Evolution and Regulation of a Large Effector Family of *Pyricularia oryzae*

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Plant pathogen effectors play important roles in parasitism, including countering plant immunity. However, investigations of the emergence and diversification of fungal effectors across host-adapted populations has been limited. We previously identified a gene encoding a suppressor of plant cell death in *Pyricularia oryzae* (syn. *Magnaporthe oryzae*). Here, we report the gene is one of a 21-member gene family and we characterize sequence diversity in different populations. Within the rice pathogen population, nucleotide diversity is low, however; the majority of gene family members display presence-absence polymorphism or other null alleles. Gene family allelic diversity is greater between host-adapted populations and, thus, we named them host-adapted genes (*HAGs*). Multiple copies of *HAGs* were found in some genome assemblies and sequence divergence between the alleles in two cases suggested they were the result of repeat-induced point mutagenesis. Transfer of family members between populations and novel *HAG* haplotypes resulting from apparent recombination were observed. *HAG* family transcripts were induced in planta and a subset of *HAGs* are dependent on a key regulator of pathogenesis, *PMK1*. We also found differential intron splicing for some *HAGs* that would prevent ex planta protein expression. For some genes, spliced transcript was expressed in antiphase with an overlapping antisense transcript. Characterization of *HAG* expression patterns and allelic diversity reveal novel mechanisms for *HAG*

regulation and mechanisms generating sequence diversity and novel allele combinations. This evidence of strong in planta-specific expression and selection operating on the *HAG* family is suggestive of a role in parasitism.

Keywords: blast disease, gene duplication, horizontal gene transfer (HGT), MGG_17227, parasexual, RIP mutagenesis

Plant pathogens produce effector proteins that can interact with multiple host proteins, and key host proteins may be recognized by many different pathogen effectors (Weßling et al. 2014). Overall, the cumulative action of many individual effectors contributes to lesion number and lesion size in *Pyricularia oryzae* (Liao et al. 2016). Effector proteins can be recognized by plant resistance proteins to activate plant immunity and such effectors are then also called avirulence (Avr) proteins. Pathogen effectors often have substantial redundancy, and disruption of effector/Avr protein–resistance protein interaction (e.g., by AVR gene loss or silencing) restores virulence, often without obvious loss of aggressiveness. This can result in a host-adapted pathogen population with presence-absence or other null allele polymorphism for effector/AVR genes.

P. oryzae parasitizes a wide range of cereal and grass hosts with genetically distinguishable populations adapted to different hosts (Gladieux et al. 2018a and b). Although most isolates belong to these distinct lineages, population analyses using whole-genome sequencing (WGS) have shown that gene flow between populations occurs (Gladieux et al. 2018b). A number of WGS analyses have been carried out to assess mechanisms of evolution driving pathogen diversity and to identify candidate effector genes based on patterns of evolution that suggest effector/AVR gene coevolution with host resistance genes (Chiapello et al. 2015; Kim et al. 2019; Latorre et al. 2020; Petit-Houdonot et al. 2020; Yoshida et al. 2016). Host shifts of *P. oryzae* to wheat (Inoue et al. 2017) and from rice to *Panicum repens* (Torpedograss) (Couch and Kohn 2002) have been documented. In the case of the shift to wheat, after the initial invasion of wheat lines lacking resistance, gene loss in the pathogen allowed adaptation to the presence of resistance genes present in other cultivars.

AVR1-CO39 is a gene identified as conferring Avr to rice carrying the *Pi-CO39* resistance gene. AVR1-CO39 is common among grass pathogens but functional alleles are absent from rice pathogens. The *Pi-CO39* resistance gene is not found in all

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rice cultivars, but it has been suggested that it may have been predominant in the location of rice domestication where *P. oryzae* first adapted to rice, thereby selecting for lineages lacking the gene (Tosa et al. 2005).

P. oryzae Eleusine and rice pathogen populations contain *PWL1* and *PWL2*, respectively, and, when transferred to weeping lovegrass isolates, these genes cause a loss of pathogenicity to weeping lovegrass (Kang et al. 1995; Sweigard et al. 1995). In nature, one *Eleusine* pathogen lineage contains *PWL1* and this differentiates the two major *Eleusine* lineages by differentiating host range (Asuke et al. 2020b). At least five AVR genes recognized by wheat were identified in *Eleusine* isolates (Asuke et al. 2020a). Considering these examples, it is clear that host-adapted pathogen populations may harbor effector genes that function as AVR genes on a different host and lack functional effector/AVR genes recognized by resistance genes in its own host population.

We previously sequenced a population of rice isolates (Zhong et al. 2018), and, in that analysis, we identified a *P. oryzae* gene (gene ID MGG_17227) that displayed presence-absence polymorphism. We further showed the gene is expressed during colonization and delivered into the host cell. Transient coexpression of MGG_17227 and Bax1 protein in tobacco suppressed Bax1-mediated cell death, as has been found for other effectors (Park et al. 2012). As MGG_17227 is one member of a gene family, we hypothesize that all members of the gene family play a role in the interaction with the plant host.

Sequence divergence of effector genes may allow adaptation to different hosts by optimizing interactions with target proteins in the new host (Dong et al. 2014). We do not know if gene family members are involved in host adaptation, but we named the gene family host-adapted genes (*HAGs*) to describe that different host-adapted populations tended to have different combinations of alleles for the genes. In this study, we examined allelic diversity of the gene family in *P. oryzae* and found evidence for generation of new alleles and found *HAG* movement between host-adapted populations. The patterns of gene distribution are similar to those described above for cultivar-specific and host species-specific AVR genes.

Effectors are often preferentially expressed during host colonization (Jeon et al. 2020; Torres et al. 2016). We found

differential expression of the *HAG* family in planta and differential regulation by a regulator of pathogenesis, *PMK1*. In addition, we identified stage-specific intron splicing and detected expression of overlapping antisense RNA for at least three family members. These findings suggest multiple modes of transcriptional and posttranscriptional regulation of *HAG* family expression.

RESULTS

The *HAG* effector gene family in *P. oryzae*.

The *HAG* family encodes approximately 100- to 120-amino acid proteins with predicted secretion signal peptides of 16 to 24 amino acids. The proteins share motifs that are unique to the gene family (Fig. 1), which were used to identify the family members by alignment (Altschul et al. 1990; Brown et al. 1998; Madeira et al. 2019). The order of the motifs is conserved with a cysteine-rich region containing four or five cysteine residues, a GCD tripeptide containing the sixth conserved cysteine, a GRxV motif, and a C-terminal F(I/V)GC(A/V) motif containing the eighth conserved cysteine. Not all family members have eight cysteines, excluding the predicted signal peptide (Almagro Armenteros et al. 2019), the proteins contain six or eight cysteine residues with the exception of *HAG17*, which lacks the fifth conserved cysteine residue and so has seven cysteines. Proteins with six cysteine residues lack the fourth and seventh cysteine residues relative to the proteins with eight cysteines (Fig. 1).

All 21 members of the *P. oryzae HAG* family are present in rice isolate P131 (Xue et al. 2012) (Table 1). The genes are dispersed in the genome (>20 Kb apart), with the exception of *HAG19* and *HAG20*, which are separated by less than 2 Kb. A comparison of *HAG* locations in the reference *P. oryzae* strain 70-15 (Dean et al. 2005), *Eleusine* isolate MZS-1-6 (Gómez Luciano et al. 2019), and *Triticum* isolate B71 (Peng et al. 2019) showed a good degree of synteny (Fig. 2). It was shown that the S8 segment of the 70-15 reference genome corresponds to one end of chromosome 7 in B71, indicating that *HAG18* is syntenic in these isolate genomes (Peng et al. 2019). Supplementary Table S1 provides the locations, orientation, and distances between the genes. A translocation of a 1.9-Mb segment of chromosome 6 to chromosome 1 in 70-15 was noted previously (Gómez Luciano et al. 2019; Peng et al. 2019) and this

Fig. 1. Alignment of *Pyricularia oryzae* host-adapted gene (*HAG*) proteins. Sequence identity to Hag1p is shown (pid). Smaller arrowheads point to the predicted signal peptide cleavage sites. Indicated regions highlight a cysteine-rich, GCD tripeptide, GRxV, and C-terminal F(I/V)GC(A/V) motifs. Consensus sequences with 100 to 70% identity are indicated in the lines below the alignment with conserved cysteines numbered below. Circled residues highlight three types of proteins. The six-cysteine group has substitutions at position 4 (circled) and lack the cysteine at position 7 relative to those with eight cysteines (position 7 circled). Hag17p lacks cysteine 5 (circled alanine substitution) and has just seven cysteines. An intron in the genomic sequences lies precisely between the codons specifying cysteines 4 and 5 in all genes (large arrowhead above Hag1p sequence). A WebLogo graphic representation is at bottom.

accounts for *HAG6* residing on chromosome 1 in 70-15 and chromosome 6 in MZ5-1-6 and B71. We noted copies of *HAG4* (*HAG4b* and *HAG4c*) on chromosomes 1 and 6 are roughly near the new and old location of *HAG6* in 70-15 (Fig. 2). The absence of some family members in 70-15 (*HAG19* to *HAG21*), MZ5-1-6 (*HAG4*, *HAG21*), and B71 (*HAG4*), and the multiple copies of *HAG4* in 70-15 did not alter the conclusion that *HAG* organization in the genome is largely maintained. The *HAG4c* allele differs from *HAG4a* and *HAG4b* by one SNP (single nucleotide polymorphism) and has the same sequence as the reference allele in P131. Supplementary Table S2 provides the DNA and protein sequences used as reference sequences and Supplementary Table S3 is a compendium listing the genome assemblies assessed and the SNP differences from the reference alleles for each gene for each genome.

There is considerable sequence variation between paralogs, for example, alignments of Hag1p with other family members

were found to have sequence identity ranging from 22 to 56% (Fig. 1), and, in overall pairwise comparisons, the greatest identity is 73% between Hag4p and Hag10p.

Of the 18 family members present in the 70-15 genome, automated annotation of the 70-15 reference assembly correctly predicted nine of them, three were not predicted as coding regions, and six gene models were incorrect. Thus, previous transcriptome studies generally would not have been able to fully assess expression of all members of the family. In addition to manual annotation of the two exons and intron for each gene, the assemblies for the genes that had rare SNPs or indels were assessed, if possible. For example, the sequence assemblies for *HAG3* required correction in two important genome assemblies. The *HAG3* sequence in the 70-15 reference genome assembly GCF_000002495.2 has an erroneous extra cytosine (C) residue yielding a stretch of eight C residues that predicts a frameshift in the coding sequence. Examination of the

Table 1. Gene family location in the P131 (SAMN02981399; AHZT01) assembly

Broad ID ^a	Gene name	Contig	Orientation ^b	Start codon	Stop codon
MGG_17227	<i>HAG1</i>	AHZT01002070.1	F	14,867	15,292
MGG_17240	<i>HAG2</i> ^c	AHZT01002549.1	R	1,573	1,178
MGG_16603	<i>HAG3</i>	AHZT01000287.1	R	1,701	1,262
MGG_17587	<i>HAG4</i>	AHZT01000776.1	R	852	472
MGG_16814	<i>HAG5</i> ^c	AHZT01000450.1	R	13,393	12,974
MGG_16404	<i>HAG6</i> ^c	AHZT01001918.1	F	770	1,208
MGG_17155	<i>HAG7</i>	AHZT01000892.1	R	1,095	1,516
MGG_10732	<i>HAG8</i>	AHZT01002379.1	R	41,170	40,740
MGG_16401	<i>HAG9</i>	AHZT01001367.1	F	2,637	3,078
MGG_05403	<i>HAG10</i>	AHZT01001637.1	R	8,198	7,803
MGG_07352	<i>HAG11</i>	AHZT01000147.1	F	19,573	20,022
MGG_15924	<i>HAG12</i>	AHZT01001332.1	F	7,505	7,914
MGG_16703	<i>HAG13</i>	AHZT01002050.1	R	4,322	3,894
None	<i>HAG14</i>	AHZT01001360.1	R	15,882	15,449
None	<i>HAG15</i>	AHZT01001973.1	R	2,696	2,265
MGG_17205	<i>HAG16</i>	AHZT01000006.1	R	1,189	800
None	<i>HAG17</i>	AHZT01001450.1	F	1,417	1,819
MGG_18102	<i>HAG18</i>	AHZT01002202.1	F	12,801	13,238
None	<i>HAG19</i>	AHZT01000264.1	R	1,310	928
None	<i>HAG20</i>	AHZT01000264.1	R	3,241	2,856
None	<i>HAG21</i>	AHZT01000088.1	F	2,929	3,362

^a Broad ID from 70-15 assembly GCF_000002495.2.

^b F and R indicate forward or reverse gene orientation.

^c Contains frameshift mutations in all rice isolates.

Fig. 2. Schematic of host-adapted gene (*HAG*) distribution in the reference genome assemblies and lineage assessment of chromosome 1 of isolate 87-120. Rice isolate 70-15 lacks *HAG19* and *HAG20*, which are located near *HAG5* in MZ5-1-6 and B71. Isolates 70-15 and MZ5-1-6 both lack *HAG21*, which is located near the tip of chromosome 5 in B71. Isolate 70-15 has three copies of *HAG4* (designated *HAG4a*, *HAG4b*, and *HAG4c*). MZ5-1-6 and B71 lack *HAG4*. Rice isolate 87-120 was shown to be a recombinant with DNA aligning best to sequences of different lineages (Gladieux et al. 2018a and b). Chromosome 1 aligns with segments of single-copy sequence with near identity to 70-15 (solid color), G17 (light shading), MZ5-1-6 (medium shading), or different from these three sequences (no shading near tip). *HAG10* lies within a 263-Kb region of near identity with G17 sequence.

deposited sequence chromatograms and fastq data unambiguously shows seven Cs at that position in all deposited reads, yielding the correct reading frame (discussed below). The B71 genome assembly GCA_004785825.1 (200× genome coverage) misplaced approximately 120 bp covering the 5' end of *HAG3* (present in the raw reads, absent in the assembly). However, the smaller GCA_001675625.1 assembly of B71 (157× genome coverage) was correct for the *HAG3* region. We manually annotated each predicted rare SNP or indel if sequencing reads were deposited with the assemblies.

Rice isolate allelic diversity.

The major rice pathogen lineage is proposed to have diverged from a *P. oryzae* *Setaria*-adapted population about 10,000 years ago (approximately 1,200 to 22,000) (Couch and Kohn 2002; Gladieux et al. 2018a), and the current population is estimated to have had a common origin as recently as about 1,000 years ago (175 to 2,700) (Gladieux et al. 2018a; Latorre et al. 2020; Zhong et al. 2018). Not surprisingly, there is generally low sequence diversity within the *P. oryzae* rice-adapted population, although there is clear evidence for genetic exchange between populations (Gladieux et al. 2018b).

Frameshift alleles for *HAG2*, *HAG5*, and *HAG6* are fixed in the rice pathogen population, except for a small number of isolate sequences from which the genes are absent. *HAG2* has a deletion of a CTTT sequence at nucleotide 19 in the protein coding sequence, *HAG5* has an insertion of a CCCT sequence after nucleotide 78, and *HAG6* has a deletion of a CA dinucleotide at position 308. The *Setaria viridis* isolate SV9610 genome sequence does not have frameshifted alleles but otherwise is identical to rice isolate alleles for *HAG2* and *HAG5* and has one synonymous SNP for *HAG6*. The SV9610 isolate

sequences for these genes were used for protein sequence comparison with other family members (Fig. 1; Supplementary Table S2) and were used as the reference sequence for SNP analysis. We confined our SNP analysis to the coding regions to simplify the analysis, and intron variation was not considered in defining alleles.

Rice isolate 87-120 was previously shown to be a recombinant (Gladieux et al. 2018b) and this isolate is discussed later. Of the remaining 150 rice isolate genome sequences across all 21 genes, only 13 different SNPs were identified (Supplementary Table S3). Disregarding four SNPs in the *HAG2*, *HAG5*, and *HAG6* pseudogenes, altered protein products would result from four nonsynonymous substitutions: N53D ($n = 1$) in *HAG7*, A47V ($n = 3$) in *HAG12*, and S3F ($n = 2$) and F8I ($n = 35$) in *HAG13*.

A WGS SNP-based tree was used to place 90 rice isolates into three clades (Fig. 3) (Zhong et al. 2018). Clade 3 represents a clonal lineage of *Mat1-1* isolates and Clade 2 represents a clonal lineage of *Mat1-2* isolates. Clade 1 was assigned to capture the remaining genetic diversity of the rice pathogens and contains isolates of both mating types. Just ten of these 90 genome sequences contain all 21 members of the gene family, and these are all in clade 3. Thirty-one of the 37 remaining clade 3 isolates belong to a lineage lacking *HAG15*. *HAG1* and *HAG21* are absent in all 28 clade 2 isolate sequences. The 15 clade 1 isolates each lack from one to six family members. Overall, 13 family members (*HAG1* to *HAG5*, *HAG9*, *HAG12*, *HAG15*, *HAG16*, *HAG18* to *HAG21*) display presence-absence polymorphism among these 90 assemblies. The phylogenetic pattern of gene presence and absence indicates that deletions of some genes (e.g., *HAG21*) have resulted from independent events (Fig. 3).

Fig. 3. Relationship between host-adapted gene (*HAG*) family deletions and rice isolate phylogeny. Whole-genome sequencing (WGS) single nucleotide polymorphism tree with isolates assigned to three clades. Gene deletions are indicated (horizontal bars indicate multiple isolates containing the same deletion). Other deletions are labeled over vertical lines. Genes deleted in addition to those indicated by horizontal bars are indicated with a plus (+) sign. Genes occurring in triplicate copies are indicated (3×). Isolates related to FJ81278 in clade 3 containing more than one copy of *HAG12* are indicated with a gray box (+*HAG12*).

Five genes (*HAG7*, *HAG8*, *HAG10*, *HAG13*, and *HAG17*) were present in all 151 rice isolate genome sequences examined, *HAG18* was absent in one sequence, and *HAG4*, *HAG6*, *HAG12*, and *HAG14* were each absent from two genome sequences (Supplementary Table S3). In FJ81278, the *HAG9* and *HAG18* deletions (Fig. 3) are each captured within single contigs and the genes were replaced by transposon insertions. Relative to Guy11 as a reference, an approximately 1,800-bp *Fot1* repetitive element (Kito et al. 2003) replaced a 23,418-bp region containing *HAG9*. A single approximately 1,860-bp copy of a *Pot2* transposon (Kimura and Yamaguchi 1998)

replaced a 8,345-bp region containing *HAG18*. Contigs often terminate at repetitive element sequences so that deletion endpoints are unclear in most cases.

The median number of *HAG* deletions per rice isolate is 1 (average 1.7, standard deviation 1.3). The greatest number of deleted *HAGs* in an isolate is six for 13-FM-16-1. We noted that the B157 and RMg-DI genome assemblies lacked seven and 12 *HAGs*, respectively, however, we excluded those assemblies from this analysis (discussed below).

In isolates from other host populations, frameshifts, repetitive element insertions, and gene deletions are also observed, yielding

Table 2. Analysis of the KJ201 infection time course and Guy11 *PMK1*-dependent *HAG* expression data^a

Gene	Data from Jeon et al. (2020)						Reanalysis					
	Hours postinoculation						Hours postinoculation					
	Mycelia	18	27	36	45	72	Mycelia	18	27	36	45	72
FPKM values of the KJ201 infection time course												
<i>HAG1</i>	0.0	2.5	6.5	7.1	28.5	5.9	0.1	2.9	16.0	6.7	29.7	7.2
<i>HAG2</i>	0.0	42.0	67.6	54.9	59.3	9.2	0.0	42.6	177	57.7	62.3	12.2
<i>HAG3</i>	0.0	43.2	54.1	103	244	121	0.0	58.4	163	123	307	186
<i>HAG4</i>	0.0	9.4	38.5	4.3	4.2	3.2	0.0	43.9	140	16.6	14.7	14.0
<i>HAG5</i>	0.1	14.2	6.1	14.7	10.5	6.4	0.0	13.5	23.7	14.5	15.9	11.3
<i>HAG6</i>	0.0	0.0	0.3	0.0	0.4	0.8	0.1	0.5	0.6	0.4	0.5	1.3
<i>HAG7</i>	0.2	20.9	16.4	24.4	13.1	6.3	0.7	21.8	43.0	19.7	11.4	1.9
<i>HAG8</i>	0.0	2.3	1.1	1.7	3.4	1.0	0.0	2.3	4.6	2.0	3.5	1.4
<i>HAG9</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.6	79.6	27.0	2.9	0.3
<i>HAG10</i>	0.0	9.4	20.2	6.1	11.6	2.6	0.0	12.9	38.0	10.6	16.5	4.5
<i>HAG11</i>	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.2	0.0	0.0
<i>HAG12</i>	0.0	11.1	31.9	85.8	247	64.4	0.0	9.6	72.0	65.4	196	61.8
<i>HAG13</i>	0.0	44.8	88.2	5.9	8.8	5.1	0.5	39.9	130	5.4	8.5	7.5
<i>HAG14</i>	N/A	N/A	N/A	N/A	N/A	N/A	0.0	7.4	22.1	3.0	2.5	2.3
<i>HAG15</i>	Gene absent						Gene absent					
<i>HAG16</i>	0.1	15.4	12.0	23.9	16.9	17.5	0.1	16.1	36.1	23.3	16.1	22.2
<i>HAG17</i>	N/A	N/A	N/A	N/A	N/A	N/A	0.0	20.2	50.2	6.4	3.7	2.6
<i>HAG18</i>	0.0	0.0	0.0	0.0	9.7	10.6	0.0	14.7	25.4	17.3	41.0	56.5
<i>HAG19</i>	N/A	N/A	N/A	N/A	N/A	N/A	0.0	286.8	369	156	368	155
<i>HAG20</i>	N/A	N/A	N/A	N/A	N/A	N/A	0.0	113.1	359	123	164	54.9
<i>AvrPi9</i>	3.4	855	1,930	1,560	1,650	462	9.3	1,050	3,066	1,806	2,191	833
<i>AvrPiz-t</i>	0.0	18.2	36.5	41.2	63.6	51.6	0.2	29.9	129	60.4	116	115

Gene	Data from Sakulkoo et al. (2018)				Reanalysis		
	Control	1NA-PP1	Q value	Regulated ^b	Control	1NA-PP1	Regulated ^b
FPKM values for chemical inhibition of <i>PMK1</i> 26 to 32 h postinfection							
<i>HAG1</i>	9.05	8.04	1	No	15.7	3.5	No
<i>HAG2</i>	0.00	0.00	1	No	3.3	0.0	No
<i>HAG3</i>	11.12	0.00	1	No	180	2.3	Yes
<i>HAG4</i>	0.00	0.00	1	No	130	68.8	No
<i>HAG5</i>	N/A	N/A	N/A	N/A	105	0.0	Yes
<i>HAG6</i>	0.00	0.00	1	No	0.0	0.0	No
<i>HAG7</i>	0.00	2.08	1	No	2.1	1.2	No
<i>HAG8</i>	10.09	0.00	1	No	9.3	0.0	No
<i>HAG9</i>	N/A	N/A	N/A	N/A	2.0	0.0	No
<i>HAG10</i>	11.59	0.00	1	No	9.8	0.0	No
<i>HAG11</i>	0.00	0.00	1	No	0.0	0.0	No
<i>HAG12</i>	128.24	0.00	0.0005	Yes	93.9	1.2	Yes
<i>HAG13</i>	9.88	4.84	1	No	11.4	7.6	No
<i>HAG14</i>	N/A	N/A	N/A	N/A	2.1	0.0	No
<i>HAG15</i>	N/A	N/A	N/A	N/A	0.5	0.6	No
<i>HAG16</i>	22.37	0.00	0.001	Yes	9.9	0.3	No
<i>HAG17</i>	N/A	N/A	N/A	N/A	5.4	4.3	No
<i>HAG18</i>	0.00	0.00	1	No	14.4	1.7	No
<i>HAG19</i>	Gene absent				Gene absent		
<i>HAG20</i>	Gene absent				Gene absent		
<i>AvrPi9</i>	72.83	0.00	0.444	No	439	62.6	No
<i>AvrPiz-t</i>	0.00	3.09	1	No	0	3.2	No

^a FPKM = fragments per kilobase per million reads. N/A = not analyzed, not reported, or no value.

^b *PMK1* was judged as regulated with a minimum of 30 hits to the gene across experiments and >10-fold difference.

additional null alleles for *Hag1*, *Hag4* to *Hag7*, *Hag9*, *Hag12*, *Hag13* to *Hag15*, *Hag17* to *Hag18*, and *Hag20* (Supplementary Table S3). Overall, of the 21 *HAG*s, null alleles for only *HAG8* and *HAG10* have not yet been identified in natural isolates.

Genome assemblies with multiple copies of *HAG*s were found. As with 70-15, three copies of *HAG4* were found in the FJ81278 and Guy11 genome sequences (Bao et al. 2017) (Fig. 3). Two copies of *HAG4* were also present in the V86010 assembly and all other rice isolate assemblies examined had one copy. Because the Guy11 and FJ81278 assemblies were based on long read (PacBio) sequences (Bao et al. 2017), the detection of multiple copies of *HAG4* might be due to the enhanced ability to assemble multiple gene copies surrounded by large regions of repetitive sequence. Therefore, we reevaluated the raw sequencing reads for 95 genome assemblies and found a distribution of 11 isolates with the reference allele (P131) sequence only, 52 isolates with the t213c allele only, and 32 isolates with sequencing reads showing both alleles present. All three genotypes were found distributed across clade 3, the t213c allele alone or both allele types were present in clade 1 isolates, and all clade 2 isolates uniformly contained only the t213c allele. Southern blotting was used to verify multiple copies in Guy11, 70-15, and FJ81278, and single copies in FJ98099, Sar-2-20-1, and FJ72ZC7-77 (data not shown). Three copies of *HAG12* were also found in FJ81278 in clade 3 (Fig. 3), with one copy having no SNPs relative to P131 and two copies having a c140t SNP. We examined sequencing reads from isolates most closely related to FJ81278 (FJ78-JJ to FJ81-ZP) (Fig. 3). We found both alleles present in FJ81-MH, ZJ08-41, ZJ00-10, and ZJ2011-7-1.

Deletion and duplication events may be aided by flanking repetitive elements that promote recombination (Yoshida et al. 2016), and genes for secreted effector proteins appear to be concentrated in regions with above-average density of transposon sequences in *P. oryzae* (Zhong et al. 2020). The majority of *HAG* family members in the sequences of FJ81278 and Guy11 have repetitive elements within 5 kb either upstream, downstream, or upstream and downstream of the gene. The three copies of *HAG4* in rice isolates have closely flanking repetitive elements (range around 450 to around 1,500 bp). The three copies of *HAG12* in FJ81278 also have relatively short distances to repetitive elements (around 1.4 to around 8.9 Kb).

The closeness of some repetitive sequences to the coding region may impact gene expression. For *HAG17*, repetitive sequences lie approximately 270, 6,600, and 31,000 bp upstream of the start codon in FJ81278, KJ201, and Guy11, respectively. In the case of the *HAG6* pseudogene in the sequence of KJ201 and other isolates, a repetitive sequence lies just 395 bp upstream of the start codon.

Expression and differential regulation of *HAG* family members.

As some *HAG*s were not previously annotated, we reassessed a published transcriptome time course of infection with rice isolate KJ201 (Jeon et al. 2020). *HAG15* and *HAG21* are absent from the KJ201 genome sequence and no transcripts were found for them (Table 2). Data for *HAG14*, *HAG17*, *HAG19*, and *HAG20* were not reported in the previous study, as there were no gene predictions for them. In addition, differences in our gene models for *HAG3*, *HAG5*, *HAG6*, *HAG7*, *HAG16*, and *HAG18* and our inclusion of 150-bp flanking regions affected expression values in some cases. In both analyses, *HAG6* and *HAG11* were poorly expressed.

The peak of expression for most gene family members was found at 27 or 45 h postinfection (hpi), during the main stage of biotrophic growth. This timing was consistent with expression peaks for *AvrPi9* and *AvrPiz-t* (Table 2). Transcript levels declined more rapidly from the 27 hpi peak for some genes

(>5-fold decrease in expression at 36 hpi for *HAG4*, *HAG13*, *HAG14*, and *HAG17*). *HAG1*, *HAG2*, *HAG5*, *HAG7* to *HAG10*, *HAG16*, *HAG19*, and *HAG20* showed a slower decline in expression, *HAG3* and *HAG12* peaked at 45 hpi and *HAG18* was peaking at 72 hpi. Thus, family members do not share a single common expression pattern during host colonization, suggesting they may play coordinated but distinct roles during host colonization.

To further assess regulation of the gene family, we reexamined published transcriptome data (Sakulkoo et al. 2018) to determine the requirement for *PMK1* mitogen-activated protein kinase. *PMK1* regulates pathogenicity and it was found that a number of effector genes were dependent on *PMK1* activity. The study used an engineered allele of *PMK1* sensitive to chemical inhibition by 1NA-PP1 and compared gene expression after 6 h of treatment with the inhibitor after an initial infection period of 26 h. Guy11 was the parental strain and *HAG19* to *HAG21* are absent from the genome. Expression of eight *HAG* genes, *HAG1*, *HAG3*, *HAG7*, *HAG8*, *HAG10*, *HAG12*, *HAG13*, and *HAG16* was detected previously (Sakulkoo et al. 2018). We detected expression of 16 *HAG* family members (Table 2). *HAG12* and *HAG16* were determined to be regulated by *PMK1* in the original study (Table 2). In our reanalysis, we required a minimum of 30 mRNA fragments per gene and 10-fold differential expression as a signal for regulation by *PMK1* and found *HAG3*, the *HAG5* pseudogene, and *HAG12* to be regulated by *PMK1*. *HAG8*, *HAG10*, *HAG16*, and *HAG18* may also be dependent on *PMK1*; however, the number of detected transcripts for these genes was low. *HAG4*, *HAG13*, *HAG15*, and *HAG17* were not regulated by *PMK1*, thus, *HAG* family members are regulated differentially by *PMK1*.

Unspliced and antisense *HAG* transcript abundance.

All members of the gene family contain a single intron precisely between the codons specifying the fourth and fifth consensus cysteine residues. Analysis of reads from the KJ201 infection time course that align across the junction of the intron demonstrated the presence of unspliced transcript. We also noted alternative splicing for several *HAG*s, but these were of low abundance (<1%). Overall, the lowest levels of spliced transcripts were found for *HAG1* with 84% (34 unspliced, 173 spliced), *HAG13* with 92% (34 unspliced, 402 spliced), and *HAG17* with 63% (39 unspliced, 65 spliced) combined across all timepoints. At 72 hpi, these values were 88% (65 of 74 spliced), 76% (60 of 79), and 0% (0 of 20). *AvrPi9* has an intron and this transcript displayed 99% spliced introns across the time course and 99% spliced at 72 hpi. Sixteen transcripts for *AvrPi9* were found in the in-vitro mycelial sample and we noted seven unspliced and nine spliced transcripts. There were very few *HAG* family fragments crossing the intron boundary in the mycelial samples, but for *HAG1*, *HAG7*, *HAG13*, and *HAG16*, there were a combined 12 reads and all were unspliced. For the first intron in the actin transcript, we found >98% spliced transcripts in the infection time course and >99% in mycelia.

In addition to unspliced RNA, we noted a relatively abundant antisense transcript with an 84-nucleotide (nt) intron overlapping *HAG17*. The antisense transcript was expressed in antiphase with spliced *HAG17* transcripts. *HAG17* displayed efficient splicing at 18 and 27 hpi, with 65 spliced, five unspliced, and three spliced antisense RNA fragments combined. In contrast, no spliced *HAG17* transcripts were detected at 36, 45, or 72 hpi, but 34 unspliced and 14 antisense spliced transcripts were detected. The unspliced transcripts could be either from the sense or antisense transcripts. We also noted two reads with a 97-bp antisense transcript intron for *HAG13* at 72 hpi and one antisense read at 72 hpi for *HAG1*. As with *HAG17*, we found relatively few unspliced transcripts for

HAG13 until 45 hpi (31 spliced, 11 unspliced) and 72 hpi (44 spliced, 28 unspliced, two antisense). The *HAG1*, *HAG13*, and *HAG17* antisense transcript introns are similarly positioned and overlap the *HAG* transcript introns.

We assessed two transcriptome studies of in vitro-grown cells with sufficient sequencing depth to detect intron splicing for 12 *HAG*s. One study evaluated the role of protein kinase C (PKC) on gene expression in mycelia of Guy11 (Penn et al. 2015). The second study examined the role of a transcription factor, *VRP1*, on gene expression 5 h after induction of appressoria in the 70-15 background (Cao et al. 2016). Intron splicing was efficient in the first intron of actin, with nearly 100% of transcripts spliced (Table 3). Including genes with at least 20 RNA fragment sequences aligning to the intron region,

the combined data indicate overall splicing of *HAG2*, *HAG4*, *HAG5*, *HAG6*, and *HAG9* was efficient (70 to 100% spliced). *HAG16* and *AvrPi9* were spliced with moderate efficiency (40 to 60%). Transcripts were spliced at low frequency (0 to 15%) for *HAG3*, *HAG7*, *HAG10*, *HAG13*, *HAG15*, and *HAG17*. Thus, there is differential splicing of *HAG* family member transcripts or differential production of unspliced antisense transcript, when grown ex planta.

For *HAG17*, in addition to the 373 unspliced fragments (Table 3), an additional 89 spliced antisense transcript fragments were detected, representing approximately 20% of the total transcripts. This ratio is similar to that observed in the 36- to 72-hpi timepoints in the KJ201 infection study. Analysis of the potential *HAG17* region antisense transcript based on the

Table 3. Splicing of host-adapted gene (*HAG*) introns of in-vitro mycelia and appressoria

Gene	PKC study		Vrf1 Study		Combined		
	Splice	No Splice	Splice	No Splice	Splice	No Splice	% Spliced
<i>HAG1</i>	0	1	0	0	0	1	N/A
<i>HAG2</i>	0	1	27	0	27	1	96
<i>HAG3</i>	4	26	2	137	6	163	4
<i>HAG4</i>	108	4	1,878	26	1,986	30	99
<i>HAG5</i>	115	29	461	1	576	30	95
<i>HAG6</i>	16	5	0	2	16	7	70
<i>HAG7</i>	15	307	0	6	15	313	5
<i>HAG8</i>	0	2	1	4	1	6	14
<i>HAG9</i>	1	1	15	4	16	5	76
<i>HAG10</i>	0	3	68	462	68	465	13
<i>HAG11</i>	0	0	0	0	0	0	N/A
<i>HAG12</i>	0	0	0	0	0	0	N/A
<i>HAG13</i>	0	44	0	0	0	44	0
<i>HAG14</i>	0	2	1	3	1	5	17
<i>HAG15</i>	4	20	2	182	6	202	3
<i>HAG16</i>	31	22	1	23	33	45	42
<i>HAG17</i>	0	4	2	369	2	373	0.5
<i>HAG18</i>	0	0	2	7	2	7	22
<i>AvrPi9</i>	206	150	10	10	216	160	57
Actin	2485	6	20,909	18	23,394	24	100

Table 4. Allele number, allele SNPs or indels and predicted protein changes for *HAG11* and *HAG13*^a

Allele	<i>HAG11</i>		<i>HAG13</i>	
	SNP alleles = 16	Hag11p variants = 11	SNP alleles = 15	Hag13p variants = 11
0	Full deletion	N/A	No deletion allele	N/A
1	None	No change	None	No change
2	g135a	No change	c8t	S3F
3	t279c	No change	c24g	F8L
4	c60t	No change	c8t a51g	F8L
5	c60t c261g	No change	c8t a28g	S3F I10V
6	c110a	A37E	c8t a28g Del 212a	S3F I10V frameshift
7	c110a a154g t161c t165c g170a	A37E S52G I54T R57K	c8t a28g t291c	S3F I10V
8	c110a a154g t161c t166c c177t c237g c246t	A37E S52G I54T F56I	c8t c83a a103g a119c c191g t250g g271t g320t	S3F P28Q I35V Q40P P64R F84V V91F G107V
9	t107c c110a a154g t161c g170a a289g	V36A A37E S52G I54T R57K T97A	c8t c83a a103g c191g t250g g271t	S3F P28Q I35V P64R F84V V91F
A	t107c c110a a154g t161c t213c c246t	V36A A37E S52G I54T	c8t c83a a103g c191g t250g g271t g320t	S3F P28Q I35V P64R F84V V91F G107V
B	a5g c54t c110a t127g a154g t161c g170a	H2R A37E C43G S52G I54T R57K	c8t t30c t36g a51c g52a c66g t68a c83a a131c c190a c191g t250g g271t c333t c353a	S3F E17D V18I V23E P28Q K44T P64Q F84V V91F
C	c54t c110a t127g a154g t161c g170a	A37E C43G S52G I54T R57K	c8t t30c t36g a51c g52a t68a c83a a131c c190a c191g t250g g271t c333t c353a	S3F E17D V18I V23E P28Q K44T P64Q F84V V91F
D	c54t t107c c110a a154g t161c t165c g170a	V36A A37E S52G I54T R57K	c8t a51g c83a c150g g271t a292g	SF3 E17D P28Q Y50* V91F T98A
E	c54t c110a t127g a154g t161c g170a a181g	A37E C43G S52G I54T R57K K61E	c8t a51g c83a g271t a292g	SF3 E17D P28Q V91F T98A
F	t44c c110a a154g t161c t165c g170a c261g	V15A A37E S52G I54T R57K	c8t c16t	SF3 L6F

^a Bold indicates conserved Cys or nonsense single nucleotide polymorphism (SNP).

Table 5. Alleles^a assigned for *HAG1* to *HAG21* associated with host and number of isolates in each group

Host	<i>HAG1</i>	<i>HAG2</i>	<i>HAG3</i>	<i>HAG4</i>	<i>HAG5</i>	<i>HAG6</i>	<i>HAG7</i>	<i>HAG8</i>	<i>HAG9</i>	<i>HAG10</i>	<i>HAG11</i>
<i>Oryza sativa</i> (rice)	0.1	0.2	0.1.2 .8	0.1.2	0.2.3.4.5	0.2	1.2	1	0.1.7	1.6	0.1.2.3
<i>Setaria viridis</i>	3.4	1.4	1	3	1	1	3	1	1	1	4.6
<i>Setaria italica</i>	1.4.C	1	1.4	3.4.5.6	1.B	1	3	1	1	1.8	6
<i>Panicum repens</i>	1	1	1	2	C	3	3	5	8	1	5
<i>Brachiaria distachya</i> (Bd8401)	8	1	1	0	1	4	4	5	1	1	1
<i>Eleusine coracana</i>	7	6	5	0	7.9.D	7	1	2	5	5	7.A
<i>Eleusine indica</i>	7	6	5	0	7.9.D	7.8	1.8	2.4	3.5	5	7.A
<i>Eragrostis curvula</i>	A	7	5	0	6	8	9	3	3	6	9.A
<i>Lolium perenne</i>	6	6	3	0	7	7	1	2	2	5	1
<i>Lolium multiflorum</i>	6	6	3.5	0	7	7	1	2	2	5	1
<i>Lolium</i> sp.	6	6	3	0	7	7	1	2	2	5	1
<i>Festuca arundinacea</i>	6	6	5	0	7	7	1	2	2	5	1
<i>Triticum aestivum</i> ^b	2.6	3.6	3.5	0	7.A	7	1.6	1.2	2	3	1.D*.F*
<i>Bromus tectorum</i> (P28)	2	3	5	0	7	7	1	1	2	5	1
<i>Brachiaria mutica</i> (Bm88324)	5	5	4	0	6	5	7	2	4	4	8
<i>Stenotaphrum secundatum</i>	2	3	6	0	7	6	4	2	4	3	B
<i>Cenchrus</i> sp. (MG07)	9	8	7	0	7	A	B	6	6	7	C
Uncertain host (pg1213-2)	B	9*	1	0	7	9	A	3	9	2	E

(Continued on next page)

^a Alleles (designated 0 through F) are separated by periods; bold = null alleles; italics = alleles unique to these groupings; asterisks (*) indicate nonsynonymous substitution of conserved cysteine residue; NA = incomplete sequence.

^b Contains isolates of both *Lolium* and *Triticum* genetic lineages and the P29 isolate originally isolated from *Bromus* spp.

RNA contig, assuming the 84-nt intron separates two exons, suggested the potential to encode a 98-amino acid hypothetical protein that did not possess a predicted signal peptide (Supplementary Fig. S1). Reads defining the locations of the anti-sense introns for *HAG1*, *HAG13*, and *HAG17* are listed in Supplementary Table S4.

Allelic diversity of the *HAG* family in host-adapted populations.

Different *P. oryzae* host-adapted populations form genetically distinct lineages, although cross-infection by some isolates is observed. Allelic diversity of *HAGs* across populations was assessed. Genes with the least allelic diversity affecting the protein sequences across all the isolates were *HAG19* (no nonsynonymous SNPs) and *HAG8* and *HAG14*, in which several alleles are found across populations but protein-coding regions were altered by no more than one amino acid. The largest number of alleles were found for *HAG11* (16 alleles) and *HAG13* (15 alleles), that each yielded 11 protein sequence variants. Allelic diversity across populations (Supplementary Table S3) was compiled to define the set of alleles for each gene (Supplementary Table S5). Supplementary Table S5 entries for *HAG11* and *HAG13* without designation of host population are shown in Table 4. The greatest number of amino-acid changes in one of the *HAG11* alleles was six (*Stenotaphrum* sp.) and for *HAG13* this was nine (*Stenotaphrum* and *Cenchrus* spp.). Some of these alleles may encode nonfunctional proteins as some alleles of *HAG11* encode a C43G substitution, which would alter the third conserved cysteine. *HAG13* has a frameshift allele, which was verified by manual annotation, and one allele contains a nonsense mutation, Y50*, that is not validated. The greatest number of amino-acid differences were found for alleles of *HAG3*, *HAG6*, and *HAG9*, having 12, 10, and 11 amino-acid changes (approximately 10% protein sequence divergence).

Table 5 compiles the information of Supplementary Table S5 to summarize alleles present in isolates in each of the host-adapted groups surveyed. The 12 members of the *Lolium* group (isolates from *Lolium* species and *Festuca arundinacea*) were fixed for alleles at 16 of the 20 genes present in the population. The 11 isolates in the *Eleusine* lineage were fixed for alleles at eight of the 20 genes. Five genes (*HAG2*, *HAG10*, *HAG13*, *HAG19*, and *HAG20*) were fixed for the same allele across both

Lolium and *Eleusine* lineages (Table 5). The two populations were fixed for different alleles of *HAG1* (one amino-acid change) and *HAG12* (four amino-acid changes).

The wheat-infecting population contains isolates that belong to *Triticum* and *Lolium* genetic lineages. Seven *HAGs* have fixed alleles in the wheat-infecting population, including the wheat pathogen P29 that was originally isolated from a *Bromus* host (Pieck et al. 2017). In addition to the null allele for *HAG14* found in all wheat-infecting isolates, most wheat isolates (25 of 29), have a frameshift mutation for *HAG15*. Overall, the wheat pathogens are moderately diverse, with the 29 genome sequences analyzed represented by 15 *HAG* haplotypes. Two of the 15 haplotypes are shared by the *Lolium multiflorum* isolates PL2-1 and PL3-1 (not shown).

Although there are too few isolate sequences for most populations to know if the trend of different host-adapted populations having distinguishable *HAG* haplotypes will hold, *HAG* haplotypes may be helpful in assigning isolates to major *P. oryzae* clades (Fig. 4). For example, the *HAG2*, *HAG5*, and *HAG6* pseudogenes identify rice-adapted isolates and a Pyret transposon insertion in *HAG19* is found exclusively in all *Setaria* isolate sequences examined thus far. Since gene flow between populations occurs (Gladieux et al. 2018b), exceptions to these patterns are inevitable. However, some *HAG* alleles may help in assigning isolates to populations.

Multicopy *HAG* genes arise from gene transfer processes.

Of the two copies of *HAG21* in the *Festuca* isolate TF05-1, one copy is identical to the most common allele in closely related *Lolium* isolates and most *Triticum* isolates. The second copy is a unique allele but shares 15 of 16 SNPs with the single-copy gene found in the *Triticum* isolate Br7 and in isolates from other hosts (Table 6). Other isolates do not have this second copy, including the *Triticum* isolate PY5010, which, based on WGS analysis, is closely related to TF05-1, displaying just 65 SNPs per megabase (Gladieux et al. 2018b) (Fig. 4), does not have this second copy. Chromosome painting of Br7 (Gladieux et al. 2018b) showed that chromosome 5 contains substantial similarity to the *Stenotaphrum* lineage and the *HAG21* region is nearly identical to *Stenotaphrum* sequences from about 95 Kb upstream to about 4 Kb downstream of the gene. The corresponding chromosome painting of TF05-1 does not show any

Table 5. (Continued from previous page)

Host	HAG12	HAG13	HAG14	HAG15	HAG16	HAG17	HAG18	HAG19	HAG20	HAG21	Isolates
<i>Oryza sativa</i> (rice)	0.1.2	1.2.3.4	0.1	0.1.2	0.1.2	1	0.1.8	0.1	0.1	0.1	151
<i>Setaria viridis</i>	0.1	2.6.7	1	1.2	1	9	0.1	1	1	0.1.4.5	4
<i>Setaria italica</i>	1	2.5	1	0.3	1	9	0.1	1	1.3	4.5	3
<i>Panicum repens</i>	1	E	6	0	1	8	0	4	2	4	2
<i>Brachiaria distachya</i> (Bd8401)	1	D	6	1	1	1	7	4	2	4	1
<i>Eleusine coracana</i>	4	8	3.4.5	6.8	1.3.4	4.6	4.6	3	1	0.8.B	5
<i>Eleusine indica</i>	4	8	3.4.5	6.8	1.3.4	4.6	4.6	3	1	0.3.8.	6
<i>Eragrostis curvula</i>	4	9	7	6	5	5	3	3	4	2	1
<i>Lolium perenne</i>	1	8	5	8	4.6	6	6	3	1	9	5
<i>Lolium multiflorum</i>	1	8	5	6.8	4.6	6	6	3	1	9	3
<i>Lolium</i> sp.	1	8	5	8	4.6	6	6	3	1	9	2
<i>Festuca arundinacea</i>	1	8	5	6.8	3.6	6	6	3	1	9.A	2
<i>Triticum aestivum</i> ^b	1.3	8	5	4.5.6	3.4.6	3.6	2.6.9	3	1	7.9	29
<i>Bromus tectorum</i> (P28)	1	8	5	5	3	3	6	3	1	9	1
<i>Brachiaria mutica</i> (Bm88324)	5	NA	7	7	6	2	5.C.D	4	2	7	1
<i>Stenotaphrum secundatum</i>	3	A.B	A	9*	3	3	9.C	3	1	7	2
<i>Cenchrus</i> sp (MG07)	6	C	8	A	7	7	2	5	5	6	1
Uncertain host (pg1213-2)	7	NA	B	B	6	0	A	3	4	C	1

large region of the genome with strong similarity to the *Stenotaphrum* lineage; however, a region from about 9 Kb upstream to about 2 Kb downstream of *HAG21* copy 2 is nearly identical to the Br7 and *Stenotaphrum* sequences. This finding supports the view that the second copy is an acquisition by gene transfer and not the result of gene duplication and divergence or whole genome recombination with lineages represented in this study.

Stenotaphrum isolate SSFL14-3 has two complete *HAG13* sequences. The copy 1 allele is unique but similar to an allele found in *Eragrostis* populations and somewhat similar to *Eleusine* and *Lolium* populations (Table 6). Copy 2 differs by 14 SNPs from copy 1 but differs by just 1 SNP from the *Cenchrus* isolate MG07 allele. This suggests that the copies were not generated by duplication and divergence within the *Stenotaphrum* lineage and that one copy arrived from another lineage.

There are three copies of *HAG18* in the Bm88324 assembly, two copies in *Stenotaphrum* isolate SSFL14-3 and one copy in SSFL02-1 (Table 6; Fig. 5). Copy 1 of SSFL14-3 is identical to the single copy in SSFL02-1 in a 9-Kb region of 5' flanking sequence. Copy 1 of Bm88324 shares 98% identity (8,895 of 9,057 nt) across the region. Surprisingly, the *Stenotaphrum* copy 1 allele is nearly identical (9,054 of 9,057 identical, 99.97%) to the B71 *Triticum* isolate sequence and the other *Triticum* isolates bearing this allele (25 of 29 isolates). Thus, in conflict with the WGS phylogeny (Fig. 4), the *Stenotaphrum* copy 1 and B71 *HAG18* gene regions are more closely related to each other than to any other lineages. This region of near identity for *HAG18* extends from about 70 Kb upstream to about 4 Kb downstream of the gene. Interestingly, chromosome 7 of the Br7 *Triticum* lineage isolate has substantial identity with *Stenotaphrum* across the chromosome (Gladioux et al. 2018b). The region of identity runs from about 90 Kb upstream to about 80 Kb downstream of the gene. This pattern of multiple lengths of the segments aligning with *Stenotaphrum* lines in the *Triticum* lineage is consistent with recombination between a *Stenotaphrum* lineage and a progenitor of the *Triticum* lineage and subsequent recombination within the lineage.

Rather than the 98% identity between Bm88324 and *Stenotaphrum* copy 1 alleles, copy 2 of Bm88324 *HAG18* is nearly identical to copy 2 of SSFL14-3 in a 2.5-Kb region containing *HAG18* and 5' flanking sequence (2,546 of 2,548 identical). Copy 1 and copy 2 in SSFL14-3 align only across a 500-bp sequence containing the coding region and share 95.6% identity (480 of 502 identical). The identity of *Stenotaphrum* copy 1 with the *Triticum* allele and copy 2 with copy 2 of Bm88324

(Table 6) make it clear that at least one gene transfer event has occurred. We suggest the direction of transfer was from the *Stenotaphrum* isolate population to the *Triticum* isolate population (Fig. 5).

Gene duplication and divergence through repeat-induced point mutagenesis (RIP).

We noted that all the SNPs that differentiate the Bm88324 *HAG18* copies from each other were C to T or G to A transition mutations (Table 6) and assessed this region for the possibility these alleles were generated by RIP (Ikeda et al. 2002). RIP has been characterized in several fungal species, including

Fig. 4. Whole genome sequencing (WGS) single nucleotide polymorphism (SNP) analysis-based phylogeny of selected members of *Pyricularia oryzae* populations illustrating diverse lineages. *Oryza*, *Setaria*, and *Panicum* isolates have *HAG4*, which is absent from genome sequences from all other lineages. *Triticum* and *Lolium* isolates are fixed for a null allele in *HAG14* that is variably present in the *Eleusine* lineage. *Brachiaria* isolate Bd8401, and *Panicum repens* isolates have SNP variants of the frameshift allele of *HAG6* present in the *Oryza* pathogen population. The *Oryza* population is also fixed for frameshift alleles of *HAG2* and *HAG5*. The *Setaria* population sequences are fixed for an insertional of a transposon in *HAG17*.

P. oryzae, and this process results in premeiotic CG to TA transition mutations in repeated DNA sequences during mating. We compared Bm88324 *HAG18* copies 2 and 3 to each other and they align across a 2.1-Kb region with 95.5% identity (2,036 of 2,100 identities) and we found 61 CG to TA transitions and three other types of SNPs. Although the number of SNPs is low, the ratio of CG to TA transitions is elevated relative to the SNPs between Bm88324 copy 1 and SSFL14-3 copy 1, which share 98% identity (93 CG to TA versus 65 other). We propose that copies 2 and 3 are products of RIP mutagenesis. Since the alleles appear to have originated in the Bm88324 lineage, copy 2 must have been transferred to the *Stenotaphrum* lineage (Fig. 5).

Setaria isolate MG08 has three distinct copies of *HAG4* (Table 7). MG05 is a closely related isolate that has three similar copies, but none of these were completely contained in a single contig. The three copies of the gene in MG08 had eight to 11 SNPs in the coding region relative to *Setaria* isolate US71 and most were GC to TA transitions. To better assess the SNPs in the region, we aligned sequencing reads from the MG05 and MG08 sequencing projects, using the *HAG4* region of the US71 genome assembly as a template. An approximate 3,420-bp region bounded by repetitive sequences about 2,650 bp upstream and about 590 bp downstream of the *HAG4* start codon in US71 resolved to three copies for each isolate. The coding regions for copies 1 and 2 of each isolate were identical but copy 3 of MG05 differed from copy 3 of MG08 at five positions (Table 7). SNPs relative to US71 that were not fixed across alleles were counted. For MG08, there were 138 CG to TA SNPs versus five other SNPs and, for MG05, this was 130 to five (Table 7). The preponderance of CG to TA transitions in these alleles is indicative of RIP. The alleles have intact coding regions with three to four nonsynonymous

SNPs each, however, one allele has an intron splice site consensus sequence mutation.

Recombinant *HAG* haplotypes.

Using chromosome painting analysis (Lawson et al. 2012), it was previously shown that rice isolate 87-120 is a recombinant mostly similar to the typical rice isolate but having large blocks of DNA with high identity to *Eleusine* (e.g., MZ5-1-6) and *Eragrostis* (e.g., G17) genome sequences (Gladieux et al. 2018b). Twenty of the 21 *HAGs* in 87-120 were identical to rice isolate alleles. The *HAG10* allele is identical to the allele from *Eragrostis* isolate G17, which has eight SNPs relative to the rice isolate allele. Consistent with the chromosome painting analysis, we found single-copy DNA sequences of 87-120 chromosome 1 aligned to a nearly 5-Mb region that is highly similar (about 100% identity) to *Eleusine* isolate MZ5-1-6, and this region is flanked on one side by an approximately 250-Kb region of interleaved *Eleusine* and *Eragrostis* lineage segments and on the other side by an approximately 263-Kb segment highly similar (approximately 100% identity) to the *Eragrostis* isolate G17 sequence. The *HAG10* gene (position 5.106 Mb in 70-15) aligns within this region of high identity to G17 genome sequence (Fig. 2). The recombination junction between *Eleusine* lineage sequence to *Eragrostis* lineage sequence near *HAG10* occurs in single-copy sequence at about position 5,345,260 in MZ5-1-6 chr 1 (approximately 5.017 Mb in 70-15 chr 1, approximately 96.25 Kb in 87-120 contig PQBK01001703.1). The recombination junction between *Eragrostis* lineage DNA to *Oryza* lineage DNA occurs in single-copy sequence at about position 5.2802 Mb in 70-15 (14.23 Kb in 87-120 contig PQBK-01001922.1). Thus, *HAG10* is located in the approximately 263-Kb *Eragrostis* lineage segment.

Table 6. Sequence comparison of multicopy gene alleles for *HAG21*, *HAG13*, and *HAG18*

Gene/host	Isolate (copy #)	Allele (SNPs relative to P131) ^a
<i>HAG21</i>		
<i>Triticum</i> (28 of 29 isolates) <i>Festuca arundinacea</i>	PY5010 TF05-1 (copy 1)	t252c
<i>F. arundinacea</i>	TF05-1 (copy 2)	g8c t19c t21c c33t g54a c55a t57g c58t a61c t144c c153t c156t g165c a204g a301c g339a
<i>T. aestivum</i> , <i>Brachiaria mutica</i> , <i>Stenotaphrum secundatum</i>	Br7, SSFL14-3, Bm88324	g8c t19c t21c c33t g54a c55a t57g c58t a61c t144c c153t c156t g165c a204g g339a
<i>Cenchrus</i>		g8c t19c t21c c33t g54a c55a t57g c58t a61c t144c c153t c156t g165c a204g c251g g339a
<i>HAG13</i>		
<i>Eleusine</i> , <i>Lolium</i> , <i>Triticum</i>	All	c8t c83a a103g a119c c191g t250g g271t g320t
<i>Eragrostis</i>	G17	c8t c83a a103g c191g t250g g271t
<i>S. secundatum</i>	SSFL14-3 (copy 1)	c8t c83a a103g c191g t250g g271t g320t
<i>S. secundatum</i>	SSFL14-3 (copy 2)	c8t t30c t36g a51c g52a c66g t68a c83a a131c c190a c191g t250g g271t c333t c353a
<i>Cenchrus</i>	MG07	c8t t30c t36g a51c g52a t68a c83a a131c c190a c191g t250g g271t c333t c353a
<i>HAG18</i>		
<i>Triticum</i> (<i>Lolium</i> lineage)	PY5010	c41t a61g c96g t156c
<i>B. mutica</i> , <i>S. secundatum</i>	Bm88324 (copy 2) SSFL14-3 (copy 2)	c41t g166a g199a g232a g237a c243t g253a g280a c318t
<i>B. mutica</i>	Bm88324 (copy 3)	c41t g196a g232a g237a c243t g244a g278a g280a c318t
<i>S. secundatum</i> , <i>T. aestivum</i>	SSFL14-3 (copy 1) SSFL02-1 25/29 <i>Lolium</i> and <i>Triticum</i> wheat isolates	c243t c255g c318t
<i>B. mutica</i>	Bm88324 (copy 1)	c318t

^a Bold type added to assist visual alignment of sequences. SNP = single nucleotide polymorphism.

HAG4 is present in *Setaria* and *Oryza* pathogen populations and was also found in two *Panicum repens* isolate genome sequences (BTTrp-5 and BTTrp-6) (Soanes et al. 2017). WGS SNP analysis show that these *Panicum repens* isolates are outside of the *Oryza* and *Setaria* pathogen clade (Fig. 4) and more closely related to the *Brachiaria* Bd8401 isolate, sharing identical alleles for ten *HAGs*, of which two are unique to these isolates (Table 5). This suggests the two lineages (Fig. 4) may be derived from a common ancestor. Chromosome painting analysis of the Bd8401 sequence (Gladieux et al. 2018b) is consistent with genome-wide recombination involving the *Setaria/Oryza* lineage and apparently involving a partner with novel *HAGs*. *HAG4* could have entered the *Panicum repens* lineage in a recombination event that generated the lineage containing Bd8401 and the *Panicum repens* isolates. However, the *HAG4* allele found in BTTrp-5/BTTrp-6 isolates is the t213c allele identical to a common allele in rice isolates. In the region from approximately 2,600 bp upstream to approximately 760 bp downstream of the *HAG4* start codon, most rice isolates have two to three SNPs relative to the *Panicum repens* isolates. This same region differed by a minimum of 10 SNPs from the seven *Setaria* isolate sequences. It is possible the *HAG4* allele exists or existed in the *Setaria* pathogen population when recombination between *Setaria* and another lineage led to this

Panicum repens lineage; however, the close sequence identity between *Panicum* and *Oryza* isolate *HAG4* sequences also suggests a possible recent movement of *HAG4* from the rice isolate lineage into the *Panicum repens* lineage.

As mentioned before, the Br7 *Triticum* isolate has a different allele of *HAG21* from all other members in the *Lolium/Triticum* group sequenced (Table 6). *HAG21* is located approximately 124 Kb from the beginning of the chromosome 5 scaffold of the B71 *Triticum* isolate. Br7 DNA aligning to this region is most related to the *Stenotaphrum* sequence, based on chromosome painting analysis (Gladieux et al. 2018b). Using B71 as a scaffold, SSFL14-3 and BR7 aligned with high sequence identity from the beginning of the chromosome 5 scaffold until it began to diverge in the region from 2.5 to 10 Kb downstream of *HAG21*. Thereafter, BR7 sequence aligned with high identity with B71 for at least 100 Kb. Recombination that replaced a tip segment of chromosome 5 containing *HAG21* appears to be the origin of this *HAG21* allele in Br7.

DISCUSSION

Expression and regulation of *HAG* family members.

Updated annotation of the *HAGs* allowed us to refine prior genome-wide transcriptome studies to assess *HAG* expression patterns more comprehensively. Most genes were induced in

Fig. 5. Model for *HAG18* evolution and movement. **A**, Populations diverge yielding *Brachiaria* and *Stenotaphrum* pathogen lineages with *HAG18* orthologs (copy 1) with 98% sequence identity. In the Bm88324-containing lineage, duplication generates three copies of the gene and copies 2 and 3 underwent repeat-induced point (RIP) mutagenesis. **B**, Copy 2 is transferred to a member of the *Stenotaphrum* lineage yielding the lineage containing SSFL14-3, resulting in the observed nearly 100% identity between copy 2 of Bm88324 and copy 2 of SSFL14-3. **C**, Recombination of the *Stenotaphrum* lineage with a progenitor of the *Triticum* lineage delivers the *Stenotaphrum*-like *HAG18* copy 1 found in most *Triticum* lineage isolates, such as isolate Br7 (*HAG18^{Br7}*) having nearly 100% identity with the *Stenotaphrum* isolate sequence. The *Lolium* population and some members of the *Triticum* population have an allele common to *Lolium* isolates with approximately 97% identity to the Br7 allele.

Table 7. Allele sequence and CG to TA transition counts for *HAG4* indicative of repeat-induced point mutagenesis

Isolate	<i>HAG4</i> allele (SNPs relative to P131) ^a	SNPs versus US71 ^b		
		CG to TA	MG08	Other
US71	t213c t267c			
MG08 (copy 1)	c22t c27t c32t g57a t66c g130a a147c t213c		MG08	
	c254t t267c			
MG08 (copy 2)	c22t c27t c32t c63t t66c g118a g130a g131a	138		5
	t132g a147c t213c c231t t267c			
MG08 (copy 3)	g52a t66c g82a g109a a147c c210t t213c			
	t267c c270t g279a c297t			
MG05 (copy 1)	Identical to MG08 (copy 1)		MG05	
MG05 (copy 2)	Identical to MG08 (copy 2)	130		5
MG05 (copy 3)	g57a a147c c210t t213c t267c c270t g279a			
	c297t			

^a Bold type added to assist visual alignment of sequences.

^b Number of CG to TA versus other classes of single nucleotide polymorphisms (SNPs) across the three copies relative to US71 in a 3.4-Kb region.

KJ201 (Jeon et al. 2020), with a time course similar to well-studied effectors such as *AvrPiz-t* and *AvrPi9* (Li et al. 2009; Wu et al. 2015). *PMK1* acts in planta to regulate critical functions required for host colonization (Sakulkoo et al. 2018). Combining analyses, *HAG3*, *HAG12*, *HAG16*, and the *HAG5* pseudogenes are strongly dependent on *PMK1*, and other family members (*HAG8*, *HAG10*, and *HAG18*) are probably regulated by *PMK1*, but deeper sequencing and analyses of additional strains are needed. *HAG4*, *HAG13*, and *HAG17* were not strongly influenced by *PMK1*. Additional expression data combined with comparisons of promoter regions of the genes within and between populations would help to define candidate *cis*-acting elements responsible for the different patterns of regulation.

Our analysis uncovered the potential for additional transcriptional and posttranscriptional mechanisms controlling *HAG* expression. Antisense RNA of the key regulator of the *Neurospora* circadian clock is regulated in antiphase to the sense transcript and leads to dicer-independent small interfering RNA production, stalling of RNA polymerase, and methylation of both histone H3 and DNA in the promoter of the gene (Dang et al. 2016; Xue et al. 2014). This process involves a conserved exonuclease (ER-1, enhanced RNA interference) that also functions in the cytoplasm to regulate stability of target RNAs. If a similar mechanism applies to the genes in which antisense transcripts were detected, *HAG17* and, possibly, *HAG1* and *HAG13* ER-1 mutants would be predicted to have defects in their expression. Naturally occurring antisense RNAs are one form of long non-coding RNA. Long noncoding RNAs are found in the genomic regions associated with effector genes in *Phytophthora* spp. (Wang et al. 2018) and *Ustilago maydis* (Goulet et al. 2020). In *U. maydis*, antisense RNAs were implicated in regulating expression at unlinked loci, and disruption of one antisense RNA reduced aggressiveness of the pathogen.

The transcripts of several family members were mostly unspliced during growth in vitro. Since transcription of *HAGs* is very low in mycelia, it seems redundant or unnecessary to regulate intron splicing. However, if the low expression is a result of an antisense RNA regulatory mechanism, the unspliced RNA detected may be from the antisense RNA rather than *HAG* transcript. Alternatively, intron retention has been shown to occur in plant RNAs and leads to retention in the nucleus prior to posttranscriptional splicing (Jia et al. 2020). This has been proposed to serve as a means of environmental or developmental regulation. Quantification of sense versus antisense transcripts in vitro and during colonization will help to address this question. Protein arginine methylation has been shown to impact splicing in *P. oryzae* and might therefore be involved in the regulated splicing of the *HAGs* or their antisense transcripts. Mutants of the protein arginine methyltransferase in *P. oryzae* have reduced aggressiveness (Li et al. 2020). The *HAGs* provide candidate genes to further characterize the role of unspliced and antisense RNAs in transcriptional and posttranscriptional control of effector gene expression in fungal pathogens.

HAG pseudogenes were found in several *P. oryzae* populations. The *HAG2* and *HAG5* pseudogenes were expressed and regulated similarly to other genes. Pseudogene RNA along with transcripts with alternatively spliced or unspliced introns generate premature termination codons. Transcripts with premature termination codons have been found to take part in a genetic compensation response involved in transcriptional regulation of other family members within gene families in zebrafish (Ma et al. 2019). Thus, the possibility exists for selection to maintain certain null alleles to modulate expression of gene family members.

In addition to identifying that gene family members display expression patterns expected for effector genes, our analysis has suggested several novel mechanisms that may impact effector expression and regulation.

Gene family null alleles.

We expect at least partial functional redundancy among gene family members, and we found the primary variation in the rice pathogen population was that of presence-absence polymorphism. Effector/Avr genes may operate at a host cultivar level (presence-absence polymorphism within a host-adapted population) or host species level (presence-absence polymorphism between different host-adapted populations). We observed both of these patterns for members of the *HAG* family. As this pattern is often associated with the presence of resistance genes in the host, *HAGs* may be useful tools for screening grass and cereal germplasm for resistance genes, and identification of such genes may facilitate effective resistance gene deployment strategies for blast diseases.

Allelic variation.

Duplication and divergence is an important mechanism for innovation of new gene function. Because RIP yields multiple CG to TA transition mutations, candidate recent RIP events may be identified. RIP has been detected in other fungal plant pathogens, as illustrated in *Leptosphaeria maculans* (Fudal et al. 2009), in which RIP events led to stop codons in an Avr gene and was responsible for generation of virulent isolates in the field. They found 4% to 9% nucleotide divergence with nearly all SNPs being GC to TA transitions.

Two of the copies of *HAG18* in Bm88324 carry the signature of RIP, a process that occurs during mating. That one of these is almost identical to one of the *HAG18* copies in SSFL14-3 suggests that the copy moved into the *Stenotaphrum* isolate lineage after the RIP event occurred within the Bm88324 lineage. Whether the RIP allele provided a selective advantage to the *Stenotaphrum* lineage isolates bearing it is an interesting question that could be addressed by functional comparison of *HAG18* alleles. If the RIP allele does provide a fitness advantage, it would define one mechanism for generation of new alleles that eventually might replace the older version of the gene or expand the gene family. The exclusive presence of *HAG4* in the *Setaria/Oryza* lineage and apparent subsequent entry into the *Panicum repens* lineage suggests such an expansion is possible.

A second apparent example of RIP in three copies of *HAG4* in two *Setaria* isolates suggests that RIP may be a common mechanism for diversification of *HAG* family members. We observed that about 4% of the nucleotides were altered in the *HAG4* region relative to US71 and 96.5% of these were CG to AT transitions, similar to the level of RIP mutation found for *Leptosphaeria maculans*. For RIP to contribute to sequence divergence, the occurrence of duplicated copies of effector genes may not be required. Many effector genes, including the *HAGs*, lie near repetitive sequences. RIP can spread beyond repetitive regions into single-copy sequences (Vyas and Kasbekar 2005). Multiple synonymous and nonsynonymous substitutions will arise simultaneously in RIP, complicating evaluation of gene evolution based on an assumption of one nucleotide change at a time.

Recombination to generate *HAG* haplotype diversity.

In the rice population, evidence was found for sexual recombination at the center of origin, although the frequency of sexual recombination is low (Saleh et al. 2012; Zeigler 1998). Parasexual recombination in *P. oryzae* has been demonstrated in the laboratory (Noguchi et al. 2006) and strongly suggested to occur in the field (Zeigler 1998). Recombination between host-adapted populations also occurs (Gladieux et al. 2018a and b; Zeigler 1998).

Movement of mini-chromosomes can carry virulence determinants between strains (Peng et al. 2019). Movement of smaller regions associated with transposons has been shown for

Avr-Pita, a gene that occurs in zero to three copies in *P. oryzae* (Chuma et al. 2011). The involvement of transposons in virulence factor transfers also has been shown in other fungal pathogen systems (McDonald et al. 2019). The *Avr-Pita* case suggests movement between sites within the genome and shuttling with mini-chromosomes. This mechanism is consistent with the cases where multicopy *HAG* genes were observed, including *HAG4*, *HAG12*, *HAG13*, *HAG18*, and *HAG21*.

In *Emericella nidulans*, parasexual recombination frequency is much lower than meiotic recombination frequency (Souza-Júnior et al. 2007), and a low frequency of crossovers will result in larger segments of DNA being exchanged than during meiosis. The approximately 5-Mb block noted for the chromosome 1 region of isolate 87-120 is consistent with parasexual recombination. Vegetative incompatibility could limit hyphal fusion of isolates and inhibit exchange of mini-chromosomes or the frequency of parasexual events; however, vegetative incompatibility appears to be weak in rice isolates and rice-*Eleusine* isolate pairings (Crawford et al. 1986; Zeigler 1998); thus, there may be no significant barrier to fusion in planta in mixed infections or during saprophytic phases (Avila-Adame 2014).

The *HAG* family illustrates many of the mechanisms documented for movement of known fungal effector/*Avr* genes and provides specific examples that further amplifies studies showing that gene flow between *P. oryzae* populations occurs at easily measured frequency (Gladieux et al. 2018b). In addition to the examples illustrated here, examples of *HAG* movement or recombination are evident in the *HAG* haplotypes listed in Supplementary Table S3. Assuming that acquisition of an additional copy of a *HAG* family member or a new *HAG* allele provides, at most, a small advantage to the recipient, it is surprising that examples of RIP and *HAG* movement between populations were so readily detected. It is possible that additional genes contributing to pathogen fitness may also be transferred with *HAGs* and this may facilitate the success of the recipient lineages. However, the observation of movement of *HAG* alleles between populations suggest that *HAGs* do play a role in parasitism, and the finding that combinations of *HAG* alleles appear preferentially in some populations likely reflects that these combinations of alleles contribute to adaptation to that host. Competition between *HAGs* and their alleles could lead to replacement of less-fit alleles, and perhaps that is happening with the *Stenotaphrum HAG18* allele observed in the *Triticum* population.

MATERIALS AND METHODS

Identification of *HAG* genes, SNPs, and repetitive sequences.

We analyzed alignments of protein sequences to define gene family members and aligned DNA sequences to identify SNPs against the P131 rice isolate sequences for most genes, except we used SV9610 isolate sequences for *HAG2*, *HAG5*, and *HAG6*. SNPs identified in alignments of genomic DNA were mapped to the coding sequence (intron removed) to define alleles. SNPs were detected using an in-house PERL script to scan sequence alignments and to output the SNPs in the notation used here, e.g., a18c indicates the 'a' residue at position 18 of the coding sequence was changed to a 'c' residue. Small insertions and deletions were noted as position with the string of nucleotides inserted or deleted (indel). This is designated in Table 4, for example, as Del 212a to indicate a deletion at position 212 of a single 'a' residue. Indels were rare and alignments were examined in these cases to manually verify the calls and, if available, deposited sequencing reads for those genomes were queried to validate indels in the primary reads. Although rare mutations in introns that would prevent RNA

splicing and thus represent null alleles were noted, we did not use this information in defining alleles for each gene. Repetitive sequences were defined as alignment of five or more copies of a sequence of at least 200 bp in length.

Analysis of RNA expression and intron splicing.

Magic-BLAST (Boratyn et al. 2019) was used to align transcriptome sequence reads (Supplementary Table S6) obtained from the National Center for Biotechnology Information Short Read Archive database and these results were used to quantitate expression level and to assess introns. An in-house PERL script was used to capture Magic-BLAST output and process the read information with respect to paired-end reads and intron/exon boundary overlaps. Fragments (paired-read fragments plus unpaired reads) were counted, and expression was assessed as fragments per kilobase of sequence (*HAG* coding region plus 150 bp of flanking sequence) per million mapped reads (Jeon et al. 2020; Sakulkoo et al. 2018). Fragment sequences that crossed at least one intron boundary were counted as a splice or 'unsplice' event. Reads that either captured the spliced intron (called by Magic-BLAST) or sequence alignments that either read through the intron boundary or were arrested at the intron/exon junction and had overhangs of unaligned sequence were captured. The captured information on these paired reads were compared and the read overlapping the intron was collected and counted as an intron overlapping read. When both reads overlapped the intron boundaries, only one read was counted as an intron overlapping read. Information about these reads combining Magic-BLAST output and intron/exon boundary information were then imported into a spreadsheet and were scanned manually to accept or reject intron overlapping reads. The main cause of rejection was due to sequences with arrested alignment at an intron/exon boundary that had overhangs that were ambiguous (e.g., single nucleotide overhang that could match to either spliced or unspliced intron) or the overhang beyond the intron/exon boundary failed to align with the predicted properly spliced sequence. Other than antisense intron reads, which were retained separately, all other reads with overhang sequence that did not align to the properly spliced intron were rejected. These rare, rejected reads were due to alternative splicing or, for some overhang sequences, an alignment to nearby sequence was not found. The error rate for manual retention and rejection of intron overlapping reads was not assessed.

There was no false positive signal for *HAG* genes from alignments to uninfected rice tissue in the KJ201 time course data. We empirically determined that rejecting Magic-BLAST alignments matching less than 50% of the read length and alignments that displayed less than 92% identity across the alignment prevented cross-alignment of reads to different *HAG* family members.

To assess intron splicing efficiency and antisense transcripts, raw counts of spliced and unspliced reads were used. Compared with controls, *HAG* family expression was not significantly different in response to PKC inhibition or in the *vrfl* mutant versus wild type. The control and test transcriptome data for mycelia in the PKC study and for appressoria of both 70-15 and *vrfl* were combined to assess spliced versus unspliced RNA (Table 3).

Characterization of genome sequences and assemblies.

Errors in genome assemblies are difficult to avoid due to repetitive elements and duplicated regions. Rare alleles were checked where possible, e.g., sequencing read archive data (Supplementary Table S6) were used as needed to assess sequence quality of SNPs, indels, and the B71 assembly by manual inspection and assembly.

In cases in which the coding region was only partially covered by a single contig, or coverage involved multiple contigs where an individual contig did not completely contain the coding region, we assessed whether this was due to insertion or deletion and these cases are noted in Supplementary Table S3. If no determination could be made with the assembly, these alleles were indicated in Supplementary Table S3 as ‘incomplete.’ Sixteen of the 20 incomplete genes were found in four of the genome assemblies. *HAG4* was absent from the B157 genome assembly; however, we probed the fastq sequence files and identified a single read that had a perfect match for *HAG4*. Therefore, we cannot be certain about the presence or absence of *HAG4* in this isolate. Twelve *HAGs* were absent from the RMg-DI genome assembly. However, transcriptome data submitted along with the genome assembly for the isolate was assessed and we identified RNA for eight of the missing 12 genes in those data. This is noted in Supplementary Table S3. Chromatogram and sequence read IDs used to assess the *HAG3* sequence of 70-15 are listed in Supplementary Table S7.

Visualization of HAG genes on chromosomes was done using PhenoGram (Wolfe et al. 2013). The consensus graphic was prepared using WebLogo (Schneider and Stephens 1990).

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LITERATURE CITED

- Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., and Nielsen, H. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* 37:420-423.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Asuke, S., Nishimi, S., and Tosa, Y. 2020a. At least five major genes are involved in the avirulence of an *Eleusine* isolate of *Pyricularia oryzae* on common wheat. *Phytopathology* 110:465-471.
- Asuke, S., Tanaka, M., Hyon, G. S., Inoue, Y., Vy, T. T. P., Niwamoto, D., Nakayashiki, H., and Tosa, Y. 2020b. Evolution of an *Eleusine*-specific subgroup of *Pyricularia oryzae* through a gain of an avirulence gene. *Mol. Plant-Microbe Interact.* 33:153-165.
- Avila-Adame, C. 2014. Transmission of the G143A QoI-resistance point mutation through anastomosis in *Magnaporthe grisea*. *Pest Manag. Sci.* 70:1918-823.
- Bao, J., Chen, M., Zhong, Z., Tang, W., Lin, L., Zhang, X., Jiang, H., Zhang, D., Miao, C., Tang, H., Zhang, J., Lu, G., Ming, R., Norvienyeku, J., Wang, B., and Wang, Z. 2017. PacBio sequencing reveals transposable elements as a key contributor to genomic plasticity and virulence variation in *Magnaporthe oryzae*. *Mol. Plant* 10:1465-1468.
- Boratyn, G. M., Thierry-Mieg, J., Thierry-Mieg, D., Busby, B., and Madden, T. L. 2019. Magic-BLAST, an accurate RNA-seq aligner for long and short reads. *BMC Bioinformatics* 20:405.
- Brown, N. P., Leroy, C., and Sander, C. 1998. MView: A web-compatible database search or multiple alignment viewer. *Bioinformatics* 14:380-381.
- Cao, H., Huang, P., Zhang, L., Shi, Y., Sun, D., Yan, Y., Liu, X., Dong, B., Chen, G., Snyder, J. H., Lin, F., and Lu, J. 2016. Characterization of 47 Cys2-His2 zinc finger proteins required for the development and pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *New Phytol.* 211:1035-1051.
- Chiappello, H., Mallet, L., Guérin, C., Aguilera, G., Amselem, J., Kroy, T., Ortega-Abboud, E., Lebrun, M. H., Henrissat, B., Gendral, A., Rodolphe, F., Tharreau, D., and Fournier, E. 2015. Deciphering genome content and evolutionary relationships of isolates from the fungus *Magnaporthe oryzae* attacking different host plants. *Genome Biol. Evol.* 7:2896-2912.
- Chuma, I., Isobe, C., Hotta, Y., Ibaragi, K., Futamata, N., Kusaba, M., Yoshida, K., Terauchi, R., Fujita, Y., Nakayashiki, H., Valent, B., and Tosa, Y. 2011. Multiple translocation of the AVR-Pita effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species. *PLoS Pathog.* 7:e1002147.
- Couch, B. C., and Kohn, L. M. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94:683-693.
- Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. 1986. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. *Genetics* 114:1111-1129.
- Dang, Y., Cheng, J., Sun, X., Zhou, Z., and Liu, Y. 2016. Antisense transcription licenses nascent transcripts to mediate transcriptional gene silencing. *Genes Dev.* 30:2417-2432.
- Dean, R. A., Talbot, N. J., Ebbole, D. J., Farman, M. L., Mitchell, T. K., Orbach, M. J., Thon, M., Kulkarni, R., Xu, J. R., Pan, H., Read, N. D., Lee, Y. H., Carbone, I., Brown, D., Oh, Y. Y., Donofrio, N., Jeong, J. S., Soanes, D. M., Djonovic, S., Kolomiets, E., Rehmeier, C., Li, W., Harding, M., Kim, S., Lebrun, M. H., Bohnert, H., Coughlan, S., Butler, J., Calvo, S., Ma, L. J., Nicol, R., Purcell, S., Nusbaum, C., Galagan, J. E., and Birren, B. W. 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* 434:980-986.
- Dong, S., Stam, R., Cano, L. M., Song, J., Sklenar, J., Yoshida, K., Bozkurt, T. O., Oliva, R., Liu, Z., Tian, M., Win, J., Banfield, M. J., Jones, A. M., van der Hoorn, R. A., and Kamoun, S. 2014. Effector specialization in a lineage of the Irish potato famine pathogen. *Science* 343:552-555.
- Fudal, I., Ross, S., Brun, H., Besnard, A. L., Ermel, M., Kuhn, M. L., Balesdent, M. H., and Rouxel, T. 2009. Repeat-induced point mutation (RIP) as an alternative mechanism of evolution toward virulence in *Leptosphaeria maculans*. *Mol. Plant-Microbe Interact.* 22:932-941.
- Gladioux, P., Condon, B., Ravel, S., Soanes, D., Maciel, J. L. N., Nhani, A., Jr., Chen, L., Terauchi, R., Lebrun, M. H., Tharreau, D., Mitchell, T., Pedley, K. F., Valent, B., Talbot, N. J., Farman, M., and Fournier, E. 2018b. Gene flow between divergent cereal- and grass-specific lineages of the rice blast fungus *Magnaporthe oryzae*. *MBio* 9:e01219-17.
- Gladioux, P., Ravel, S., Rieux, A., Cros-Arteil, S., Adreit, H., Milazzo, J., Thierry, M., Fournier, E., Terauchi, R., and Tharreau, D. 2018a. Coexistence of multiple endemic and pandemic lineages of the rice blast pathogen. *MBio* 9:e01806-17.
- Gómez Luciano, L. B., Tsai, I. J., Chuma, I., Tosa, Y., Chen, Y. H., Li, J. Y., Li, M. Y., Lu, M. J., Nakayashiki, H., and Li, W. H. 2019. Blast fungal genomes show frequent chromosomal changes, gene gains and losses, and effector gene turnover. *Mol. Biol. Evol.* 36:1148-1161.
- Goulet, K. M., Storfie, E. R. M., and Saville, B. J. 2020. Exploring links between antisense RNAs and pathogenesis in *Ustilago maydis* through transcript and gene characterization. *Fungal Genet. Biol.* 134:103283.
- Ikeda, K., Nakayashiki, H., Kataoka, T., Tamba, H., Hashimoto, Y., Tosa, Y., and Mayama, S. 2002. Repeat-induced point mutation (RIP) in *Magnaporthe grisea*: Implications for its sexual cycle in the natural field context. *Mol. Microbiol.* 45:1355-1364.
- Inoue, Y., Vy, T. T. P., Yoshida, K., Asano, H., Mitsuoka, C., Asuke, S., Anh, V. L., Cumagun, C. J. R., Chuma, I., Terauchi, R., Kato, K., Mitchell, T., Valent, B., Farman, M., and Tosa, Y. 2017. Evolution of the wheat blast fungus through functional losses in a host specificity determinant. *Science* 357:80-83.
- Jeon, J., Lee, G. W., Kim, K. T., Park, S. Y., Kim, S., Kwon, S., Huh, A., Chung, H., Lee, D. Y., Kim, C. Y., and Lee, Y. H. 2020. Transcriptome profiling of the rice blast fungus *Magnaporthe oryzae* and its host *Oryza sativa* during infection. *Mol. Plant-Microbe Interact.* 33:141-144.
- Jia, J., Long, Y., Zhang, H., Li, Z., Liu, Z., Zhao, Y., Lu, D., Jin, X., Deng, X., Xia, R., Cao, X., and Zhai, J. 2020. Post-transcriptional splicing of nascent RNA contributes to widespread intron retention in plants. *Nat. Plants* 6:780-788.
- Kang, S., Sweigard, J. A., and Valent, B. 1995. The PWL host specificity gene family in the blast fungus *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 8:939-948.
- Kim, K. T., Ko, J., Song, H., Choi, G., Kim, H., Jeon, J., Cheong, K., Kang, S., and Lee, Y. H. 2019. Evolution of the genes encoding effector candidates within multiple pathotypes of *Magnaporthe oryzae*. *Front. Microbiol.* 10:2575.
- Kimura, M., and Yamaguchi, I. 1998. Convergent transcription units and their promoters at both ends of pot2, an inverted repeat transposon from the rice blast fungus. *J. Biochem.* 124:268-273.
- Kito, H., Takahashi, Y., Sato, J., Fukiya, S., Sone, T., and Tomita, F. 2003. Occan, a novel transposon in the F0t1 family, is ubiquitously found in several *Magnaporthe grisea* isolates. *Curr. Genet.* 42:322-331.
- Latorre, S. M., Reyes-Avila, C. S., Malmgren, A., Win, J., Kamoun, S., and Burbano, H. A. 2020. Differential loss of effector genes in three recently expanded pandemic clonal lineages of the rice blast fungus. *BMC Biol.* 18:88.
- Lawson, D. J., Hellenthal, G., Myers, S., and Falush, D. 2012. Inference of population structure using dense haplotype data. *PLoS Genet.* 8:e1002453.

- Li, W., Wang, B., Wu, J., Lu, G., Hu, Y., Zhang, X., Zhang, Z., Zhao, Q., Feng, Q., Zhang, H., Wang, Z., Wang, G., Han, B., Wang, Z., and Zhou, B. 2009. The *Magnaporthe oryzae* avirulence gene AvrPiz-t encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene Piz-t. *Mol. Plant-Microbe Interact.* 22: 411-420.
- Li, Z., Wu, L., Wu, H., Zhang, X., Mei, J., Zhou, X., Wang, G. L., and Liu, W. 2020. Arginine methylation is required for remodelling pre-mRNA splicing and induction of autophagy in rice blast fungus. *New Phytol.* 225:413-429.
- Liao, J., Huang, H., Meusnier, I., Adreit, H., Ducasse, A., Bonnot, F., Pan, L., He, X., Kroj, T., Fournier, E., Tharreau, D., Gladieux, P., and Morel, J. B. 2016. Pathogen effectors and plant immunity determine specialization of the blast fungus to rice subspecies. *eLife* 5:e19377.
- Ma, Z., Zhu, P., Shi, H., Guo, L., Zhang, Q., Chen, Y., Chen, S., Zhang, Z., Peng, J., and Chen, J. 2019. PTC-bearing mRNA elicits a genetic compensation response via Upf3a and COMPASS components. *Nature* 568:259-263.
- Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A. R. N., Potter, S. C., Finn, R. D., and Lopez, R. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* 47 (W1):W636-W641.
- McDonald, M. C., Taranto, A. P., Hill, E., Schwessinger, B., Liu, Z., Simpfendorfer, S., Milgate, A., and Solomon, P. S. 2019. Transposon-mediated horizontal transfer of the host-specific virulence protein ToxA between three fungal wheat pathogens. *MBio* 10:e01515-19.
- Noguchi, M. T., Yasuda, N., and Fujita, Y. 2006. Evidence of genetic exchange by parasexual recombination and genetic analysis of pathogenicity and mating type of parasexual recombinants in rice blast fungus, *Magnaporthe oryzae*. *Phytopathology* 96:746-750.
- Park, C. H., Chen, S., Shirsekar, G., Zhou, B., Khang, C. H., Songkumarn, P., Afzal, A. J., Ning, Y., Wang, R., Bellizzi, M., Valent, B., and Wang, G. L. 2012. The *Magnaporthe oryzae* effector AvrPiz-t targets the RING E3 ubiquitin ligase APIP6 to suppress pathogen-associated molecular pattern-triggered immunity in rice. *Plant Cell* 24: 4748-4762.
- Peng, Z., Oliveira-Garcia, E., Lin, G., Hu, Y., Dalby, M., Migeon, P., Tang, H., Farman, M., Cook, D., White, F. F., Valent, B., and Liu, S. 2019. Effector gene reshuffling involves dispensable mini-chromosomes in the wheat blast fungus. *PLoS Genet.* 15:e1008272.
- Penn, T. J., Wood, M. E., Soanes, D. M., Csukai, M., Corran, A. J., and Talbot, N. J. 2015. Protein kinase C is essential for viability of the rice blast fungus *Magnaporthe oryzae*. *Mol. Microbiol.* 98:403-419.
- Petit-Houdenot, Y., Langner, T., Harant, A., Win, J., and Kamoun, S. 2020. A clone resource of *Magnaporthe oryzae* effectors that share sequence and structural similarities across host-specific lineages. *Mol. Plant-Microbe Interact.* 33:1032-1035.
- Pieck, M. L., Ruck, A., Farman, M. L., Peterson, G. L., Stack, J. P., Valent, B., and Pedley, K. F. 2017. Genomics-based marker discovery and diagnostic assay development for wheat blast. *Plant Dis.* 101:103-109.
- Sakulkoo, W., Osés-Ruiz, M., Oliveira Garcia, E., Soanes, D. M., Littlejohn, G. R., Hacker, C., Correia, A., Valent, B., and Talbot, N. J. 2018. A single fungal MAP kinase controls plant cell-to-cell invasion by the rice blast fungus. *Science* 359:1399-1403.
- Saleh, D., Xu, P., Shen, Y., Li, C., Adreit, H., Milazzo, J., Ravigné, V., Bazin, E., Nottéghem, J. L., Fournier, E., and Tharreau, D. 2012. Sex at the origin: An Asian population of the rice blast fungus *Magnaporthe oryzae* reproduces sexually. *Mol. Ecol.* 21:1330-1344.
- Schneider, T. D., and Stephens, R. M. 1990. Sequence logos: A new way to display consensus sequences. *Nucleic Acids Res.* 18:6097-6100.
- Soanes, D., Ryder, L., Islam, M., and Talbot, N. J. 2017. Genome assemblies of *Magnaporthe oryzae* isolated from Bangladesh in 2016 and 2017. *figshare*. Published online.
- Souza-Júnior, S. A., Becker, T. C., and Castro-Prado, M. A. 2007. Asexual recombination in a *uvsH* mutant of *Aspergillus nidulans*. *Biol. Res.* 40:65-71.
- Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F. G., and Valent, B. 1995. Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. *Plant Cell* 7:1221-1233.
- Torres, M. F., Ghaffari, N., Buïate, E. A., Moore, N., Schwartz, S., Johnson, C. D., and Vaillancourt, L. J. 2016. A *Colletotrichum graminicola* mutant deficient in the establishment of biotrophy reveals early transcriptional events in the maize anthracnose disease interaction. *BMC Genomics* 17:202.
- Tosa, Y., Osue, J., Eto, Y., Oh, H. S., Nakayashiki, H., Mayama, S., and Leong, S. A. 2005. Evolution of an avirulence gene, AVR1-CO39, concomitant with the evolution and differentiation of *Magnaporthe oryzae*. *Mol. Plant-Microbe Interact.* 18:1148-1160.
- Vyas, M., and Kasbekar, D. P. 2005. Collateral damage: Spread of repeat-induced point mutation from a duplicated DNA sequence into an adjoining single-copy gene in *Neurospora crassa*. *J. Biosci.* 30:15-20.
- Wang, Y., Ye, W., and Wang, Y. 2018. Genome-wide identification of long non-coding RNAs suggests a potential association with effector gene transcription in *Phytophthora sojae*. *Mol. Plant Pathol.* 19:2177-2186.
- Webbing, R., Eppe, P., Altmann, S., He, Y., Yang, L., Henz, S. R., McDonald, N., Wiley, K., Bader, K. C., Gläßer, C., Mukhtar, M. S., Haigis, S., Ghamsari, L., Stephens, A. E., Ecker, J. R., Vidal, M., Jones, J. D., Mayer, K. F., Ver Loren van Themaat, E., Weigel, D., Schulze-Lefert, P., Dangel, J. L., Panstruga, R., and Braun, P. 2014. Convergent targeting of a common host protein-network by pathogen effectors from three kingdoms of life. *Cell Host Microbe* 16:364-375.
- Wolfe, D., Dudek, S., Ritchie, M. D., and Pendergrass, S. A. 2013. Visualizing genomic information across chromosomes with PhenoGram. *BioData Min.* 6:18.
- Wu, J., Kou, Y., Bao, J., Li, Y., Tang, M., Zhu, X., Ponaya, A., Xiao, G., Li, J., Li, C., Song, M. Y., Cumagun, C. J., Deng, Q., Lu, G., Jeon, J. S., Naqvi, I., and Zhou, B. 2015. Comparative genomics identifies the *Magnaporthe oryzae* avirulence effector AvrPi9 that triggers Pi9-mediated blast resistance in rice. *New Phytol.* 206:1463-1475.
- Xue, M., Yang, J., Li, Z., Hu, S., Yao, N., Dean, R. A., Zhao, W., Shen, M., Zhang, H., Li, C., Liu, L., Cao, L., Xu, X., Xing, Y., Hsiang, T., Zhang, Z., Xu, J. R., and Peng, Y. L. 2012. Comparative analysis of the genomes of two field isolates of the rice blast fungus *Magnaporthe oryzae*. *PLoS Genet.* 8:e1002869.
- Xue, Z., Ye, Q., Anson, S. R., Yang, J., Xiao, G., Kowbel, D., Glass, N. L., Crosthwaite, S. K., and Liu, Y. 2014. Transcriptional interference by antisense RNA is required for circadian clock function. *Nature* 514:650-653.
- Yoshida, K., Saunders, D. G., Mitsuoka, C., Natsume, S., Kosugi, S., Saitoh, H., Inoue, Y., Chuma, I., Tosa, Y., Cano, L. M., Kamoun, S., and Terauchi, R. 2016. Host specialization of the blast fungus *Magnaporthe oryzae* is associated with dynamic gain and loss of genes linked to transposable elements. *BMC Genomics* 17:370.
- Zeigler, R. S. 1998. Recombination in *Magnaporthe grisea*. *Annu. Rev. Phytopathol.* 36:249-275.
- Zhong, Z., Chen, M., Lin, L., Chen, R., Liu, D., Norvienyeku, J., Zheng, H., and Wang, Z. 2020. Genetic variation bias toward noncoding regions and secreted proteins in the rice blast fungus *Magnaporthe oryzae*. *mSystems* 5:e00346-20.
- Zhong, Z., Chen, M., Lin, L., Han, Y., Bao, J., Tang, W., Lin, L., Lin, Y., Somai, R., Lu, L., Zhang, W., Chen, J., Hong, Y., Chen, X., Wang, B., Shen, W. C., Lu, G., Norvienyeku, J., Ebbola, D. J., and Wang, Z. 2018. Population genomic analysis of the rice blast fungus reveals specific events associated with expansion of three main clades. *ISME J.* 12:1867-1878.