The Plant Cell, Vol. 10, 1095–1105, July 1998, www.plantcell.org

A Porphyrin Pathway Impairment Is Responsible for the Phenotype of a Dominant Disease Lesion Mimic Mutant of Maize

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The maize lesion mimic gene *Les22* is defined by dominant mutations and characterized by the production of minute necrotic spots on leaves in a developmentally specified and light-dependent manner. Phenotypically, *Les22* lesions resemble those that are triggered during a hypersensitive disease resistance response of plants to pathogens. We have cloned *Les22* by using a *Mutator*-tagging technique. It encodes uroporphyrinogen decarboxylase (UROD), a key enzyme in the biosynthetic pathway of chlorophyll and heme in plants. *Urod* mutations in humans are also dominant and cause the metabolic disorder porphyria, which manifests itself as light-induced skin morbidity resulting from an excessive accumulation of photoexcitable uroporphyrin. The phenotypic and genetic similarities between porphyria and *Les22* along with our observation that *Les22* is also associated with an accumulation of uroporphyrin revealed what appears to be a case of natural porphyria in plants.

INTRODUCTION

A large class of mutations exists in maize that is characterized by the spontaneous formation of discrete or expanding lesions of varying size, shape, and color on leaves (Walbot et al., 1983; Johal et al., 1995). Because lesions associated with some of these mutants resemble symptoms of certain diseases of maize, they have been collectively called disease lesion mimics (Neuffer and Calvert, 1975). To date, >40 independent lesion mimics, both recessive (designated *les*) and dominant (designated *Les*), have been identified in maize (Johal et al., 1995; G.S. Johal, unpublished data). Similar mutations have been reported for other plants (Dangl et al., 1996). In Arabidopsis, they have been referred to as lesions simulating disease (*lsd*) or accelerated cell death (*acd*) mutants (Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994). Most lesion mimics (>70%) inherit as Mendelian dominants, prompting speculation that they may constitute the largest class of gain-of-function mutations in plants (Johal et al., 1995; Dangl et al., 1996).

Our current understanding of what goes wrong in lesion mimics is meager. The expression of most, if not all, lesion mimics is developmentally programmed and is readily affected by the genetic background of the plant (Hoisington et al., 1982; Walbot et al., 1983; Johal et al., 1995; Dangl et al., 1996). Cell death is a ubiquitous feature of lesion mimics; its extent is often increased by high-intensity light, raising the possibility that reactive oxygen species are involved in the etiology of lesion mimics (Johal et al., 1995; Dangl et al., 1996). In fact, superoxide has been shown to be responsible for the expression of lesions in the Arabidopsis *lsd1* mutant (Jabs et al., 1996). Another common feature of many mimics is that they are associated with defense responses that are normally triggered in response to pathogens (Wolter et al., 1993; Dietrich et al., 1994; Greenberg et al., 1994; Dangl et al., 1996). This has led researchers to propose that lesion mimics represent defects in genes that normally control or regulate the hypersensitive response (HR) or are responsible for the highly contained nature of this cell death reaction (Dangl et al., 1996; Morel and Dangl, 1997). Although some direct evidence for this proposal has been obtained (Hu et al., 1996), it is unlikely that all lesion mimics are aberrations of plant responses to pathogens.

Both determinate and propagative-type lesion mimics exist in plants, and this has been interpreted to imply that cell death is either initiated precociously or is not contained adequately in these mutants (Walbot et al., 1983; Johal et al., 1995; Dangl et al., 1996). Because cell death in plants, like in animals, has relevance to development, differentiation, and maintenance of tissue (Johal et al., 1995; Dangl et al., 1996; Greenberg, 1996; Jones and Dangl, 1996), lesion mimics are an excellent model for understanding how cell death is regulated and executed in plants. Recently, three recessive lesion mimic genes have been cloned from three plant

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species: Arabidopsis, barley, and maize (Buschges et al., 1997; Dietrich et al., 1997; Gray et al., 1997). As would be expected from their recessive loss-of-function phenotypes, they all appear to encode cell death–suppressible functions that are unique to plants. For instance, the *LSD1* gene of Arabidopsis, which encodes a zinc finger transcription factor, may negatively regulate cell death (Dietrich et al., 1997). Likewise, the *Mlo* gene of barley appears to encode a membrane protein whose function may be to regulate negatively both cell death and disease resistance responses (Buschges et al., 1997). The maize *Lls1* gene inhibits cell death, albeit apparently by degrading a phenolic mediator of cell death (Gray et al., 1997).

Although understanding the nature of the defect in a recessive loss-of-function mutation may be relatively straightforward, the mechanistic basis of a dominant mutation may be virtually impossible to predict from the phenotype. To elucidate the molecular basis of a dominant lesion mimic mutation of maize, we selected *Les22* (previously designated *Les*-2552*; Johal et al., 1994). Dominant mutations of *Les22* are characterized by the formation of discrete, tiny, whitish-gray bleached or necrotic spots on leaf blades. The lesions resemble those observed during the HR, not only in appearance but also in their highly contained or discrete nature (Figure 1A). Here, we report the isolation of the *Les22* gene and show that it encodes uroporphyrinogen decarbox-

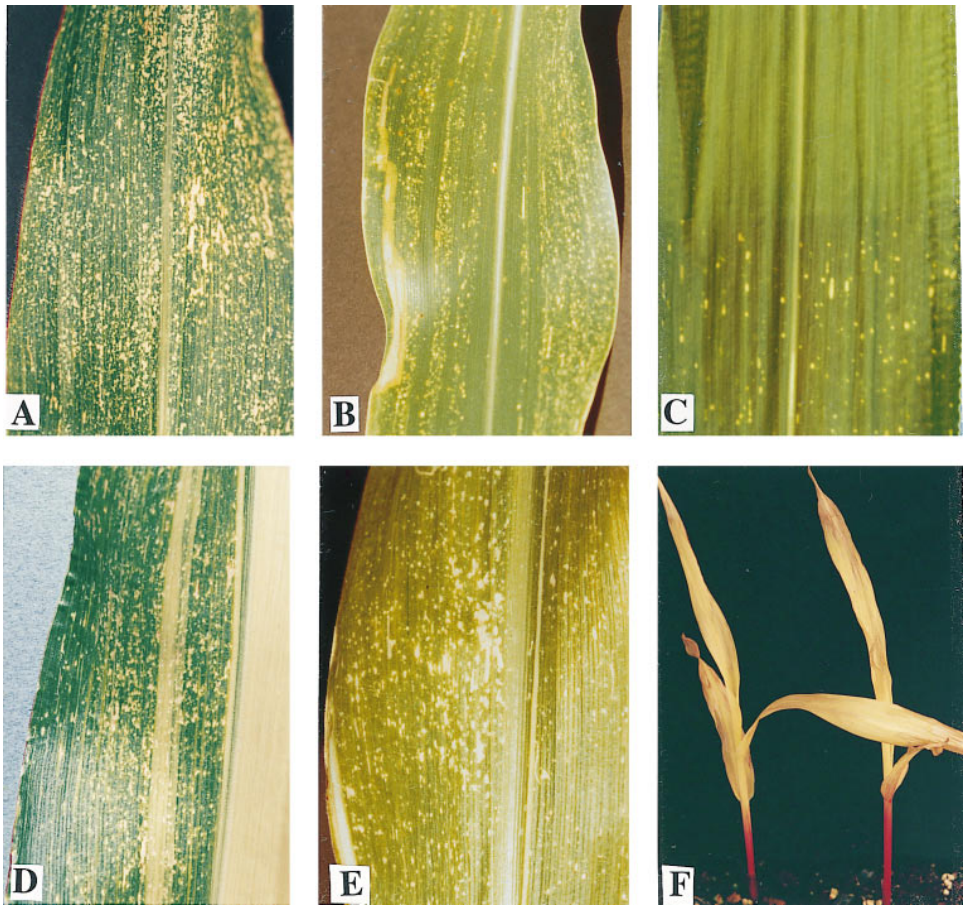

Figure 1. Phenotypic Features of *Les22*.

(A) Typical morphology of *Les22* lesions on a leaf blade.
(B) Somatic revertant sectors (green stripes) on a leaf, illustrating the *Mu*-suppressible nature of *Les22-7*.
(C) The upper half of the leaf, which was covered with aluminum foil, failed to form *Les22* lesions.
(D) A double mutant of *Les22* and *ij1* showing lesions in both green and albino tissues.
(E) *Les22* lesions on an *Oy1-700* mutant leaf.
(F) The ysl mutant.

ylase (UROD), a key enzyme of the porphyrin pathway. In addition, we present evidence suggesting that the phenotype of the dominant lesion mimic *Les22* is the result of porphyria, which develops as a consequence of null mutations in one of the alleles of *Urod.*

RESULTS

The original mutation leading to the identification of the *Les22* locus was isolated by D.S. Robertson (Iowa State University, Ames, IA) from an active *Mutator* (*Mu*) population and was kindly provided to us for study. Because *Les22* is a dominant mutant, it is easy to spot in the field. As a result, 16 additional cases of *Les22*-like mutants that originated independently of each other were collected from various *Mu* populations; these populations had been generated to tag various genes with *Mu* by using both targeted and random approaches. Three of the new *Les22* mutants were tested for linkage with the original mutant (Johal et al., 1994). Because all of them were found to be tightly linked, it was tentatively concluded that they all originated from the same locus (Johal et al., 1994). These putative alleles of *Les22* have been named *Les22-1* through *Les22-16*, whereas the original mutant has been designated *Les22-17.*

Les22 Expression Is Developmentally Controlled

Like most maize lesion mimic mutants, the onset of *Les22* lesions is developmentally regulated and appears to be dictated by an age-related gradient of unknown nature (Johal et al., 1995). Lesions first appear as tiny round or elliptical spots near the tip of the primary leaf on the upper side when the plants turn 3 or 4 weeks old. These lesions extend somewhat downward and out in the next 1 to 2 days and produce grayish white flecks of dead necrotic cells that become visible on both sides of the leaf (Figure 1A). Meanwhile, new spots appear toward the base as the plant matures and on successive leaves as they attain the required developmental competence. As a result, at the time of flowering, blades of all leaves are covered with *Les22* lesions. Although most of these lesions remain tiny ($<$1 mm in diameter), occasional streaks of dead tissue can also be seen on *Les22* mutants (Figure 1A).

Two other dominant mutations that exhibit a lesion mimic phenotype identical to that of *Les22* are *Les2* (Neuffer and Calvert, 1975) and *Les28* (Martienssen and Baron, 1994). Interestingly, both *Les22* and *Les2* map to the short arm of chromosome 1 (1S) (Johal et al., 1994). To measure the linkage of *Les22* to *Les2*, a mapping population was generated by pollinating *Les22* plants with pollen from a *Les2* plant, followed by outcrossing a few *Les22*/*Les2* double mutants that displayed a relatively severe phenotype with the inbred Mo20W. Of the 340 plants screened, three were found to be wild type or recombinants. This result suggests that these mutations either originate from tightly linked loci or represent defects of the same locus. The latter possibility, however, relies on the assumption that the three recombinants recovered represented *Les* plants whose phenotype was somehow suppressed. In fact, the expression of *Les22* is readily influenced by the genetic background of the plant; the phenotypic severity of *Les22* progressively diminished as it was successively backcrossed to the inbred B73.

Although the map location of *Les28* has not yet been reported, *Les22* shares two additional features with *Les28.* First, both express in a cell-autonomous manner (Johal et al., 1994; Martienssen and Baron, 1994). Second, some mutant alleles of *Les22*, including *Les22-7* (Figure 1B), exhibit an epigenetic, *Mu*-suppressible phenomenon that has been described previously for *Les28* (Martienssen and Baron, 1994). As expected from a *Mu*-suppressible mutant, *Les22-7* plants sometimes lose their *Les* phenotype in a developmentally progressive manner (Figure 1B), only to revert back to the lesioned phenotype after a reactivation cross with an active *Mu* line (Martienssen and Baron, 1994).

Expression of *Les22* Lesions Is Mediated by Incident Light

The phenotype of *Les22*, like most other maize mimics, is light dependent (Hoisington et al., 1982; Johal et al., 1995). When leaves or parts of leaves are protected from light (e.g., by covering with aluminum foil), they fail to develop lesions (Figure 1C). If such areas are kept hidden from light for prolonged periods ($>$1 week after the onset of lesions), lesions almost never appear on them, suggesting that there is a developmental window during which the maize leaf tissue is competent for expressing *Les22* lesions, provided that light is available.

To investigate what aspect of light was responsible for the expression of *Les22* lesions, double mutants of *Les22* were generated with two photosynthetically compromised mutants of maize, *iojap1* (*ij1*) and *oil yellow1-700* (*Oy1-700*). The *ij1* mutant carries a recessive defect in chloroplast development, manifested by the formation of variegated leaves with alternate green and albino stripes (Han et al., 1992). The dominant *Oy1-700* mutant is defective in chlorophyll accumulation (Mascia, 1978), resulting in plants with an oily greenish yellow hue. As shown in Figures 1D and 1E, the expression of *Les22* lesions was not significantly altered in either mutant background. This is in contrast with the expression of *lls1* in these mutants; *lls1* lesions require green, photosynthetically active tissue for both initiation and continued development (Close et al., 1995). Because the status of the leaf tissue with regard to chloroplast development or photosynthesis does not seem to be important in the expression of *Les22* lesions, the ontogeny of *Les22* lesions would seem to be mediated primarily by incident light. The quantity of light, however, seems to be important, because

Les22 expression is much more severe under field conditions than in the greenhouse. In addition, the severity of *Les22* lesions in the greenhouse is inversely proportional to the distance of the plant from the light source (Johal et al., 1994).

Yellow Seedling Lethal Appears to Be the Homozygous Phenotype of *Les22*

Restriction fragment length polymorphism (RFLP) markers were used to determine the phenotype of a plant homozygous for *Les22.* To identify RFLP markers that flank *Les22*, an outcross population was constructed between *Les22-9* and the inbred A632. Two hundred plants from this progeny were used to map a number of RFLP markers from 1S in relation to *Les22.* Two flanking markers, *UMC194* and *UMC76*, were identified that mapped 2.6 centimorgans (cM) distal and 9.8 cM proximal, respectively, to *Les22.* These markers were used to genotype an F_2 population (36 plants) derived from the *Les22-7* mutant. Contrary to what was thought previously (Johal et al., 1994), densely lesioned F_2 plants were not homozygous for *Les22.* Instead, a yellow seedling lethal (ysl) mutant (Figure 1F), which scalded easily in sunlight, was found to segregate completely with both flanking RFLP markers, raising the possibility that this ysl mutant may very well be the phenotype of a *Les22* homozygote.

Tagging and Cloning of *Les22*

No deliberate effort was made to tag the *Les22* gene. Instead, as mentioned earlier, 16 independent cases of *Les22* were recovered by serendipity from various gene-tagging projects with *Mu* (Johal et al., 1994). In addition, a number of plants with somatic sectors (representing forward insertional mutation), exhibiting lesion morphology and color typical of *Les22*, were also observed in these *Mu*-active populations. Because *Les22* mutants or somatic sectors were not found in plant populations lacking *Mu* activity, the *Les22* phenotype probably does not reflect an inherent instability of the *Les22* locus, as has been the case with the *Rp1* locus of maize (Hulbert and Bennetzen, 1991), but is likely the consequence of *Mu* insertions.

To identify *Mu* elements that may have caused these mutations, each mutant was backcrossed three times with either B73 or A632 (Johal et al., 1994), and the progeny from the last cross was subjected to a gel blot–based analysis that examined the linkage of each mutant allele with each of the nine known *Mu* elements (Walbot, 1992; Bennetzen et al., 1993). From the *Les22-7* family, a *Mu1*-hybridizing, 6.5-kb XhoI restriction fragment was identified that was present in the DNA of all 39 mutants and absent in the DNA of all 30 wild-type siblings (Figure 2A). This restriction fragment either carries at least a part of the *Les22* gene or contains a *Mu1* element that is closely linked to the *Les22* locus. To characterize it further, the 6.5-kb XhoI restriction fragment was cloned in λ ZAPII and then rescued as a phagemid.

Cloning Confirmation of *Les22*

To verify the cloning of *Les22*, a polymerase chain reaction (PCR) approach was used (Gray et al., 1997). An ~500-bp fragment, designated LF7, was amplified from the 6.5-kb XhoI clone using a *Mu* terminal inverted repeat (*Mu*-TIR) primer (Gray et al., 1997) and the M13 reverse primer and then cloned and sequenced. Two oppositely orienting PCR primers (LF7-A and LF7-B) were designed from the se-

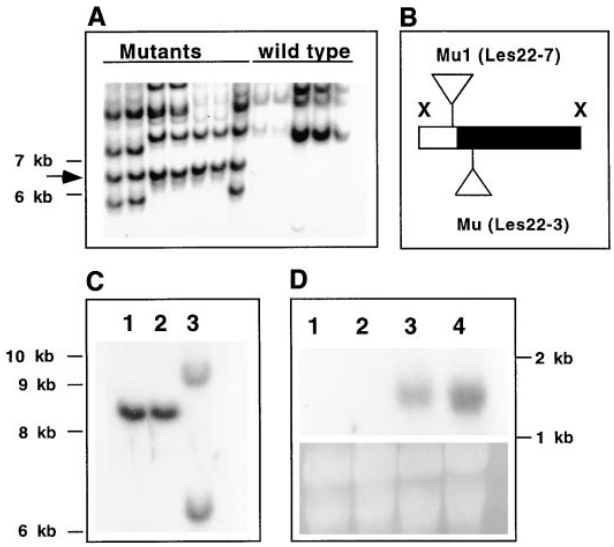

Figure 2. Cloning and Confirmation of *Les22.*

(A) A DNA gel blot showing the cosegregation of a *Mu1*-hybridizing, 6.5-kb XhoI restriction fragment (arrow) with the mutant allele of *Les22-7*.

(B) Schematic representation of the 6.5-kb XhoI (X) clone showing the relative insertion of *Mu* elements (triangles) in *Les22-7* and *Les22-3* mutants. The open and shaded boxes represent regions of the clone 5′ and 3′, respectively, of the *Les22* translation start codon.

(C) A DNA gel blot showing the RFLP (the length difference between the band in lanes 1 and 2 and the top band in lane 3 is 1.4 kb) between the mutant allele of *Les22-7* (upper band, lane 3) and its wild-type progenitor allele (lanes 1 and 2). DNAs were digested with XbaI, which does not cut within *Mu1*, and the blot was hybridized with LF7.

(D) RNA gel blot analysis (top) of total RNA extracted from the ysl segregants of *Les22-7* (lane 1) and *Les22-15* (lane 2) F_2 populations and from *Les22-7* (lane 3) and its wild-type sibling (lane 4). The blot was hybridized with LF7 and was prepared from the gel (ethidium bromide stained) shown at bottom.

Numbers at left and right in **(A)**, **(C)**, and **(D)** represent the lengths in kilobases of DNA and RNA standards, respectively.

quence of LF7, and each was used in combination with the *Mu*-TIR primer in a PCR reaction in which the template DNA was derived from each of the 16 *Les22* mutants. A 300-bp product, which hybridized with LF7, was obtained from the DNA of the *Les22-3* mutant when PCR amplified with *Mu*-TIR and LF7-B, demonstrating that a *Mu* element was present in the vicinity of the LF7 region in the *Les22-3* mutant allele as well. Subsequent sequence analysis of this PCR product showed that a *Mu* element had inserted in the *Les22-3* mutant allele 95 nucleotides away from the *Mu1* insertion in *Les22-7* (Figure 2B). Such multiple insertions in independent mutants are considered proof for the correct cloning of a gene (Walbot, 1992; Gray et al., 1997).

Unequivocal evidence that *Les22* had been cloned came from two additional experiments. First, we sought to detect polymorphism between the *Les22-7* mutant allele and its wild-type progenitor or ancestor. The *Les22-7* mutant was found as a single plant in the progeny of a cross between the inbred Pr1 (Johal and Briggs, 1992) and a *Mu* active line. DNA from 50 wild-type siblings of the original *Les22-7* mutant was compared with DNA of the *Les22*-7 mutant allele, which was obtained from one of the advanced generations of *Les22-7* with A632 (described above). A DNA polymorphism (Figure 2C) of the size expected from a *Mu1* insertion (Bennetzen et al., 1993) was detected between the mutant allele of *Les22-7* and its wild-type progenitor.

Second, RNA gel blot analysis showed that the steady state level of a 1.5-kb, LF7-specific transcript, which was found fairly abundantly in wild-type plants (Figure 2D), was reduced to ~50% of the wild-type level in *Les22.* This reduction was observed not only in *Les22-7*, as shown in Figure 2D, but also in *Les22-3* and *Les22-15;* the nature of the mutational defect in *Les22-15* remains unknown. Furthermore, the LF7-specific transcript was missing completely in the ysl mutants that segregated recessively in the self-pollinated populations of *Les22-3*, *Les22-7*, and *Les22-15*; the data on both *Les22-7* and *Les22-15* are presented in Figure 2D. Not only does this transcript analysis serve to confirm beyond doubt that the correct gene has been cloned (Walbot, 1992), but it also provides unequivocal evidence that in homozygous form, *Les22* manifests as a ysl. In addition, these results indicate that all three of the mutant alleles characterized here by RNA gel blot analysis are the result of null mutations of *Les22.* Furthermore, progeny from crosses of *Les22-7* with *Les22-3* or *Les22-15* produced ysl plants that segregated as Mendelian recessives. This result indicated that *Les22-7* is allelic to both *Les22-3* and *Les22-15.*

Les22 Encodes UROD

To ascertain the molecular nature of *Les22*, a 1.5-kb cDNA clone corresponding to the sequence of LF7 was recovered from the maize expressed sequence tag collection at Pioneer Hi-Bred International Inc. and sequenced (Figure 3). BLAST analysis indicated that *Les22* encodes UROD, the fifth enzyme of the C-5 porphyrin pathway. This enzyme is required in plants to produce the tetrapyrrole rings of both chlorophyll and heme (Beale and Weinstein, 1990; von Wettstein et al., 1995). Consistent with the identification of UROD as the product of *Les22* is the finding that *Les22* homozygotes exhibit a chlorophyll-less, ysl phenotype (Figure 1F). In addition, *Les22* mutants also appear to be deficient in heme, because the activity of catalase, a heme-containing protein (Labbe-Bois et al., 1977; Anderson et al., 1995), is reduced by ~50 and 100% in *Les22* mutants and homozygotes (ysl plants), respectively, as compared with the level detected in wild-type siblings (Figure 4).

The *urod* gene and the porphyrin pathway, in which UROD catalyzes the sequential decarboxylation of uroporphyrinogen III to coproporphyrinogen III (Elder and Roberts, 1995; von Wettstein et al., 1995), have been highly conserved during evolution (Beale and Weinstein, 1990; Mock et al., 1995; Reinbothe et al., 1996; Zoladek et al., 1996). Whereas the predicted protein of the maize *urod* gene exhibits a 97 and 93% amino acid similarity to the corresponding proteins from barley and tobacco (Mock et al., 1995), respectively (Figure 3), it exhibits 54% similarity with the human UROD (Romeo et al., 1986). The maize *urod* gene encodes a 393–amino acid protein, compared with the 391–amino acid protein of tobacco. The first 62 amino acids encoded by the maize *urod* gene have significant divergence from the first 60 amino acids encoded by the tobacco UROD and may constitute the transit peptide that is expected to localize the enzyme to chloroplasts (Mock et al., 1995). In the mutant alleles *Les22-7* and *Les22-3*, *Mu* elements had inserted 37 nucleotides upstream and 58 nucleotides downstream, respectively, from the middle of the start codon (Figure 2B). Considering the locations of both of these *Mu* insertions, it is not surprising that they cause null mutations in the *Les22* gene, as has been demonstrated by transcript analysis (Figure 2D). In addition, the *Mu1* element in *Les22-7*, which appears to be between the transcription and translation start sites of *urod*, is in a position to create a *Mu*-suppressible mutant. A pseudo–wild-type phenotype occurs when *Mu* activity is lost, because there is a methylation-dependent, read-out promoter in the *Mu1*-TIR (Barkan and Martienssen, 1991).

A Block in the Porphyrin Pathway (Porphyria) May Account for the *Les22* Phenotype

Although it is apparent from these data that the molecular basis of *Les22* resides in the disruption of *urod*, how do null mutations result in the *Les22* lesion mimic phenotype, especially because it exhibits a dominant mode of inheritance? Examination of human *urod* mutations shows that like *Les22*, they inherit dominantly, depend on light for phenotypic manifestation, and result from a loss of function of the *urod* gene (Romeo, 1977; De Verneuil et al., 1986; Moore et al., 1987). These *urod* defects are responsible for a genetic

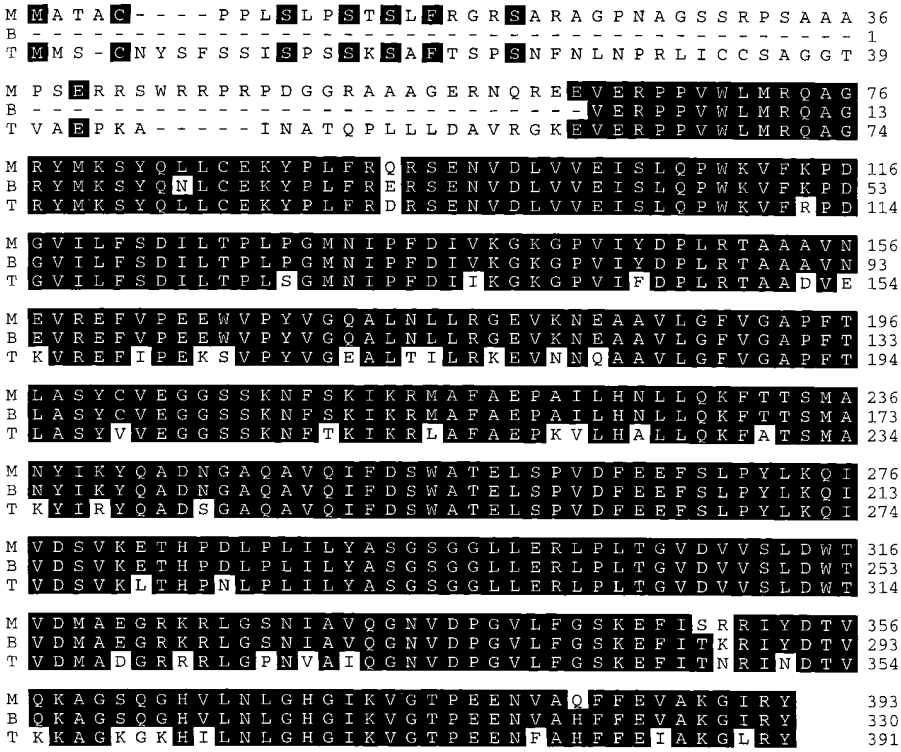

Figure 3. Comparison of the Maize UROD Sequence with That of Barley and Tobacco.

Sequence alignment of the predicted maize UROD (M; GenBank accession number AF058763) protein with the predicted URODs of barley (B; GenBank accession number X82832) and tobacco (T; GenBank accession number X82833). Identical amino acid residues are shaded black. Dashes represent gaps introduced to optimize alignment.

disorder in humans, called *porphyria cutanea tarda*, or porphyria in general, and are associated with light-stimulated skin morbidity, apparently caused by an excessive accumulation of uroporphyrin III (Moore et al., 1987; Straka et al., 1990; Moore, 1993; McCarrol, 1995). This substrate, like all other porphyrin intermediates, becomes highly reactive upon photoexcitation and results in the production of cell-damaging oxygen free radicals (OFRs) (Moore et al., 1987; Straka et al., 1990; Zoladek et al., 1996).

To evaluate whether the pathological basis of *Les22* also has its roots in porphyria, we extracted uroporphyrin(ogen) and its natural product, coproporphyrin(ogen), from both *Les22* heterozygotes (with the lesion mimic phenotype) and homozygotes (ysl mutants) and compared them with those of their wild-type siblings. Compared with wild-type controls, uroporphyrin levels were found to be elevated in *Les22* plants. Whereas *Les22* mutants exhibited a two- to threefold increase in uroporphyrin levels (Table 1), as would be expected from their heterozygous genotype with only one functional copy of the *urod* gene, *Les22* homozygotes had as much as 60 times the amount of uroporphyrin compared with wild-type siblings (Table 1). In contrast, compared with wild-type siblings, the levels of coproporphyrin were either not affected or completely diminished in *Les22* mutants (heterozygotes) and homozygotes, respectively (data not shown). These results are consistent with the interpretation that the porphyrin pathway is partly blocked at the step catalyzed by UROD in the *Les22* lesion mimic mutants and that this disorder is responsible for the etiology of *Les22* lesions. This conclusion is further substantiated by the finding that

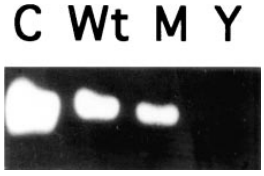

Figure 4. Catalase Activity of *Les22* Mutants and Homozygotes.

The activity of catalase in the seedling extracts of wild type (Wt), *Les22* mutant (M), and ysl (Y) plants. Four units of authentic catalase (Sigma) were loaded in lane C; 30 μg of total protein was loaded in each of the other lanes.

Table 1. Uroporphyrin III Levels in Selected Leaf Tissues of a *Les22* Mutant, Its Homozygote (ysl), and a Wild-Type Sibling

Tissue	Uroporphyrin III (nmol/g Fresh Weight)[a]
Wild-type apical	0.259 ± 0.008
Wild-type basal	0.216 ± 0.005
Les22 apical	0.608 ± 0.018
Les22 basal	0.476 ± 0.014
ysl total leaf	14.042 ± 0.421

[a] The data represent the mean of four replications (±SD).

transgenic tobacco plants expressing antisense *urod* accumulated high levels of uroporphyrin that resulted in an age-dependent necrosis of leaves under high-light regimes (Mock and Grimm, 1997).

DISCUSSION

We have successfully cloned *Les22*, a dominant disease lesion mimic gene of maize. This gene encodes UROD, the fifth enzyme of the C-5 porphyrin pathway, which is important in the production of both chlorophyll and heme in plants (Beale and Weinstein, 1990; von Wettstein et al., 1995). This finding is consistent with the fact that *Les22* homozygotes are devoid of chlorophyll and possibly heme, too, because they lack the activity of catalase, a heme-requiring enzyme (Labbe-Bois et al., 1977; Beale and Weinstein, 1990; Anderson et al., 1995). The identity of this lesion mimic gene as *urod* implies that *Les22* mutants constitute an example of an inborn error of metabolism in maize. However, the dominant inheritance of this disorder is caused not by a gain of a new function but rather by a null (loss-of-function) mutation in one copy of the *urod* gene, thereby revealing a rare case of true haploinsufficiency in plants (Birchler, 1993).

How does a null mutation in *Urod* account for a dominantly inheriting lesion mimic phenotype? A compelling explanation is provided by the *Urod* mutations of humans, which are associated with porphyria, a genetic disorder that results from a metabolic impairment of the porphyrin pathway (Moore et al., 1987; Straka et al., 1990; Moore, 1993; McCarrol, 1995). One major and consistent clinical manifestation of porphyria is hypersensitivity of skin to sunlight, caused by an accumulation of uroporphyrin. The reason is that when an allele of *Urod* becomes inactive (as a result of a null mutation), the activity of UROD is reduced to half of its normal level, leading to a partial block in the pathway; this results in a uroporphyrin increase (Romeo, 1977; De Verneuil et al., 1986; Straka et al., 1990; Moore, 1993). Because uroporphyrin becomes highly reactive upon photoexcitation, it donates energy or electrons to molecular oxygen, resulting in the formation of singlet oxygen and OFRs that damage skin cells. This characteristic of porphyrins to turn into killer molecules upon photoactivation or UV activation has been successfully used as a method for the clinical treatment of skin cancer (Fijan et al., 1995).

Several features of *Les22* suggest that it has much in common with human *porphyria cutanea tarda* and therefore may be caused by the same mechanism. For instance, the phenotypic manifestation of both *Les22* and porphyria is conditioned by sunlight. They both inherit as dominant mutations, and quite intriguingly, this dominance is not the result of a gain of a new function, as is usually the case with most dominant mutations (Hodgkin, 1993), but is the consequence of a loss of function of one copy of the *Urod* gene. In addition, both conditions show elevated levels of uroporphyrin. This raises the possibility that a derangement of porphyrin metabolism, which we have termed phytoporphyria, may also be the basis for the etiology of *Les22.* OFRs have been implicated in various kinds of cell death in plants, including the HR (Rebeiz et al., 1990; Foyer et al., 1994; Levine et al., 1994; Hammond-Kosack and Jones, 1996; Greenberg, 1997; Lamb and Dixon, 1997). Because the activity of both catalase and peroxidase, two heme-containing enzymes that degrade H_2O_2 (Foyer et al., 1994; Anderson et al., 1995), is likely to be compromised in *Les22*, these mutants may be even more sensitive to OFRs. The chemistry of a photosynthetically active leaf is certainly consistent with the interpretation that OFRs produced as a result of phytoporphyria may cause the cell death lesions associated with *Les22.* Not only are the leaf cells exposed to direct sunlight throughout the day, but they are also in an environment rich in oxygen and lipids, making them especially prone to photooxidative damage elicited by excessive porphyrins. This vulnerability of the leaf tissue and the damaging effects of the blocked porphyrin pathway have been exploited in the design of photodynamic herbicides (Rebeiz et al., 1990).

That *Les22* is the result of phytoporphyria is further strengthened by a recent study (Mock and Grimm, 1997) in which the activity of tobacco UROD was artificially altered by antisense technology. Transgenic plants with reduced UROD activity accumulated high levels of uroporphyrin and developed necrotic patches on older leaves under high-light conditions. Two problems, however, have prevented this study from gaining due recognition and appreciation. First, the gene knockouts accomplished by an antisense approach often are not straightforward and precise and therefore are difficult to interpret. For example, irrespective of photodynamic leaf necrosis, UROD-deficient transgenic tobacco plants exhibited aberrant growth phenotypes, including stunted growth of shoots and roots, reduction in total leaf area and weight, and delayed flowering. This contrasts with the *Les22* mutants that are identical to their wild-type siblings in every respect of growth and development. Second, similar transgenic plants that exhibited a light-dependent necrotic phenotype were also obtained when a number of other genes, including those that encode catalase (Chamnongpol

et al., 1996), phenylalanine ammonia-lyase (Elkind et al., 1990), or ubiquitin (Bachmair et al., 1990), were inactivated by the transgenic approach, thereby preventing a rigorous cause and effect relationship from being established.

The involvement of the porphyrin (chlorophyll) pathway in *Les22* etiology may also explain the developmental pattern of *Les22* lesions. In plants, this pathway is light inducible and operates at highest efficiency only in developing leaves when the demand for chlorophyll is maximal (von Wettstein et al., 1995; Reinbothe and Reinbothe, 1996). Any block in the pathway during this period will surely result in photooxidative damage, as has been witnessed with the developmental course of *Les22* lesion expression. In contrast, the influx of metabolites in the porphyrin pathway is tremendously reduced in fully expanded leaves (He et al., 1994; Reinbothe and Reinbothe, 1996), likely causing any partial blocks in the pathway to be relieved after the peak chlorophyll biosynthesis is over. This may explain why parts of the *Les22* leaf that are kept hidden from light for prolonged times become recalcitrant to developing lesions. Thus, both the developmental timing and developmental window during which *Les22* leaves are competent for forming lesions are largely dictated by the developmental interval during which the porphyrin pathway is fully engaged.

Unexplained by this study, but a truly remarkable feature of *Les22* as well as a number of other lesion mimic mutants of maize (Walbot et al., 1983; Johal et al., 1995) and other plants (Greenberg and Ausubel, 1993; Wolter et al., 1993; Dangl et al., 1996), is the determinate or discrete nature of the cell death lesions, despite the fact that the entire tissue is mutant. One possibility, suggested by Martienssen (1997), is that factors involved in lesion initiation may also serve as signals to enhance the effectiveness of mechanisms that degrade these signals in adjoining cells. OFRs, which appear to mediate the expression of *Les22* as well as other lesion mimics (Johal et al., 1995; Jabs et al., 1996), fit this proposed role. It is known that the extent to which OFRs may be problematic depends on the balance between their rates of production and removal by various mechanisms (Foyer et al., 1994; Anderson et al., 1995; Lamb and Dixon, 1997). In addition, all cells of a leaf, despite having the same genetic makeup, may not be identical in terms of their developmental or metabolic status and may therefore differ in their ambient redox milieu and thus their sensitivity to damage associated with OFRs.

Besides providing a molecular explanation for the genesis of a dominant lesion mimic mutation and unveiling a natural case of phytoporphyria, this study has a number of other interesting implications. First, this represents a rare case of a mutation of a conserved gene that has similar phenotypic manifestations in both humans and plants. The dominant nature of this defect suggests that the porphyrin pathway, although it is expected to operate in different subcellular locations in plant and human cells (Moore et al., 1987; von Wettstein et al., 1995), may be regulated very similarly in both organisms. Because mutations of most genes of the porphyrin pathway in humans result in porphyria (Moore et al., 1987; Moore, 1993; McCarrol, 1995), it raises the possibility that maize mutations phenotypically identical to *Les22* may also be the result of defects in other genes of the porphyrin pathway. In fact, this seems to be the case. Genetic allelism tests, which can be done with *Les22* because of the ysl phenotype of its homozygotes, have provided evidence that our 17 putative *Les22* mutants (*Les22-1* to *Les22-17*) are actually mutations of three different but genetically linked loci (G. Hu and G.S. Johal, unpublished data).

Second, *Les22* is the only known mutation affecting an enzyme of porphyrin biosynthesis in plants and thus provides an excellent tool for understanding the regulation of chlorophyll and heme production. A wealth of physiological and biochemical evidence indicates that flow of substrates into the porphyrin pathway is controlled by the synthesis of δ–aminolevulinic acid (ALA), the first committed precursor of the porphyrin pathway (von Wettstein et al., 1995; Reinbothe and Reinbothe, 1996). Although light is required to trigger the synthesis of ALA and the differentiation of chloroplasts (Reinbothe and Reinbothe, 1996), a feedback inhibition of ALA synthesis by an end product of the porphyrin pathway is thought to be involved in the regulation of influx into the pathway (von Wettstein et al., 1995; Reinbothe and Reinbothe, 1996). Both the nature of the product and the mechanism involved in effecting feedback inhibition remain unknown, probably because there have been no porphyrin pathway mutants that affect both chlorophyll and heme biosynthesis. Thus, the availability of *Les22* mutants may fill this void and allow researchers to address these questions of long-standing importance.

Third, *Les22* is cell autonomous, visually discernible, and nonlethal, therefore providing an elegant molecular tool to probe into the phenomenon whereby plants keep the activity of transposons in check. The mutant phenotype of *Les22-7*, which is caused by an insertion of *Mu1* in the 5′ end of the *Urod* gene upstream of the translation start site, requires *Mu* activity in the plant. The enigmatic *Mu*-suppression phenomenon of dominant-negative regulation was originally discovered with *hcf106* (Martienssen et al., 1990) and later shown to suppress coordinately both *hcf106* and *Les28* (Martienssen and Baron, 1994). Somehow, it results in the loss of *Mu* activity during development and after inbreeding, even though functionally intact *MuDR* elements are present in the genome. Although loss of *Mu* activity correlates with the methylation of *Mu* elements, the mechanisms involved in the triggering and execution of *Mu* element methylation are currently unknown.

This study illustrates one final point: that the homozygous phenotype of all dominant lesion mimics, which in maize represent 32 of the known 47 mimics (G.S. Johal, unpublished data), should be determined if possible. Although the dominant phenotype (in heterozygous form) of a lesion mimic mutant is unlikely to provide a clue—even a wrong clue—as to where the biochemical lesion might be, knowing its homozygous phenotype might prove to be quite rewarding. As

we have witnessed with *Les22*, the homozygous phenotype may bear no similarity to the heterozygous phenotype.

METHODS

Plant Materials

The origin of the *Les22* maize mutants and the way they were propagated has been described previously (Johal et al., 1994) as well as specified in Results. The *ij1* and *Oy1-700* mutants were provided by M.G. Neuffer (University of Missouri, Columbia).

Cosegregation Analysis and Cloning

Genomic DNA from maize seedlings was extracted by the cetyltrimethylammonium bromide–based method, as described by Hulbert and Bennetzen (1991). DNA gel blot analysis to identify restriction fragment length polymorphism (RFLP) markers and to perform cosegregation analysis was done as described by Gardiner et al. (1993). Cosegregation analysis, to look for *Mutator* (*Mu*) elements linked to various *Les22* mutant alleles, was first performed with pooled (involving at least 15 plants) DNAs from either the mutant or wild-type siblings of each mutant. DNA samples were digested with seven restriction enzymes, and gel blots were hybridized with each of the nine *Mu* elements, as described earlier (Gray et al., 1997). Identification of the *Mu1*-hybridizing, 6.5-kb XhoI restriction fragment, which was detected only from mutant samples of *Les22-7*, was followed by examining its linkage with the *Les22*-7 mutant allele in 69 plants.

The λ ZAPII vector (Stratagene, La Jolla, CA) was used to clone the *Mu1*-containing 6.5-kb XhoI restriction fragment, followed by rescuing of this fragment as a phagemid by use of in vivo excision. The DNA fragment (LF7, ~500 bp long) flanking the left side of the *Mu1* insertion in this clone was amplified using a *Mu*-TIR primer (5′-CGCCAACGCCTCCATTTCGTCGAATCC-3′) and the vector-specific reverse primer (Gray et al., 1997). LF7 was subcloned in the TA Cloning vector (Invitrogen, Carlsbad, CA) and then sequenced. Two oppositely orienting LF7-specific primers were designed that were used to confirm the identity of the cloned fragment as *Les22* by a polymerase chain reaction (PCR)–based method (Gray et al., 1997). These primers were LF7-A, with the sequence 5′-CTTGCCTTCATGTACCTCCCG-3′, and LF7-B, with the sequence 5′-CGGGAGGTACATGAAGGCAAG-3′. The PCR conditions were as described by Gray et al. (1997).

RNA Gel Blot Analysis

For expression analysis, total RNA was extracted from seedlings by using the Trizol reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer's instructions. RNA samples containing ~30 μg of total RNA per lane were electrophoresed through formaldehyde agarose gel, as described earlier (Gray et al., 1997). RNA blots were hybridized with a probe made from the LF7 fragment in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), and 0.1% SDS at 65°C for 18 to 20 hr and washed (three times for 20 min each) in a solution containing 0.2 × SSC and 0.1% SDS at 65°C.

Protein Extraction and Catalase Assay

Total proteins were extracted from maize seedlings, as described previously (Anderson et al., 1995). Protein concentration was quantified using the Bradford (1976) assay with a protein assay kit (Bio-Rad). Catalase activity was assayed according to the method described by Anderson et al. (1995), using 30 μg of total protein per sample. Four units of catalase (Sigma) were used as a positive control.

Uroporphyrin Extraction and Analysis

Uroporphyrin(ogen) and coproporphyrin(ogen) were extracted from 10-day-old maize seedlings obtained from an F_2 population of *Les22-15.* The methods used to extract and analyze these porphyrin intermediates by using HPLC were as described by Mock and Grimm (1997) and Kruse et al. (1995). The entire foliar tissue (pooled) was used for ysl mutants. For *Les22* mutants (heterozygotes), only the second leaf (from the bottom), partitioned into lesion-containing (apical) and lesion-lacking (bottom) parts and pooled from a number of plants, was used. Pooled tissues from wild-type siblings were equivalent to the corresponding tissue from *Les22* mutants.

DNA Sequencing and Analysis

DNA sequences were determined by automated sequencing on an ABI377 sequencer (Perkin-Elmer, Foster City, CA) at the DNA Core Facility of the University of Missouri. DNA sequence analysis was performed using ALIGN and MEGALIGN programs of the DNASTAR software package (DNASTAR Inc., Madison, WI). Searches of the GenBank database were performed using the BLAST WWW server of the National Center for Biotechnology Information (Bethesda, MD).

ACKNOWLEDGMENTS

We thank John Gray for technical assistance and Brent Buckner, Dilbag S. Multani, and John Gray for helpful comments and discussions. This work was supported in part by grants from the National Science Foundation (NSF) and Pioneer Hi-Bred International Inc. to G.S.J. G.H. was supported by a postdoctoral fellowship from the University of Missouri Maize Biology Training Program, a unit of the NSF/Department of Energy/U.S. Department of Agriculture Collaborative Research in Plant Biology Program.

Received February 16, 1998; accepted May 1, 1998.

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