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# REVIEW

# Fungal Avirulence Genes: Structure and Possible Functions

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Laugé, R., and de Wit, J. G. M. 1998. Fungal avirulence genes: Structure and possible functions. *Fungal Genetics and Biology* 24, 285–297. Avirulence (*Avr*) genes exist in many fungi that share a gene-for-gene relationship with their host plant. They represent unique genetic determinants that prevent fungi from causing disease on plants that possess matching resistance (*R*) genes. Interaction between elicitors (primary or secondary products of *Avr* genes) and host receptors in resistant plants causes induction of various defense responses often involving a hypersensitive response. *Avr* genes have been successfully isolated by reverse genetics and positional cloning. Five cultivar-specific *Avr* genes (*Avr*4, *Avr*9, and *Ecp*2 from *Cladosporium fulvum; nip1* from *Rhynchosporium secalis;* and *Avr2-YAMO* from *Magnaporthe grisea*) and three species-specific *Avr* genes (*PWL1* and *PWL2* from *M. grisea* and *inf1* from *Phytophthora infestans*) have been cloned. Isolation of additional *Avr* genes from these fungi, but also from other fungi such as *Uromyces vignae*, *Melampsora lini, Phytophthora sojae,* and *Leptosphaeria maculans,* is in progress. Molecular analyses of nonfunctional *Avr* gene alleles show that these originate from deletions or mutations in the open reading frame or the promoter sequence of an *Avr* gene. Although intrinsic biological functions of most *Avr* gene products are still unknown, recent studies have shown that two *Avr* genes, *nip1* and *Ecp*2, encode products that are important pathogenicity factors. All fungal *Avr* genes cloned so far have been demonstrated or predicted to encode extracellular proteins. Current studies focus on unraveling the mechanisms of perception of avirulence factors by plant receptors. The exploitation of *Avr* genes and the matching *R* genes in engineered resistance is also discussed. © 1998 Academic Press

*Index Descriptors:* avirulence; gene-for-gene; elicitor; cultivar specificity; species specificity; resistance; plant receptor; two-component system.

Many fungal plant diseases occur in nature. Unlike the limited number of species that cause disease in animals, several thousands of fungal species are responsible for plant diseases. It is known that every plant species is a potential host for various fungi. However, disease is an exception rather than a rule and most natural interactions between fungi and plants that live in the same biotope do not lead to a disease (Agrios, 1997). Most of these interactions are of the "nonhost" type, in which the fungus is not known to be pathogenic on a given plant, probably because the fungus lacks the basic pathogenicity factors that would enable it to cause disease on a plant (Heath, 1991). The remaining interactions are of the "host" type, in which the fungus is known to be a pathogen on a given plant. However, for the latter type of interaction, not all combinations of fungal strains and plant cultivars will lead

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to a disease. The host type of interactions are therefore divided into "compatible interactions" in which a "susceptible plant" becomes diseased upon attack by a "virulent pathogen" and "incompatible interactions" in which a "resistant plant" does not develop disease upon attack by an "avirulent pathogen." The first report of genetic plant resistance against a fungal disease goes back to the end of the last century when Farrer described resistance in wheat against yellow rust (Farrer, 1898). The resistant character was subsequently demonstrated to be a dominant monogenic trait (Biffen, 1905, 1907, 1912). In the following decades, several reports appeared on monogenic and polygenic resistances to various diseases in different plant species. Introgression into cultivars confirmed that monogenic resistances were usually dominant.

Disputes about whether specific interactions occur during compatible or incompatible interactions were finally resolved in the early forties. Flor, working on the *Melampsora lini*–flax interaction (Flor, 1946, 1955), and Oort, working on the *Ustilago tritici*–wheat interaction (Oort, 1944), made a genetic breakthrough by studying the genetics of both host plant and fungus. Heritability of virulence (the ability to cause a disease) and avirulence (the inability to cause disease on particular cultivars) was investigated. Flor made hybrids between strains of *M. lini* virulent or avirulent on a given flax cultivar and scored the resulting dikaryotic offspring. Virulence appeared to be recessive and avirulence dominant. This laid the basis for the gene-for-gene concept that states that for every dominant gene determining resistance in the host there is a matching dominant avirulence gene in the pathogen. This concept is illustrated in Table 1. The avirulence (*Avr*) gene of a pathogen exists by virtue of its matching resistance (*R*) gene in the host plant. Although gene-for-gene interactions have been described for many fungus–plant interactions, with examples from all classes of fungi, they occur most frequently among obligate and biotrophic fungi (Day, 1974). While originally proposed for plant-pathogenic fungi, other pathogen–plant interactions involving bacteria, viruses, nematodes, and even insects have been shown to comply with the gene-for-gene concept (Agrios, 1997). The gene-for-gene concept has also been proposed to account for species-specificity in fungi that are known to infect several different plant species while individual strains are often restricted to one or few host plants. A few exceptions have been reported in which resistance and avirulence do not follow the typical inheritance patterns as described by Flor (1946). Examples include: (i) recessive inheritance of resistance and avirulence, (ii) resistance controlled by two genes, and (iii) additional control of resistance by modifiers or suppressors (Barrett, 1985; Christ *et al.*, 1987). Since the majority of *Avr* and *R* genes are dominant and likely to correspond to positive functions, the elicitor–receptor model has been proposed to explain the biochemical basis of the gene-for-gene concept (Gabriel and Rolfe, 1990; Keen, 1990; De Wit 1992, 1997). According to this model, a specific elicitor (putative product of an *Avr* gene) is recognized by a receptor (putative product of the matching *R* gene) of the resistant plant. This interaction would activate a signal transduction pathway that leads to active resistance often involving a hypersensitive response (HR) (Schell and Parker, 1990; Lamb, 1994).

TABLE 1
The Gene-for-Gene Hypothesis Illustrated for Two Matching *Avr*/*R* Gene Pairs

| | Host genotype | |
|---|---|---|
| Pathogen genotype | *R1– r2r2* | *r1r1 R2–* |
| Haploid | | |
| *A1 a2* | Incompatible | Compatible |
| *a1 A2* | Compatible | Incompatible |
| Diploid/dikaryotic | | |
| *A1– a2a2* | Incompatible | Compatible |
| *a1a1 A2–* | Compatible | Incompatible |

This review focuses on fungal *Avr* genes. Recent results on *Avr* gene sequences, *Avr* gene expression, structure and activity of *Avr* gene products, and evolution of *Avr* genes are presented. The intrinsic biological function of *Avr* genes for the pathogen, the mechanisms of perception of AVR factors by plants carrying the matching *R* genes, and the exploitation of *Avr* and matching *R* genes in molecular resistance breeding will be discussed. Fungal elicitors that do not induce plant defense responses in a cultivar- or species-specific manner (for review see Ebel and Scheel, 1997), in addition to plant defense responses triggered after perception of *Avr* gene-encoded elicitors (Vera-Estrella *et al.*, 1994; Hammond-Kosack and Jones, 1996) will not be discussed here.

## STRUCTURE AND PROPERTIES OF *Avr* GENES AND THEIR PRODUCTS

By definition *Avr* genes restrict the host range of plant pathogenic fungi. Therefore, *Avr* genes have received

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TABLE 2
Currently Cloned Fungal Avirulence Genes

| Pathogen | *Avr* gene | Specificity | Homology | References |
|---|---|---|---|---|
| *Cladosporium fulvum* | *Avr*9 | Tomato/*Cf-9* genotypes | Cystine-knot peptide (structural) | Van den Ackerveken *et al.* (1992)<br>Vervoort *et al.* (1997) |
| | *Avr*4 | Tomato/*Cf-4* genotypes | None | Joosten *et al.* (1994) |
| | *Ecp*2 | Tomato/*Cf-ECP2* genotypes | None | Laugé *et al.* (1998) |
| *Rhynchosporium secalis* | *nip1* | Barley/*Rrs1* genotypes | Toxin/Hydrophobin? | Rohe *et al.* (1995)<br>Wevelsiep *et al.* (1993) |
| *Magnaporthe grisea* | *AVR2-YAMO* | Rice/Yashiro-mochi cultivar | Neutral $Zn^{2+}$ protease (sequence motif) | Valent (1997) |
| | *PWL2, PWL1* | Weeping lovegrass | None | Sweigard *et al.* (1995)<br>Kang *et al.* (1995) |
| *Phytophthora parasitica* | *para1* | *Nicotiana tabacum?* | None | Ricci *et al.* (1992)<br>Kamoun *et al.* (1994) |
| *Phytophthora infestans* | *inf1* | *Nicotiana benthamiana* | None | Kamoun *et al.* (1998) |

considerable attention from molecular biologists studying the interactions between fungi and plants. Consequently, in the past decade, much research has been focused on cloning and characterizing fungal *Avr* genes. To date eight *Avr* genes have been isolated (Table 2). Five *Avr* genes (*Avr*9, *Avr*4, *Ecp*2, *nip1*, and *AVR2-YAMO*) govern cultivar specificity fitting the original gene-for-gene hypothesis as proposed by Flor for the *M. lini*–flax interaction (Flor, 1946), while three *Avr* genes (*PWL1, PWL2*, and *inf1*) govern species specificity in fungus–plant interactions.

## *Avr* GENES WITH CULTIVAR-SPECIFICITY

### *Avr Genes Avr 9, Avr 4, and Ecp 2 of Cladosporium fulvum*

*Cladosporium fulvum* causes leaf mold on tomato and interacts with its host in a gene-for-gene manner (Figs. 1A, 1B, and 1C) (De Wit, 1992). Several monogenic dominant genes that confer resistance against *C. fulvum* (*Cf*–) have been identified in wild relatives of tomato and were introgressed into commercial cultivars. The presence of *Avr* genes *sensu stricto* Flor (1946) in *C. fulvum* could not be genetically demonstrated as the sexual form of this fungus is unknown. Histological studies on incompatible interactions involving various *Cf* genes showed that they are mainly of the HR type (Lazarovits and Higgins, 1976; De Wit, 1977). *C. fulvum* does not produce specialized feeding structures during colonization of tomato leaves and fungal growth remains confined to the apoplast (Lazarovits and Higgins, 1976; De Wit, 1977). All molecules involved in communication between the fungus and its host plant are therefore present in apoplastic fluids (AFs) isolated from *C. fulvum*-infected plants, including the putative AVR molecules that act as specific elicitors on plants carrying the matching *R* genes.

The putative *Avr*9 avirulence gene of *C. fulvum*, matching the *Cf-9* resistance gene in tomato, was the first *Avr* gene to be investigated in the *C. fulvum*–tomato interaction. AFs were prepared from susceptible plants inoculated with strains that cannot overcome the *Cf-9* gene and from races that can overcome the *Cf-9* gene. Upon injection of these AFs into leaves of *Cf-9*-containing and *Cf-9*-lacking plants, it was confirmed that a protein elicited specific HR in the injected area of *Cf-9*-containing plants (Table 3). This proteinaceous elicitor is present in AFs from all interactions involving races that cannot overcome the *Cf-9* gene, while it is absent in AFs from races that can overcome the *Cf-9* gene (Fig. 1D) (Scholtens-Toma *et al.*, 1989). Therefore, this elicitor was proposed to be the product (AVR9) of the putative *Avr*9 gene of *C. fulvum*. The AVR9 elicitor has been purified (Figs. 1E and 1F) and was found to be a 28-amino-acid (aa) peptide (Scholtens-Toma and de Wit, 1988). The *Avr*9 structural gene has been subsequently cloned (Van den Ackerveken *et al.*, 1992). It encodes a preproprotein of 63 aa with a characteristic signal peptide for extracellular targeting. The 40-aa secreted peptide is further processed by endogenous and plant proteases into the mature 28-aa AVR9 elicitor (Van den Ackerveken *et al.*, 1993). Transfer of the *Avr*9 gene into a wild-type $Avr9^-$ strain is sufficient to render the resulting $Avr9^+$ transgenic strain avirulent on *Cf-9*-containing plants (Van den Ackerveken *et al.*, 1992).

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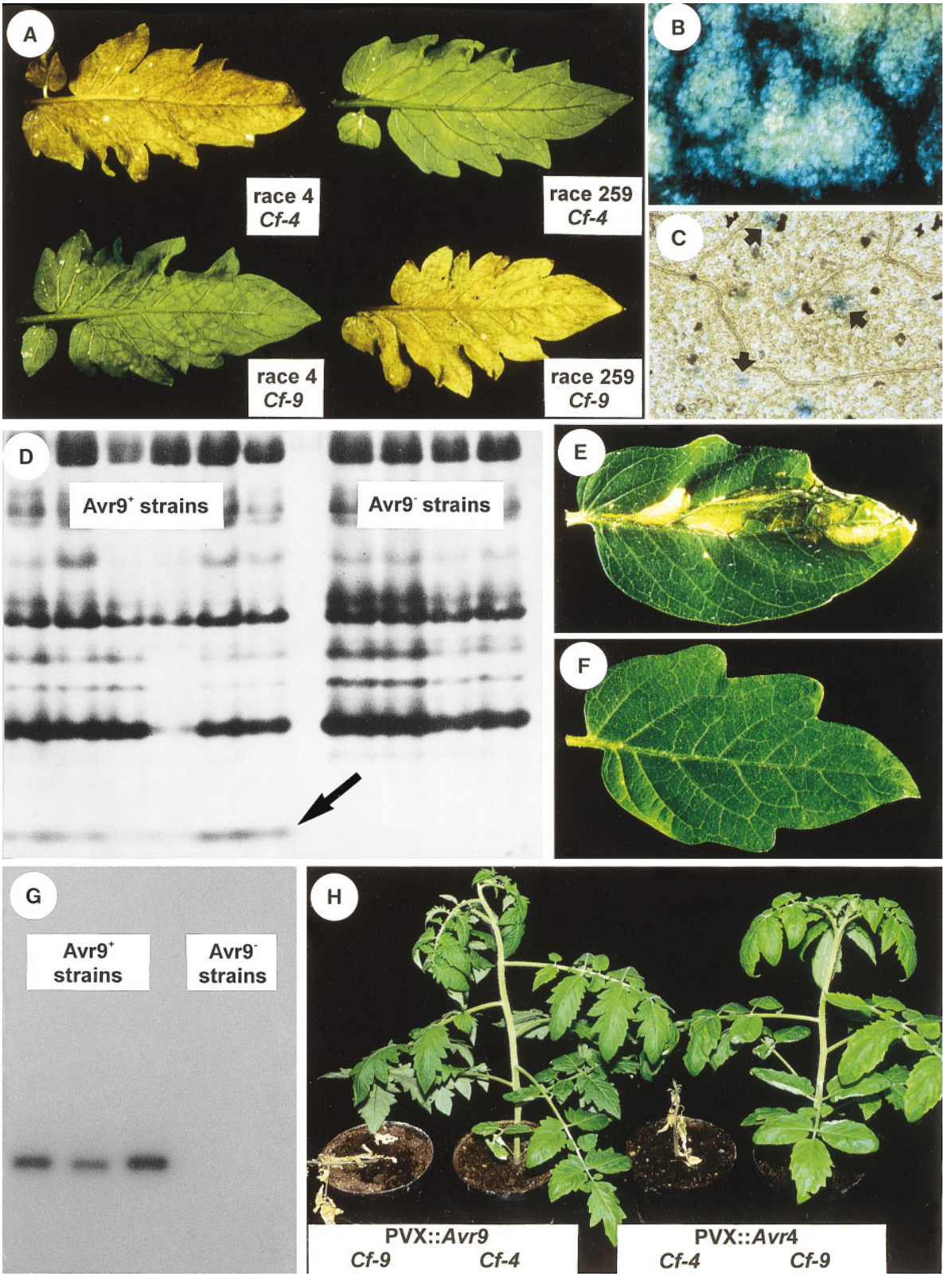
A
race 4
Cf-4
race 259
Cf-4
race 4
Cf-9
race 259
Cf-9
B
C
D
Avr9+ strains
Avr9- strains
E
F
G
Avr9+
strains
Avr9-
strains
H
PVX::Avr9
Cf-9
Cf-4
PVX::Avr4
Cf-4
Cf-9

TABLE 3
HR-Inducing Activity of Elicitors from Various *Cladosporium fulvum* Strains on Different Tomato Genotypes

| *C. fulvum* strains | Elicitor(s) produced | Tomato genotypes | | | |
|---|---|---|---|---|---|
| | | *Cf-0* | *Cf-4* | *Cf-9* | *Cf-ECP2*[a] |
| Wild-type races | | | | | |
| Race 5 | AVR4, AVR9, ECP2 | —[b] | HR | HR | HR |
| Race 4 | AVR9, ECP2 | — | — | HR | HR |
| Race 2.4.5.9 | ECP2 | — | — | — | HR |
| Mutant races | | | | | |
| Race 5-Δ*Avr*9[c] | AVR4, ECP2 | — | HR | — | HR |
| Race 5-Δ*Ecp2*[d] | AVR4, AVR9 | — | HR | HR | — |

[a] Laugé *et al.* (1998).
[b] —, no HR, the plant is susceptible.
[c] Marmeisse *et al.* (1993).
[d] Marmeisse *et al.* (1994).

Transformation-mediated disruption of *Avr*9 in two wild-type *Avr*9$^+$ strains changes the strains from avirulent to virulent on *Cf-9* plants (Marmeisse *et al.*, 1993). These results prove that the *Avr*9 gene is the *Avr* gene matching the tomato *Cf-9* resistance gene. Expression studies have shown that the *Avr*9 gene is strongly induced during infection (Fig. 1B). Furthermore, the *Avr*9 gene is induced *in vitro* by nitrogen starvation (Van den Ackerveken *et al.*, 1994). In agreement with this finding, many (TA)GATA regulatory sequences described as targets for the *Aspergillus nidulans* AREA and *Neurospora crassa* NIT2 transcription factors involved in nitrogen metabolism are present in the promoter of *Avr*9. Current studies aim to evaluate the role of nitrogen in *Avr*9 expression during the colonization of the host plant. No sequence variation has been detected among wild-type *Avr*9$^+$ strains tested so far, and all wild-type strains known to overcome the *Cf-9* resistance gene have been demonstrated by Southern blot analysis to lack the entire *Avr*9 ORF (Fig. 1G) (Van Kan *et al.*, 1991). Thus wild-type *Avr*9$^-$ strains avoid recognition in *Cf-9* plants by not producing the AVR9 elicitor (Fig. 1D).

The *Avr*4 gene that matches the tomato *Cf-4* resistance gene has been isolated in a way similar to the *Avr*9 gene (Table 3) (Joosten *et al.*, 1994). The *Avr*4 gene encodes a 135-aa preproprotein with a characteristic signal peptide for extracellular targeting. Like AVR9, the AVR4 product is further processed into a mature elicitor protein of 86 aa by plant and/or fungal proteases. Transformation of a wild-type *Avr*4$^-$ strain with the *Avr*4 gene renders the transgenic strain avirulent on *Cf-4*-containing plants. *Avr*4 gene expression is strongly induced during infection (Fig. 1C); however, specific conditions that induce *Avr*4 gene expression *in vitro* have not been found so far. The sequence of the *Avr*4 gene in various wild-type *Avr*4$^+$ strains shows no polymorphism. All *avr*4 alleles from wild-type *Avr*4$^-$ strains exhibit various single-point mutations, mainly single nucleotide substitutions resulting in the exchange of one amino acid in the AVR4 elicitor. In contrast to *Avr*9, all wild-type strains which are virulent on *Cf-4* plants contain *avr*4 alleles that are strongly expressed during infection. However, none of the proteins encoded by the *avr*4 alleles could be detected in the AFs of infected plants. Overexpression of the *avr*4 alleles using the potato virus X expression vector (Chapman *et al.*, 1992) (Fig. 1H) showed that some *avr*4 alleles encode a protein that is still recognized by *Cf-4* plants (Joosten *et al.*, 1997). Thus, different from wild-type *Avr*9$^-$ strains, wild-type *Avr*4$^-$ strains avoid recognition on plants containing the *Cf-4* gene by encoding unstable and/or mutated homologues of the AVR4 elicitor.

The *Avr*4 and *Avr*9 genes do not share homology with each other or with sequences present in databases. These

FIG. 1. (A) Leaves of *Cf-4* and *Cf-9* tomato genotypes inoculated with race 4 and race 2.5.9, resulting in a reciprocal gene-for-gene check; *Cf-4*/race 4 and *Cf-9*/race 2.5.9 give a compatible interaction while *Cf-4*/race 2.5.9 and *Cf-9*/race 4 give an incompatible interaction. Note the abundant brownish sporulating mycelium at the lower side of the leaf in the two compatible interactions. (B and C) Micrographs of tomato leaves inoculated with transgenic fungal strains containing promoter–GUS constructs. (B) *Avr*9–promoter–GUS transgenic strain inoculated on a susceptible plant; the interaction is compatible and strong staining develops around the vascular tissue. (C) *Avr*4–promoter–GUS transgenic strain inoculated on a resistant plant; the interaction is incompatible and limited staining occurs at the penetration sites (arrows) of the resistant plant. (D) Protein profiles of apoplastic fluids isolated from susceptible plants inoculated with six different *Avr*9$^+$ strains of *C. fulvum* (note the presence of the AVR9 elicitor indicated by the arrow) and four different *Avr*9$^-$ strains (note the absence of the AVR9 elicitor). (E and F) Tomato leaflets injected with elicitor of *C. fulvum*. (E) Leaflet of *Cf-9* genotype injected with purified AVR9 elicitor; HR visualized as necrosis occurs in the injected area. (F) Leaflet of *Cf-0* genotype injected with purified AVR9 elicitor; no necrosis occurs. (G) Southern blot of three *Avr*9$^+$ strains and two *Avr*9$^-$ strains probed with *Avr*9 cDNA; note the presence of an *Avr*9 signal in *Avr*9$^+$ strains and the absence of signal in *Avr*9$^-$ strains. (H) Symptoms that have developed on *Cf-4* and *Cf-9* genotypes after inoculation with recombinant PVX viruses containing the cDNA of either the *Avr*9 or the *Avr*4 gene; note that for matching *R*/*Avr* gene pairs strong necrosis has developed following systemic viral spread.

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two avirulence genes have in common that they encode low-molecular-weight cysteine-rich proteins that are secreted by *C. fulvum* during infection of tomato. Many additional low-molecular-weight proteins are present in AFs isolated from tomato leaves infected by *C. fulvum*. These additional proteins have been named ECPs, for extracellular proteins, and have been proposed to act as avirulence factors on yet unidentified resistant tomato genotypes. The two proteins, ECP1 (Joosten and de Wit, 1988) and ECP2 (Wubben *et al.*, 1994), have been assayed for HR-inducing activity on a collection of tomato breeding lines prescreened for resistance against *C. fulvum*. While none of these tomato lines display HR upon infiltration of purified ECP1, a few lines display HR upon infiltration of purified ECP2 (Table 3). In addition, it has been demonstrated that the ECP2-encoding gene acts as an *Avr* gene of *C. fulvum* on these lines that contain a monogenic resistance against *C. fulvum* operating through recognition of ECP2 (Laugé *et al.*, 1998). This indicates that avirulence of *C. fulvum* operates through recognition of its excreted proteins by different tomato genotypes, after which a HR is induced.

### *Avr Gene nip1 of Rhynchosporium secalis*

*Rhynchosporium secalis* causes leaf scald on barley. This fungus is assumed to interact in a gene-for-gene manner with its host, although, as for *C. fulvum*, lack of a sexual cycle of *R. secalis* prevents the genetic demonstration of *Avr* genes. After penetration of the cuticle, fungal mycelium remains subcuticular, and fungal growth is arrested after collapse of a few epidermal cells in incompatible interactions. Resistance of barley cultivars to *R. secalis* is assumed to result from early and strong induction of plant defense responses after perception of the fungal avirulence molecules.

Three low-molecular-weight necrosis-inducing peptides, NIP1, NIP2, and NIP3, have been purified from culture filtrate of the fungus grown *in vitro* and have been reported to act as aspecific toxins on barley, other cereals, and bean (Wevelsiep *et al.*, 1991, 1993). However, NIP1 also triggers the expression of two barley genes that encode pathogenesis-related (PR) proteins in a cultivar-specific manner. This elicitation of PR protein genes occurs specifically in barley cultivars that contain the *Rrs1* gene (resistance to *R. secalis* 1) and not in barley cultivars that do not contain *Rrs1*. The two mRNAs accumulate with similar timing when *Rrs1* barley cultivars are either treated with purified NIP1 or inoculated with strains of *R. secalis* that are avirulent on *Rrs1* barley cultivars (Hahn *et al.*, 1993). The *nip1* gene of *R. secalis* that encodes NIP1 has been obtained via reverse genetics and has been proposed to represent the putative avirulence gene matching *Rrs1*, the *AvrRrs1* gene. Transfer of *nip1* to a wild-type *AvrRsr1*$^-$ strain gave transformants that are avirulent on *Rrs1* barley cultivars, which proves that *nip1* and *AvrRsr1* are the same gene (Rohe *et al.*, 1995). Two *nip1* sequences encoding NIP1 proteins differing from each other by three amino acids are found among wild-type strains of *R. secalis* that are avirulent on *Rrs1* barley cultivars. Strains of *R. secalis* that are virulent on *Rrs1* barley cultivars have additional single-point mutations or have lost the *nip1* sequence completely.

### *Avr Gene AVR2-YAMO of Magnaporthe grisea*

*Magnaporthe grisea* is a foliar pathogen on more than 50 different grass species, including rice, on which it causes blast disease. Since the sexual stage of *M. grisea* is controlled under laboratory conditions (Herbert, 1971), the genetics of virulence and avirulence have been studied extensively.

Crosses between strains of *M. grisea* that are differentially pathogenic on various rice cultivars have revealed the existence of many gene-for-gene relationships with cultivar specificity between the fungus and rice (Valent *et al.*, 1991; Silué *et al.*, 1992). The avirulence gene *AVR2-YAMO*, which prevents infection of the Yashiro-mochi cultivar, has been isolated by positional cloning (Valent, 1997). It encodes a 223-aa protein with a putative signal peptide. The sequence of the *AVR2-YAMO* gene does not share significant homology with other known proteins, except for a small domain which shares homology with the active site of neutral $Zn^{2+}$ proteases. Since the gene is located at the tip of chromosome 1, many spontaneous virulent mutants result from DNA deletions or insertions. Virulent isolates carrying point mutations in the putative active site of neutral $Zn^{2+}$ proteases have also been found.

## *Avr* GENES WITH SPECIES-SPECIFICITY

### *Avr Genes PWL1 and PWL2 of M. grisea*

Despite a wide host range, individual isolates of *M. grisea* are often restricted to one or a few plant species.

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The existence of *Avr* genes governing species specificity has been demonstrated (Valent, 1997). Crosses between strains of *M. grisea* that either could or could not infect weeping lovegrass (*Eragrostis curvula*) have led to the identification of two *PWL* genes (govern pathogenicity toward weeping lovegrass) that showed characteristics of *Avr* genes in preventing *M. grisea* from causing disease on this plant species. *PWL1* originates from a strain virulent on finger millet (*Eleusine coracana*), while *PWL2* has been identified in a strain virulent on rice. The *PWL2* avirulence gene has been isolated by positional cloning (Sweigard *et al.*, 1995). Transformation of a wild-type *PWL2*$^-$ strain with the *PWL2* gene renders the strain avirulent on weeping lovegrass. The encoded product is predicted to be a hydrophilic protein of 145 aa with a putative signal peptide. A search for *PWL2* homologues in various *M. grisea* isolates has shown the *PWL* genes to be members of a small gene family. *M. grisea* isolates that are virulent on weeping lovegrass contain *PWL2* homologues (Kang *et al.*, 1995). *PWL1* has been cloned by homology to *PWL2*. Interestingly, although the *PWL1* gene shares only 75% aa identity with *PWL2*, it is still a functional homologue of *PWL2*, as it prevents infection of weeping lovegrass. In contrast, another *PWL* homologue, *PWL3* (51% aa identity with *PWL2*), from the finger millet isolate is nonfunctional, as it does not prevent infection of weeping lovegrass. A fourth *PWL* homologue, *PWL4*, has been isolated from a weeping lovegrass isolate. The latter is more related to *PWL3* (72% aa identity) than to *PWL2* (57% aa identity) and, like *PWL3*, it does not confer avirulence on weeping lovegrass. Surprisingly, when the open reading frame (ORF) of *PWL4* is placed under the control of either the *PWL1* or the *PWL2* promoter, it confers avirulence on weeping lovegrass, while similar constructions with the *PWL3* ORF do not. This suggests that *PWL4* is nonfunctional due to a defective promoter, while *PWL3* is nonfunctional due to mutations within its ORF (Kang *et al.*, 1995).

### *The Genes Encoding Elicitins of Phytophthora spp.*

For a long time the involvement of the elicitins as avirulence determinants at the species level has been proposed (Ricci *et al.*, 1992; Kamoun *et al.*, 1994). This family of low-molecular-weight proteins from species of *Phytophthora* and *Pythium* (Kamoun *et al.*, 1997) induces specific necrosis in all *Nicotiana* species tested so far. The main reason for considering these proteins as avirulence determinants on *Nicotiana* species came from studies on *Phytophthora parasitica*. The level of elicitin production has been analyzed for isolates of *P. parasitica* obtained from tobacco and other host plants. A strong negative correlation was found between virulence on tobacco and the level of elicitin production (Ricci *et al.*, 1992; Bonnet *et al.*, 1994). Consequently, the *para1* gene encoding the elicitin parasiticein of *P. parasitica* has been proposed to act as a species-specific *Avr* gene (Kamoun *et al.*, 1993). Lack of an efficient genetic transformation system and the diploid nature of this fungus have prevented so far confirmation of the role of *para1* in species specificity of *P. parasitica*. Recent studies on the elicitin infestin encoded by the *inf1* gene of the closely related oomycete *Phytophthora infestans* give molecular support for the role of elicitins in species specificity. Wild-type strains of *P. infestans* are avirulent on *Nicotiana* species and are arrested at an early stage of infection on these plants. *inf1*$^-$ strains of *P. infestans*, obtained through silencing of *inf1* in wild-type *inf1*$^+$ strains, can complete the infection cycle up to sporulation on *Nicotiana benthamiana* (Kamoun *et al.*, 1998). Therefore, at least for this interaction, the *inf1* gene appears to act as an *Avr* gene with species specificity in preventing infection of *N. benthamiana* by *P. infestans*.

### *Avr Genes That Are Currently Being Cloned*

The cloning of additional fungal *Avr* genes is in progress. These include *Avr* genes such as *AvrCo39*, *AVR1-MARA*, *AvrIrat7–1*, *AvrMednoï-1*, and *AvrKu86–1* of *M. grisea* giving cultivar specificity on rice (Leong *et al.*, 1994; Mandel *et al.*, 1997; Dioh *et al.*, 1996) and *AvrLm1* of *Leptosphaeria maculans* giving cultivar-specificity on canola (Ansan-Melayah *et al.*, 1995). *Avr* genes from *Phytophthora sojae*, *P. infestans*, and *M. lini* giving cultivar specificity on soybean, potato and flax, respectively, are currently being mapped (Whisson *et al.*, 1994, 1995; Van der Lee *et al.*, 1997; Timmis *et al.*, 1990). Reverse genetics is applied to clone the genes encoding two recently reported cultivar-specific elicitors of *Uromyces vignae* acting on cowpea (D'Silva and Heath, 1997).

## INTRINSIC FUNCTION OF *Avr* GENES

Scientists have always been intrigued by the presence of *Avr* genes in the genome of many plant pathogens. The definition of a fungal *Avr* gene concerns the inability of a

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particular fungal strain to cause disease on a particular plant species or cultivar. The definition does not imply an intrinsic function of the *Avr* gene for the fungus itself. As *Avr* genes restrict the host range of pathogens, one would expect these genes to disappear quickly through selection pressure exerted by a resistant plant. Therefore, an intrinsic pleiotropic function(s) for the fungus itself has been hypothesized in order to explain the maintenance of *Avr* genes in a population of fungal pathogens (Knogge, 1996). *Avr* genes could have either housekeeping or pathogenicity functions, encoding products such as enzymes involved in the degradation of plant substrates, toxins, or suppressors of the host defense responses. Examination of phenotypes of wild-type *Avr*$^-$ strains might give clues to a putative intrinsic function of a given *Avr* gene. However, in most cases only a few wild-type *Avr*$^-$ strains have been described. In addition, in nature *Avr*$^-$ strains may have evolved to compensate for the loss of the intrinsic *Avr* gene functions by complementation through a functional homologue(s). A reliable way to test the putative intrinsic function of an *Avr* gene is to compare, *in vitro* and/or *in planta*, the phenotype of an isogenic *Avr*$^-$ strain created by gene disruption with the phenotype of the corresponding near-isogenic wild-type *Avr*$^+$ parental strain.

Currently, little information is available concerning the putative functions of the *Avr* genes that have been described above. *Avr*9 disruption mutants are not affected in their growth pattern. They do not display an altered phenotype *in vitro* compared with the isogenic wild-type parental strains, neither do they seem to be affected in their pathogenicity on tomato (Laugé *et al.*, 1998). Putative involvement of the *Avr*9 gene in the nitrogen metabolism of *C. fulvum* has been proposed, as discussed before. Phenotypes of wild-type *Avr*4$^-$ strains are not different from those of *Avr*4$^+$ strains *in vitro* or *in planta* (M. H. A. J. Joosten, pers. comm.), but *Avr*4 disruption mutants have yet to be created to confirm the lack of an important role of *Avr*4 for *C. fulvum*. In contrast, *Ecp*2 was originally characterized as a gene with an important role in pathogenicity of *C. fulvum*, as an *Ecp*2 disruption mutant displayed reduced leaf colonization and reduced conidiation compared with the wild-type parental strain (Laugé *et al.*, 1997).

Wild-type *nip1*$^-$ strains as well as *nip1* disruption mutants of *R. secalis* have also been shown to exhibit a significant reduction in pathogenicity on barley (Rohe *et al.*, 1995; Knogge, 1996). The toxic activity of NIP1 that acts through stimulation of plant plasmalemma ATPase is likely to account for the pathogenicity function of NIP1. The spacing of cysteine residues in the NIP1 protein was found to be similar to that found in the family of the fungal hydrophobins, but no such function for NIP1 has been demonstrated yet.

A putative intrinsic function for *PWL1* and *PWL2* of *M. grisea* and *inf1* of *P. infestans* cannot be proven easily. These genes are members of gene families with potential functional homologues (Sweigard *et al.*, 1995; Kamoun *et al.*, 1997). Cumulative disruption of all genes separately and/or in combination will be required to assess the possible functions of *PWL1*, *PWL2*, and *inf1*.

## AVIRULENCE PERCEPTION

The mechanisms of recognition of *Avr* gene products by plants carrying the matching resistance gene are still largely unknown. The simplest model predicts that the *Avr* gene product is the elicitor molecule which is directly perceived by the resistant plant via the action of an *R*-gene-encoded receptor (Keen, 1990; De Wit, 1992). Several research groups are trying to find experimental evidence to prove or disprove this model.

The *Avr* genes that have been obtained through the reverse genetics approach (*Avr*4, *Avr*9, *Ecp*2, *nip1*, and *inf1*) have been isolated based on a specific induction of defense responses (HR or induction of PR proteins) by their encoded products in plants carrying the matching *R* gene. These *Avr* genes encode the eliciting AVR product, which is perceived by the resistant plant directly or after processing. Some of the *Avr* genes that have been obtained through positional cloning might not directly encode the eliciting compound. The *PWL2* gene product is predicted to be extracellular. However, injection of weeping lovegrass with the purified recombinant PWL2 protein did not elicit any detectable plant response (Sweigard *et al.*, 1995). The *AVR2-YAMO* gene product, which has potential protease activity, might be responsible for the release of an active elicitor molecule. The latter has been described for the avirulence gene *avrD* of the bacterium *Pseudomonas syringae* pv. *tomato*, the product of which is responsible for the synthesis of syringolide elicitors (Keen *et al.*, 1990).

Biochemical studies have been initiated to unravel the molecular mechanisms of recognition of the HR-eliciting AVR9 protein by *Cf-9* plants. Binding studies using $^{125}$I-labeled AVR9 showed the presence of a high-affinity binding site for the AVR9 protein in membrane fractions of

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tomato. Surprisingly, this high-affinity binding site for AVR9 appeared to be present in both *Cf-9*-containing and *Cf-9*-lacking plants (Kooman-Gersmann *et al.*, 1996). Therefore this binding site could not represent the *Cf-9* gene product. Consequently, the biochemical model that predicts that the *Cf-9* resistance gene encodes a unique receptor for AVR9 perception has to be refined. Most probably the AVR9 elicitor binds to a coreceptor that is part of a receptor complex including the *R*-gene-encoded product (Kooman-Gersmann *et al.*, 1998). Similar studies are under way for the AVR4 and NIP1 elicitors (Joosten *et al.*, 1997; Knogge, 1996).

## EXPLOITATION OF *Avr* GENES IN MOLECULAR RESISTANCE BREEDING

The interest in studying *Avr* genes is obvious for understanding mechanisms of plant resistance. However, as stated above, *Avr* genes exert their function in concert with their host counterpart, the matching *R* genes. Concomitantly with the growing interest in cloning *Avr* genes, *R* genes have also received much attention. Apart from fundamental interest in the gene pairs, engineering resistance by genetic transfer of *Avr*/*R* gene pairs to plants has become an object of study for biotechnological applications. This method has been introduced as the two-component sensor system (De Wit, 1992). The strategy consists of transferring an *Avr*/*R* gene pair to a given crop plant. By regulating both in time and in space the expression of the *Avr*/*R* gene pair, one can envisage artificial resistance of the transgenic crop as taking place against any pathogen which can be inhibited by HR. Along these lines, the *Avr*9/*Cf-9* gene pair is now being tested for suitability in molecular resistance breeding. Constructs have been made in which either the *Avr*9 gene or the *Cf-9* gene (Jones *et al.*, 1994) is under control of a pathogen-inducible promoter, P*gst1* (Strittmatter *et al.*, 1996). These constructs have been transferred to tomato plants (Fig. 2). Progenies obtained after selfing of primary transformants were subsequently inoculated with a wild-type *Avr*9$^-$ strain of *C. fulvum*. Several transgenic lines showed resistance to this strain, with HR induction at the site of infection, thus pointing to transient induction of the *Avr*9 transgene. As a result of the HR, fungal growth is arrested and the plant becomes resistant (De Wit, 1997). Use of the *Avr*9/*Cf-9* gene pair only in a homologous system like the

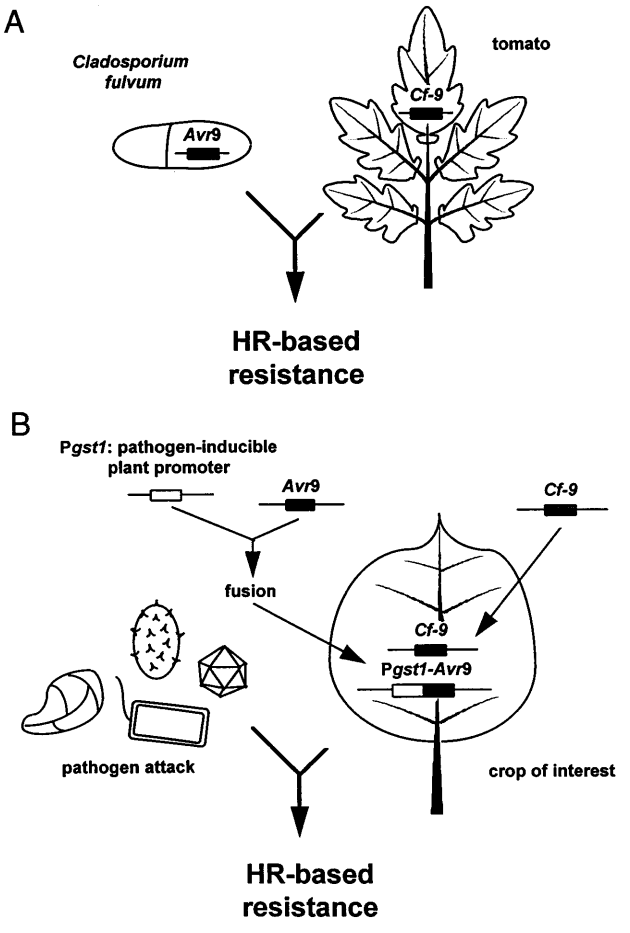


FIG. 2. (A) Natural resistance: The gene-for-gene system. The *Avr*9/*Cf-9* gene pair governs natural HR-based resistance in the *C. fulvum*–tomato interaction. (B) Engineered resistance: The two-component system. Upon transfer of *Cf-9* and *Avr*9, which is placed under control of the pathogen-inducible promoter P*gst1*, to a recipient crop; engineered HR-based resistance occurs against pathogens that can infect this plant.

*C. fulvum*–tomato interaction would give only limited application. Fungi have various modes of infection, and similarly plants may have various modes of defense responses. Signal transduction pathways involved in defense of plants may vary from one family to another or even from one genus to another. Nevertheless, HR seems to be a common resistance mechanism in many plants. Experiments involving the transfer of the *Avr*9/*Cf-9* gene pair to solanaceous plants other than tomato, to nonsolanaceous plants such as *Arabidopsis*, and to monocots such as rice are presently in progress. Resulting transgenic plants are currently being tested for resistance toward their pathogens.

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## OUTLOOK

Our molecular genetic knowledge of fungal *Avr* genes is presently based on the analysis of only eight genes that have little in common. They share sequence homologies neither at the nucleotide level nor at the amino acid level. Some are only expressed *in planta* while others are expressed *in vitro* as well as *in planta*. However, they are all known or predicted to encode extracellular proteins. The plant *R* genes *Cf-4* (Thomas *et al.*, 1997) and *Cf-9* (Jones *et al.*, 1994) acting against *C. fulvum* are the only *R* genes directed against a fungus for which the matching *Avr* genes, *Avr*4 and *Avr*9, respectively, have been cloned. *Cf-4* and *Cf-9* encode predicted extracytoplasmic glycoproteins with a short intracellular anchor which would fit a model for extracellular perception of the AVR4 and AVR9 elicitors excreted by the fungus in the apoplast. Most plant pathogenic fungi develop intimate contact with the host plasma membranes through structures such as haustoria that are required for uptake of nutrients (Agrios, 1997). The other cloned *R* genes conferring resistance against fungi; *L6* (Lawrence *et al.*, 1995) and *M* (Anderson *et al.*, 1997), directed against *M. lini; RPP5* (Parker *et al.*, 1997), directed against *Peronospora parasitica;* and *I2* (Ori et al, 1997), directed against *Fusarium oxysporum* f. sp. *lycopersici*, appear to encode intracellular proteins. The matching *Avr* genes have not been characterized yet; however, the putative cytoplasmic localization of the products of these *R* genes suggests that fungal elicitors may actively enter plant cells through the haustorial membrane. Such a possibility is illustrated by plant pathogenic bacteria. Most bacterial *Avr* gene products are cytoplasmic and are subsequently injected actively into the cytoplasm of the host cell through a type III secretion system (Alfano and Collmer, 1996; Leach and White, 1996). Accordingly, most of the bacterial *R* genes that have been cloned encode cytoplasmic proteins (De Wit, 1997). Extracellular perception of elicitors such as AVR4 and AVR9 of *C. fulvum* may eventually be an exception rather than a rule. However, it is too early to draw conclusions on the mode of perception of fungal avirulence factors by plants since only few have been characterized in detail and no direct interaction between a fungal avirulence factor and an *R* gene product has been demonstrated unequivocally.

*Avr* gene products detected by the surveillance system of the plant must embody important pleiotropic functions; otherwise the encoding genes would have been lost from the pathogen population quickly. If an *Avr* gene does not have a clear direct effect on pathogenicity while colonizing the host plant, it may still play an important role in spore dispersal or survival (overwintering or bridging periods when there are no host plants available). These latter features are difficult to qualify or quantify under laboratory conditions. So far, five of the cloned bacterial *Avr* genes, *avrA*, *avrB*, *avrE*, *avrRPM1*, and *avrBs2* (Alfano and Collmer, 1996; Leach and White, 1996), and two fungal *Avr* genes, *Ecp*2 and *nip1*, have been shown to encode proteins that function as factors of pathogenicity. This demonstrates that plants have evolved monitoring systems to recognize pathogenicity factors of pathogens, which turn them into elicitors of HR-based resistance. Although most pathogen *Avr* genes cloned so far show no or little homology to each other, one would expect that *Avr* genes that encode important pathogenicity factors could have homologous counterparts in closely or even distantly related pathogens. The matching *R* genes would be potentially durable genes as the pathogen should not lose or adapt important pathogenicity factors easily.

The homology in sequence and presumably also in the structure of the proteins encoded by plant *R* genes targeted against viruses, bacteria, fungi, and nematodes, that have been cloned from different plant species such as *Arabidopsis*, flax, rice, tobacco, and tomato, is intriguing (Jones and Jones, 1996; De Wit, 1997). It is hypothesized that *R* genes, in addition to being part of the surveillance system, might embody additional functions for plants. They might act as receptors for yet unidentified endogenous ligands and might be involved in physiological and/or developmental regulation. The *R* gene homology also suggests that their products represent versatile (co)receptor molecules for binding ligands as monomers, dimers, or heteromers as has been reported in mammals for receptors of growth factors (Heldin, 1995).

## CONCLUSION

The fine tuning between pathogen *Avr* genes and matching plant *R* genes seems to be the outcome of coevolution between a pathogen and its host over a long time. In natural ecosystems plants will generate new recognition specificities targeted against deleterious pathogens. Simultaneously, the pathogen will develop strains that overcome the resulting resistances. The accumulation of surveillance specificities is expected to have its cost for the plant. In a similar way, loss or mutational adaptation of an *Avr* gene which has a crucial intrinsic function has its cost for the pathogen. It is probably the balance between

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the advantage of having the intrinsic function(s) and the drawback of having a restricted host range that decides whether a particular *Avr* gene is kept or eliminated. Thus some avirulence genes will occur only briefly in the populations of fungal pathogens, while others could persist for a longer time. What we currently observe is presumably only a snapshot of a very dynamic process in the interactions between pathogenic fungi and their host plants.

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