A guardian of grasses: Specific origin and conservation of a unique disease-resistance gene in the grass lineage

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The maize *Hm1* gene provides protection against a lethal leaf blight and ear mold disease caused by *Cochliobolus carbonum* race 1 (CCR1). Although it was the first disease-resistance (DR) gene to be cloned, it remains a novelty because, instead of participating in the plant recognition and response system as most DR genes do, *Hm1* disarms the pathogen directly. It does so by encoding an NADPH-dependent reductase, whose function is to inactivate *Helminthosporium carbonum* (HC) toxin, an epoxide-containing cyclic tetrapeptide, which the pathogen produces as a key virulence factor to colonize maize. Although CCR1 is strictly a pathogen of maize, orthologs of *Hm1* and the HC-toxin reductase activity are present in the grass family, suggesting an ancient and evolutionarily conserved role of this DR trait in plants. Here, we provide proof for such a role by demonstrating its involvement in nonhost resistance of barley to CCR1. Barley leaves in which expression of the *Hm1* homologue was silenced became susceptible to infection by CCR1, but only if the pathogen was able to produce HC toxin. Phylogenetic analysis indicated that *Hm1* evolved exclusively and early in the grass lineage. Given the devastating ability of CCR1 to kill maize, these findings imply that the evolution and/or geographical distribution of grasses may have been constrained if *Hm1* did not emerge.

host–pathogen specificity | nonhost resistance | host-specific toxin | *Cochliobolus* | *Helminthosporium carbonum* toxin reductase

An important goal of research on disease is to establish how specificity, a hallmark of most infectious diseases, is established. A given pathogen normally can cause disease in only a single or limited number of species, while the species outside of its host range are immune to it. Although it remains largely unknown how such specificity is established in most plant diseases, one exception is provided by fungal pathogens that use host-specific (host-selective) toxins (HSTs) as key mediators of pathogenicity (1, 2). Most HSTs are low-molecular-weight fungal metabolites that inflict damage on only those plants that are susceptible to the producing pathogen. HSTs facilitate disease development in a genotype-specific fashion, thus determining both the host range of the pathogen and the specificity of the disease. It is generally assumed that the presence of a corresponding molecular target in the host and not in nonhost species underlies all cases of HST selectivity (1, 2).

A classic example of a plant disease involving an HST is that of the maize leaf spot and ear mold (3). This lethal disease is caused by *Cochliobolus carbonum* race 1 (CCR1), a fungal ascomycete whose asexual form (anamorph) is known as *Helminthosporium carbonum* (HC) (synonym *Bipolaris zeicola*). CCR1 is among the most destructive pathogens of maize. It can kill susceptible maize plants at any stage of development (Fig. 1) (4). Unlike most other plant pathogens, CCR1 can invade every part of the host, causing blight of the leaves, rot of the roots and the stalk, and mold of the ear (Fig. 1 *A–H*).

The ability of CCR1 to cause disease depends on two conditions. The first requires the host to lack *Hm1*, a disease-resistance (DR) gene present almost ubiquitously in maize (5, 6). Second, CCR1 must produce HC toxin (3), an archetypal HST of the structure cyclo(D-Pro-L-Ala-D-Ala-L-2-amino-8-oxo-9,10-epoxy decanoic acid) (Fig. 1*I*) (7–10). Genetic variants of CCR1 that lack the ability to produce HC toxin are nonpathogenic and unable to invade beyond the penetration site (Fig. 1*J*). However, they do resume growth and colonization of the host tissue if HC toxin is administrated exogenously to the infection site (11). Maize lines that are resistant to CCR1 are tolerant of HC toxin compared with susceptible lines (11). On these resistant lines, CCR1 is contained at the infection site in the same fashion as the HC-toxin-deficient CCR1 does in a susceptible host (Fig. 1*J*).

Interestingly, the *Hm1*-based resistance mechanism that evolved naturally in maize targeted HC toxin for detoxification (12). It is mediated by an enzyme encoded by *Hm1* (13). Named HC-toxin reductase (HCTR), this enzyme is an NADPH-dependent reductase that bears significant homology to dihydroflavonol reductase (DFR) involved in the biosynthesis of flavonoids and anthocyanins throughout the plant kingdom. In addition to the *Hm1* gene, which confers complete protection in every part of the plant, certain lines of maize contain a second DR gene *Hm2*, which confers effective resistance only in adult plants (5, 6). The cloning of *Hm2* has demonstrated that it encodes a structural, albeit truncated, duplicate of HM1 [supporting information (SI) Fig. 5] (36).

Like most pathogens of plants, CCR1 exhibits a high degree of host specificity and can cause disease only in maize. All other plant species, including those that are closely related to maize, are virtually immune to CCR1. Notwithstanding the absolute requirement of maize as a host for CCR1, all grass species tested possess candidate genes or sequences with high homology to *Hm1*. These include sorghum, rice, barley, wheat, rye, oats, millet, fescue, bluegrass, reed canarygrass, and bamboo (5, 14). In barley, rice, and sorghum, these homologs are syntenic with that of the maize *Hm* genes (5, 14), indicating that they are truly orthologous and derived from a common ancestor. HCTR activity has also been detected in all grasses tested, including barley, wheat, sorghum, rice, and oats, implying that these genes are not relics of the past but still maintained functionally in

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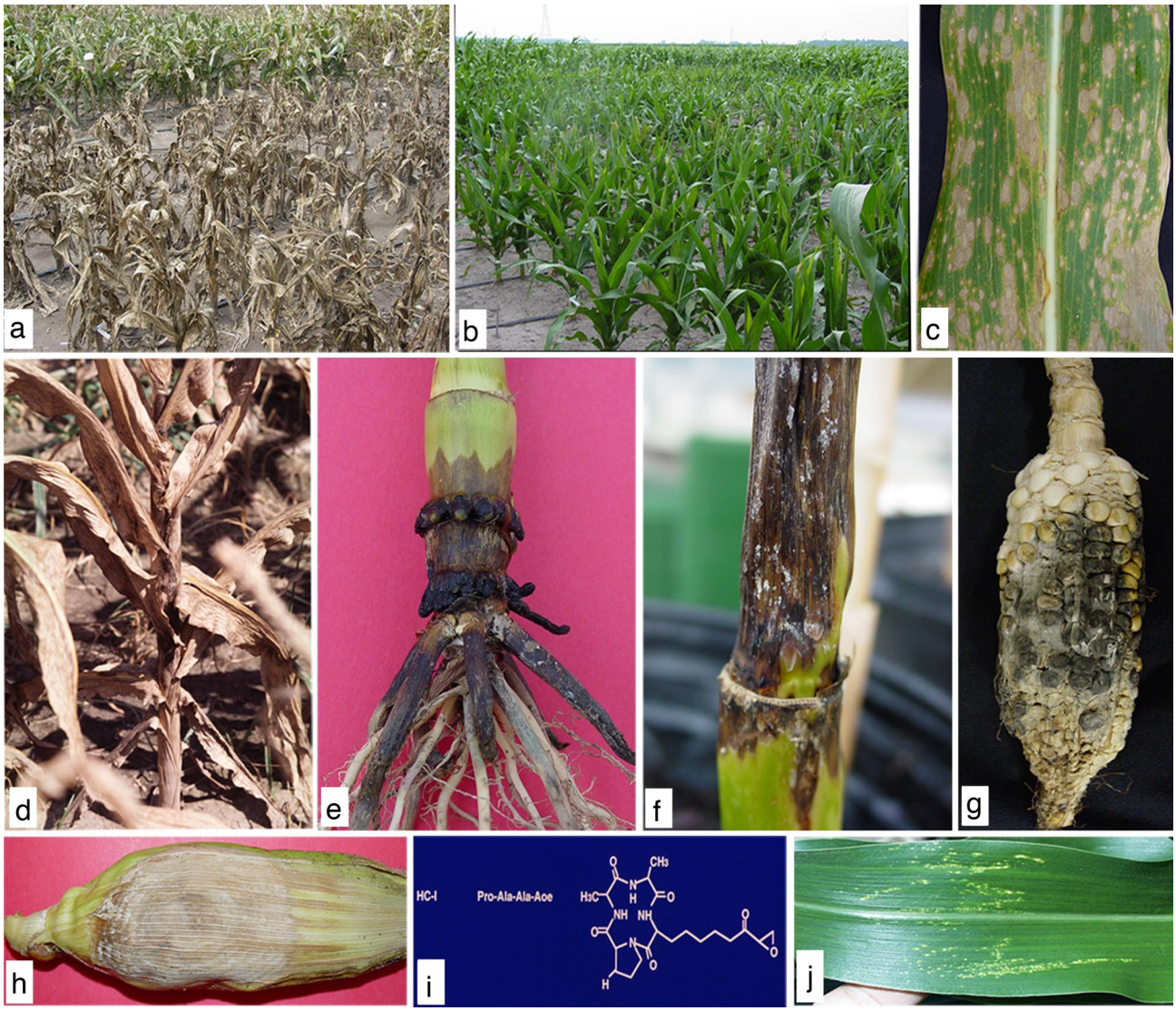

Fig. 1. Exceptional ability of CCR1 to invade and devastate susceptible maize. (*a*) A field of maize plants killed after infection with CCR1. (*b*) The same field ≈4 weeks earlier at the time of inoculation. (*c–g*) CCR1 can invade every part of the maize plant, causing spots on leaves (*c*), blight of the foliage (*d*), and rot of the roots (*e*), the stalk (*f*), and the ear (*g*). (*h*) Aerial hyphae are often produced on infected tissues if climate is warm and humid. (*i*) Structure of HC toxin. (*j*) Typical size and appearance of leaf lesions incited by CCR1 if it lacks the ability to produce HC toxin or the host harbors HCTR to degrade HC toxin.

nonmaize cereals (10, 15). In contrast, no HCTR activity, nor the sequences that could be considered truly homologous to *Hm1*, has been detected outside of the grass lineage. This includes the model dicot *Arabidopsis*, whose genome has been sequenced fully. These results and observations suggest that the need to detoxify HC toxin probably arose only in grasses.

The widespread presence of HCTR-encoding sequences in the grass family raises the question: Why are functional homologs of *Hm1* present and maintained in plant species that are outside of the host range of CCR1? One possibility is that HCTR performs another essential function, in addition to reducing HC toxin. In this scenario, the ability of HCTR to inactivate HC toxin would only be incidental, happening entirely by serendipity. No evidence exists yet to support such a role for HCTR in plants, although it was reported recently that a rice homolog of *Hm1* was able to protect plants from multiple stresses when overexpressed ectopically as a transgene (16, 17). An alternative hypothesis for the ubiquitous presence of *Hm1* in all grasses is that they all perform the same function as the maize *Hm1* gene and serve to guard their hosts against a pathogen such as CCR1. The results presented here support such a role for *Hm1* and show that that the HCTR function evolved exclusively to contend with HC toxin.

Results and Discussion

Conserved Role of *Hm1* in Barley. To address the possibility that the threat imposed by CCR1 is responsible for the conserved maintenance of HCTR across all cereals, we took advantage of barley as an experimental system. This decision was dictated by two criteria. First, the barley *Hm1* homologs were already cloned and characterized, at least from the line Morex, which was found to harbor a tandemly duplicated pair of transcriptionally active *Hm1* genes (14). Second, virus-induced gene silencing (VIGS) has been developed as a reverse genetics tool for functional characterization of genes in this cereal (18), including those involved in DR (19). The tripartite genome of the barley stripe mosaic virus (BSMV) has been harnessed as a vector to implement VIGS in this cereal (18). The line that responds the best to VIGS in barley is Black Hulless. To examine whether it contains one or more copies of *Hm1*, sequences corresponding to this

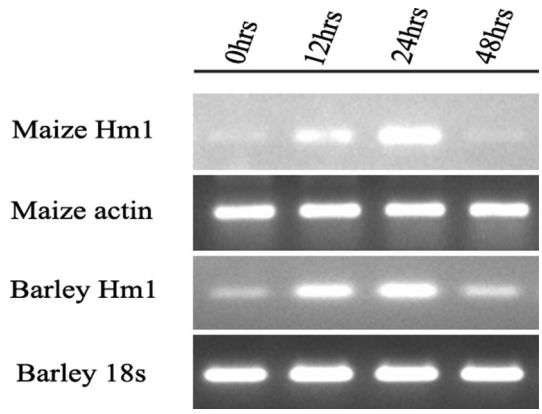

Fig. 2. Expression of the maize and barley *Hm1* genes in response to CCR1 infection. RT-PCR amplification of total RNA isolated from maize and barley seedlings was conducted at different times after inoculation with CCR1. The controls (maize *actin* and barley *18S* genes) show equal amplification in all samples.

gene were PCR-amplified and sequenced. The results obtained showed that *Hm1* exists as a single copy gene in Black Hulless, which, like the Morex homologs, encodes a peptide that is 72% identical and 85% similar to that of maize HM1 (14) (SI Fig. 6).

The transcriptional behavior of the barley *Hm1* gene was examined in response to inoculation with CCR1 and compared with that of the maize *Hm1* gene (Fig. 2). Interestingly, both the maize and barley genes behave in a similar manner in response to infection by CCR1. Although both are constitutively expressed at very low levels in seedling leaves, their transcript levels rapidly and transiently increase many fold in response to infection. This increase is detectable by 12 h after infection (hai), reaches peak levels ≈36 hai, and begins to decline by 48 hai. Because the transcriptional activity of a gene often corresponds to its biological function, these results strengthened the hypothesis that the function of the barley *Hm1* was the same as that of the maize *Hm1*, i.e., to negate the disease-inducing ability of HC toxin.

To silence the barley *Hm1* by VIGS, three separate constructs were made containing different parts of the barley *Hm1* gene in the BSMV vector (Fig. 3*a*). These constructs were used individually and in combination with the *phytoene desaturase* (*Pds*) gene to infect leaves of 7-day-old barley seedlings. Silencing of *Pds*, which results in photobleaching of the affected tissue (18, 20), was used as a visible marker for VIGS. Plants infected with the *Pds* construct alone (BSMV:Pds) or an empty vector (BSMV:00) served as controls.

A week after infection with BSMV vectors, the plants were challenged with either the wild-type (HC toxin-producing) or the HC toxin-nonproducing isolate of CCR1. Similar to the reaction of resistant maize, BSMV:00 plants inoculated with either isolate of CCR1 responded by producing minute chlorotic flecks or lesions at the site of infection (Fig. 3*b*). Microscopic examination of the interaction revealed that pathogen behavior and growth were similar. For instance, the fungus germinated and produced appressoria within 8–10 h after inoculation, allowing the pathogen to directly penetrate epidermal cells (Fig. 3*h*). However, subsequent growth of the pathogen ceased, and the pathogen was contained within the chlorotic fleck. The BSMV:Pds plants responded similarly to pathogen inoculation, as did the empty vector controls, and restricted the pathogen to the infection site (Fig. 3*c*).

By contrast, plants whose *Hm1* gene was silenced exhibited spreading disease lesions when their leaves were sprayed with the HC toxin-producing isolate of CCR1 (Fig. 3 *d–f*). These lesions

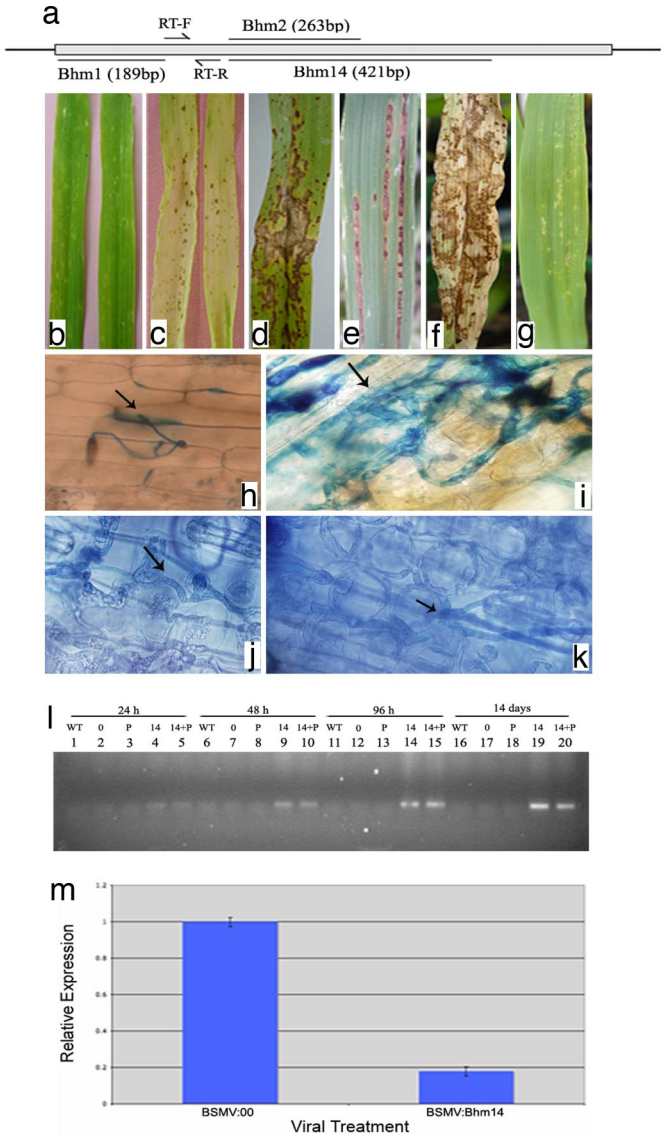

Fig. 3. VIGS-mediated suppression of *Hm1* results in barley susceptibility to CCR1. (*a*) Schematic representation showing the size and relative locations of barley *Hm1* fragments designed to produce VIGS vectors. (*b* and *c*) Typical appearance of lesions caused by CCR1 on normal barley leaves (*b*) or leaves in which the *Pds* gene is suppressed via VIGS (*c*). (*d–f*) CCR1-caused expanding lesions on barley leaves in which *Hm1* was suppressed alone (*d* and *e*) or in combination with *Pds* (*f*). (*g*) Resistant lesions of *Hm1*-suppressed barley to an HC toxin-deficient isolate of CCR1. (*h–k*) Microscopic demonstration showing that while CCR1 fails to grow beyond the site of penetration in normal barley leaves (*h*), it is able to grow and invade substantially inside the *Hm1*-suppressed barley leaves (*i–k*). Arrows indicate an appressorium (*h*) and fungal hyphae (*i–k*) ramifying through the barley leaf tissue. (*l*) Increase in CCR1 biomass over time in *Hm1*-suppressed barley leaves as demonstrated by PCR amplification of its 5.8S rRNA ITS sequence. WT indicates no viral infection; others denote infection with BSMV:00 (0); BSMV:Pds (P); BSMV:Bhm14 (14); and BSMV:Pds plus BSMV:Bhm14 (14+P). (*m*) Real-time RT-PCR of barley *Hm1* transcripts after infection of WT and *Hm1*-suppressed leaves with CCR1. (Magnification: *h*, ×20; *i–k*, ×100.)

continued to enlarge over time and coalesced to cause extensive tissue damage typical of symptoms associated with maize leaf blight (Figs. 3 *d–f* and 1*c*). Microscopic analysis indicated that the pathogen continued to invade after penetration and grew through barley epidermal and mesophyll cells as it does in maize (Fig. 3 *i–k*). This resulted in substantial growth of the pathogen,

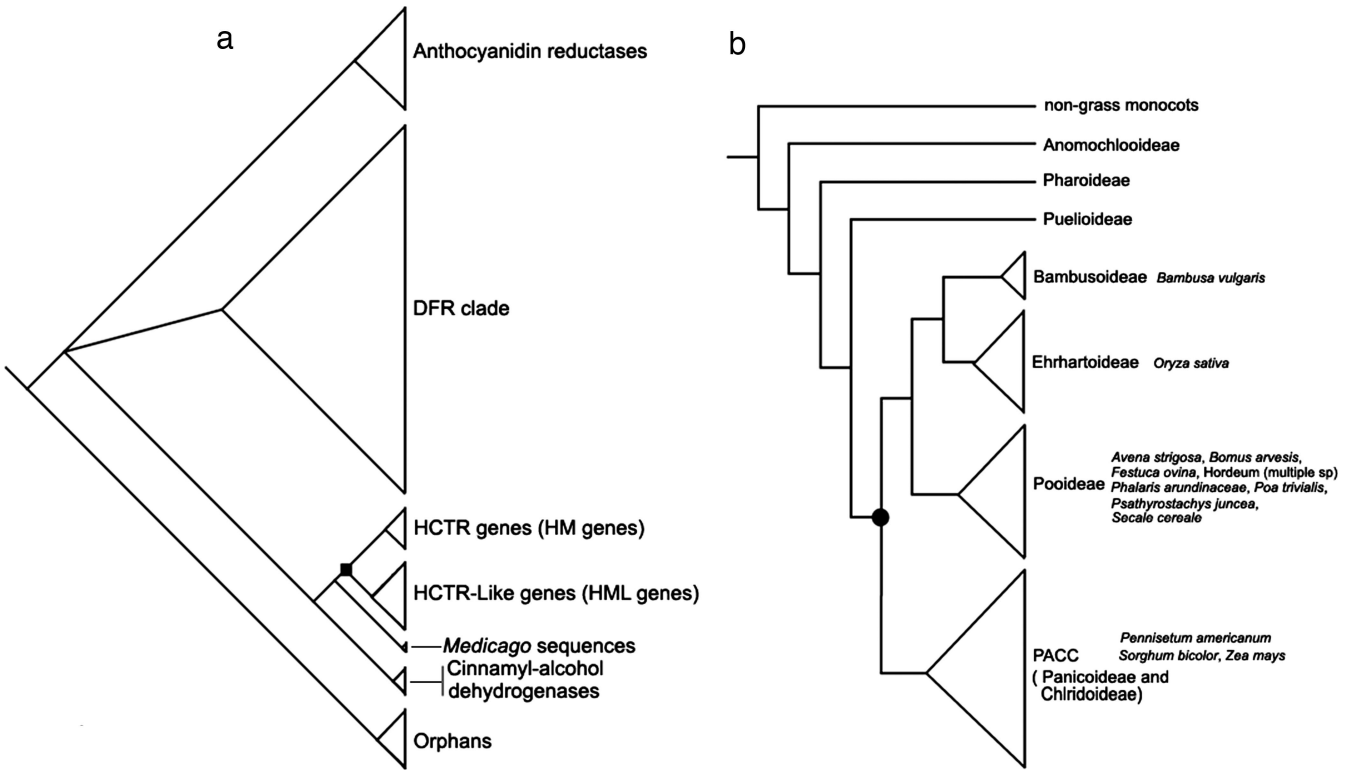

Fig. 4. Evolutionary origin of *Hm1*. (*a*) Summary phylogenetic tree, based on a Bayesian analysis of DNA sequences. This tree shows relationships between *Dfr*, Anthocyanidin reductases, *Cad*, HCTR (*Hm1 and Hm2*), and HCTR-like (*Hml*) genes. Both Monocot and eudicot sequences are found in the *Dfr*, Anthocyanidin reductases, and *Cad* clades. Two sequences from *Medicago* are sister to the HCTR and HCTR-like clades. The black square indicates gene duplication event resulting in the grass-specific HCTR and HCTR-like gene sequences. (*b*) Grass phylogeny [modified from Barker *et al.* (26)]. Listed are species for which HCTR genes have been sequenced or detected by Southern blot hybridization (14). The black dot indicates theb minimum point at which HCTR gene function may have originated, although the function may be found in all grasses.

as is also evidenced by a PCR-based fungal mass analysis of CCR1 (Fig. 3*l*) (21). We were able to reisolate CCR1 from surface-sterilized barley leaves containing disease lesions, and these isolates retained full pathogenicity toward susceptible maize.

All three BSMV:Hm1 constructs were equally effective in suppressing barley resistance to CCR1, indicating that the VIGS studies are reproducible and that susceptibility to CCR1 is not a result of off target silencing. Thus, CCR1 has the ability to colonize barley, provided its *Hm1* gene is silenced. But this happens only if the *Hm1*-silenced plants are challenged with an HC toxin-producing isolate of CCR1; HC toxin-deficient isolates fail to extend beyond the site of penetration regardless of whether *Hm1* is silenced or not (Fig. 3*g*). These results are consistent with a specific role of the barley *Hm1* homolog against HC toxin. To address whether the loss of resistance to CCR1 was, in fact, the result of down-regulation of the *Hm1* gene, the expression of this gene was examined by real-time PCR in the silenced tissue after infection of barley leaves with BSMV:Bhm14. As expected, the expression of *Hm1* significantly decreased in silenced leaves (Fig. 3*m*), providing a cause and effect relationship between the suppression of *Hm1* and the induction of susceptibility to CCR1.

These results provide compelling evidence for the role of *Hm1* in nonhost resistance of barley to CCR1. When the function of this gene is blocked, barley becomes susceptible to CCR1. This is essentially what happened in maize naturally. Like all plants, maize initially was not a host of CCR1. However, when mutant alleles of both *Hm1* and *Hm2* assorted together during inbred development, the resulting lines were susceptible to CCR1 (5). This led to the genesis of a new disease of maize by a pathogen that was not previously known to exist (4, 5, 22). The present study sheds light on this subject and implies that the breakdown of HCTR-based resistance in maize unveiled an ancient case of parasitism in which HC toxin played a decisive role. Although we may never know the identity of the pathogen that caused this disease of the past and forced the grass lineage to acquire *Hm1*, it is feasible that it was either CCR1 itself or its immediate ancestor. This notion is based on the fact that CCR1 is the only pathogen known to produce HC toxin (10), and it also exhibits the ability to colonize dead or senescing tissues of a wide range of grasses (23, 24).

***Hm1* Evolved Exclusively in Grasses.** The conserved structure and function of *Hm1* in maize and barley predict the existence of this DR gene in the grass family before its radiation into major cereals 50 million to 60 million years ago (25, 26). To gain an insight into the origin and subsequent evolution of *Hm1*, we constructed a large data set consisting of representatives of HCTR (*Hm1* and *Hm2*), *Hml*, *Dfr*, *Cad*, and other related flavonoid biosynthesis genes from a range of land plant species (SI Fig. 7). A Bayesian phylogenetic analysis revealed the following about the evolutionary history of *Hm1* and its paralogs or homeologs. First, *Hm1* is an ancient gene preserved in all major grasses. It had a single (monophyletic) origin rather early in the evolution of the grass lineage (Fig. 4). Second, the HCTR gene family represents a distinct lineage separate from the *Hml* clade, both of which are present exclusively in grasses. This clearly contrasts with all other genes included in the analysis, which are represented widely throughout the plant kingdom. Third, *Hm2* emerged specifically in the maize lineage probably

as a result of a whole genome duplication event that occurred in this lineage after divergence from the sorghum lineage (Fig. 4).

Fourth, this analysis led to the identification of an additional *Hm1*-like (*Hml*) gene whose encoded peptide bears >70% identity with HM1 and HM2 (SI Fig. 5). *Hm1* and *Hml* appear to have resulted from a duplication event before the radiation of major clades of grasses (Fig. 4) (26). The exact placement of this duplication event remains unresolved, as does the question of which evolved first, *Hm1* or *Hml*. Interestingly, the *Hml* clade has undergone significant expansion in rice (SI Fig. 7). *Hml* maps to an area of the genome (chromosome 7S) that has never been shown to be associated with resistance to CCR1, making it unlikely to encode HCTR. The function of the *Hml* gene remains to be addressed.

Implications. The maintenance of HCTR gene function in maize and barley, coupled with the unique phylogenetic position of the *Hm1* gene (with no closely related orthologs in eudicots), suggests that *Hm1* may have played a critical role in the evolution of most of our cereal crops. Given the devastating potential of CCR1 to kill susceptible corn, it is likely that this fungus or its ancestral form would have threatened the existence of grasses, or at least severely constrained their geographical distribution, had *Hm1* not evolved to detoxify HC toxin. Thus, it seems likely that *Hm1* served as a guardian of the grass family, allowing it to survive, thrive, and evolve into crops that feed the world.

In addition to revealing the antiquity and ubiquity of the *Hm1*-encoded DR function in grasses, this study has broad implications for the field of plant pathology. The first concerns the definition of HC toxin as an HST. Our results show that HC toxin is not a HST, as is traditionally assumed. Rather, it is the malfunction of *Hm1* that renders HC toxin host-specific. Consistent with this idea are the findings that all plant histone deacetylases, which appear to be the targets of HC toxin's action, are sensitive to HC toxin (10, 27). However, this raises another question: Why are dicots that do not possess HCTR activity resistant to CCR1? Second, this study helps establish a paradigm for nonhost resistance in plants and shows that this kind of resistance, which often exists at the species level, could result from the operation of an active, evolutionarily conserved DR mechanism. In this regard, *Hm1* can be viewed as a nonhost resistance gene that protects the entire grass family from CCR1. Finally, the success of *Hm1* in keeping a deadly pathogen like CCR1 in check since the dawn of grasses provides a good example for designing similar protection strategies against other diseases.

Materials and Methods

Plant and Fungal Materials. Maize and barley seedlings were grown and maintained in the greenhouse as described (5, 20). The maize line used for expression analysis was B73. Fungal strains were cultured and maintained as described (5).

VIGS Experiments. Three separate fragments, Bhm1 (189 bp), Bhm2 (263 bp), and Bhm14 (431 bp), were used to silence the barley *Hm1* gene. These were generated by PCR amplification from barley genomic DNA as follows: Bhm1 was amplified with the forward primer 5′-TCG AGG CAG GCT ACA CCG TCC-3′ and reverse primer 5′-TGG CGA CGA GGA AGA CGA AGT GG-3′. Bhm2 was amplified with the forward primer 5′-TCA TCT CTG AAT CTT GTT GGA CTC-3′ and reverse primer 5′-GAT CCT CGG CAG CAT GAA G-3′. Amplification of Bhm14 was accomplished by using the same forward primer as for Bhm2 but the reverse primer was 5′-TAC TTG CTG CCG TAG TGG TCC AAG-3′. All of these fragments were cloned into the BSMV vector as described (20). The silencing control construct (BSMV:Pds) contained an 185-bp fragment of the *Pds* gene (18, 20). *In vitro* transcription of viral RNAs and subsequent inoculation of 7-day-old barley seedlings was done as described (20). Challenge inoculations were carried out by spraying seedlings with either an HC toxin-producing or -nonproducing isolate of CCR1. Inoculated leaves were sampled daily for microscopic observations and stained with trypan blue in lactophenol before visualization (11). Pictures were taken with an Olympus BX41 with DP70 digital camera. For fungal biomass analysis, CCR1-specific primers and PCR protocols were used as described (21). All VIGS experiments involved a minimum of eight plants, and each experiment was performed at least three times.

RNA Analysis. Total RNA from maize and barley leaves was extracted by using TRIZOL (Invitrogen) and treated with RNase-free DNase I (Promega). For analysis by RT-PCR, 0.2 μg of total RNA of each of maize and barley was reverse-transcribed to generate first-strand cDNA by using the one-step RT-PCR kit (Qiagen) in accordance with the manufacturer's instructions. PCR conditions were 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s (34 cycles for the *Hm1* genes and 28 cycles for the control genes). The primers *Hm1F* (5′-CGATCGCTGGGTGCCAGTTC-3′) and *Hm1R* (5′-TGAAGTCTCTGTACCCGACG-3′) were used to amplify the maize *Hm1* transcript. The primers *ActinF* (5′-GCATCCTGACACTGAAGTAC-3′) and *ActinR* (5′-GATAGCAACATACATTGCTGG-3′) were used to amplify the maize *Actin* gene transcript as a control. The barley primers used to amplify the *Hm1* transcript were RT-F (5′-TTC GTC TTC CTC GTC GCC AAC-3′) and RT-R (5′-CGT CCT AGA CTC CGC GCA TA-3′), and the *18S* control primers were the same as before (20). Real-time RT-PCR quantification of the Barley *Hm1* expression in VIGS-silenced leaves was performed at 36 h after CCR1 inoculation as described (20). The experiment was replicated three times, and each replicate contained an RNA pool of four plants infected with either BSM:00 or BSMV:Bhm14.

Molecular Cloning and Analysis. DNA was extracted from maize seedlings as described (5). The maize *Hm2* gene was cloned as overlapping PCR fragments from a maize tester homozygous for this allele by using the following primer sets: *Hm2F1*, 5′-TCGCAGAAACCGGATTAGTGGGTA-3′ and *Hm2R1*, 5′-TTTGTACCCATCGCCGGAACC-3′; *Hm2F1*, 5′-AGTGCCCTAGTCCATCGAGTAGCA-3′ and *Hm2R2*, 5′-TAGTGGTCCGTGATGTCGTGGATG-3′; *Hm2F3*, 5′-CCTTCGCAGCGCACACTTCGA-3′ and *Hm2R3* 5′-AGTGGTTCTGCTTGGTTGAAAGGA-3′. PCR conditions were as described (5). The PCR amplicons were subcloned into the pGem-T easy vector system (Invitrogen), and three separate clones were sequenced at the Purdue Genomic Facility. *Hml* was isolated from a genomic library of B73 as described (28). *Hml* was mapped to the maize chromosome 7 by using oat-maize addition lines DNA (29). The barley *Hm1* homolog was cloned by RT-PCR from total RNA isolated from Black Hulless seedling as described above. The primers were: *BHm1F* (5′-CGATCGCTGGGTGCCAGTTC-3′) and *BHm1R* (5′-TGAAGTCTCTGTACCCGACG-3′).

Phylogenetic Analysis. Sequences for phylogenetic analysis were obtained from GenBank and PlantGDB (30, 31). Bayesian phylogenetic analyses were conducted by using MrBayes version 3.1.2 (32, 33). MrModeltest v. 2.2, with Akaike Information Criterion and hierarchical likelihood ratio testing, was used to determine the best-fitting model of molecular evolution, GTR + I + Γ (34, 35). Two independent runs were done with MrBayes. Using random starting trees, Markov Chain Monte Carlo using three heated chains and one cold chain and a flat Dirichlet prior on nucleotide frequencies and relative rate parameters, the Bayesian analysis was run for 1,000,000 generations sampling every 100 generations, resulting in 10,000 trees. Chain stationarity was achieved after 154,500 generations. A total of 8,455 trees were used to create a 50% majority rule consensus tree; the percentage of times clades occurring in this sample of trees reflects clade posterior probability values.

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