Mutagenesis – the Key to Genetic Analysis

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Abstract Mutagenesis is a major key to understanding gene function. Most chapters in this book take advantage of mutant alleles to advance the knowledge of maize traits. The chemical mutagen, EMS, has been particularly important because it has a very high efficiency and can be used in any genetic background. EMS also generates half-plant chimeras, which have interesting consequences for lethal dominant mutations. Although dominant mutants are often considered gain-of-function abnormalities, from analysis of thousands of mutants, it appears that most dominants mimic a set of recessive mutants. Examples in which the genes have been cloned demonstrate that a gene defined by a dominant mutation often functions in the same pathway as the gene defined by a recessive mutation with similar phenotype. We present an historical perspective of EMS mutagenesis and discuss frequencies of different types of mutations. Two types of dominant mutants that appear frequently and have recessive counterparts are described in more detail.

1 An Historical Perspective of EMS mutagenesis

Plant breeders and geneticists have long sought ways to increase mutation frequencies so as to acquire unique and useful mutant types. The pioneering work of L. J. Stadler at Missouri University established that ionizing radiation from X-rays and

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atomic energy, applied to maize, was not a productive source of heritable changes but, instead, caused mostly re-arrangements or destructive deletions of genetic material. Some wave-lengths of UV light, on the other hand, did produce small changes in the gene which led to an early proof (13 years before Watson and Crick) that DNA was the basic genetic substance (Stadler and Uber, 1942). At the same time, McClintock showed that considerable variation could be produced by the transposable elements that she discovered and characterized (McClintock, 1950). Once DNA was established as the molecular basis for inheritance, the use of chemical agents that could change the nature of DNA was an appropriate strategy. The problem then became one of developing techniques for applying powerful and dangerous chemicals to the DNA of living germ cells without damaging them or the surrounding cells. Early efforts with radiation and harsh chemicals failed because they usually killed surrounding tissue before penetrating to the nuclei of the germ line. The paraffin oil technique for treating corn pollen (Neuffer and Coe, 1978) was ideal because it brought the chosen chemical (ethylmethane sulphonate, EMS) into close proximity with the chromosomes and, unlike seed treatment, reached the germ line at the one cell stage so that the consequences could be unambiguously identified in the progeny.

A large-scale experiment was initiated to determine the efficiency of different mutagens (Neuffer, 1966). Neuffer set out to test the stability of the colorless *a1-m* allele in the absence of *Dt,* knowing that, in the presence of *Dt*, hundreds of dots could be seen on each kernel (Nuffer, 1961). From looking at thousands of kernels with literally millions of aleurone cells, each carrying three *a1-m* alleles, it was estimated that the frequency of reversion from *a1-m* to *A1* was less than 10^{-7}. Mutagenesis was carried out using X-rays, UV, and EMS on *a1-m dt* (lacking *Dt*) material. The subsequent 10,000 kernels produced in each treatment were screened for color phenotypes. No individual dots or colored kernels were found in any of the treatments. However, one or more sectors of dots were observed in each treatment (Figure 1). These can be interpreted as newly induced trans-acting *Dt* loci, not as excision of the suppressing *rDt* element from the *A1* locus. The newly induced *Dt* loci could have been predicted as McClintock reported that new transposon activity often appears as a consequence of chromosome breakage. Thus, it was possible to show that while the three mutagenic agents were able to produce new transposon activator elements, none were able to dislodge or deactivate the *rDt* element at the *A1* locus. Now that we know the molecular basis for transposable elements, the stability of *a1-m dt* is not surprising.

The M1 kernels from each treatment were planted to look for mutant seedlings as a comparative control measure of their mutagenic potential. M1 progeny from both X-rays and UV had a few small, weak, and abnormal aneuploid types. The UV treatment also produced two seedling mutants (one pale green and one dwarf). In contrast, the M1 from EMS treatment produced a large number of clear, mutant seedling phenotypes (such as white, yellow, yellow green, necrotic, dwarf, rolled leaf, virescent, adherent). Given that these mutants were dominant, it suggested that EMS-mutagenized progenies were a rich source of new mutants. More than half of the selfed ears also segregated for recessive heritable phenotypes. These spectacular

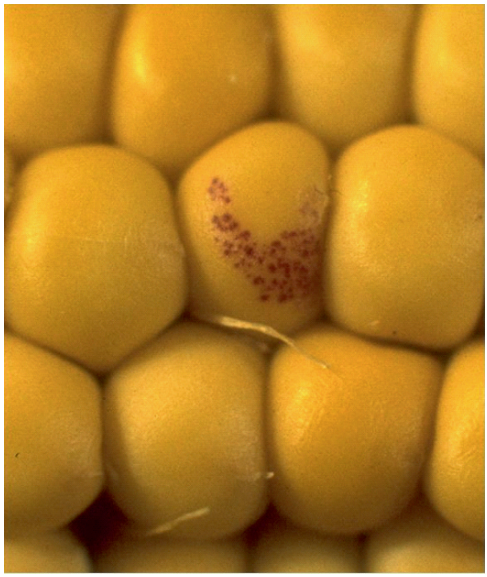

Fig. 1 Section of an *a1-m dt* ear crossed by *a1-m dt* pollen treated with EMS showing normal colorless kernels and one exceptional kernel with a sector of purple dots

results led to a focus on EMS as a mutagen to produce the variation needed for crop improvement and for a better understanding of gene function.

2 Frequency and Types of Mutations with EMS

The protocol for EMS mutagenesis of maize pollen in paraffin oil is outlined in Mutants of Maize (Neuffer et al., 1997) and is elaborated in the chapter by Weil and Monde. One can optimize the variables for success of the mutagenesis prior to pollination by plating a subset of the pollen on media and checking for a reduction in germination frequency (Neuffer and Coe, 1978). Despite adherence to the protocol, considerable variability in results can occur. In some treatments, such as the one described below, the results have been truly startling while, in others, all the pollen died or very few mutants were found. Over the years, attempts have been made to understand the variables, one of which is the inbred background. For example, Mo17 has large pollen grains that survive the treatment very well but produce few mutants while B73 has small grains that are easily killed. A longer treatment for Mo17 and shorter for B73 improved the mutagenesis. Other variables, such as temperature and humidity on the day of treatment, have a big effect. Ideally, multiple treatments can be carried out to optimize the conditions in each genetic stock.

In addition to the very high mutation rates of EMS, it can be specifically applied to a single germ cell. Thus, when one sees multiple occurrences of a particular phenotype following pollen EMS treatment, it is clear that each one is a unique

event. This is not the case for treatment of seeds in any plant and especially in plants where the male and female gametes are in separate flowers. With seed treatment, the germline is multicellular, thus leading to the formation of offspring with a mixture of mutant and non-mutant cells. The multiple copy mutant progeny that arise are often misread as multiple events leading to the conclusion of much higher frequencies and confusing results. The same is true for mutagenesis experiments with transposable elements where transposon insertions may occur at many stages of development.

In order to determine the frequency of dominant and recessive mutations, a particularly fruitful mutagenesis experiment was followed in detail (Figure 2).

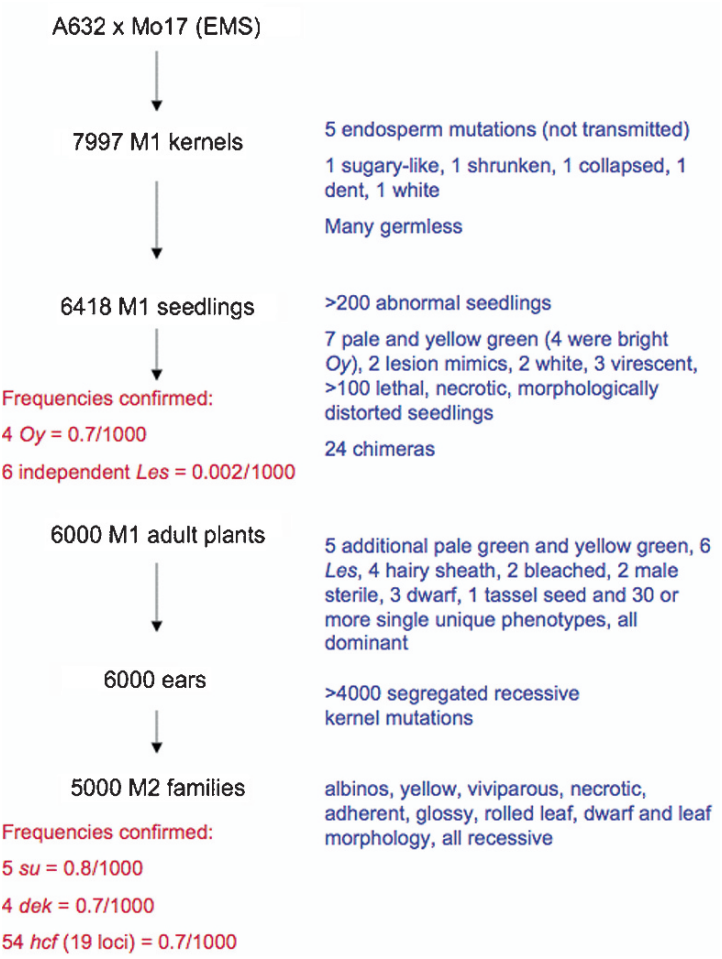

Fig. 2 Frequency and types of mutations in one particular treatment. The frequencies of mutations were confirmed by progeny testing

The progeny of a cross of A632 ears by EMS-mutagenized pollen of Mo17, including 7,997 M1 kernels, was screened for mutant phenotypes expressed in the triploid endosperm, which is derived from two untreated nuclei from the female parent and one EMS-treated nucleus from the male parent. As expected, kernels with mutant endosperm had normal embryos since it would be extremely rare for the same gene to be mutated in both sperm cells of a single pollen grain. In addition, a larger number of germless kernels with normal endosperm that failed to germinate were found. These potential mutants obviously had no progeny but provided an indication of the types of mutations that could affect the development of the embryo.

The M1 kernels were planted in the field and 6,418 M1 seedlings were screened for seedling mutant phenotypes. The germination frequency (80%) was significantly lower than that of the untreated control (96%). The kernels that failed to germinate were assumed to carry a significant but unknown number of lethal mutations, including the germless class described above. Treatment of pollen with EMS routinely causes this reduction in M1 seed viability. Over 200 dominant mutations were seen in the M1 seedlings, including 7 pale and yellow green (4 of which were bright *Oil yellow* (*Oy*) mutants), 2 lesion mimic, 2 white, 3 virescent, and more than 100 lethal, necrotic, morphologically distorted seedlings. The white, lethal and necrotic mutants died as seedlings, while the virescent mutants gradually became more normal and others persisted as mutant to maturity. Twenty-four seedlings with longitudinal stripes or chimeras of distinct mutant phenotypes, similar to those observed in whole seedling mutants, were also seen. Having normal adjoining tissue often sustained the mutant tissue, even in the case of lethal phenotypes, allowing these chimeras to persist till maturity.

Some phenotypes, such as tasselseed or male sterile, were seen only in adult plants (Figure 2). As with the seedling mutants, a corresponding number of half plant chimeras were also seen for these adult phenotypes. These chimeras occurred at approximately the same frequency as the whole plant mutants. More than thirty unique phenotypes were documented, as well as a considerable number of weak, slender or morphologically abnormal plants. At least that many more were seen but could not be confirmed either because no progeny were obtained or because they failed to transmit their mutant phenotype. Among those chimeras that were viable but failed to transmit the mutant phenotype, a large number of small slender plants that looked like aneuploids and haploids were found. The putative haploids were of two types; one of which looked like and had the glume bar allele (at the *b1* locus) of A632 and the other looked like and had the non-bar *b1* allele of Mo17. It was therefore inferred that they were maternal and paternal haploids, respectively. In total, 57 dominant mutants from the M1 seedling and plant screens were saved and assigned an identifying name and number.

Self-pollinated ears from 6,000 normal-appearing M1 plants were examined for kernel mutants segregating in a recessive 3:1 ratio. Two thirds segregated for at least one kernel mutant phenotype, essentially covering most of the known kernel phenotypes that could be expected to appear in a yellow dent background. Viviparous white kernel mutants were found with albino embryos that germinate precociously while still in the ear. Also included were various new types of defective

kernel mutants; those with phenotypes in the endosperm, the embryo, or both. In addition, a wide range of semi-sterile ears were missing one quarter of the kernels or segregated tiny vestiges of what may have started out to be a kernel. Some ears had normal kernels on one side but less than one-quarter mutants of a particular type on the other side. These ears almost certainly corresponded to a half plant chimera for a recessive mutation. Given the recessive nature of these mutations, the chimeras had to include the ear and tassel of the M1 parent.

Twenty M2 seed samples of 5000 M1 ears were planted in sand benches and observed from emergence to the four-leaf stage. Most of the known mutant phenotypes were seen repeatedly along with some new types. The most frequent types were those relating to the absence of chlorophyll; the white, yellowish-white, and yellow seedlings that remained so until they died. This chlorophyll-less class appeared in approximately 10% of the progenies. The next largest group included those which showed variation in the type, quantity, and timing of chlorophyll production; the yellow green, pale green and virescent seedling mutants. Other common types were necrotic, adherent, glossy, rolled leaf, dwarf and leaf morphology, all with frequencies above 1%. Fifty-four *high chlorophyll fluorescence* (*hcf*) mutants were found from this population, which included 19 loci (Miles and Daniel, 1974).

To determine the prevalent recessive mutation frequency, two kernel mutants (*su1* and *dek1*) that were easily and unambiguously recognized were selected, as well as one group of seedling mutants (*hcf*) for which we were able to obtain precise data quickly (Figure 2). For these recessive phenotypes, we arrived at a frequency of 0.7 mutants per locus per 1,000 pollen grains. Thus, a 20 seed sample from 3,000 of our 5,000 ear collection would have a 95% chance to carry almost any desired mutant.

The dominant mutants fell into two classes. One class has a few unique loci with mutation rates as high as those seen for recessives. *Oy* is one such mutation (0.7/1000). The frequency for the majority of dominant mutants and a few unique recessive loci, is much lower than 0.1 per 1000 pollen grains per locus. For example, dominant *Lesion mimic* (*Les*) mutants are found at a frequency of 0.002/1000. Certainly, there are phenotypic classes that are recalcitrant to EMS. The dominant *Knotted1* (Hake et al., 2004) and recessive *tasselseed4* (Chuck et al., 2007b) mutations are two examples for which no EMS-induced alleles are known, all the mutant alleles result from chromosomal rearrangements or transposon insertions. In addition, some phenotypes are dependent on the genetic background, as will be discussed for the *Les* mutants. This dependence provides a very important reason for carrying out mutagenesis in multiple genetic backgrounds and with both EMS and transposons.

The frequency obtained from EMS mutagenesis is high enough that one could expect to find mutations in duplicate genes. For example, a 15:1 segregation ratio was seen on the self-fertilized M1 ear that led to the discovery of the *orange pericarp* (*orp*) loci (Figure 3A). The orange color is in the pericarp, which is the genetically identical maternal tissue covering all the kernels and should not differ in phenotype from kernel to kernel. When planted, these kernels produced very weak seedlings that survived only with ultimate care and grew into small morphologically defective plants smelling of indole (Figure 3C). Indole was identified as the substance accumulating in the endosperm that diffused into the

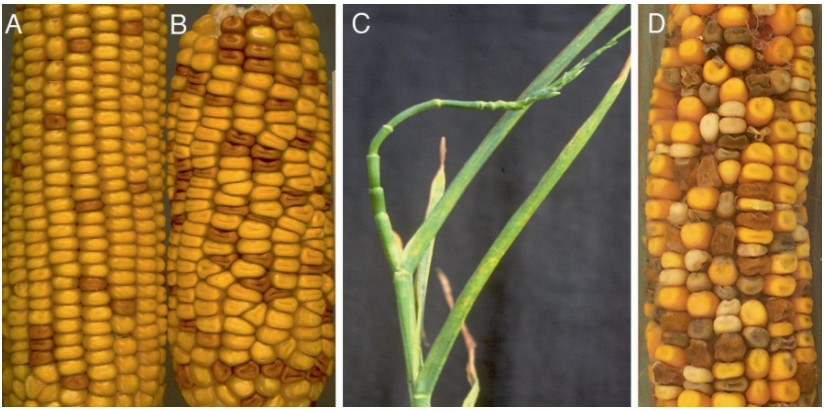

Fig. 3 Multiple mutations with EMS. A) A self-fertilized M1 ear segregated for two mutations, *orp1-1186A* and *orp2-1186B* at the ratio of 1:15. The pericarp is orange due to the secretion of indole from the underlying filial endosperm. B) A single factor ratio is obtained for an ear homozygous recessive for one factor and segregating for the other. C) Phenotype of the *orp1; orp2* homozygote showing orange color on green, narrow leaves and failure to develop properly. D) A selfed M1 ear segregating for four different recessive mutations (sugary, white, viviparous, and brown kernel)

maternal pericarp and turned it orange, explaining the unexpected phenotypic differences in the genetically identical pericarp (Wright and Neuffer, 1989). Analysis of *orp* led to an improved understanding of the tryptophan pathway (Wright et al., 1991; Wright et al., 1992).

Probably the most abundant class of recessive mutants was the *defective kernel* (*dek*) class, seen in selfed ears where 1/4 of the kernels failed to advance beyond the vestige of a kernel to produce a tiny empty shell of pericarp. If we consider only those mutants recognized as having a semblance of seed form, the *dek* class still constitutes a sizeable portion of the mutants produced (Neuffer and Sheridan, 1980; Sheridan and Clark, 1987; Clark and Sheridan, 1988, 1991). Chimeras have been useful to study the *dek* mutants as it allows one to see the recessive phenotype beyond the embryo lethal stage. A good example is the analysis of *colorless floury defective, dek1*. This mutant was found to be colorless because it lacked the aleurone layer (Cone et al., 1989), had a vigorous root but no shoot growth and was albino as determined in chimeral shoot tissue that was also morphologically altered (Neuffer, 1995; Becraft et al., 2002).

In total, the EMS Mutation Project has produced and observed more than 100,000 mutagenized M1 kernels. From 45,000 M1 plants, over 1,000 promising dominant mutants were found. Of these, 307 have proven heritable and were given name and number, 54 of which were located to chromosome arm. From 32,000 M2 ears, 52% had visible recessive variations segregating on the ear. Several ears had four visible kernel phenotypes (Figure 3D) and one of these proved to have three additional visible phenotypes in the seedling and the mature plant. A total of 5,737 putative recessive mutants of all types were observed, 706 of which have been assigned a chromosomal position.

3 The importance of Dominant Mutations

Dominant mutations exert a special attraction on the geneticist, as they are recognized in the F1, thereby simplifying pedigrees. Although rare, the high mutation rates of EMS provide the possibility of finding such alleles. Mutations that have been important for agriculture are often dominant, as is the case for the *Rht* dwarfing mutations of wheat (Peng et al., 1999) and some alleles that confer disease resistance. Dominant mutations have also had important consequences in evolution. Five major quantitative trait loci (QTL) account for most differences between maize and teosinte, two of which correspond to the *teosinte branched1* (*tb1*) and *teosinte glume architecture* (*tga*) loci (Doebley et al., 1997; Wang et al., 2005). The maize *tb1* and *tga* alleles are dominant over their respective teosinte alleles (Dorweiler et al., 1993; Doebley et al., 1995; Wang et al., 2005). For members of gene families, dominant mutations are often the only mutation that is visible.

3.1. Dominant Morphological Mutants with Recessive Counterparts

Gibberellin mutants. A classic example of dominant and recessive mutations in a biological pathway comes from study of dwarf mutants in the maize gibberellin pathway (Phinney, 1956). The dominant dwarf, *D8*, and five recessive dwarfs all have short stature, dark green leaves, and a failure of stamen arrest in the ear. Recessive mutants can be rescued with exogenous GA and define genes that encode enzymes in the gibberellin (GA) biosynthetic pathway. In contrast, dominant *D8* mutants have high levels of GA and are not responsive to exogenous GA (Phinney, 1956). D8 is a member of the GRAS family of transcription factors that is unstable in the presence of GA. The dominant mutants have deletions in the DELLA domain, rendering the protein stable and thus unable to transduce the GA signal (Peng et al., 1999). This pattern of recessive mutations in biosynthetic genes and dominant mutations in receptor or signaling proteins is also seen with other hormones in Arabidopsis such as ethylene (Wang et al., 2002).

Leaf mutants. The *Knotted1* (*Kn1*) and related *knox* (*knotted1 homeobox*) mutants, *Roughsheath1*, *Gnarley1*, *Liguleless3* and *Liguleless4* mutants provide good examples of phenotypes that are due to misexpression. The genes encode a family of homeodomain transcription factors (Kerstetter et al., 1994) that are strongly expressed in vegetative and inflorescence meristems (Jackson et al., 1994). The dominant mutant phenotypes are due to misexpression in the leaf (Vollbrecht et al., 1991 Schneeberger et al., 1995; Foster et al., 1999; Muehlbauer et al., 1999; Bauer et al., 2004) and show defects in proximal-distal patterning, as discussed by Foster and Timmermans. Recessive mutants were found by screening for loss of the dominant phenotype. Because of functional redundancy, loss-of-function mutants may have no phenotype as in *Lg3* (Bauer et al., 2004) or may be background dependent (Vollbrecht et al., 2000).

Kn1 mutants have not been found in EMS screens although they appear frequently in *Mutator* lines (our observations). In twelve characterized *Kn1* mutations, two alleles have *Mutator* elements inserted 5′ of the transcription start site (Ramirez, 2007), and nine *Mutator* elements and one *Ds2* element are in a small region of the third intron (Greene et al., 1994; Vollbrecht et al., 2000). In two other mutants, an uncharacterized insertion is also in this third intron and an *rDt* element is found in the 4th intron. The original allele, *Kn1-O* (Bryan and Sass, 1941; Gelinas et al., 1969; Freeling and Hake, 1985), is a tandem duplication (Veit et al., 1990) and a new allele also appears to be a duplication (Ramirez, 2007). The position of the insertions in the intron suggests that intronic sequences are likely to be important for regulation. Indeed, several conserved non-coding sequences were found in this intron (Inada et al., 2003). The position of the insertions in the 5′ region and the break point between the copies of the tandem repeat in *Kn1-O* highlight promoter sequences that may be needed to keep the gene from being expressed in the leaf. Studies of homologous *kn1* genes in Arabidopsis have identified conserved sequences that are important for keeping *knox* expression out of the leaf (Uchida et al., 2007).

A number of recessive mutants show displaced sheath/blade boundary and misexpress *knox* genes, suggesting that their function is to negatively regulate *knox* genes in the leaf. The first studied was *roughsheath2* (*rs2*) (Timmermans et al., 1999; Tsiantis et al., 1999), which encodes a MYB transcription factor. KNOX proteins are misexpressed in *rs2* mutant leaf primordia. *indeterminate gametophyte* (*ig*) mutants have a leaf phenotype in addition to the gametophyte phenotype. Ectopic leaf flaps occur that are more reminiscent of abaxial/adaxial polarity defects. *ig* encodes a LOB domain protein, a homolog of which is implicated in negatively regulating Arabidopsis *knox* genes (Evans, 2007). Other genes that misexpress *knox* genes are not yet cloned. *corkscrew* mutants have displaced blade/sheath boundaries, altered phyllotaxy and show misexpression of *kn1*, *rs1* and *lg3* (Alexander et al., 2005). *semaphore* mutants misexpress *gn1* and *rs1* in the leaf and endosperm and show pleiotropic defects (Scanlon et al., 2002). These mutants also have reduced polar auxin transport. Whether this last defect is due to misexpression of *knox* or other genes is unknown.

Rolled leaf1 (*Rld1*) is another dominant leaf mutant for which recessive mutants with a related phenotype have been identified. *rld1* encodes a homeodomain-leucine zipper transcription factor that is normally expressed adaxially (Juarez et al., 2004b). In the dominant *Rld1* mutant, the gene is expressed throughout the leaf and the leaf is adaxialized (Figure 4B). Four *Rld1* alleles have been identified through *Mutator* and EMS screens and they all result in the same base pair substitution in the microRNA complementarity site of *mir166* (Juarez et al., 2004b). The recessive *milkweed pod1* (*mwp1*) mutant has similar patches of adaxialization (Figure 4A) and *rld1* is misexpressed in *mwp1* leaves (Candela et al., 2008). *mwp1* encodes a member of the KANADI family of transcription factors, which are known to promote abaxialization in Arabidopsis. The double mutant shows a more severe phenotype than either single mutant, suggesting that *mwp1* has additional functions besides the regulation of *rld1*. Other dominant mutants affecting leaf development are *Rough sheah4* and *Morph*, both identified in EMS screens (Figure 4C, D).

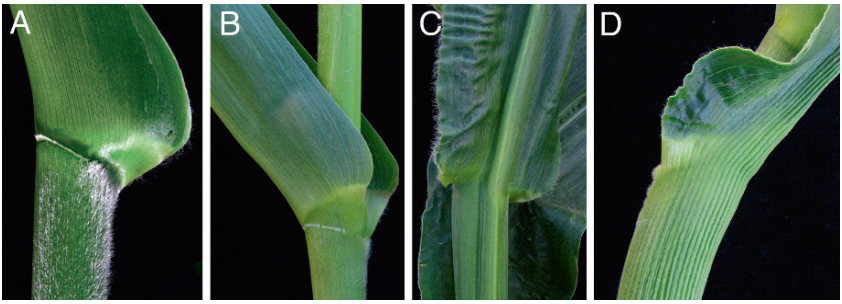

Fig. 4 The sheath and ligule region of leaf mutants. A) *milkweed pod*. B) *Rolled*. C) *Morph* D) *Roughsheath4*. (photos courtesy of Hector Candela)

A related recessive phenotype is seen in *leafbladeless1* (*lbl1*) mutants. While *Rld1* leaves are adaxialized (Nelson et al., 2002; Juarez et al., 2004a), *lbl1* leaves are abaxialized (Timmermans et al., 1998; Nogueira et al., 2007). Double mutants of *lbl* and *Rld1* show a suppressed phenotype. In *lbl1* mutants, *rld1* expression is decreased. *lbl1* encodes a protein involved in the small interfering RNA (siRNA) pathway and mutants have an increase in *mir166* RNA levels (Nogueira et al., 2007).

Inflorescence mutants. The *tasselseed* mutants provide another example of similar dominant and recessive mutants (Figure 5A, B). As mentioned by Vollbrecht and Schmidt, *tasselseed6* encodes an *AP2* gene that was previously identified by its recessive phenotype, *indeterminate spikelet* (*ids1*) (Chuck et al., 1998). *ts4* is one of the maize *miR172* genes and regulates the expression of *ts6/ids1* posttranscriptionally (Chuck et al., 2007b). Like *Rld1*, the lesion in the dominant *Ts6* allele is a base pair substitution in a microRNA complementarity site. The presence of a mutant phenotype in *ts4* is impressive given the fact that there are at least five *miR172* genes in the maize genome. Two other dominant *tasselseed* mutants have been described in the literature. It will be interesting to determine if they also encode targets of *ts4*.

thick tassel dwarf (*td1*) and *fasciated ear2* (*fea2*) are two recessive mutations that have an enlarged tassel rachis and fasciated ear tips. They respectively encode a leucine rich receptor kinase and a leucine rich receptor (Taguchi-Shiobara et al., 2001; Bommert et al., 2005), whose Arabidopsis orthologs *CLAVATA1* and *CLAVATA2* are well studied (Clark et al., 1993; Kayes and Clark, 1998). Dominant *Fascicled* (*Fas*) mutants have a similar phenotype (Haas and Orr, 1994; Orr et al., 1997). *Fas* ears differ from *td1* and *fea2* in branching from the base of the ear. The main rachis of the tassel also splits. It will be interesting to determine if the *Fas* gene product encodes a component of the CLAVATA pathway.

The dominant *Barren inflorescence1* (*Bif1*) and recessive *bif2* mutations result in similar phenotypes. *bif2* encodes a kinase with similarity to *PINOID* in Arabidopsis (McSteen et al., 2007). The inflorescence is barren, although there are a few spikelets in some inbred backgrounds. Leaf development is normal (McSteen

and Hake, 2001). Because PINOID is known to function in the regulation of auxin transport (Friml et al., 2004), we hypothesize that *Bif1* carries a dominant mutation that perturbs auxin transport regulation. Interestingly, the *bif* phenotypes are reminiscent of the *orange pericarp* double mutant phenotype (Figure 3). Indole is an intermediate in both the biosynthesis of tryptophan and auxin (indole-acetic acid), so both phenotypes are likely to be auxin-related.

Heterochronic mutants A group of mutants, referred to as heterochronic, shows delayed transition from the juvenile to the adult phase of vegetative development (Poethig, 1988a). The *Corngrass1* (*Cg1*) phenotype is most dramatic, the plant producing many tillers that continue to produce tillers (Figure 5C) (Singleton, 1951). In *Cg1* mutants, leaves are juvenile and roots are produced at all nodes. The defect extends into the inflorescence (Galinat, 1954a, b). In wild-type inflorescences, bract leaves are small and reduced. In contrast, *Cg1* bract leaves are large and vegetative in appearance (Figure 5D). Spikelet meristems and spikelet pair meristems are not apparent and floral meristems appear on the inflorescence in *Cg1* mutants. The tassel is also unbranched (Chuck et al., 2007a).

Cg1 was cloned and shown to encode *mir156*, a microRNA that targets transcripts of *Squamosa Promoter Binding Like* (*SPL*) genes. *Cg1* carries a transposon insertion in the 5′ region, which causes the misexpression of the microRNA (Chuck et al., 2007a). A second allele, identified by activation tagging, carries a T-DNA insertion that activates transcription of the microRNA gene. Twelve different *SPL* genes showed reduced expression in *Cg1* mutants. An analysis in Arabidopsis demonstrated that a parallel pathway of *mir156* regulation of *SPL* genes controls phase change in that species (Wu and Poethig, 2006). It is not likely that a single recessive mutation will mimic all of the *Cg1* phenotypes, however, there may be mutations that mimic one or two of the *Cg1* traits. One example is *tassel sheath*, which has elongated bract leaves, similar to those found in *Cg1* mutants. Another example is *unbranched* (see chapter 2). Given the nature of the lesion, it is not surprising that an EMS-induced *Cg1* mutation has never been identified.

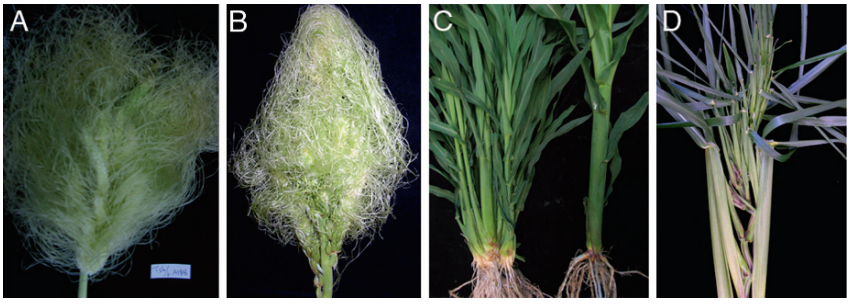

Fig 5 Mutations in microRNA regulated pathways. A) *tasselseed4*, B) *Tasselseed6*, C) *Corngrass* mutants (left) make multiple tillers that have juvenile phenotypes compared to wild type (right). D) In *Corngrass*, vegetative features continue into the inflorescence. (photos courtesy of George Chuck)

3.2 Disease Lesion Mimic Mutants

Disease lesion mimic mutants show symptoms mimicking disease or the resistance response in the absence of disease agents (Walbot et al., 1983). Both dominant and recessive lesion mimic mutants exist, which have been designated *Les* and *les* respectively. Although disease lesion mimic mutants are known to exist ubiquitously in plants (Dangl et al., 1996; Lorrain et al., 2003), they were initially recognized in maize as a unique class of mutants (Neuffer and Calvert, 1975). More than 50 loci have been identified in maize that cause *Les/les* phenotypes when defective (Johal, 2007) with a few represented by multiple alleles. Extrapolating from the general lack of confirmed allelic pairs at many of these loci, it has been suggested that more than 200 lesion mimic loci might exist in maize (Neuffer et al., 1983). Since more than half of these mutants are inherited in a partially- or completely-dominant fashion, *Les* loci constitute the largest class of gain-of-function mutations in maize (Johal, 2007).

Although every lesion mimic mutant is unique in some aspects, they fall into two general categories, determinative and propagative (Johal et al., 1995; Dangl et al., 1996). In determinative mutants, lesions are initiated frequently but their expansion is often curtailed. This gives the appearance of a massive hypersensitive response (HR), which is a programmed cell death reaction unleashed in resistant host cells in response to a diverse array of pathogens (Martin et al., 2003). In propagative mutants, lesions are initiated rarely, they tend to expand uncontrollably, covering large areas of the host tissue (Dangl et al., 1996; Lorrain et al., 2003). It is presumed that lesions in the determinative class arise from impairments that lower the threshold for cell death initiation (Walbot et al., 1983; Dangl et al., 1996). In contrast, propagative mutants are thought to represent defects in genes that encode negative regulators of cell death in plants (Walbot et al., 1983; Dangl et al., 1996).

The production of lesions in most maize *Les/les* mutants is developmentally programmed and influenced by genetic background (Neuffer et al., 1983; Johal, 2007) (also see MaizeGDB). Environmental factors, such as light and temperature, also have a significant effect on their etiology (Hoisington et al., 1982; Gray et al., 1997; Hu et al., 1998). Another unique aspect of most lesion mimic mutants is that they display their phenotype in a cell-autonomous manner (Fig. 6A). This characteristic, along with the fact that many are partially dominant, light-sensitive and developmentally programmed, suggests that there may be common factors contributing to the phenotypic manifestation of lesion mimic mutants.

Two features of lesion mimic mutants have triggered a great deal of interest. First, lesion mimic mutations often sensitize the host to pathogens, resulting in heightened defense responses (Dangl et al., 1996; Hu et al., 1996; Lorrain et al., 2003). This association has led to the belief that these mutants represent a valuable resource to study plant defense signaling and response in the absence of compounding effects from the pathogen. However, unlike the lesion mimic mutants of Arabidopsis and other dicots, maize *Les/les* mutants do not elicit a heightened systemic acquired response, even though some of the markers associated with such a response are upregulated in some of the maize lesion mimic mutants (Morris et al., 1998). A local resistance response in the immediate vicinity of lesions of some maize *Les*

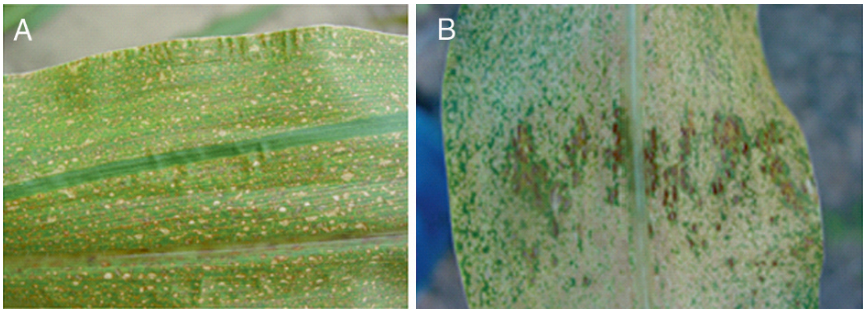

Fig. 6 A) A normal green somatic sector caused by insertion of a *Mutator* element in the *Les10* dominant mutant allele. B) Suppression of cell death underlying *Les17* lesions in the vicinity of common rust pustules

loci has been observed in a few cases (Johal, 2007). Curiously, the common rust pathogen, an obligate biotroph, can also suppress lesions associated with *Les17*, thereby producing areas on the leaf that are often referred to as 'green islands' in plant pathology literature (Fig. 6B). Second, tissue damage is a normal part of *Les/les* mutants. Cell death underlying this damage happens either precociously in these mutants or is not contained following normal onset (Johal, 2007). This has led many to suggest that *Les/les* mutants represent defects in genes and mechanisms that control programmed death of cells and tissues in plants (Johal et al., 1995; Dangl et al., 1996; Lorrain et al., 2003). In this regard, *Les/les* mutants appear to hold great promise because they may provide insights into mechanisms that control and signal cell death pathways in plants.

Why are there so many lesion mimic loci in plants? True to their name, one mechanism underlying some of these mimics involves defects in plant disease resistance genes (Johal et al., 1995). These R genes encode proteins that respond to pathogen ingress by triggering a rapid cell death response, HR (hypersensitive response) in affected host cells (Martin et al., 2003). Each R gene triggers HR only in response to a specific set of races of a single pathogen. An R gene can become defective such that it triggers an HR even in the absence of the pathogen (Johal et al., 1995; Martin et al., 2003), as first observed with maize *Rp1* that conditions resistance to common rust, caused by *Puccinia sorghi*. Occasionally, intragenic recombination within the *Rp1* locus, which is composed of tandemly duplicated copies of individual R gene paralogs, leads to the creation of novel genes, some of which confer a *Les* phenotype in which HR is triggered constitutively in the absence of pathogen ingress (Hu et al., 1996). Both dominant and recessive les mutants, differing in severity, have been identified at the *Rp1* locus (Hu et al., 1996). This suggests that weak alleles may behave as recessives and strong alleles may behave as dominants.

Notably, a majority of the maize *Les/les* mutants, however, do not seem to be involved in plant defense responses. Two other *Les/les* genes that have been cloned suggest errors or impairments in metabolism that lead to the lesion mimic phenotype. The maize mutant *Les22* is a key example of this (Hu et al., 1998). It is defective in a single copy of the *Urod* gene that encodes a tetrapyrrole biosynthetic enzyme

required for the production of both heme and chlorophyll in plants. But why does *Les22* behave as a dominant mutant? The reason lies in the haplo-insufficient nature of the *urod* gene. When one copy of this gene is defective, the pathway runs into a bottleneck, causing the accumulation of a highly photodynamic intermediate, uroporphyrinogen. In the presence of light, this molecule leads to the production of singlet oxygen, which, in turn, leads to the *Les22* phenotype. However, if both copies of *urod* are defective, the mutants are albino due to lack of chlorophyll (Hu et al., 1998). Thus, the phenotype of a gene that leads to a *Les* phenotype as a heterozygote could be quite different from its homozygous phenotype.

Mutations in the chlorophyll degradative pathway, as well as in the biosynthetic pathway, also lead to a *les* phenotype (Johal, 2007). A good example is *lls1*, which controls the first committed step of the chlorophyll degradation pathway (Gray et al., 1997; Gray et al., 2002), and the maize ortholog of the Arabidopsis *acd2* mutant (G. Johal, unpublished results), which controls the next step following *lls1*. Again, cell death associated with both of these mutants is caused by the accumulation of phytotoxic intermediates that leads to cellular damage.

Among all the factors that impact the etiology of a maize *Les/les* mutant, the genetic background is perhaps the most important. A *Les/les* mutant may have a lethal phenotype in one genetic background but a largely benign phenotype in another (Neuffer et al., 1983). Among the inbreds that tend to be suppressive is Mo20W, a 'stay-green' line that can withstand high heat and intense light (Neuffer et al., 1983). The W23 inbred, in contrast, enhances the severity of many mimics to the point that they become lethal when introgressed into its genome (Neuffer et al., 1983). Studies on *Les1* showed that the suppressible effect of Mo20W was dominant (over its enhanced expression in W23) and under the control of multiple factors (Neuffer et al., 1983).

A QTL approach involving an F_2 population between *les23*::Va35 and Mo20W was used to identify the modifiers responsible for the background dependence (Penning et al., 2004). A strong QTL, *slm1*, was identified which controlled more than 70% of the *les23* phenotypic variation in this population. *Slm1* has been mapped to the long arm of chromosome 2 (2L) in maize (Penning et al., 2004). A similar QTL capable of suppressing the phenotype of *Rp1-D21*, a constitutively active allele of *Rp1*, has been mapped to 10S (P. Balint-Kurti, personal communication). Suppressors of *Les/les* loci appear to be rather common in the maize genome, and can cause a lesion mimic mutant to become cryptic. Such is the case with Mo17, which fails to manifest the lesioned phenotype of the severe *les*-mo17* mutation because it carries two unlinked suppressors of *les*-mo17*. When these suppressors segregate away from *les*-mo17,* as happens in the IBM RILs or in the F2 populations of Mo17 with various inbreds, *les-Mo17* reveals itself (G. Johal, unpublished results).

3.3 Half Plant Chimeras

Half plant chimeras have been found for most of the dominant phenotypes observed, occurring at approximately the frequency of their whole plant equivalents in all the

treatment progenies studied. Sometimes the difference is very subtle, such as a slightly different level of green, which can only be detected in side-by-side tissue comparisons of the chimera (Figure 71). Similarly, dominant mutants that grow

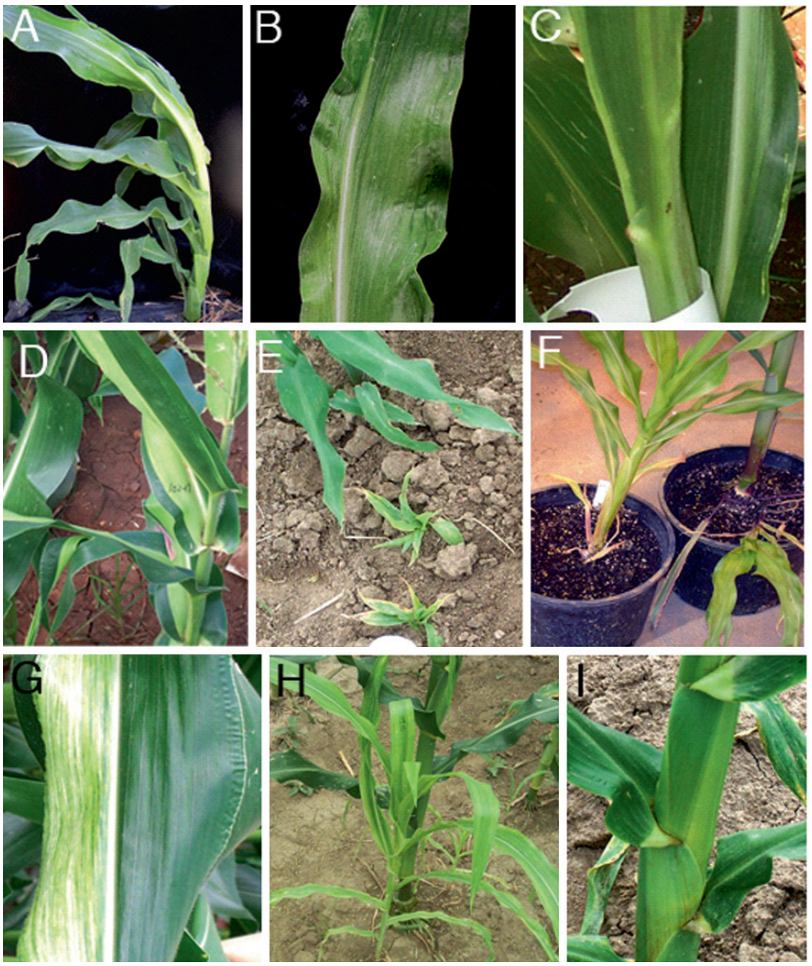

Fig. 7 Examples of half-plant chimeras. **A**) In this plant, half of each leaf is narrow, causing a bent posture (SH6842-141). **B**) Leaf from (A). **C**) Original *Liguleless narrow* chimera in which one half of the plant had narrower leaves and a displaced ligule. **D**) Half plant, pale green chimera (Ppg*Chi 2542). **E**) Progeny from the chimera in (D) segregated 1:1 for small pale green plants that made no tassels or ears. **F**) Progeny from (D) grown in the greenhouse show pale green plants that fall over because of very poor root growth but were able to make some pollen. **G**) Half plant chimera (Vsr*-2595) with yellowish-white tissue with tiny yellow green streaks that enlarge and merge to give a yellow green plant. **H**) Progeny from Vsr*-2595 that was pale green and infertile. Crosses to *R1-rsc* suggested a tight association with anthocyanin expression in the aleurone, but not necessarily with linkage to *r1*. This mutant may be allelic to one or more of the following similar mutants reported to be on chromosome 10L: *dek21, v29, Vsr1*, and *w2* (Neuffer et al., 1997). **I**) Pale sheath chimera (PlSh*-2562) showing the clear distinction between mutant and normal tissue

slower or faster than normal siblings are not easily recognized, but the mutation can be seen in a chimera. Such chimeras are often recognized as bent or curved plants depending on the nature of the gene product (Figure 7A, B). By examining the border between mutant and normal tissue, one may be able to determine if there is a sharp boundary, suggesting that the gene product acts autonomously. Alternatively, a blending gradient along the border may be seen indicating diffusion of gene product from one tissue into the other; such as is the case for *colorless floury defective (dek1)* and the *floury* endosperm (Neuffer, 1995). One may also fail to distinguish between mutant and wild-type tissue as in the case of *Cg* (Poethig, 1988b) and most sectors involving *D8* (Harberd and Freeling, 1989).

When the mutation occurs in an essential gene that is effectively lethal, the wild-type half often rescues the lethal mutant half. Some mutations turn out to be conditionally lethal, such that the chimera can be crossed to a different inbred and may survive in a vigorous hybrid background or survive when grown in the greenhouse. The *Liguleless narrow* (*Lgn*) chimera shown in Figure 7C was obvious at the ligule and auricle, which normally serves as a sharp boundary between blade and sheath. On one half of the leaf, the ligule and auricle were in their normal position and form, while the other half had a reduced auricle and displaced ligule. The chimera, which originated following B73 EMS pollen treatment onto B73, was crossed to A632 and produced vigorous plants with a mild ligule defect. Crosses of *Lgn* back to B73 produced very weak, liguleless plants that were nearly sterile. It is likely that this mutation would have been lost had it not been identified as a chimera.

PgV-2542* originated as a very light yellow green chimeric plant in the M1 of A619 × B73 treated pollen (Figure 7D). Pollen from this chimeric plant crossed on a standard stock produced progeny that segregated 1:1 for tiny, yellow green, and dwarf-like plants that failed to make a tassel or ear (Figure 7E). Replanting progeny under intensive care produced short, pale green mutant plants (Figure 7F), which fell over because they lacked normal root development. They also failed to make viable ears but did make one tassel with enough pollen for outcrossing and viable offspring for further analysis. A review of this mutant's history suggests that it would not have survived except as a chimera on a normal plant.

Vsr-2595*, originated as a chimeric plant with a large yellowish white half leaf sector on one side of the plant (Figure 7G), in a cross of Mo17 × A632 treated pollen. At the seedling stage it was almost white with tiny yellow green streaks, typical of those seen in recessive *v29* and dominant *Vsr1*. These streaks greened up to near normal green. Pollen from this chimeral plant crossed onto B73/A619 segregated 1:1 for yellowish white virescent seedlings that slowly greened up to produce small striped yellow green plants (Figure 7H), a few of which survived to maturity.

3.4 Lethal Dominants

Normally in genetic studies, mutants for which no progeny can be obtained are not described. However, with repeated occurrences obtained through chemical mutagenesis,

such examples are worth noting. One example is the white or yellowish albino seedling (Figure 8A), the rare dominant phenotypic equivalent of the most frequent of all the recessive seedling mutants. Several of these have been seen both as chimeras and as whole seedling lethal cases. The same is true for the tannish necrotic seedling lethals. Another good example is a mutant that has small fleshy leaves, whose surface

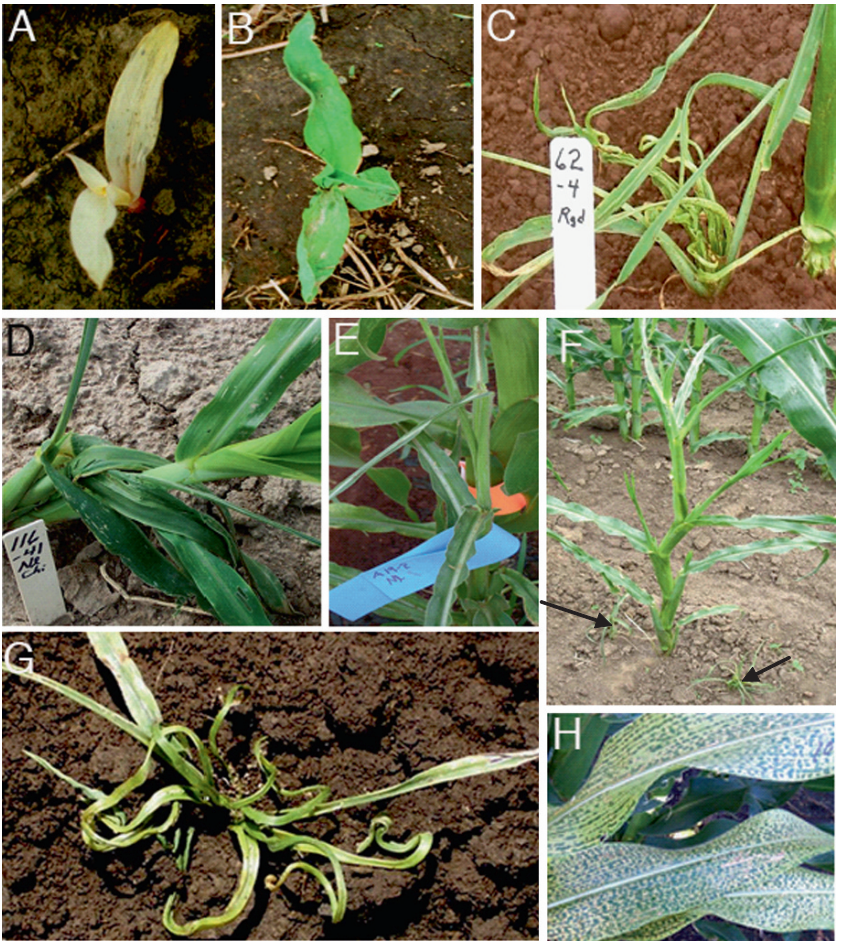

Fig. 8 Dominant lethal mutations. **A**) Dominant yellowish, white lethal M1 seedling (W*-33:1022–58). **B**) Dwarf with fleshy sheen heart shaped leaf (DfShn*-33:1018–33). **C**) M1 plant, tangled midrib only (84:62–4). **D**) Putative DfShn type lethal chimera (Chi*79:116–4). **E**) Original Nl*-2598 mutant in Mo17 with narrow leaves and zig-zag culm. **F**) The progeny from the sib cross of heterozygous Nl-2598 plants segregated original mutant type and small, midrib only types (arrows) that were probably the homozygotes but looked just like the original heterozygote of another midrib only mutant (C above). **G**) Close-up of one of these small plants in F. **H**) *Leopard spot*. Unusual mutant with pale yellow background and green spots. The mutant arose in Mo17×A632 (81:ll108–1) but has no progeny

glistens like paint with metallic particles (Figure 8B). The leaf is broad and heart shaped. This phenotype has not been seen as a chimera but has been seen four times as a whole seedling, which does not usually grow beyond the four-leaf stage.

A third example that has been seen repeatedly is a narrow leaf mutant whose leaves are loosely tangled like cords of twine, and consists of mostly midrib (Figure 8C). These occur at a frequency as high as 1/1000 pollen grains for some treatments and not in others. They are similar to the recessive *leafbladeless* mutants discussed in Chapter 9. The presumed chimeras (Figure 8D) are distorted by the pulling of normal and mutant tissues against each other such that no normal tassels or ears are produced. We have seen the same phenotype in sib-crosses of Nl*-2598 (Figure 8F, G). This mutant originated as a whole plant, with narrow leaf blades, hairy leaf margins and sheath, zigzag stalk and a few branched tassels with viable pollen, in an M1 from the cross of Mo17 by treated Mo17 pollen (Figure 8E). Pollen from this mutant plant crossed onto W22/W23 gave a 1:1 segregation for narrow leaf and normal plants. Subsequent sib progeny gave a wide range of phenotypes (Figure 8F, G) from bladeless tangled leaves to plants that looked like the original mutant parent. The extreme class (probably the homozygotes) appear to be identical with the heterozygotes of the dominant no progeny mutant, *Leafbladeless* (Figure 8C).

4 Conclusions

The ease of mutagenesis, mutant discovery and genetic analysis has kept maize at the forefront of plant genetics for decades. Many genes have been cloned thanks to transposable elements used as gene tags. The synteny of the maize genome with sequenced genomes of rice and sorghum has now made positional cloning also possible. In fact, the first maize gene cloned by position was a QTL and its identity was confirmed using EMS mutagenesis (Wang et al., 2005). Once the maize genome sequence is completed, positional cloning will become even more robust. To clone a gene defined by EMS mutagenesis one has only to develop a segregating population. The high frequency of mutation generated by EMS provides the chance of having multiple alleles. New alleles can also be obtained with reverse genetics resources described in this volume. The recent breakthroughs in high throughput sequencing technology suggest that it may even be possible to determine the mutated gene that results in lethal dominants, which precludes the creation of segregating populations. Half plant chimeras would be especially useful as the DNA from normal and mutant half of the leaf could be compared. Although there would be dozens of mutations, theoretically, there would be one that is only found in one half of the leaf. Future screens should keep careful phenotypic records of half-plant chimeras and lethal dominants along with a sample of the DNA for sequence analysis. In summary, EMS is an efficient, effective tool that differs from other mutagens in its production of valuable dominant lethals and half plant chimeras allowing for the study of genes that are recalcitrant to other forms of genetic analysis.

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