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Multiple Avirulence Paralogues in Cereal Powdery Mildew Fungi May Contribute to Parasite Fitness and Defeat of Plant Resistance

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Powdery mildews, obligate biotrophic fungal parasites on a wide range of important crops, can be controlled by plant resistance (*R*) genes, but these are rapidly overcome by parasite mutants evading recognition. It is unknown how this rapid evolution occurs without apparent loss of parasite fitness. R proteins recognize avirulence (AVR) molecules from parasites in a gene-for-gene manner and trigger defense responses. We identify AVR_{a10} and AVR_{k1} of barley powdery mildew fungus, *Blumeria graminis* f sp *hordei* (*Bgh*), and show that they induce both cell death and inaccessibility when transiently expressed in *Mla10* and *Mlk1* barley (*Hordeum vulgare*) varieties, respectively. In contrast with other reported fungal *AVR* genes, AVR_{a10} and AVR_{k1} encode proteins that lack secretion signal peptides and enhance infection success on susceptible host plant cells. AVR_{a10} and AVR_{k1} belong to a large family with >30 paralogues in the genome of *Bgh*, and homologous sequences are present in other formae speciales of the fungus infecting other grasses. Our findings imply that the mildew fungus has a repertoire of *AVR* genes, which may function as effectors and contribute to parasite virulence. Multiple copies of related but distinct AVR effector paralogues might enable populations of *Bgh* to rapidly overcome host *R* genes while maintaining virulence.

INTRODUCTION

Obligate biotrophic parasites cause the most serious and widespread diseases of crop plants but are challenging to investigate because they cannot grow outside their host. The three major groups of biotrophic filamentous parasites are the powdery mildew and rust fungi and the downy mildews, which are oomycetes. Powdery mildews cause economic losses to most crops in temperate areas, infecting >9000 dicot and >650 monocot plant species (Chaure et al., 2000). Infection of host plants during the growing season results from wind-dispersed conidiospores that are the asexual (haploid) state of the fungus. Many powdery mildews also have a sexual phase resulting in the formation of ascospores (Figure 1A). Most powdery mildews show a high degree of host specialization, a feature well exemplified in *Blumeria graminis* f sp *hordei* (*Bgh*), which displays gene-for-gene interactions with its host plant, barley (*Hordeum vulgare*) (Schulze-Lefert and Panstruga, 2003).

In gene-for-gene interactions (Flor, 1971), the recognition of an avirulence (AVR) molecule by a host resistance (R) protein triggers a localized cell death, known as the hypersensitive response, and other defense responses that prevent further parasite growth (Greenberg and Yao, 2004; Skamnioti and Ridout, 2005). Bacterial AVR proteins are introduced into host cells by the type-three secretion system and can contribute to successful infection in susceptible host varieties (Alfano and Collmer, 2004; Janjusevic et al., 2006). Thus, bacterial AVR proteins are often described as effectors since they have both elicitor (avirulence) and virulence activities. Some fungal AVR proteins could potentially function as effectors. For example, Avr4 from *Cladosporium fulvum* binds to chitin, so it may function to protect the fungus from plant chitinases during infection (van den Burg et al., 2003). Avr2, also from *C. fulvum*, binds to Rcr3, a Cys protease required specifically for the function of the resistance gene Cf-2 (Rooney et al., 2005). However, there is no proof that these or any other fungal AVR proteins contribute directly to infection success.

More than 25 independent *AVR* genes have been described in *Bgh* isolates (Brown and Jessop, 1995; Jensen et al., 1995), but none are yet isolated. Although *AVR* genes are distributed throughout the *Bgh* genome, a cluster containing AVR_{k1}, AVR_{a10}, and AVR_{a22} is linked by 1 to 2 centimorgans (cM) (Brown and Jessop, 1995; Jensen et al., 1995; Caffier et al., 1996). More than 85 barley *R* genes, each conferring resistance to specific *Bgh* AVR elicitors, have been described, including *Mlk1* and 28 alleles at the *Mla* locus on barley chromosome 5 (Jørgensen, 1994). The six isolated *Mla* alleles (*Mla1*, *Mla6*, *Mla7*, *Mla10*, *Mla12*, and *Mla13*) are predicted to share >90% amino acid sequence identity (Zhou et al., 2001; Halterman et al., 2003; Shen et al., 2003; Halterman and Wise, 2004). Mla proteins have

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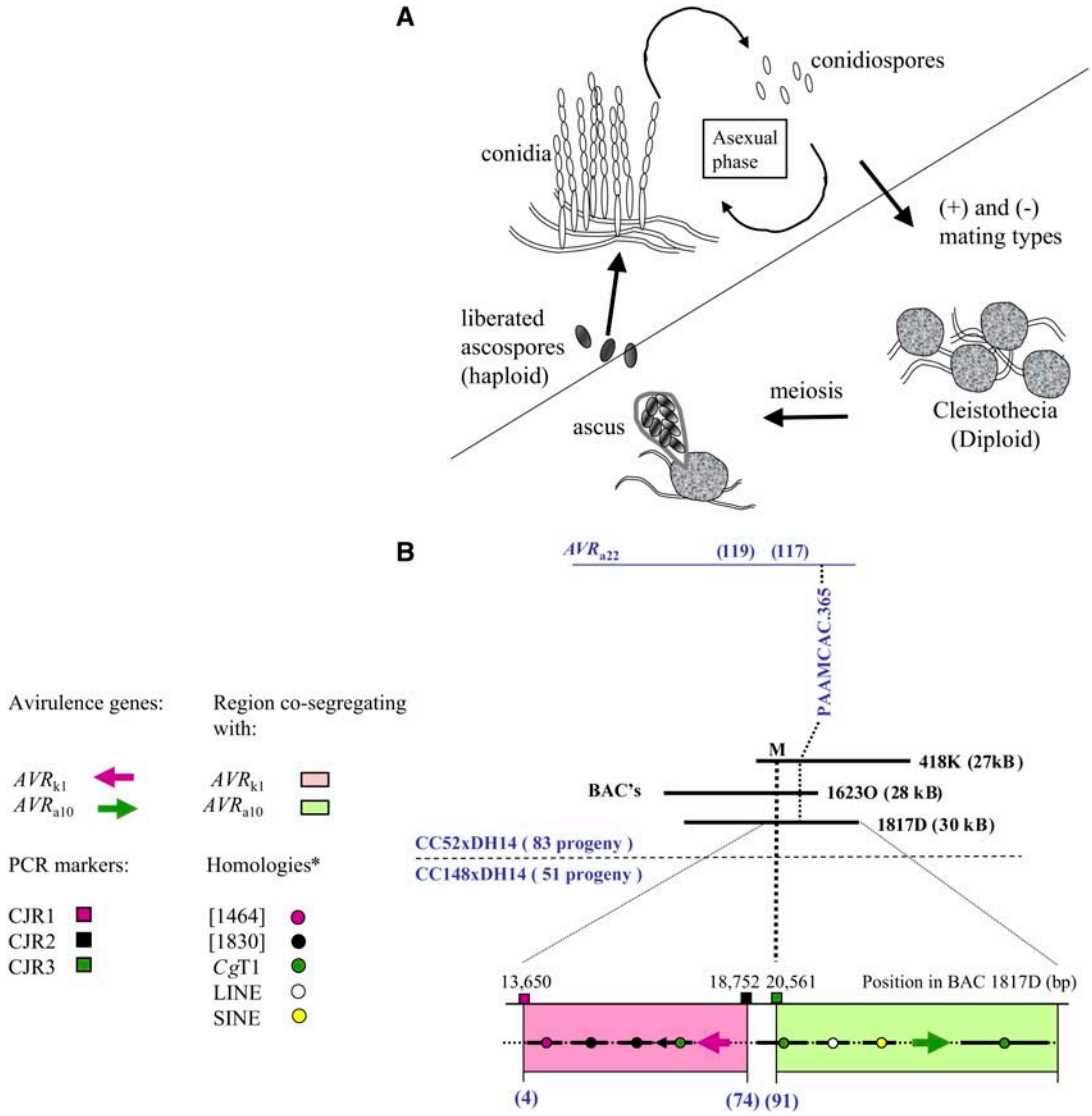


Figure 1. Lifecycle of *Bgh* and the Isolation of AVR_{k1} and AVR_{a10} by Map-Based Cloning.

(A) Compatible isolates of opposite mating types can be crossed to produce single ascospore cultures. These can be maintained as individual cultures in the haploid asexual phase on detached barley leaves, and DNA for mapping can be obtained from conidiospores.

(B) Genetic and physical map of the genomic region of *Bgh* containing AVR_{k1} and AVR_{a10}. Genetic mapping data for two crosses is shown in blue, and recombinant progeny isolate numbers are in parentheses. Primer sequences for PCR markers are shown in Methods. M is a microsatellite mapped with marker CJR3. Marker CJR1 is a size polymorphism (541 bp inserted in DH14) and defines the left border of the region cosegregating with AVR_{k1}. The right border of the AVR_{k1} cosegregating region was defined by sequencing PCR amplicons at CJR2. Homologies were established by BLASTX (Altschul et al., 1997) against ESTs in the NCBI and COGEME databases. LINE and SINE elements are as previously described (He et al., 1996; Wei et al., 1996). Numbers in brackets are COGEME accession numbers.

conserved coiled-coil N-terminal regions, nucleotide binding (NB) sites, variable leucine-rich repeat (LRR), and C-terminal regions that can confer recognition specificity for different *Bgh AVR* gene products (Shen et al., 2003). Mla proteins are related to other NB-LRR proteins, such as leaf rust and powdery mildew resistance genes Lr10 and Pm3b in wheat (*Triticum aestivum*), and to RPM1 in *Arabidopsis thaliana* (Feuillet et al., 2003; Yahiaoui et al., 2004). Mla and these related NB-LRR resistance proteins are likely to be located within the cytoplasm, where they recognize pathogen AVR molecules that enter the host cell (Schulze-Lefert and Panstruga, 2003). The powdery mildew resistance gene *Mlk1* is also located on chromosome 5, ~5 cM from the *Mla* locus (Maroof et al., 1994).

AVR genes have now been isolated from the two other groups of obligate biotrophs, represented by the flax rust fungus *Melampsora lini* and the oomycetes *Hyaloperonospora parasitica*, *Phytophthora infestans*, and *Phytophthora sojae* (Allen et al., 2004; Dodds et al., 2004; Shan et al., 2004; Armstrong et al.,

2005; Rehmany et al., 2005; Catanzariti et al., 2006). Although nothing is known about the function of these *AVR* genes, all of them are subject to diversifying selection, which implies that they are maintained in populations because they play a role in pathogenicity. All biotrophs penetrate host plant cells and establish within them haustoria, specialized feeding structures for nutrient uptake. Some of the *AVR* genes identified in rust fungi are upregulated within the haustoria, suggesting a role in the establishment of biotrophy (Catanzariti et al., 2006).

Our objective was to isolate *AVR* genes from *Bgh*, a representative of the powdery mildews as the third major group of obligate biotrophic parasites. Isolation of *AVR* genes is a vital step in understanding the biology of *Bgh* in at least two respects. First, the large repertoire of *AVR* and *R* genes makes *Bgh* and barley excellent models for investigating how variation in molecular structures can account for specificity of parasite recognition. To understand the function of barley *R* genes in eliciting plant defenses, it is also essential to investigate the function of *AVR* genes corresponding to isolated *R* genes (Schulze-Lefert and Panstruga, 2003). Second, *Bgh* is an important model for research on the population biology of host–parasite coevolution in agriculture (Brown and Høvmoller, 2002). Cereal powdery mildews can rapidly evolve to overcome host *R* genes without apparent loss of fitness (Bronson and Ellingboe, 1986; Brown, 2003). The isolation of *Bgh AVR* genes would therefore permit investigation into the molecular basis for host adaptation and parasite evolution.

RESULTS

Genetic Delimitation of a Candidate AVR_{k1} Gene

To clone *AVR* genes from *Bgh*, we targeted the cluster containing AVR_{k1}, AVR_{a10}, and AVR_{a22}, which are linked within 1 to 2 cM of each other. Two genetic crosses were made: one between *Bgh* isolates DH14 and CC52 and another between DH14 and CC148 (see Methods). The infection types of *AVR* genes segregating in these crosses are shown in Table 1. DNA from progeny and parental isolates was used for genetic mapping by amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995), and a BAC library was made using DNA from the common parent isolate DH14. An AFLP marker PAAMCAC-365 present in DH14 DNA and mapping 2.4 cM (two recombinants out of 83) from the AVR_{a22} locus in the cross between *Bgh* isolates DH14 and CC52 was identified, providing a starting point for physical mapping at the $AVR_{k1}/AVR_{a10}/AVR_{a22}$ cluster. PAAMCAC-365 was used to isolate three BACs by hybridization (Figure 1B). BAC 1817D was sequenced by shotgun cloning and primer walking. Comparative sequencing of BAC 1817D and parental DNA by long-range PCR identified a microsatellite (M) that consisted of 26 repeats of the DNA sequence (ATGTGG) in DH14 and 4.5 repeats in CC52 and CC148. M was therefore mapped in both DH14 × CC52 and DH14 × CC148 as PCR marker CJR3 with primers PCR3F and PCJR3R. In the cross DH14 × CC148, there were two recombinants between the AVR_{k1} and M loci, providing additional confirmation that BAC 1817D was located close to the $AVR_{k1}/AVR_{a10}/AVR_{a22}$ cluster. Further sequence comparisons between DH14 and CC148 DNA near the M locus were made by PCR with primers designed from the sequence of BAC 1817D. Two further polymorphisms were identified at PCR loci CJR1 and CJR2 (Figure 1B), enabling a 5102-bp region cosegregating with the AVR_{k1} gene to be delimited. The entire cosegregating region was sequenced in both DH14 and CC148 by long-range PCR.

Table 1. Infection Types of *Bgh* Isolates

Barley Variety	*R* Gene[b]	Infection Type[a]		
		CC52	CC148	DH14
Pallas or Siri	*Mla8*	4	4	4
Midas and P03	*Mla6*	0	4	0
P09 and S09[c]	*Mla10*	4	0	4
P10	*Mla12*	4	4	0
P17 and S17	*Mlk1*	4	1	4
Hordeum 1063	*Mlk1*	4	1–2	4
W37/136	*Mlh*	1–2	4	0
P12	*Mla22*	0	4	4
Rupal	*Mla13*	4	0	0
P08B	*Mla9*	4	0	0
P06	*Mla7*	4	1–2	1–2

[a] Infection types are based on Brown and Wolfe (1990): 0, fully resistant, no visible symptoms; 1, necrotic flecks, no sporulation; 2, heavy necrotic flecks, scarce sporulation; 3, light necrotic flecks, moderate sporulation; and 4, fully susceptible, no necrosis or chlorosis.
[b] *R* gene designations as in Jørgensen (1994).
[c] P and S series of near-isogenic lines derived from Pallas and Siri, respectively (Kølster et al., 1986; Kølster and Stølen, 1987).

Within the cosegregating region, an open reading frame (ORF) was identified that was complete in three *Bgh* isolates with the phenotype Ak1 (avirulent on *Mlk1* plants). In four Vk1 isolates (Vk1, virulent on *Mlk1* plants), however, the amino acid sequence contained either stop codons or a frame shift (Figure 2A). This DNA sequence for this ORF was therefore a candidate for the AVR_{k1} gene (AVR_{k1}-*CAND*). A retrotransposon sequence with homology to Cg T1 from *Colletotrichum gloeosporoides* (He et al., 1996) was located immediately 3′ to AVR_{k1}-*CAND*. Other sequences with homology to *Bgh* ESTs were located within the cosegregating region but were discounted as candidates for the AVR_{k1} gene because no DNA sequence polymorphism between Ak1 and Vk1 isolates was detected.

The AVR_{k1}-*CAND* sequence is transcribed in Ak1 isolates of *Bgh*. cDNA transcripts containing the same stop codon were isolated by rapid amplification of cDNA ends (RACE)-PCR from barley leaf epidermis infected with the Ak1 *Bgh* isolate CC148. In one transcript, the polyadenylation site occurred shortly after the stop codon, whereas in the other, this occurred after the 3′ Cg T1 homologue sequence (Figure 2B).

AVRk1-CAND Causes Mlk1-Specific Cell Death

An AVR protein is predicted to elicit host cell death specifically in plants with the corresponding *R* gene. As no reliable transformation procedure is available for *Bgh*, we tested Mlk1-specific recognition of AVR_{k1}-CAND by a cell death assay after transient expression in planta. This biolistic method has been used to

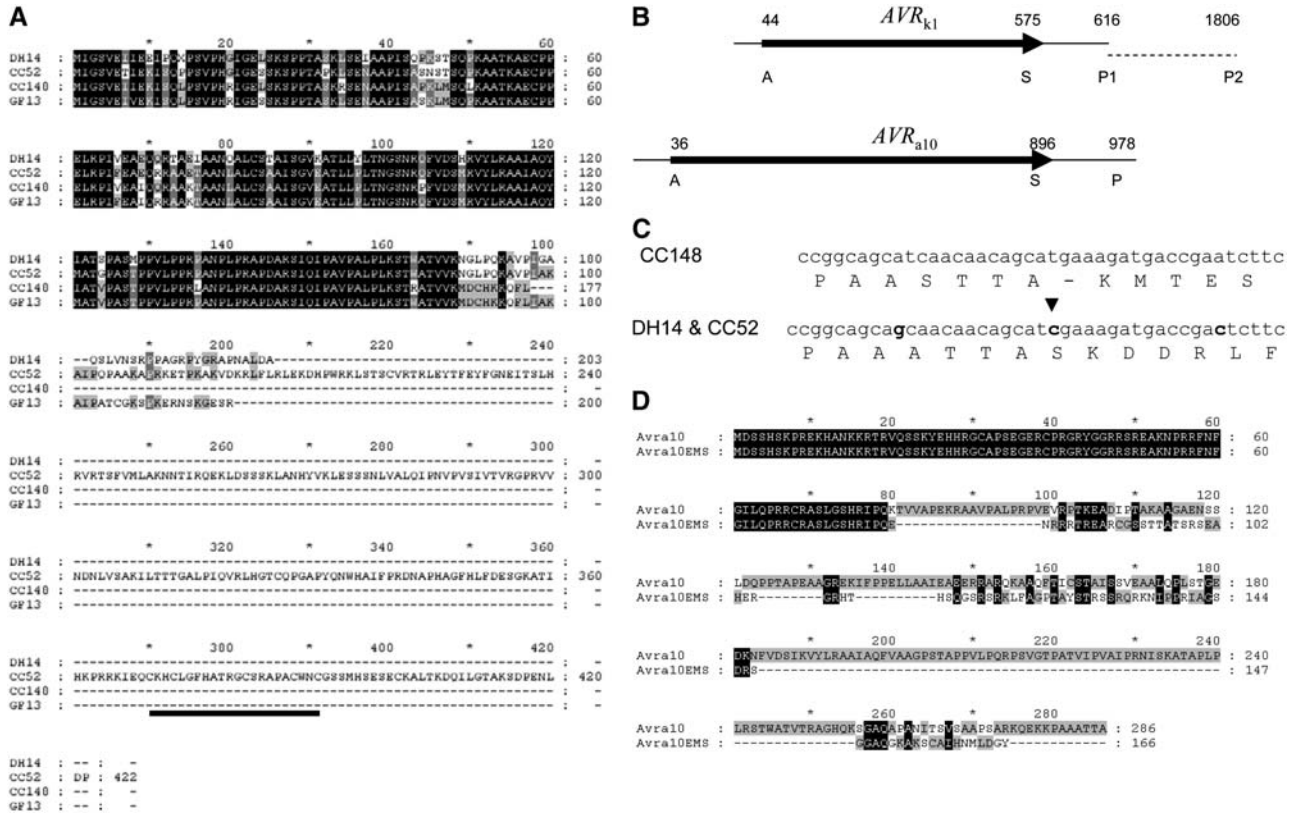


Figure 2. AVR_{k1} and AVR_{a10} Sequence Variants and Transcripts.

(A) Alignment of amino acid residues of AVR_{k1} isolated from Akl (CC148, GF13, and HLA) and Vk1 (CC52, DH14, GF4, and C24R2) isolates. The most conserved residues are boxed in black, and less conserved residues are in lighter gray. The sequence of isolate HLA is the same as CC148. The stop codon at position 14 in the amino acid alignment of DH14 is denoted by an X. The sequence of isolate CC52, which is the same as all the other Vk1 isolates, is extensively different to that of the Ak1 isolate CC148. This is caused by nucleotide polymorphisms that result in a frame shift and fusion with the downstream Cg T1 retrotransposon. Underlined residues are those characteristic of the NB domain of retrotransposons related to Cg T1 (He et al., 1996).
(B) Diagrams illustrating cDNA transcripts of AVR_{k1} and AVR_{a10} obtained by RACE-PCR. Numbers are the position of features in base pairs from the 5′ mRNA cap. A is ATG start, S is stop codon, and P is the location of the polyadenylation site. Two transcripts with the same stop codon and terminating at P1 and P2 were obtained for AVR_{k1}.
(C) The nucleotide and translated amino acid sequence of AVR_{a10} is the same in isolates CC148 (Aa10) and DH14 and CC52 (both Va10), except at the C terminus, where variant nucleotides are shown in bold. The insertion of cytosine (arrowhead) in Va10 isolates results in a frame shift and fusion with a Cg T1 retrotransposon.
(D) Comparison of sequences of AVR_{a10} in isolate CC148 (Aa10) with the Vk1 isolate obtained from CC148 after EMS mutagenesis.

verify other fungal, oomycete, and bacterial *AVR* genes (Leister et al., 1996; Jia et al., 2000; Allen et al., 2004; Rehmany et al., 2005). The method relies on *R* gene–dependent cell death causing a reduction in reporter protein accumulation when reporter and candidate *AVR* genes are coexpressed in cells of host plant leaves. We cobombarded a green fluorescent protein (GFP)–expressing reporter plasmid with an AVR_{k1}-CAND–expressing plasmid and compared the results to a null plasmid containing a nonfunctional allele of AVR_{k1}-*CAND* isolated from the Vk1 strain DH14. Near-isogenic barley lines with specific resistance genes backcrossed into varieties Pallas (P series) or Siri (S series) (Kølster et al., 1986; Kølster and Stølen, 1987) were used. A GFP index was measured as the proportion of GFP-expressing cells visible in *Mlk1*-resistant barley lines (P17 and S17) or lines with other resistance genes (P09, *Mla10*; S09, *Mla10*; P22, *Mla22*) relative to *mlk1*-susceptible lines (Pallas or Siri).

Using this method, we demonstrated that the AVR_{k1}-CAND–expressing plasmid caused a highly significant ($P > 0.001$) *Mlk1*-dependent reduction in the GFP index to 59% (Figure 3A). This result is consistent with the recognition of AVR_{k1}-CAND by Mlk1 causing cell death and a consequent reduction in GFP protein. No significant reduction in the GFP index was detected when AVR_{k1}-*CAND* was cobombarded into leaves containing other *Mla* resistance alleles, confirming that recognition of AVR_{k1}-CAND is specific to Mlk1. As expected, the GFP index was not reduced when the null plasmid was used on *Mlk1* leaves.

AVRk1-CAND Induces Inaccessibility in *Mlk1* Leaves

We further characterized the function of AVR_{k1}-*CAND* by exploiting a feature of powdery mildew gene-for-gene interactions known as induced inaccessibility, whereby virulent isolates can

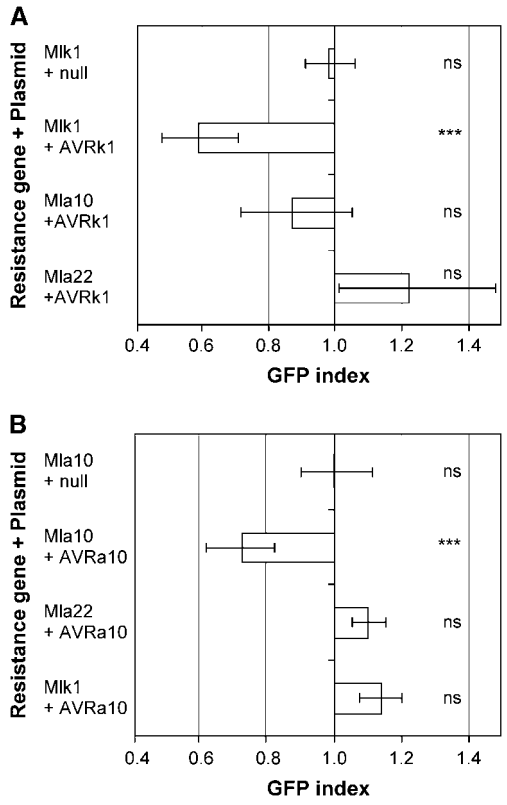


Figure 3. AVR Recognition Measured with a Cell Death Assay.

GFP index measured in barley leaves with *Mla10*, *Mlk1*, and *Mla22* alleles compared with Pallas or Siri near-isogenic parental lines after expression of AVR_{k1} or the null plasmid **(A)** and AVR_{a10} or the null plasmid **(B)**. Each data point summarizes five experiments with two replicates of each lisolate + plasmid combination per experiment and seven leaves of each variety per variety + plasmid replicate. Predicted means were calculated by generalized linear modeling of the logarithm of GFP index with the model experiment + plasmid × near-isogenic series. The plasmid effect was highly significant (F-test, $P < 0.001$). As there was no significant effect of the interaction between plasmid and near-isogenic series (Pallas or Siri), means were predicted for varieties and plasmids pooled across near-isogenic series. Significance test for difference of GFP index from 1: ***, $P < 0.001$; ns, $P > 0.05$. Bars indicate mean ± 1 SE.

no longer infect living host cells first attacked by avirulent or nonhost isolates (Prats et al., 2006). We developed an infection assay based on the facts that GFP accumulated in 59% of AVR_{k1}-*CAND* cobombarded *Mlk1* cells (Figure 3A) and that live infection attempts on those cells could be viewed by microscopy. Leaves were first cobombarded with the GFP-expressing reporter plasmid and either the AVR_{k1}-CAND–expressing or the null plasmid and then infected with either of two Vk1 isolates of *Bgh*, DH14 and CC52. Infection success or failure was determined by microscopy examination to establish whether GFP-expressing cells were penetrated by the fungus and whether such cells remained alive (Figures 4A to 4C).

There was a highly significant reduction in the number of AVR_{k1}-CAND–expressing host cells successfully penetrated by the parasite on *Mlk1* leaves compared with those expressing the null plasmid (Figure 4D). At failed infection sites, cytoplasmic aggregation typical of hypersensitive response–induced cell death, and live host cells on which the fungus had failed to penetrate, were observed. Our results show that transiently expressed AVR_{k1}-CAND induces inaccessibility to virulent isolates attacking cells on *Mlk1* lines and that this effect is specific to the AVR_{k1}-Mlk1 interaction. Ten days after infection, necrotic lesions were observed on *Mlk1* leaves cobombarded with *GFP* and AVR_{k1}-*CAND* and subsequently infected by either of the two Vk1 isolates (Figure 5). The lesions resulted in diminished fungal growth, which was measured as a significant reduction in spore production.

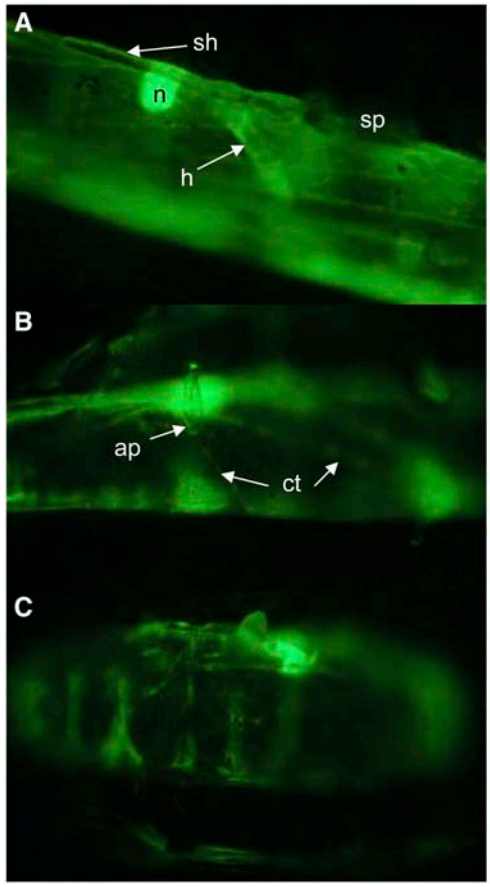
AVRk1-CAND Enhances Infection on Susceptible Varieties

The ability to examine single, infected, living epidermal cells expressing AVR_{k1}- CAND enabled us to make another important and unexpected observation. When expressed in the susceptible cultivar Pallas, AVR_{k1}-CAND significantly increased the number of cells successfully infected by the Vk1 isolates (Figure 4D). This suggests that AVR_{k1}-CAND has an effector function that enhances infection in compatible interactions. The number of spores produced on Pallas leaves was similar after bombardment with either *AVRk1-CAND* or the null plasmid (Figure 5). Infection is therefore probably enhanced only in the very small number of individual AVRk1-CAND–expressing cells obtained after the biolistic treatment, the rest of the leaf remaining unaffected.

In summary, AVR_{k1}-*CAND* cosegregates with the AVR_{k1} phenotype, has a functional ORF sequence in Ak1 but not Vk1 isolates, is expressed by Ak1 isolates, elicits (1) host cell death, (2) induced inaccessibility, and (3) reduced fungal sporulation specifically in *Mlk1* plants, and has an effector function in promoting susceptibility to disease in plants lacking *Mlk1*. Together, these seven points confirm that AVR_{k1}-*CAND* is indeed AVR_{k1} with dual AVR and disease effector functions.

AVR_{a10} Is a Paralogue of AVR_{k1}

We identified a paralogue of AVR_{k1} located 7.5 kb from AVR_{k1} and reasoned that this may encode another *AVR* gene within the $AVR_{k1}/AVR_{a10}/AVR_{a22}$ cluster (Figure 1B). In the cross between *Bgh* isolates CC148 (Aa10, avirulent on Mla10 plants) and DH14 (Va10, virulent on Mla10 plants), this paralogue cosegregated with the Aa10 phenotype and so was a candidate for the gene (AVR_{a10}-*CAND*). Sequences of AVR_{a10}-*CAND* from natural Va10 isolates revealed mutations within the gene (Figure 2C). Furthermore, when CC148 was mutagenized with ethyl methanesulfonate (EMS) and selected for growth on *Mla10* leaves, a derivative Va10 mutant isolate had a mutation in this nucleotide sequence resulting in a premature stop codon in the AVR_{a10}-CAND protein (Figure 2D). In the cell death assay, we measured an AVR_{a10}-*CAND*–dependent reduction in the GFP index to 73% on *Mla10* leaves (Figure 3B). No reduction in the GFP index was measured on leaves containing *Mlk1* or *Mla22* genes or with the null plasmid on *Mla10* leaves. In the infection assay, inaccessibility to DH14 and another Va10 isolate, CC52, was induced in an AVR_{a10}-*CAND*–dependent, *Mla10*-specific manner (Figure 4D). Reduced



D

Variety	Test plasmid AVR_{a10}	AVR_{k1}	Null
P09 (*Mla10*)	35.8**	-	53.1
P17 (*Mlk1*)	-	24.4***	57.3
Pallas (*mla10, mlk1*)	73.9***	70.6***	44.8

Figure 4. Scoring Criteria and Results of Infection Assay.

(A) to **(C)** Photomicrographs of GFP-expressing barley epidermal cells infected with *Bgh*, photographed rapidly to minimize tissue damage by UV irradiation.

(A) Germinated spore (sp) of *Bgh* showing successful infection on a living cell with secondary hyphae (sh), haustorium (h), and plant cell nucleus (n).

(B) Failed infection on a living cell. The fungus has attempted to penetrate the cell with an appressorium (ap), a specialized infection structure, but this has withered and lost turgor. The streaming cytoplasmic threads (ct) indicate that the cell is still alive.

(C) Failed infection with a dead cell and static granular cytoplasm.

(D) Percentage of infection success of *Bgh* isolate CC52 or DH14 (both Vk1 and Va10) in barley cells following cobombardment of plasmids expressing AVR_{k1} or AVR_{a10} with a GFP-expressing plasmid. Each data point summarizes three experiments (AVR_{k1}) or two experiments (AVR_{a10}), with seven replicate leaves per experiment on average of each variety + isolate + plasmid combination. Predicted means were calculated by generalized linear modeling of the number of successful infections. The model was experiment + variety × isolate × plasmid, and a logit link function was used to analyze binomial data of successful infections as a proportion of total infections. The variety × plasmid interaction was highly significant ($P < 0.001$). As there was no significant effect of isolate (DH14 or CC52) on the variety × plasmid interaction, means were predicted for varieties and plasmids pooled across isolates. Significance test for difference between null and test plasmids: **, $P < 0.01$; ***, $P < 0.001$.

infection was associated with both failed penetration and cell death. We also measured reduced spore production, consistent with diminished fungal growth, with both of the Va10 isolates 10 d after infecting *Mla10* plants bombarded with AVR_{a10}-*CAND* (Figure 5). The number of successful infections increased after expression of AVR_{a10} in Pallas cells (Figure 4D), indicating that AVR_{a10}-CAND has an effector function of enhancing infection in susceptible lines (Pallas has *Mla8*, an ineffective *Mla10* allele).

Our results confirm that AVR_{a10}-*CAND* is indeed AVR_{a10}. The seven lines of evidence adduced for the identity of AVR_{k1} and for the dual elicitor/effector function of that gene apply equally to AVR_{a10}. In addition, EMS-induced point mutation of AVR_{a10} to produce premature termination of the coding sequence caused loss of AVR_{a10} activity.

AVR_{k1} and AVR_{a10} Are Members of a Gene Family in Powdery Mildew Fungi of Grasses

Six paralogues of AVR_{k1}, with an E value $<10^{-7}$, were identified by BLAST searching in GenBank as ESTs from *Bgh* (accession numbers BQ283794 and BQ283703) and from *Bgh*-infected barley (accession numbers DN83308, CK567720, CK568673, and CK568570). Three complete paralogous cDNAs were also amplified by RACE-PCR from barley leaves infected with *Bgh*

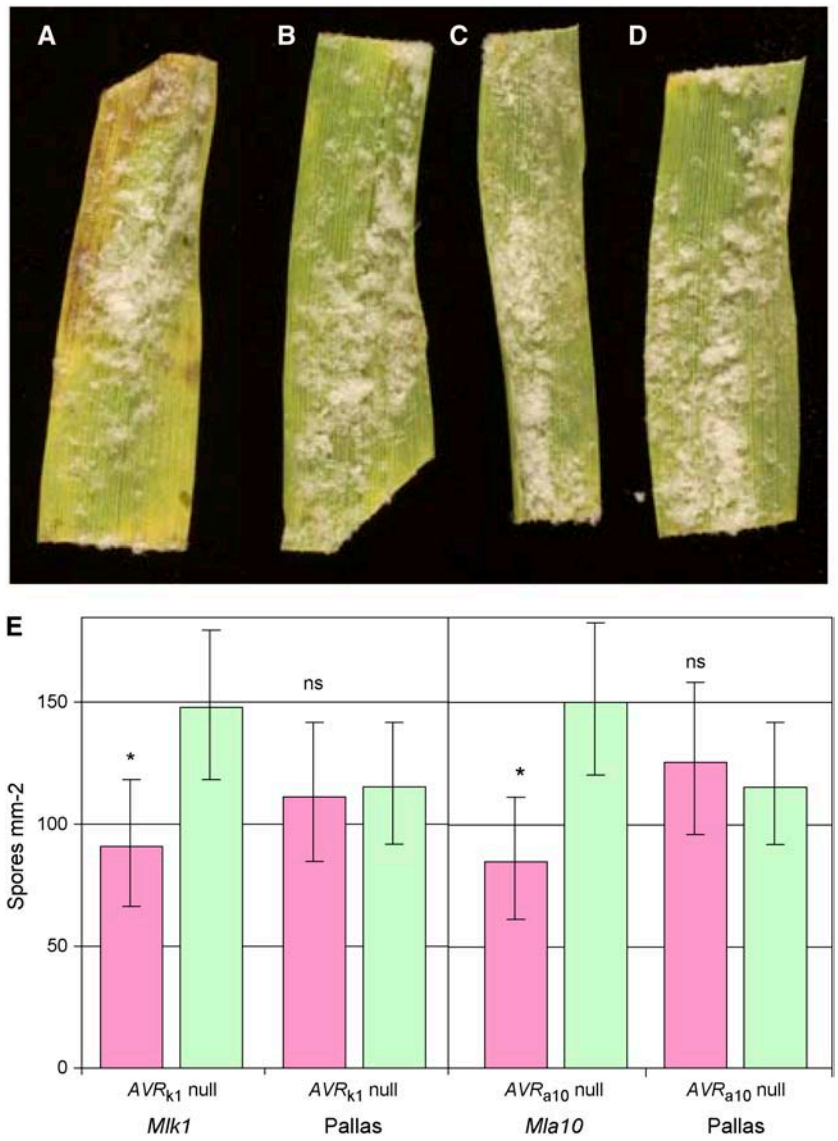


Figure 5. AVR_{k1} and AVR_{a10} Induce Delayed Necrosis after Infection.

(A) Necrosis phenotype observed on P17 (*Mlk1*) leaves bombarded with AVR_{k1}.
(B) P17 leaves bombarded with null plasmid.
(C) Pallas (*mlk1*) leaves with AVR_{k1}.
(D) Pallas leaves with null plasmid. All leaves in **(A)** to **(D)** are shown 10 d after infection with DH14.
(E) Spore production (conidia mm^{-2}) determined 10 d after infection following bombardment of AVR_{k1} or AVR_{a10} or the null plasmid as for **(D)** measured on a hemocytometer slide. Each data point summarizes three experiments with four replicates of each isolate + plasmid combination per experiment and seven leaves of each variety per variety + plasmid replicate. Predicted means were calculated by residual maximum likelihood analysis of the square root of the number of spores produced per leaf. The random effects model was experiment/isolate/replicate, and the fixed effects model was variety × plasmid. The variety × plasmid interaction was highly significant (Wald test, $P < 0.001$). As there was no significant effect of isolate (DH14 or CC52) on the variety × plasmid interaction, means were predicted for varieties and plasmids pooled across isolates. Significance test for difference between differential variety and Pallas: *, $P < 0.05$; ns, $P > 0.05$. Bars indicate mean ± 1 SE.

isolate CC148 (Figure 6A). DNA gel blot hybridization experiments revealed that sequences homologous to AVR_{k1} are present in other formae speciales of *B. graminis* isolated from the Graminae hosts, wheat, rye (*Secale cereale*), oat (*Avena sativa*), and *Agropyron* sp (Figure 6B). PCR with degenerate primers also amplified bands from the DNA of these formae speciales. The AVR_{k1} sequence hybridized to 98 of 12,288 clones in the *Bgh* BAC library (Figure 6C). Taken together, the DNA gel blot and BAC filter hybridizations indicate that there are likely to be >30 paralogues within the genomes of *Bgh* and each of the other

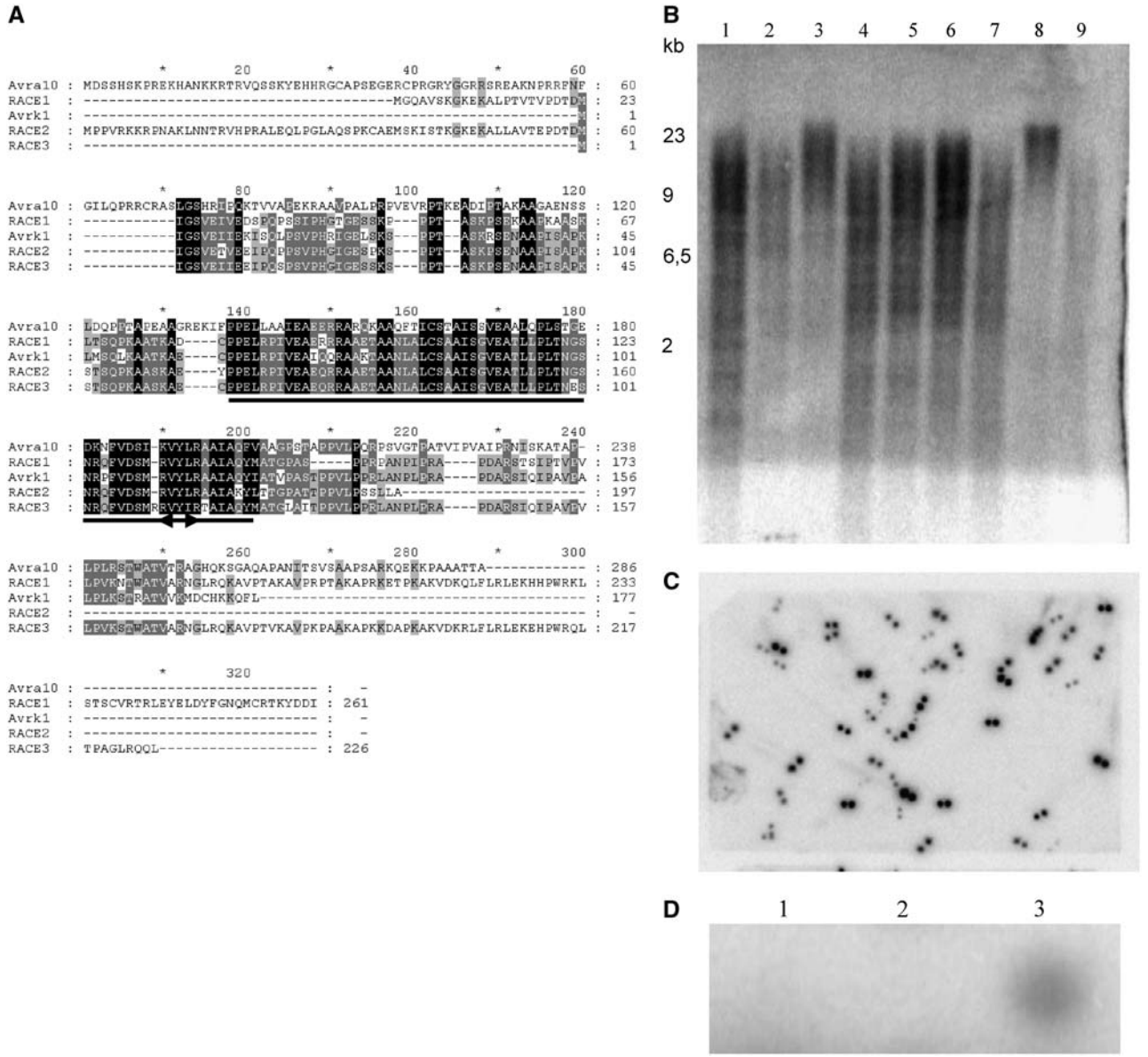


Figure 6. $AVR_{k1}$and AVR_{a10} Belong to a Gene Family in Cereal Powdery Mildews.

(A) Sequences paralogous to AVR_{k1} were isolated by RACE-PCR from *Bgh* isolate CC148. The most conserved residues are boxed in black, and less conserved residues are in lighter gray. The conserved core region is underlined and contains a motif (between arrowheads) resembling that found in oomycetes and implicated in targeting avirulence proteins into host cells (Armstrong et al., 2005; Rehmany et al., 2005; Bhattacharjee et al., 2006).
(B) DNA gel blot hybridization of the conserved core sequence of AVR_{k1} (shown in **[A]**) against DNA (10 μg), restricted with *Eco*RI, obtained from *B. graminis* formae speciales isolated from grasses: *Agropyron repens* isolate Agr2 (lane 1), oat isolates M089-12 (lane 2) and OFR2 (lane 3), rye isolates RESP4 (lane 4) and PMAS (lane 5), wheat isolates P4RC (lane 6) and PIRIB (lane 7), and barley isolates CC52 (lane 8) and CC148 (lane 9). The approximate size of restriction fragments is shown.
(C) Hybridization of the AVR_{k1} core probe against a one genome equivalent of the *Bgh* BAC library. Hybridizing clones are visible as two replicate dots at each location on the filter. The DNA gel blot and BAC library hybridizations indicate >30 paralogues present in the genomes of *B. graminis* formae speciales.
(D) Dot blot of whole genome amplifications of (1) wash from noninfected *Arabidopsis* leaves, (2) DNA from noninfected *Arabidopsis* leaves, and (3) DNA from conidiospores from *E. cichoracearum* infecting *Arabidopsis* (all 10 μg of amplified DNA).

B. graminis formae speciales. In all, 87% (85/98) of the BAC clones hybridized to both AVR_{k1} and a Cg T1 sequence, revealing a close association between this *AVR* gene family and this class of retrotransposons. The AVR_{k1} sequence also weakly hybridized to DNA from *Erysiphe cichoracearum*, which infects *Arabidopsis* (Figure 6D), but the degenerate PCR primers failed to amplify a product indicating that more distantly related sequences may be present in this parasite. Apart from the *Bgh* and *Bgh*-infected barley ESTs, there was no significant (E value $<10^{-5}$) homology to the sequence or structure of AVR_{k1} paralogues in any nucleotide or protein database or in the protein homology/analogy recognition engine (http://www.sbg.bio.ic.ac.uk/phyre).

The central core of AVR_{k1}, AVR_{a10}, and the three complete RACE-PCR paralogues were highly conserved in terms of the transcribed amino acid sequence (Figure 6A). There was a deficit of nonsynonymous nucleotide changes (*Ka*, leading to amino acid changes) over synonymous nucleotide changes (*Ks*), implying that purifying selection has maintained the amino acid sequence, possibly owing to functional constraints on the protein. The ratio *Ka*:*Ks* within the core region ranged from 0.08 to 0.49 in pairs of comparisons between the five sequences. *Ka*:*Ks* = 1 would imply an absence of either purifying or diversifying selection. By contrast, the amino acid sequences outside the core and toward the N- and C-terminal regions of the AVR proteins and the RT-PCR paralogues were highly diverse. It is not possible to calculate *Ka*:*Ks* reliably for such diverse sequences, as its value depends on the alignment chosen; indeed, the process of sequence alignment causes a downwards bias in the estimate of *Ka*:*Ks*. Nevertheless, it is evident that great sequence diversity has evolved at the N- and C-terminal parts of the protein.

DISCUSSION

We have identified avirulence genes AVR_{k1} and AVR_{a10} and demonstrated that they belong to a gene family present in *Bgh* and other formae speciales of the grass powdery mildew fungi. It is possible that other paralogues may be different *AVR* genes; this could be tested by mapping and cell death assays in host plants with corresponding *R* genes, if these are available. We show that the AVR_{k1} paralogues are located near retrotransposons. Close physical association with retrotransposons and other repetitive sequences in both bacterial and fungal genomes may contribute to effector and *AVR* gene expansion and diversification (Rohmer et al., 2004; Skamnioti and Ridout, 2005; Gout et al., 2006).

Since powdery mildews infect single host epidermal cells, we were able to directly measure the effect of transiently expressing AVR_{k1} and AVR_{a10} on penetration success. Our results indicate that the *AVR* genes can enhance infection in susceptible varieties, indicating that they may function as effectors like some AVR proteins of bacterial pathogens (Alfano and Collmer, 2004). Further experiments will be required to validate these initial observations, for example, by localizing the AVRk1 proteins during infection and identifying potential host virulence targets. Effectors of *Pseudomonas syringae* and other pathogenic bacteria show considerable interstrain variation, implying that they play a role in host specialization (Guttman et al., 2002; Roden et al., 2004). If this also applies to powdery mildew fungi, the polymorphism between avirulence gene paralogues may be involved in host specialization and the origin of formae speciales.

When AVR_{k1} was expressed in the resistant barley varieties containing *Mlk1*, GFP reporter expression was reduced to 59% compared with the control plasmid. This value is comparable to the ~50% reduction in reporter protein when *avrRpt2* from *P. syringae* was expressed in *Arabidopsis* leaves containing the corresponding *RPS2* resistance gene (Leister et al., 1996). Up to ~95% reduction in reporter protein accumulation has been measured using similar methods with other *AVR* genes (Leister et al., 1996; Allen et al., 2004; Rehmany et al., 2005). These results indicate that cell death can proceed more rapidly in some *AVR*/*R* gene combinations. We were also able to measure induced inaccessibility in GFP-expressing cells that remained alive. This indicated that cell death does not always occur in cells expressing the *AVR* gene and that other changes have prevented the establishment of a successful infection. Race-specific resistance that does not result in cell death has been reported previously in both wheat and barley powdery mildew (Schiffer et al., 1997; Li et al., 2005).

Our results show that AVR_{k1} and AVR_{a10} are recognized when transiently expressed within host cells, which is consistent with the predicted cytoplasmic location of Mla proteins (Schulze-Lefert and Panstruga, 2003). *Bgh* AVR proteins must therefore be able to enter the host cell during infection, and we are currently developing immunolocalization procedures to investigate these predictions. The *Uromyces fabae* rust protein Uf RTP1p is known to enter host cells and localize to the nucleus (Kemen et al., 2005). However, the function of Uf RTP1p and the mechanism of its entry into the host cell are not known. Other fungal and oomycete AVR proteins are also predicted to enter the host cell, where they are recognized by cytoplasmic R proteins. A conserved amino acid motif Gx_1x_2R (where x_1 is a hydrophobic or aromatic amino acid) common to four flax rust AVR proteins predicted to enter host cells has been identified (Catanzariti et al., 2006), but this sequence was not present in the *Bgh* AVR proteins. A conserved motif (RXLR, where X is any amino acid) has been implicated in the transport into host cells of AVR proteins and effectors from the oomycetes *H. parasitica* and *P. infestans* (Armstrong et al., 2005; Rehmany et al., 2005). This implication is based on amino acid sequence similarity to the PEXEL element, which directs exported *Plasmodium* virulence proteins into host cell erythrocytes (Marti et al., 2004). Indeed, the RXLR motif can substitute for the PEXEL element and drive the export and host targeting of GFP chimera proteins in *Plasmodium* sp (Bhattacharjee et al., 2006). However, there is no experimental proof that the RXLR motif can direct virulence proteins from oomycete parasites into host plant cells. Although AVR_{k1} and its paralogues do not contain the RXLR motif, we noted a similar sequence ([R/K]VY[L/I]R) within the conserved central core of the proteins (Figure 6A). However, searches of sequenced fungal genomes with this motif did not identify any proteins known to be involved in pathogenicity. Intriguingly, a motif (RMLLR), which closely resembles that found in the AVR_{k1} paralogues, has been identified as a small subset of RXLR proteins from *P. sojae* (R.H.Y. Jiang and B.M. Tyler, personal communication).

The AVR_{k1} paralogues are not predicted to contain signal peptides for secretion via the endoplasmic reticulum (ER). AVR-ACE1 isolated from *Magnaporthe grisea* is also not predicted to be secreted, but this intracellular protein is involved in the formation of a secondary metabolite that is the presumed avirulence elicitor (Böhnert et al., 2004). All other fungal proteins isolated so far function directly as AVR elicitors and are predicted to contain signal peptides for secretion via the ER. The cell death and infection assays demonstrate that AVR_{k1} and AVR_{a10} are functional inside the host cell, suggesting that an alternative route for secretion of these proteins may exist in *Bgh*. Increasing numbers of virulence and other proteins exported by non-ER-dependent routes have been identified in animal parasites, including *Candida albicans*, *Aspergillus fumigatus*, and *Leishmania* sp (Denny et al., 2000; Denikus et al., 2005; Nombela et al., 2006). Specialized delivery mechanisms may have evolved in these parasites, or the virulence proteins may contain features required for secretion and targeting to host cells. Our results indicate that searches for AVR proteins and effectors in other fungal plant parasites should not be restricted to those that contain signal peptides.

Powdery mildew fungi are vigorous parasites that rapidly adapt to overcome host recognition. We have shown that AVR_{k1} and AVR_{a10} are avirulence genes encoding proteins that elicit host defenses and contribute to successful infection. If *Bgh* AVR proteins are effectors that can functionally substitute for each other, parasite vigor and host compatibility could be maintained by the combined action and constant selection of multiple AVR_{k1} paralogues. This would explain why individual *AVR* genes can be lost without a fitness penalty (Bronson and Ellingboe, 1986; Brown and Wolfe, 1990) and why *R* genes are so easily defeated.

METHODS

Fungal Isolates and Genetic Analysis

Cultures of *Bgh* were grown on barley leaf segments (~2-cm long) kept on agar supplemented with benzimidazole (0.1 gL^{-1}) in plastic boxes (120 mm × 80 mm × 15 mm) (Brown and Wolfe, 1990). *Bgh* isolates CC52 and CC148 were crossed individually with DH14, which has the opposite mating type, resulting in the formation of cleistothecia. Single ascospore cultures were obtained from the cleistothecia (Brown and Wolfe, 1990), producing 83 progeny isolates from the cross CC52 × DH14 and 51 progeny from CC148xDH14. The parental and progeny cultures were scored for their avirulence phenotypes on Hordeum 1063 and P17 (*Mlk1*), P09 (*Mla10*), and P12 (*Mla22*). DNA was also extracted from the parental and progeny cultures as previously described (Robinson et al., 2002). Genetic mapping was performed using AFLPs (Vos et al., 1995). Parent isolates were screened with AFLP primer combinations to identify polymorphic sequences, which were then mapped with the progeny set. A selected AFLP marker band (PAAMCAC-365) was used for probing a BAC library.

Construction and Probing of a BAC Library

DNA from 7-d-old conidia (10 g) of DH14 was extracted in 10 mM Tris, pH 8.0, 100 mM EDTA, RNase (50 μg/mL), SDS (0.5% [w/v]), and proteinase K (to 100 μg/mL) and purified by ultracentrifugation on a 10 to 40% sucrose gradient. The DNA was restricted with *Sau*3a, centrifuged at 40,000 rpm for 2 h to purify high molecular weight DNA, ligated into SACBII vector (Bendahmane, 1999), and transformed into Electromax (Invitrogen) competent cells. The 12,288 clones were organized into 384 well plates and gridded onto Hybond nylon membranes for probing. A selected AFLP marker band (PAAMCAC-365) was excised from a polyacrylamide gel, rehydrated in water for 16 h at 4°C, reamplified with primers P11 (5′-GACTGCGTACATGCAGAA-3′) and M48 (5′-GATGAGTCCTGAGTAACAC-3′), labeled, and used for probing. Probes were labeled with the kit Rediprime (Amersham Biosciences) using 20 μCi [^{32}P]dCTP. Hybridization was performed overnight at 65°C (Sambrook et al., 1989). Probes were also prepared from the AVR_{k1} and *Bgh* homologues of Cg T1 (5′-ATCCAACGAAACCGCCCTCCCATACAGCAATGTAGGCGATGTCTCGGTTTCCACGCCACCCGCGGATGTTCTCGCGCACCTGCCTGCTGGAACTGTGGATCCACCATGCACTCAGCCTTCGAGTGTAAAGCTCCAACCAAGTGCCGAAACTGCGGAGGGCCCCACCAATCTGGCAGTCGAG-3′).

Hybridization Experiments with Other Formae Speciales of *B. graminis* and *Erysiphe cichoracearum*

DNA was extracted from conidiospores of *B. graminis* f sp *tritici* (isolated from wheat [*Triticum aestivum*]), f sp *secalis* (isolated from rye [*Secale cereale*]), f sp *avenae* (isolated from oat, [*Avena sativa*]), and an isolate from *Agropyron* sp (Wyand and Brown, 2003). DNA from conidiospores of *E. cichoracearum* infecting *Arabidopsis thaliana* was extracted using the Bio-Rad Chelex 100 method, developed for the isolation of DNA from minute quantities of tissue (Hirata and Takamatsu, 1996), and amplified by the GenomePhi DNA amplification kit (Amersham Biosciences). As negative control for the amplification of DNA from other microorganisms, the same procedure was performed with the wash of noninfected *Arabidopsis* leaves. Samples were denatured and blotted onto a Hybond membrane (Amersham Biosciences) using a Biodot apparatus (Bio-Rad) and hybridized with an AVR_{k1} probe as described above. PCR with primers AVRDEGF (5′-GTCGARGCMRCCCTTCWWCC-3′, where R = A+G, M = A+C, and W = A+T) and AVRDEGR (5′-GTGGCMCSWGTGCTTYTGAG-3′, where Y = C+T and S = G+C) was performed with DNA from the *B. graminis* formae speciales and *E. cichoracearum*.

Analysis of Polymorphism and Transcripts

Following sequencing, primer pairs from regions within the BAC 1817D were designed and used to amplify sequences from the isolates DH14, CC52, and CC148 with Expand long template (Roche Diagnostics) or Herculase (Stratagene) DNA polymerases. Nucleotide polymorphisms were mapped at loci CJR1-CJR3 by PCR with primers CJR1F (5′-ATATTGCCTTAATTGGTATG-3′), CJR1R (5′-TTCCAACCAGCATCATCTAC-3′), CJR2F (5′-ATTCGAAGATGACTCGAGTTTGA-3′), CJR2R (5′-GTCCAAGTGATCTATTGAATTCT-3′), CJR3F (5′-GTGAATTGTAGATGTGGATGTGG-3′), and CJR3R (5′-CCCTATTCTTGGAGGTGTTTG-3′). For analysis of polymorphism, DNA was amplified from the additional isolates GF13 and HLA (both Ak1) and GF4 and C24R9 (both Vk1) with primers CJR2F and CJR2R, which produces a 1775-bp amplicon spanning the AVR_{k1} gene. RNA was extracted from leaves of barley cultivar Golden Promise with an RNAeasy kit (Qiagen) 3 d after inoculation with CC148 and cDNA prepared with a SMART RACE cDNA kit (BD Biosciences). Nested primers to amplify AVR_{k1} by RT-PCR were R1 first, then R2 (for 5′ RACE-PCR) and R3 then R4 (for 3′ RACE-PCR). Nested primers for amplification of AVR_{a10} were R5 then R6 (5′ RACE-PCR) and R7 then R8 (3′ RACE-PCR). Sequences of these primers were as follows: R1 (5′-ACGGCGGGAATTTGTATGCTCCT-3′), R2 (5′-AGGAGCCCTTGGGAGAGGGTT-3′), R3 (5′-CTATACAACAACGCGCCGCCA-3′), R4 (5′-GCGTCG AAGCCACCCTTCTTT-3′), R5 (5′-GCCGAAACCGAGGTGATATTTG-3′) R6 (5′-GGACATCGTTCTCCTTCGCTTG-3′), R7 (5′-ATCCTCCAGCCCAGAAGATGCA-3′), R8 (5′-AAGGCAGCAGGAGCCGAAAAC-3′).

Nucleotide and Protein Characterization and Analysis

Nucleotide sequence analysis and contig assembly were performed with the STADEN package (http://www.mrc-lmb.cam.ac.uk/pubseq). Homologies were detected by probing nucleotide or protein sequences against the NCBI and EMBL databases (http://www.ncbi.nlm.nih.gov/BLAST/) and fungal sequence databases at COGEME (http://cogeme.ex.ac.uk/blast.html), the Broad Institute (http://www.broad.mit.edu/), and The Institute for Genomic Research (http://www.tigr.org/). Searches for the short amino acid motif [RK]VY[IL]R were performed with MOTIF search (http://motif.genome.jp/MOTIF2.html) using the COGEME EST database and the Broad Institute fungal sequences. Potential ORFs were detected by ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence alignment was performed with MUSCLE (Edgar, 2004) and edited with Genedoc (distributed by K.B. Nicholas, H.B. Nicholas, and D.W. Deerfield, http://www.psc.edu/biomed/genedoc/gdfeedb.htm). The relative rates of nonsynonymous and synonymous nucleotide substitutions (*Ka*:*Ks*) were calculated by the program K-Estimator 6.0 (Comeron, 1999).

EMS Mutagenesis

Isolate CC148 was grown on barley (cv Golden Promise) leaf segments for 3 d, and the leaves were transferred onto agar containing 0.2% EMS for 16 h. In each experiment, 20 plastic boxes were used, each containing ~50 leaf segments. Conidia were collected after 10 d and inoculated onto leaves of barley cv P09 (*Mla10*). A single colony was isolated after performing the experiment eight times. The colony was tested on P09 to confirm that it had the phenotype VIR_{a10} and on a set of 13 other differential varieties (Brown and Wolfe, 1990) to confirm that it had the same avirulence determinants as the original isolate, CC148.

Cell Death Assay Procedures

The cell death assay was performed by cobombarding two plasmids into plant cells, one expressing the reporter gene GFP under the control of maize (*Zea mays*) ubiquitin promoter (Pu hGFP-C3-N; Neilsen et al., 1999) and another expressing the test *AVR* gene. To prepare the test plasmids, AVR_{k1} and AVR_{a10} coding sequences were first amplified from genomic DNA of *Bgh* isolate CC148 by PCR with primers containing *Pst*I and *Sac*I restriction sites at the N and C termini, respectively. The amplification products were cloned into pGEMTeasy (Promega) and the sequences checked. After removal of GFP coding sequence by *Pst*I and *Sac*I, pUbi-GFP-Nos (for maize ubiqitin 1 promoter-GFP-Nos, obtained from K. Shirasu; Shen et al., 2003) was used as a backbone for cloning candidate *AVR* genes. AVR_{k1} and $AVRr_{a10}$ were removed from the pGEMTeasy vector by the restriction enzymes *Pst*I and *Sac*I and inserted into pUbi-GFP-Nos to create plasmids pCJR37 (AVR_{k1}) and pPS1 ($AVRr_{a10}$). A null *AVR* plasmid containing the virulence (DH14) allele of AVR_{k1} with the mutation L14stop was also prepared (pCJR17). The *AVR* constructs were applied to gold particles (8.33 μg/mg gold) with Pu hGFP-C3-N reporter (1.8 μg/mg gold). This ratio of AVR to reporter plasmid was selected after preliminary trials to achieve the maximum number of GFP-expressing cells without diluting too far the concentration of AVR-expressing plasmid. The particles were bombarded onto leaves of the barley cultivars Pallas and Siri (both susceptible to all isolates; Kølster et al., 1986; Kølster and Stølen, 1987) and the near-isogenic resistant lines P17 and S17 (both *Mlk1*), P09 and S09 (both *Mla10*), P08B (Mla9), and P12 (*Mla22*) with a particle gun (Bio-Rad). Pairs of leaves (~2 cm each in length) comprising Pallas or Siri and the test variety from the same near-isogenic series (P series is derived from Pallas, for example P09; S series is derived from Siri) were placed in each of seven replicate positions under a hepta manifold such that both varieties received a similar quantity of gold particles. The position of all leaves was marked before bombardment, and the number of cells expressing GFP in resistant varieties relative to susceptible varieties (GFP index) was determined after 44 h by viewing through a UV dissecting microscope (Leica). Statistical analysis was done by generalized linear modeling, and each experiment was performed five times.

Infection Assays

To determine the effect of *AVR* expression on infection, resistant and susceptible leaves were cobombarded at the same time as for cell death assay, with pCJR37 (AVR_{k1}), pPS1 (AVR_{a10}), or the null plasmid (pCJR17). The bombarded leaves were inoculated 24 h later with the isolates DH14 or CC52 (both Vk1 and Va10). Cells expressing GFP were scored for successful or failed infection after a further 40 h using the criteria described in Figures 4A to 4C using a UV microscope (Leica). These illustrative photomicrographs of living tissue were taken rapidly to minimize deterioration of the structures by UV light. The experiment was performed three times for AVR_{k1} and two times for AVR_{a10}. Since there was no significant difference between results for the two isolates, means were predicted for varieties and plasmids pooled across isolates. The viability of fungal colonies was also determined by a spore-counting procedure. The infection assay was performed without the inclusion of the GFP-expressing plasmid (since no UV microscopy was involved). Ten days after infection, the concentration of conidiospores on the leaf was determined by tapping them onto a hemocytometer slide with the aid of a tube (1.5-cm diameter) positioned over the counting grid. The number of spores/mm^2 in the hemocytometer grid directly represents the spores produced on the leaf. The experiment was performed three times.

Accession Numbers

Nucleotide and protein sequences of AVR_{k1} and AVR_{a10} mRNA have been deposited in the GenBank data library under accession numbers DQ679912 and DQ679913, respectively, and the genomic region cosegregating with AVR_{k1} under accession number DQ679914.

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