chromatin is supported by the observation that polyploid-induced silencing can be relieved when the *ddm1* SWI/SNF chromatin remodeling protein is absent.

How is the silent state transferred in *trans* to the paramutable alleles? In the F1, the paramutagenic allele is transcriptionally silent and the paramutable allele is expressed. Silencing requires meiosis, but in the case of *r* paramutation, does not require pairing. It is possible that during meiosis, changes in chromatin structure and accompanying activation of meiosis-specific transcription could allow for transient expression of the silenced allele. This might produce an aberrant RNA, with homology to coding regions or promoters, which could then trigger the RNA silencing pathway and lead to RNA-directed DNA methylation of homologous sequences on the paramutable allele.

There are several ways to test this hypothesis. In Arabidopsis, mutants that are defective in RNA-dependent DNA methylation could be introduced into the derived silenced diploids. If RNA is an intermediary in establishing paramutation, then the silenced alleles in mutant backgrounds would lose their paramutagenic ability. In maize, similar mutants are being developed by reverse genetics, and these could be tested for their ability to prevent *r* paramutation. A second experiment would be to look for evidence of transcription in meiotic cells. Meiotic cells could be isolated by laser capture microdissection and then RNA amplified and analyzed (Emmert-Buck et al., 1996; Higashiyama et al., 2001). The prediction is that transcripts or degradation products homologous to the silenced genes would be detected.

AN EPIGENETIC NETWORK

The analysis of paramutation presented here underscores the emerging importance of chromatin states not only as heritable but also transferable. Allele-specific communication may be mediated by noncoding RNAs that catalyze *trans*-acting alterations in chromatin structure that in turn control expression states. Mechanisms for establishing paramutation are likely to vary among different systems, but maintaining stable expression states appears to be modulated by a common network of chromatin-level regulators. Future steps into the maze of epigenetic regulation will allow us to discover how widespread allele-specific interactions like paramutation are and how universal the mechanisms controlling them prove to be.

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