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

Gongshe Hu, Nasser Yalpani, Steven P. Briggs, and Gurmukh S. Johal

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(designated les) and dominant (designated Les), have been identified in maize (Johal et al., 1995; G.S. Johal, unpublished data).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
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 decarboxy-

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.
ylose (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 test 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 tls1 in these mutants; tls1 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 F₂ population (36 plants) derived from the Les22-7 mutant. Contrary to what was thought previously (Johal et al., 1994), densely lesioned F₂ 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 Xhol 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 Xhol 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 Xhol 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-
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 tetapyrrole 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).

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
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 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.

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.
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; McCarron, 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$_2$O$_2$ (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

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

<table>
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<tr>
<th>Tissue</th>
<th>Uroporphyrin III (nmol/g Fresh Weight$^a$)</th>
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<tr>
<td>Wild-type apical</td>
<td>0.259 ± 0.008</td>
</tr>
<tr>
<td>Wild-type basal</td>
<td>0.216 ± 0.005</td>
</tr>
<tr>
<td><em>Les22</em> apical</td>
<td>0.606 ± 0.018</td>
</tr>
<tr>
<td><em>Les22</em> basal</td>
<td>0.476 ± 0.014</td>
</tr>
<tr>
<td>ysl total leaf</td>
<td>14.042 ± 0.421</td>
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$^a$ The data represent the mean of four replications (±SD).
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 etiolog also may 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 phototoxicative damage, as has been witnessed with the developmental course of Les22 lesion expression. In contrast, the influx of metabolites in the porphyrin pathway is tremendousy 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; J. ohal 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 pathways that degrade these signals in adjoining cells. OFRs, which appear to mediate the expression of Les22 as well as other lesion mimics (J. ohal et al., 1995; J. abs 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 phytoporphoryia, 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; McCarron, 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. J. ohal, 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. J. ohal, 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) flankng the left side of the Mu1 insertion in this clone was amplified using a Mu-TIR primer (5’-CCGCAACGCCCTCCATTTCGTCGAATCC-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’-CTTGCCTTCATG TACCTCCGG-3’, and LF7-B, with the sequence 5’-GGGGAGGTACATG AAAGCGGAAG-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

Uroporphyrinogen(ogen) and coproporphyrinogen(ogen) were extracted from 10-day-old maize seedlings obtained from an F2 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 (homozygotes), 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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DOI 10.1105/tpc.10.7.1095

This information is current as of August 14, 2017

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