Transposon Tagging Agronomically Important Loci in Maize

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INTRODUCTION

The ability to genetically engineer plants to make them better suited to meet existing and future human needs holds great promise. However, few agronomically significant genes have been molecularly isolated, i.e., cloned. The Schnable laboratory is currently involved in two gene isolation projects. The first, in collaboration with Basil Nikolau, involves the isolation of genes involved in the biosynthesis of novel lipids (the glossy project). The second, in collaboration with Roger Wise, has as its goal the isolation of nuclear restorer factors for cytoplasmic male sterility [(the Rf2 project)].

Rationale for the Glossy Project. Most seed oils are composed of triglyceride molecules with a mixture of fatty acids. In general, the relative proportions of the fatty acid components of the major oil sources are quite similar. In contrast, maize cuticular waxes have unique lipid components which include wax esters, alcohols, aldehydes, alkanes and very long chain fatty acids. Some of these cuticular wax components are industrially significant. In maize, the glossy (gl) genes control the accumulation and composition of cuticular wax on the surface of the first five leaves of the plant. At least 17 such mutant gl loci have been identified and more continue to be described (Sprague, personal communication). The glossy phenotype is readily distinguishable by the shiny appearance of the mutants and by the formation of water droplets on the surface of the mutant leaves when water is sprayed on them (Coe and Neuffer, 1977; Bianchi, 1978; Bianchi et al., 1985). The ultimate goal of this project is the cloning of glossy loci to facilitate the

genetic engineering of an oilseed crop so that it can better meet specialty crop needs.

Rationale for the Rf2 project. Cytoplasmic male sterility (Cms) is characterized by the failure to produce functional pollen. This failure is due to an incompatibility between nuclear and mitochondrial genotypes. Cms is usually caused by aberrant mitochondrial genes and is therefore transmitted only through the female parent. This trait is of significant value in the production of hybrids, eliminating the need for hand emasculation. In maize (Zea mays L.), there are three major groups of male-sterile cytoplasms; S (USDA), C (Charrua), and T (Texas) in addition to the N (normal) male-fertile cytoplasm. These cytoplasms are distinguished by the genetics of nuclear fertility restoration (Duvick, 1965; Beckett, 1971; Gracen and Grogen, 1974; Laughnan and Gabay-Laughnan, 1983). Restoration of pollen fertility in T cytoplasm requires the action of the two unlinked dominant nuclear restorer genes, Rf1 and Rf2 (Laughnan and Gabay-Laughnan, 1983).

Texas-type cytoplasmic male sterility (cmsT) was widely used in the United States until the 1970 epidemic of southern corn leaf blight (Ullstrup, 1972; Pring and Lonsdale, 1989). At that time approximately 85% of the U.S. maize crop was produced using cmsT. The use of cmsT was essentially discontinued following the discovery that it is highly sensitive to the host selective toxins produced by race T of Cochliobolus heterostrophus Drechsler [asexual stage Helminthosporium (Bipolaris) maydis Nisikado and Miyake], the causal organism of southern corn leaf blight and by Phyllosticta maydis, Arny and Nelson, which causes yellow leaf blight (Hooker et al., 1970; Comstock et al., 1973; Yoder, 1973).

In hybrid production a T-cytoplasm inbred female in a non-restoring (Rf1/Rf1, rf2/rf2 or rf1/rf1, Rf2/Rf2) background was typically fertilized by an inbred homozygous for the dominant allele at both restorer loci, thereby restoring fertility to the F1 progeny. The mode of restoration of T cytoplasm is

sporophytic, i.e., the genetic constitution of the diploid, sporophytic anther tissue, rather than the haploid, gametophytic pollen grain, determines pollen development. Therefore, a cmsT plant that is heterozygous for both restorer gene loci (Rf1/rf1, Rf2/rf2), will produce all normal pollen even though only one-fourth of the pollen grains carry both Rf1 and Rf2 (Laughnan and Gabay-Laughnan, 1983). Although much is known about the genetic and molecular role of Rf1 in fertility restoration, little is known about Rf2 except that in addition to Rf1, it is essential for pollen restoration.

The cloning of Rf2 is the first step in our ultimate goal of identifying the molecular mechanisms associated with fertility restoration. Ultimately, this may lead to the design of commercial alternatives of existing cytoplasmic male sterility systems.

Gene tagging via Transposons in Maize.

Transposon tagging is now an established procedure to facilitate the cloning of maize genes. It is one of the most efficient methods for isolating genes for which the gene product is unknown. Tagging means obtaining an insertion of a transposon in a gene of interest. Transposons are DNA sequences that have the capacity to move from one position in the genome to another. Because the insertion of a transposon into a gene causes a mutation, it is possible to isolate tagged alleles by screening large populations for newly generated mutant alleles. A critical component is a mutant phenotype that can unambiguously and inexpensively be distinguished from the wild-type.

Large populations of the correct genotype for scoring for new mutants can readily be generated by the isolation plot technique (Peterson, 1978). This involves growing the male and female parents of the desired cross in physical isolation from other maize plants. One of the parents carries an active transposon system and is homozygous dominant for the target locus. The other parent is homozygous for the standard recessive allele of this locus.

Prior to anthesis the female parent is detasseled. Consequently, all kernels produced on the female parent will arise as a result of pollination by the male parent. In the absence of mutation all kernels will be heterozygous and therefore they (or the plants derived from them) will express the phenotype of the dominant allele. However, if a mutation occurs at the target locu in the transposon stock it will be uncovered by the standard recessive allele contributed by the other parent. In contrast, transposon-induced mutations at the dominant allele of the target locus will be uncovered by the standard recessive allele used in the isolation plot cross. Progeny from this cross that carry newly generated transposon tagged alleles can be distinguished from non-mutant progeny because the former will express the recessive phenotype.

Approximately a dozen transposon systems (or families) have been identified in maize (reviewed by Peterson, 1988). Only three have been widely used for tagging and cloning, i.e., Mutator, Spm (or En) and Ac. Only the first two will be discussed here. The Mutator system has the highest mutation rate; Mutator lines exhibit a mutation rate 50-fold higher than the spontaneous rate and the rate observed in lines carrying other transposable element systems (Robertson and Mascia, 1981; Robertson, 1983). This elevated mutation rate is termed Mutator activity. Most new Mutator-induced mutants arise via the insertion of Mu1 transposable elements which are present in 10-50 copies per genome (Barker et al., 1984; Bennetzen et al., 1984; Brown et al., 1989; Bennetzen, 1984), although other Mu elements also cause insertion mutations at reasonable rates, e.g., Mu3 and Mu8 (Oishi and Freeling, 1988; Buckner et al., 1990; Fleenor, 1990). The high transposition rate of Mu1 makes the Mutator transposable element system an efficient tool for gene tagging and cloning (McLaughlin and Walbot 1987; McCarty et al., 1989; Martienssen et al., 1989; Buckner et al., 1990).

The Spm transposon system has also been used for gene tagging and cloning (Paz-Ares et al., 1986; Cone et al., 1986; Wienand et al., 1986; Schmidt et al., 1987). Although the mutation rate observed in Spm lines is

considerably less than that in Mutator lines (Cone et al., 1988; Robertson and Mascia, 1981; Robertson, 1983) several factors hold the potential to increase the Spm-induced mutation rate at target loci. For example, because Spm has a marked tendency to engage in intra-chromosomal transpositions (McClintock, 1962; Peterson, 1970; Nowick and Peterson, 1981) genetic constructs that place an Spm element in the vicinity of the target loci may prove advantageous.

Once a gene is tagged it can be cloned by using the inserted transposable element as a molecular probe. This approach was first utilized in Drosophila in order to clone the white locus (Bingham et al., 1981). The first report of its use in maize was by Fedoroff et al. (1984); it is now a proven technique and is widely used to isolate genes at which mutations are readily scorable. Cloning transposon tagged genes is complicated by the large number of Mu or Spm-related sequences present in maize genomes carrying active transposable element systems (Bennetzen, 1984; Cone et al., 1988); the identification of the specific Mu or Spm-related insertion that caused a tagged mutation can prove difficult. Efficient methods to circumvent this problem exist and will be described below.

RESULTS AND DISCUSSION

1. Isolation of transposon-induced mutant alleles.

There are two general approaches (directed and random) to generating tagged alleles. Both have been utilized in these gene isolation projects and will be described below.

1A. Directed Tagging

Directed tagging requires the following:

-an existing mutant allele of the targeted locus.

-plants homozygous for the mutant allele must be viable.

-for greatest efficiency, plants homozygous for the mutant allele must be capable of serving as a pollen parent in an isolation block.

-RFLP or visible markers must be available in the vicinity of the targeted gene (this means the gene must be mapped).

1A1. Directed tagging of gl loci using Mutator.

Gl1 and Gl8 were targeted in the following isolation plot crosses (the female parent is listed first in all crosses):

Cross 1: Mutator Gl1/Gl1 X gl1/gl1

Cross 2: Mutator Gl8/Gl8 X gl8/gl8

In the absence of mutation, progeny kernels from crosses 1 and 2 should be heterozygous for the wild-type allele (Gl1/gl1 and Gl8/gl8, respectively) and should therefore yield wild-type seedlings. Only if a gamete from the female (Mutator) parent carries a newly generated mutant allele (e.g., via the insertion of a Mu1 element, gl1-Mu1 or gl8-Mu1) will a glossy seedling be obtained (gl1-Mu1/gl1 or gl8-Mu1/gl8).

Approximately 1,000,000 and 324,000 kernels were obtained from the gl1 and gl8 plots respectively. About 168,000 and 96,000 progeny seedlings from each of the plots (gl1 and gl8) have been screened in greenhouse sand benches; to date, 9 gl1 and 31 gl8 mutants have been recovered. The respective mutation rates are therefore 5/100,000 (gl1) and 33/100,000 (gl8). The difference in mutation rate between the two plots (nearly an order of magnitude) is surprising because identical Mutator sources were used in the two plots. One explanation might be that the Mutator source used has a Mu element linked to gl8. In such a genetic background insertions at gl8 would occur at higher than normal rates if Mu elements have a propensity to transpose to linked sites (as is true for several other transposons).

1A2 Directed tagging of rf2 using Spm and Mutator.

Transposon tagging requires that large numbers of progeny be screened for the mutant phenotype (typically 100,000-1,000,000). For this reason, the procedure has been used primarily to tag genes affecting kernels

(and more recently seedlings) because these can be generated and screened in large numbers with relative ease. However, it is possible to tag mature plant traits such as Rf2 given sufficient determination. In brief, the procedure for tagging Rf2 was as follows. Plants carrying an active transposable element system and homozygous for the wild-type allele of Rf2 were crossed with plants homozygous for the stable recessive null allele, rf2 to expose new mutations at Rf2. The progeny from this cross were then screened for the phenotype conditioned by rf2/rf2 (i.e., male sterility in T cytoplasm). Progeny exhibiting this phenotype carry a newly mutated rf2 allele (rf2-m) contributed by the transposon donor parent and the stable recessive null allele from the female parent.

Tagging mature plant traits following these procedures requires two generations. During the first generation, cross 3 (see below) was used to generate a large population of kernels a few of which might contain transposon insertions at rf2. The female parent of cross 3 is the inbred line R213. R213 is homozygous for the standard rf2 allele (here designated rf2-std). The use of an inbred line as one of the parents in cross 3 facilitated subsequent RFLP analyses. The male parent in cross 3 carried either Spm elements or an active Mutator transposon system. The Bz locus conditions a readily scorable endosperm phenotype and serves as a linked contamination marker. In the genetic map of chromosome 9 (shown below) Rf2 is thought to lie between position 56 and 62 (Coe et al., 1991). Wx, BNL5.10 and UMC153 are RFLP markers and will be discussed later.

Bz	Wx	BNL5.10	UMC153
31	56	64	72

Genetic map of chromosome 9 (Coe et al., 1991)

Cross 3: T cytoplasm Rf1/Rf1 Bz rf2-std/Bz rf2-std X N cytoplasm rf1/rf1 Bz Rf2/Bz Rf2

In the **absence** of mutation, the progeny kernels from cross 3 will produce exclusively male-fertile plants because although they have T cytoplasm they carry one copy of each of the two dominant nuclear restorer factors, Rf1 and Rf2, (Laughnan and Gabay-Laughnan, 1983). During this first generation approximately 100,000 kernels were isolated from each transposon source for use in the tagging program.

In the second generation the two populations resulting from cross 3 were screened for rf-m mutations. Because the female parent in cross 3 carried the T cytoplasm, the progeny resulting from this cross had the correct cytoplasmic constitution for expression of either rf1/rf1 or rf2/rf2 conditioned male sterility. We screened the two populations of 100,000 kernels for insertion events at Rf2 by growing them to maturity. If the Rf2 locus was inactivated by an Spm or Mu insertion in a given progeny, that plant would be male sterile. Male-sterile plants could also arise as the result of a mutation of Rf1 in the female parent of cross 3. This event is expected to occur much less frequently than mutations at Rf2 because we did not enrich the population for mutations at this locus. In any event we can distinguish these two classes of mutations as described below.

To identify male-sterile individuals we examined each plant daily as the populations underwent anthesis. The failure of anthers to exert is the phenotype of an rf2/rf2 (or rf1/rf1) plant with T cytoplasm (Duvick, 1965). Therefore, plants that exerted their anthers did not carry an rf2-m allele and were detasseled. In contrast, plants that failed to exert their anthers putatively carried an rf2-m allele and were selected as putative mutants. All putative mutant plants from cross 3 were crossed as shown below (cross 4A or 4B).

It was not feasible to shoot bag all 200,000 plants derived from cross 3. Therefore, putative mutant plants were shootbagged as soon as they were

identified. However, because mutant plants to be used in cross 4 (A or B) could not be identified prior to anthesis some silks on putative mutant plants may have been sib contaminated before they were shoot bagged. As will be shown below (cross 5), the use of the bz marker in the pollen parent served as a contamination control. (bz was an appropriate contamination marker because no plants in the observation plot carried the bz allele).

Crosses 4A and 4B are identical except as regards the genotypes of the male-sterile female plants in these crosses. If Rf2 was tagged, the female parent will have the genotype indicated in cross 4A, while if Rf1 was mutated the genotype will be as in 4B.

Cross 4A: T cytoplasm Rf1/rf1 Bz rf2-m /Bz rf2-std X N cytoplasm rf1/rf1 bz Rf2/bz Rf2

Cross 4B: T cytoplasm rf1-m/rf1 Bz Rf2/Bz rf2-std X N cytoplasm rf1/rf1 bz Rf2/bz Rf2

The populations were screened in consecutive years because it was not possible to score all 200,000 plants for sterility during the already busy pollination season. Seven putative rf2-m alleles were isolated in 1990 from the 100,000 plants derived from the Spm parent. The 100,000 plants derived from the Mutator parent were screened for male sterility in 1991; forty putative mutants were obtained. For a cloning project it is helpful to have a large collection of tagged alleles derived from both Spm and Mutator populations (O'Rielly et al., 1985; Menssen et al., 1990).

1B. Random Tagging

Random tagging begins with a cross of a transposon stock to a standard wildtype line. Gametes produced by the transposon stock will carry newly generated transoson insertion mutations at random loci. Therefore, many of the progeny from this cross will be heterozygous for new mutations. Selfing will uncover these mutants; the resulting S1 families will segregate for

various tagged mutants. There are several advantages of the random approach over the directed.

-It is possible to isolate mutations at genes for which no existing mutant allele exists.

-Mutations can be isolated and recovered (as heterozygotes) for lethal loci.

The most serious drawback of the random approach is its low efficiency. Each S1 family represents the test of a single gamete in contrast to the directed approach where each progeny seed/plant represents the test of a gamete. Admittedly, each self uncovers all visible mutations that occurred in that gamete. In contrast, the directed scheme only uncovers mutations at the targeted locus.

Over the past several years we have screened ca. 5000 Mutator selfs for visible mutants. For example, during the spring of 1991 we screened ca. 2500 Mutator selfs and recovered up to 11 independent gl mutants.

Once mutants are recovered via random tagging it is necessary to determine the affected locus. We have conducted TB tests and allelism tests on all 13 gl mutants of unknown locus that were available to us as of spring 1991. TB tests locate mutants to chromosome arm by exploiting the fact that BA translocations fail to disjoin during the second meiotic division during microsporogenesis (Birchler, 1982). Allelism tests can be performed by crossing plants homozygous for the Mu1-tagged allele by separate tester lines, each homozygous recessive for one of the existing standard gl loci. If the tagged allele is allelic to a given standard allele all seedlings from that cross (and only that cross) will be glossy. Seedlings from all other crosses will have a wild-type phenotype. These tests have established that this collection of thirteen mutants consists of 3 gl1, 1 gl2, 1 gl4 and 8 gl8 alleles.

1C. Tagging by Mail

This third method of transposon tagging should not be overlooked.

Investigators in other labs using the random tagging method often find mutants not of direct interest to their program. For example we have found mutants that affect plant height, chlorophyll and carotenoid biosynthesis/stability, flowering (such as gender determinism and male or female flower sterility), pigment accumulation (e.g., brown midrib and golden plants), embryo development and seed dormancy. Some of these have been made available to labs with an interest in them. In turn, colleagues (D. Robertson, ISU; Cook and Miles, University of Missouri) have generously provided us with a number of tagged gl mutants.

Another available option is for investigators to exchange S1 families from Mutator stocks. This permits each investigator to screen the families directly for the trait of interest.

2. Analysis of tagged mutant alleles.

The heritability of putative mutations must be confirmed. However, most putative gl and rf2 mutants have proven heritable. To establish the heritability of rf2 mutations the following analyses were performed.

As discussed above, male-sterile plants from cross 3 could occur as a result of mutations at either Rf1 or Rf2. If a mutation at Rf1 were responsible for the male-sterile phenotype in the observation plots (progeny of cross 3) then all the plants from cross 4B will be male sterile. Mutations at Rf1 were not expected for reasons discussed above and were not found. In contrast, if a mutation at Rf2 were responsible for the male-sterile phenotype observed in the progeny of cross 3 then only half of the progeny from cross 4A would be expected to be sterile (the fertile progeny are Rf1/rf1 rf2-m/Rf2 or rf2-std/Rf2).

The female parent in cross 4A carries two rf2 alleles: the standard null and the newly induced mutant. It is possible to distinguish these two alleles by the use of RFLPs. Based upon the crossing strategy, the transposon-induced allele is "marked" by being in coupling with the closely linked RFLP markers Wx, BNL5.10 and UMC153; Rf2 and Wx are within about 5 cM of

each other (Snyder and Duvick, 1969). The ability to distinguish between the two rf2 alleles enabled us to sequester the newly tagged rf2-m allele in preparation for its analysis as described below.

Fertile plants from cross 4A that possess RFLP markers indicating that they carried the rf2-m allele rather than the rf2-std allele were selfed (cross 5). If this allele is heritable the resulting S1 families are expected to segregate 9:7 (fertile:sterile). This ratio arises due to the independent segregation of the two restorer factors (rf1 and rf2) in these T-cytoplasm plants. Non-heritability would be indicated if a 3:1 segregation were noted (segregation of only rf1).

Cross 5: T cytoplasm Rf1/rf1 rf2-m/Rf2 selfed

Of the seven putative rf2 mutants from the Spm-derived population, five displayed segregation ratios indicative of heritability of the male-sterile phenotype. However, a 9:7 was recorded for only two of these five. Two exhibited 3:13 ratios. These ratios would be expected if the newly tagged alleles are semi-dominant (i.e., Rf2/rf2-m plants are only partially restored), in contrast to rf2-std which is fully recessive. Indeed, these two 3:13 families had many plants that were difficult to classify as to their restoration status (fertile versus sterile). In the fifth instance, all 267 individuals in the S1 family were sterile. Since this family is derived from a self of a fertile plant in our winter nursery one possible explanation for this surprising result is an rf2-m allele that is environmentally sensitive, i.e., it can restore T cytoplasm in Hawaii but not in Ames. An alternative hypothesis is that this mutant arose via a mutation in the T cytoplasm such that it is restorable under Hawaii conditions but not in Ames. The putative rf2 mutants from the Mutator-derived population are currently being tested for heritability.

Some transposon-induced alleles are created by deletions or complex rearrangements rather than directly by transposon insertions. A deletion would not be directly useful in cloning. We are therefore fortunate in having multiple alleles of all loci we are interested in cloning. Because deletions

represent a small fraction of the total number of transposon-induced mutations, the probability of more than a few of our alleles arising from events other than a transposon insertion are remote. Furthermore, for most of our alleles we have no direct evidence to suggest that they actually represent deletions. For example, large deletions are not well transmitted through the male gametophyte and are not viable as homozygotes. None of our rf2 mutants and only one of our gl mutants expresses this behavior.

In previous Spm tagging experiments in which the Spm donor is the female parent, it has been necessary to score 100,000-300,000 progeny to find 1-4 new mutants (Cone et al., 1988). Our mutation rate (4/100,000) is in agreement with this value. Mutator generally has a higher mutation rate (1-10 per 100,000) than Spm. We have not yet confirmed the heritability of the putative Mutator-induced rf2 mutants. However, if the confirmation rate is similar to that observed in the Spm-derived population (5/7), then the mutation rate from the Mutator-derived population was 28/100,000, nearly an order of magnitude higher than with Spm.

3. Identification of transposons that co-segregate with mutant alleles.

Once heritable mutants have been isolated from the tagging populations the next step is to initiate the cloning of the tagged allele using the transposon as a molecular probe. This step is complicated by the high copy number of transposons in lines derived from the tagging populations.

3A. Co-segregation analysis with Mutator-tagged alleles.

Our cloning strategy relies upon the fact that a Mu insertion is responsible for most of the Mutator-induced mutations available to us. Mu1, one of the nine classes of Mu elements, is responsible for the vast majority of the insertion mutations recovered from Mutator lines (Brown et al., 1989). Therefore, we concentrated our efforts on the Mu1-specific probe in our analyses, even though diagnostic probes for each class are available in the lab.

Because Mu1 elements are present in 10-50 copies in the genomes of plants carrying an active Mutator system, the first step in cloning a gene tagged with a Mu1 element is to identify the individual Mu1 element inserted at the mutant locus. We did this by co-segregation analysis. DNA isolated from individual siblings with and without each Mutator-induced gl allele was digested with a restriction endonuclease that does not cut within the Mu1 element. These DNA samples are subjected to Southern analysis and probed with a Mu1-specific sequence. If a Mu1 element has inserted at the gl locus, the Mu1 probe will invariably hybridize to a common band in plants carrying the Mutator-induced gl allele and this band will be absent in sibling plants that do not carry this allele.

To simplify the co-segregation analysis we first undertook a serial outcrossing program to reduce the number of Mu1 elements in our gl mutant lines. The gl-Mu lines were crossed to lines which either lack or have low copy numbers of Mu1 elements in their genomes. Genomic DNA from the resulting progeny plants was prepared and digested with HindIII, which does not cut within the Mu1 element. Southern analyses of these DNAs using internal sequences from Mu1 as a probe permitted us to select the plants having lowest Mu1 copy numbers. These low-copy number plants were then backcrossed to the recurrent parent which either lacks Mu1 elements or has a low copy number. After three to four generations this procedure provided us with lines which carry the gl alleles and only 15-20 copies of the Mu1 element.

The next step was to utilize these low copy number lines in co-segregation analyses to identify the individual Mu1 element inserted at the gl locus. First, we obtained from cross 6 sibling plants with and without the gl-Mu allele (glossy and wild-type seedlings, respectively). DNA from each individual was digested with a restriction enzyme that does not cut within the Mu1 element. Mu1-specific sequences were used to probe genomic Southerns blotted with these DNAs in an effort to identify a Mu1 band which co-segregates with the gl-Mu allele, i.e., present in all glossy seedlings, but

absent in wildtype siblings.

Cross 6: gl-Mu/Gl X gl-std/gl-std

Recently, Mu1 cosegregating bands have been identified for three of the gl loci (3 gl1, 1 gl2 and 3 gl8 alleles). This finding will permit us to clone these genes, as discussed below.

3B. Co-segregation analysis with Spm tagged alleles.

Like Mu1, Spm-related sequences are present in high copy numbers (50-100 copies) and like Mu1, co-segregation analysis is used to identify the specific Spm-related sequence responsible for a given rf2-m mutation. However, unlike Mu1, serial outcrossing is not effective in reducing Spm copy numbers. This is because all maize lines have high numbers of Spm-related sequences. An alternative approach for over-coming the difficulties related to high transposon copy number is described below.

A population segregating for rf2-m versus Rf2 has been obtained from cross 7 and is currently being grown in our winter nursery. At maturity these plants will be scored for male sterility. Male-sterile and fertile plants have the following genotypes at the Rf2 locus: rf2-m/rf2 and Rf2/rf2, respectively.

Cross 7: T cytoplasm Rf1/- rf2-m/Rf2 X N cytoplasm Rf1/Rf1 rf2/rf2 (the symbol "-" refers to either Rf1 or rf1)

Once the genotypes of the individuals within the population have been clarified, genomic DNA will be prepared from each plant. Internal sequences from Spm (Schmidt et al., 1987) will be used to probe genomic Southerns blotted with DNA extracted from individual siblings with and without the Spm-induced rf2 allele and digested with restriction enzymes. If an Spm element has inserted at the Rf2 locus, the Spm probe will invariably hybridize to a common band in plants carrying the rf2-m allele and this band will be absent in sibling plants which do not carry this allele.

As discussed above, visualization by Southern analysis of a co-

segregating Spm band is often difficult because Spm-hybridizing elements are present in very high copy numbers. To overcome this difficulty, we will exploit the fact the DNA sequences flanking active transposons such as Spm are generally hypo-methylated (Cone et al., 1988). Briefly, DNA from the segregating population will be digested with restriction enzymes such as ClaI, PstI, SalI, and SstI that are sensitive to cytosine methylation. Because the bulk of the maize genome is methylated most of the DNA is poorly restricted following these digests. Therefore all transposons inserted in methylated regions remain at the top of the lane following electrophoresis. This results in a large decrease in the effective copy number and simplifies the identification of a co-segregating band.

4. Cloning co-segregating transposon bands.

As stated above, co-segregating Mu1 bands have been identified for six gl alleles representing three loci. Genomic libraries are being constructed for each of the gl mutants that display a co-segregating Mu1-hybridizing restriction fragment. These libraries are being constructed in lambda-based vectors (e.g., EMBL4 or NM1149; Frischauf et al., 1983; Murray, 1983) using DNA digested with the identified restriction enzyme and size-selected to include the co-segregating, Mu1-containing fragment. Once completed, these libraries will be screened with a Mu1 probe and positive clones will be identified via the procedure of Benton and Davis (1977). Following plaque purification, DNA from positive recombinant phage will be isolated (Yamamoto et al., 1970).

The next step will be to isolate putative gl gene DNA sequences from the recombinant phage. However, much of the maize genome consists of repetitive sequences. To isolate near-unique DNA sequences that flank the transposon positive clones will be subjected to "reverse Southerns" (Gupta et al., 1984) . This is done by size fractionating restriction digested DNA from each clone on an agarose gel, and transferring the DNA, in duplicate, to

membranes (Southern, 1975). One of the membranes will be probed consecutively with Mu1 and lambda vector sequences. This will identify the cloned fragment or fragments which contain the Mu1 element and those which correspond to the phage vector used to construct the library. The second membrane will be probed with labelled total maize genomic DNA. This is the "reverse Southern" and will identify repetitive DNA fragments; unique sequences do not give a signal in this procedure. Fragments which do not give a signal on any of the three hybridizations represent unique or near-unique sequences flanking the Mu1 element in the maize genome and putatively consist of the gl locus. These fragments will be subcloned for use in verifying their identity.

5. Verification of Putative Clones.

Although co-segregation analysis has been successfully used to clone many transposon-tagged genes from maize, the procedure suffers from the disadvantage that it is incapable of distinguishing transposons inserted at the tagged locus from those only closely linked to the tagged locus. Co-segregation analysis is after all merely a linkage test. A similar problem has been encountered previously (Chandler and Turks, 1988). It would therefore be possible to inadvertently clone a sequence that does not represent Gl or Rf2 even though it flanks a Mu1 or Spm element and cosegregates with the transposon-induced allele.

There are a number of strategies available to verify the identity of the putative cloned sequences. One of easiest is to use flanking sequences from a putative clone as a probe against a series of allelic transposon-induced mutants and their wild-type progenitor alleles. Flanking sequences from a correct clone would be expected to reveal polymorphisms between progenitor wild-type alleles and their corresponding mutant derivatives (as a result of the insertion of a transposon). This procedure underscores the importance of having available a collection of transposon-induced alleles at a given locus.

There are other methods of confirming putative clones, including molecular analysis of revertants and intra-genic recombinants. These will not be considered here.

SUMMARY

The maize transposon tagging system is an ideal means to clone qualitative genes of agronomic importance. This system is now a proven technique and is widely used in several species to isolate genes at which mutations are readily scorable. Two general tagging approaches are described and compared.

As a consequence of transposition, maize transposable elements can insert within genes leading to altered (usually null) phenotypes. The Mutator transposon system induces mutations at a very high rate (1/10,000-1/100,000). Most of these mutations arise via the insertion of Mu transposable elements (primarily Mu1). The high transposition rate of Mu1 makes the Mutator transposable element system an efficient tool for "gene tagging". Tagging means obtaining an insertion of a transposable element in a gene of interest. Because the insertion of a transposable element into a gene causes a mutation, it is possible to isolate tagged alleles by screening large populations for newly generated mutant phenotypes. We have used this approach to tag many genes.

Once tagged, mutant alleles can be cloned by using the inserted transposable element (Mu1) as a molecular probe. Because Mu1 elements are present in 10-50 copies in the genomes of plants carrying an active Mutator system, the first step in cloning a gene tagged with a Mu1 element is to identify the individual Mu1 element inserted at the mutant locus. This is done by co-segregation analysis. DNA isolated from individual siblings with and without each Mutator-induced allele is digested with a restriction endonuclease that does not cut within the Mu1 element. These DNA samples are subjected to Southern hybridization analysis and probed with a

Mu1-specific sequence. If a Mu1 element caused the mutation, the Mu1 probe will invariably hybridize to a common band in plants carrying the Mutator-induced mutant allele and this band will be absent in sibling plants which do not carry this allele. We been successful in identifying Mu1 bands which co-segregate with several of our tagged genes.

Now that co-segregating Mu1-hybridizing restriction fragments have been identified, genomic libraries are being constructed in lambda-based vectors using DNA digested with the identified restriction enzyme and size-selected to include the co-segregating, Mu1-containing fragment. Each library will then be screened with a Mu1 probe to isolate a recombinant phage which contains the tagged allele.

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