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Reductase Activity Encoded by the *HM1* Disease Resistance Gene in Maize

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The *HM1* gene in maize controls both race-specific resistance to the fungus *Cochliobolus carbonum* race 1 and expression of the NADPH (reduced form of nicotinamide adenine dinucleotide phosphate)-dependent HC toxin reductase (HCTR), which inactivates HC toxin, a cyclic tetrapeptide produced by the fungus to permit infection. Several *HM1* alleles were generated and cloned by transposon-induced mutagenesis. The sequence of wild-type *HM1* shares homology with dihydroflavonol-4-reductase genes from maize, petunia, and snapdragon. Sequence homology is greatest in the βαβ-dinucleotide binding fold that is conserved among NADPH- and NADH (reduced form of nicotinamide adenine dinucleotide)-dependent reductases and dehydrogenases. This indicates that *HM1* encodes HCTR.

Since the discovery of a race-specific compatibility factor known as HC-toxin (*1*), disease caused by *Cochliobolus carbonum* Nelson race 1 has been the subject of detailed study. The presence of HC toxin permits the fungus to infect certain genotypes of maize (*Zea mays* L.) that would otherwise be resistant. The dominant allele of *HM1* determines resistance to the fungus and reduced sensitivity to HC toxin. The structure of HC toxin is known (*2*), but its mode of action remains to be elucidated (*3*). An enzyme that inactivates HC-toxin has been identified in extracts from maize (*4*). The enzyme, HC toxin reductase (HCTR), is detectable only in extracts from resistant genotypes (*5*). These results raised the question: does *HM1* encode or act as a regulator of HCTR? In this report we describe cloning the *HM1* gene and present evidence that *HM1* encodes HCTR activity.

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Mutant alleles of *HM1* recovered from *Mutator* element stocks (*6*) were characterized. To identify co-segregation between *HM1* and restriction fragments containing a transposable element, the segregating progeny were first classified [with the use of Southern (DNA) blots] according to which *HM1* alleles they had inherited. The blots were hybridized with probes for RFLP loci that flank the locus; *PIO200644* and *PIO200044* map 5 centimorgans (cM) proximal and distal to *HM1*, respectively (*7*). Progeny that inherited intact the 10-cM block of chromosome 1 containing the mutant allele were grouped together, as were progeny that inherited the alternative allele. Recombinants were discarded. The classes were compared with each other on a Southern blot hybridized with a transposable element probe (*8*). Restriction fragments that were common to progeny that inherited the mutant allele and absent from the rest were identified. This method established linkage between restriction fragments containing a transposable element and the 10-cM block that contains the genetic locus *HM1*.

Five mutant alleles (Table 1) were analyzed. The 3.2-kb *Mu1* fragment that co-segregated with *hm1-656::Mu1* (Fig. 1) was cloned (*9*) and used to prepare a probe (designated *656) from the DNA flanking the *Mu1* insertion. The probe was used to map the clone relative to the dominant wild-type allele *Hm1*; no recombinants were observed in 60 backcross progeny. Comparison with the *Hm1-B79* allele revealed that the *Mu1* element had created an Sst I site upon insertion into *Hm1-B79*. The *656 probe was next hybridized to DNA from four different homozygous mutants all derived from the inbred line, B79. The observation of polymorphisms showed that DNA rearrangements were concomitant with mutagenesis. Similar results were obtained with *hm1-1369::Mu3*, in which *Mu3* was found to have inserted only 5 bp from the site of *Mu1* insertion in *hm1-656::Mu1*. We cloned the *hm1-1062::dHbr* allele using *656 as a probe. We mapped restriction sites in both wild-type and mutant alleles and found one restriction fragment length polymorphism. In the mutant allele, that restriction fragment contained a 315-bp insertion (designated "defective Heartbreaker," *dHBr*) with the general characteristics of a transposable element (*10*).

The *Def(HM1)-1790* allele was not transmitted through the pollen and was only poorly transmitted through the egg, typical of a chromosomal deletion. Neither the *1369 nor the *656 probes hybridized

Table 1. Properties of *hm1* alleles.

Allele*	Mutant sector†	Progenitor allele	Insertion element
hm1-656::Mu1	2/253 e	***Hm1-B79***	*Mu1*
hm1-1369::Mu3	1/230 e	***Hm1-B79***	*Mu3*
hm1-1062::dHbr	2/483 t	***Hm1-B79***	*dHbr*
Def(HM1)-1790	1/345 e	***Hm1-B79***	Deletion
hm1-1040::dSpm	63/672 t	***Hm1-4Co63***	*dSpm*

**hm1-1040::dSpm* was recovered from *P-VV/P-WW Hm1-4Co63*, whereas the other alleles arose in selfed *Mutator* plants (*Mutator Mu² per se*) that were crossed with the hybrid tester (K61/Pr) (*19*). †e = ear sector; t = tassel sector; mutants arose in progeny of crosses between plants homozygous for *Hm1* and possessing an active transposable element system and plants that were homozygous for *hm1*.

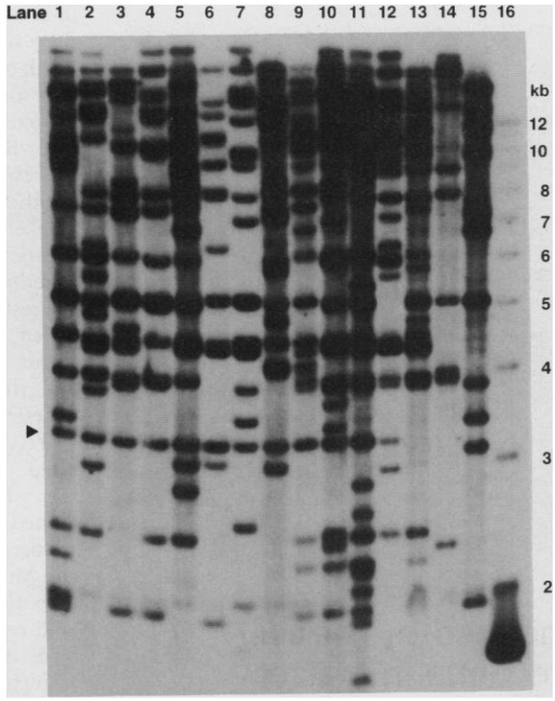

Fig. 1. Co-segregation of a *Mu1*-homologous Sst I restriction fragment with the *hm1-656::Mu1* allele. The progeny were examined on a Southern blot by hybridization with a *Mu1*-specific probe. A 3.2-kb restriction fragment (arrowhead) is evident in all of the progeny that inherited the *hm1-656::Mu1* allele (lanes 1 through 12) and is absent in all of the progeny that inherited the alternative, *hm1-1*, allele (lanes 13 through 15). Lane 16 is a size standard, multiples of 1 kb as labeled.

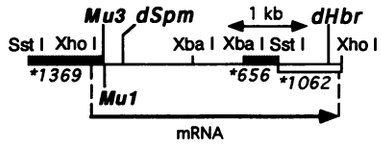

Fig. 2. Restriction map of the *Hm1-B79* allele. Restriction sites are indicated, as is region transcribed (mRNA). Sites of transposable element insertion are labeled with the transposon (*Mu3, Mu1, dSpm, dHbr*). Probes used in these experiments were **1369* (shaded box), **656* (shaded box), and **1062* (open box). Scale represents 1 kb.

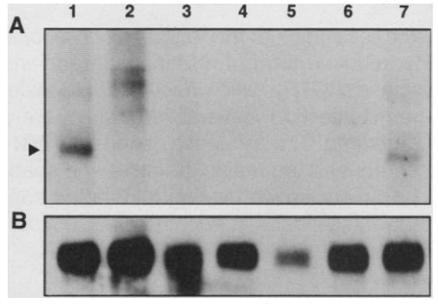

Fig. 3. Northern (RNA) blot of poly(A)$^+$ RNA isolated from etiolated mesocotyls of Pr1 (*Pr1-Hm1*; lanes 1 and 7), K61 (*hm1-2*; lane 2), *hm1-656::Mu1* (lane 3), *hm1-1062::dHbr* (lane 4), *hm1-1369::Mu3* (lane 5), *hm1-1040::dSpm* (lane 6). (**A**) An autoradiogram of the blot after it was hybridized with the **1062* probe and exposed to film for 7 days. Arrowhead indicates the wild-type 1.3-kb mRNA. (**B**) Autoradiogram of the same blot after it was stripped, hybridized with a probe for actin mRNA (*20*), and exposed to film for 12 hours.

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001 MAEKESNGVR VCVTGGAGFI GSWLVRKLLE KGYTVHATLR
041 NTGDEAKAGL LRRLVPGAAE RLRLFQADLF DAATFAPAIA
081 GCQFVFLVAT PFGLDSAGSQ YKSTAEAVVD AVRAILRQCE
121 ESRTVKRVIH TASVAAASPL LEEEVSASGV GYRDFIDESC
161 WTSLNVDYPL RSAHFDKYIL SKLRSEQELL SYNGGESPAF
201 EVVTLPLGLV AGDTVLGRAP ETVESAVAPV SRSEPCFGLL
241 RILQQLLGSL PLVHVDDVCD ALVFCMERRP SVAGRFLCAA
281 AYPTIHDVVA HYASKFPHLD ILKETEAVAT VRPARDRLGE
321 LGFQVPSTAW EEILDSSVAC AARLGSLDAS KLGLQKG
```

Fig. 4. Deduced amino acid sequence of the cloned cDNA from *Hm1-B73*. Underlined letters indicate amino acid identity (30% identity, 54% similarity determined using the GAP program of the GCG Package, Genetics Computer, Inc.) with the dihydroflavonol-4-reductase encoded by the *pallida* gene of *Antirrhinum majus* (L.) (*21*; GenBank accession number X15536). The nucleotide sequence of *Hm1-B73* has been deposited in GenBank (accession number L02540). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

with DNA from the *Def(HM1)-1790* mutant, confirming that the cloned region lies within the deletion. Test crosses with *br2* (a dwarfing mutation), which maps within 0.1 cM of *HM1* (*11*), and hybridization with RFLP probes *PIO200644* and *PIO200044* indicated that the deletion cannot encompass more than 5 cM of the chromosome within the region delimited by *br2* and one of the RFLP loci. The *hm1-1040::dSpm* allele was cloned using the **1369* probe and found to contain a *dSpm* insertion (Fig. 2).

A 1.3-kb RNA band was detected in polyadenylated [poly(A)$^+$] RNA from the resistant inbred strain Pr1 (*Hm1-Pr1*) (Fig. 3) (*12*). The susceptible strain, K61 (*hm1-2*), and the mutants either had no detectable hybridizing mRNA, or an mRNA of aberrant size resulting from hybridization to the *dHbr* sequence in the **1062* probe. Thus, resistance appears to differ from susceptibility at the transcriptional level. This makes it unlikely that susceptible genotypes possess an alternative form of *HM1* with specificity for a substrate other than HC toxin. The only function of *HM1* in young leaf tissue seems to be to provide resistance because *HM1* mutations are not obviously pleiotropic.

A 1.6-kb cDNA clone was isolated (*13*) by homology with the **1062* and **1369* probes and sequenced. The GenBank and EMBL DNA sequence databases revealed homology between the *HM1* cDNA and the NADPH-dependent dihydroflavonol-4-reductase (DFR) genes of maize (*Zea mays*), petunia (*Petunia hybrida*), and snapdragon (*Antirrhinum majus*) (Fig. 4). Homology was greatest in the region corresponding to the βαβ-dinucleotide binding fold of NADPH- and NADH-dependent reductases and dehydrogenases (that is, amino acids 16 through 32) (*14*). This homology supports the prediction that *HM1* encodes HCTR.

The cDNA clone that we isolated contains an intron. Translation of the cDNA sequence revealed stop codons in all three reading frames. The predicted translation product in frame 1 was homologous to DFRs at the 5′ end only, whereas the product in frame 2 was homologous at the 3′ end only. Between these two regions of homology is a 286-bp sequence that is flanked by consensus 5′ and 3′ mRNA splice sites. The predicted sequence that would result from splicing out this region is without stop codons, has homology to the DFRs throughout its length, and is the same size as the mRNA identified by hybridization to the **1062* probe on Northern blots. To confirm that the cloned cDNA contained an unspliced intron, reverse transcription coupled with the polymerase chain reaction (RT-PCR) was performed with RNA isolated directly from seedlings of line B73; the PCR primers were within the exons that flanked the putative intron (*15*). A 408-bp product corresponding in sequence to the predicted mature (spliced) mRNA was produced. A larger product (694 bp) corresponding to the unspliced cloned cDNA was seen only when the cloned cDNA was used as the template. The presence of an intron in this cloned polyadenylated message may represent an alternate end product of splicing or a splicing intermediate.

The discovery that *HM1* encodes a reductase that inactivates HC-toxin establishes a mode of action for plant disease resistance genes that may be widespread in

nature. Microorganisms have used antibiotic inactivation to overcome natural (*16*) and drug-related defenses of the host (*17*). The mechanism of resistance encoded by *HM1* demonstrates that the same strategy can be used by hosts to block microorganism growth. Genes that encode resistance to specific diseases may be engineered in the laboratory, as has been done for a virulence factor (*18*).

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9. DNA isolated from homozygous mutant seedlings was digested with Sst I or Xho I and the appropriate sized DNA fragments (as judged from the Southern blots) were purified by preparative gel electrophoresis and electroelution into dialysis tubing. This purified DNA was ligated to preannealed Sst I or Xho I cut arms from the bacteriophage vector λ *sep6-lac5* [E. Meyerowitz and D. Hogness, *Cell* **28**, 165 (1982)]. Packaging into Gigapack Gold (Stratagene) and screening of the libraries were carried out according to the manufacturer's instructions. All clones were subcloned into Bluescript SK$^+$ (Stratagene) and maintained in the SURE strain (Stratagene) of *Escherichia coli*.
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Structure of a Fibronectin Type III Domain from Tenascin Phased by MAD Analysis of the Selenomethionyl Protein

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Fibronectin type III domains are found in many different proteins including cell surface receptors and cell adhesion molecules. The crystal structure of one such domain from the extracellular matrix protein tenascin was determined. The structure was solved by multiwavelength anomalous diffraction (MAD) phasing of the selenomethionyl protein and has been refined to 1.8 angstrom resolution. The folding topology of this domain is identical to that of the extracellular domains of the human growth hormone receptor, the second domain of CD4, and PapD. Although distinct, this topology is similar to that of immunoglobulin constant domains. An Arg-Gly-Asp (RGD) sequence that can function for cell adhesion is found in a tight turn on an exposed loop.

Domains with amino acid (aa) sequence similarity to the type III repeats of fibronectin (FN-III domains) are found in a wide variety of proteins including adhesion molecules, cytokine receptors, muscle-related proteins, collagens, and other extracellular matrix (ECM) proteins. Although most of these ~90-aa domains have no established function, certain of them act as ligands or receptors at the cell surface. For example, the tenth FN-III repeat of fibronectin (FNfn10) interacts with integrins through its RGD sequence motif (*1*). The interaction between fibronectin and integrins is believed to anchor cells to the ECM and may also provide cells with important environmental cues.

Tenascin is a large ECM protein made up of six identical subunits in a hexabrachion structure [reviewed in (*2*)]. Each subunit comprises a string of small, globular domains, including 8 to 15 FN-III domains. Tenascin has a specific pattern of expression during embryonic development and is abundant in many tumors, but it is present in restricted locations in normal adult tissues. The functions of tenascin are still unclear, but roles in tissue growth and restructuring are suggested. The third FN-III domain (TNfn3) of both human and chick tenascin has an RGD sequence in the same location as that in FNfn10. Although the cell adhesion activity of native tenascin is still controversial (*2*, *3*), the isolated TNfn3 domain promotes strong adhesion and spreading of endothelial cells, apparently

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