Developmental Control of Promoter Activity Is Not Responsible for Mature Onset of *Cf-9B*-Mediated Resistance to Leaf Mold in Tomato

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Cf-9 confers resistance to tomato seedlings and mature plants against *Cladosporium fulvum* races expressing the Avr9 elicitor. It is the central member of a cluster of five paralogous genes in an introgressed segment of chromosome 1 derived from *Lycopersicon pimpinellifolium*. The other four genes have been named *Hcr9-9A*, *Hcr9-9B*, *Hcr9-9D*, and *Hcr9-9E*. *Hcr9-9B*, here designated *Cf-9B*, encodes weaker resistance than *Cf-9*, recognizes a different elicitor, and protects only mature plants from infection. The onset of *Cf-9B*-mediated resistance and the molecular basis for its developmental control were investigated in this study. Fungal inoculation of tomato plants containing reciprocal *Cf-9/Cf-9B* promoter-coding region swaps, analysis of tomato plants containing promoter-*gusA* fusions, and a reverse transcriptase-polymerase chain reaction study of *Cf-9* and *Cf-9B* transcripts in tomato plants suggested that transcriptional control of *Cf-9B* did not account for the late onset of *Cf-9B*-mediated resistance. Alternative explanations for the onset of *Cf-9B*-mediated resistance in mature plants are discussed.

Additional keywords: avirulence gene, guard hypothesis, Milky Way locus, resistance gene.

Many plants have specific defenses against pathogens that depend upon a dominant resistance (*R*) gene in the plant and a corresponding dominant avirulence (*Avr*) gene in the pathogen (Flor 1971). Predictions based on sequence information have suggested that resistance proteins are receptors, or subunits of receptors, that recognize specific elicitors (Dangl and Jones 2001; Staskawicz et al. 1995). These elicitors are either proteins encoded by avirulence genes or molecules that have been altered by the enzymatic action of these proteins. Recognition of an elicitor by a resistance protein triggers defense signaling that often leads to production of active oxygen species and pathogenesis-related (PR) proteins (Hammond-Kosack and Jones 1996), programmed death of plant cells in close proximity to the pathogen, and systemic acquired resistance (Hunt et al. 1996). These responses are thought to restrict the growth of plant pathogens.

Cladosporium fulvum is a biotrophic fungus that infects susceptible tomato plants under conditions of high humidity, causing leaf mold disease. Seedlings and mature plants of the

Cf9 line of *Lycopersicon esculentum* carry the *Cf-9* gene introgressed from *L. pimpinellifolium* and are resistant to fungal races secreting the Avr9 peptide (Hammond-Kosack and Jones 1993). *Cf-9* is the central member of a cluster of five paralogous genes in the *Cf9* introgression segment that spans 36.5 kb at the Milky Way locus on tomato chromosome 1; the four flanking genes are designated *Hcr9-9A*, *Hcr9-9B*, *Hcr9-9D*, and *Hcr9-9E* (Jones et al. 1994; Parniske et al. 1997). At least one of these *Hcr9* genes encodes weak resistance that protects mature tomato plants from *C. fulvum* (Lauge et al. 1998; Parniske et al. 1997). Preliminary experiments showed that *Hcr9-9B* confers this resistance and it is named *Cf-9B* in this study.

Cf-9 and *Cf-9B* both provide mature plants with race-specific resistance to *C. fulvum* but, of the two, only *Cf-9* protects seedlings. Hence, *Cf-9* and *Cf-9B* provide a useful model system for comparison of mature-plant and seedling resistance. Conceivably, *Cf-9B* provides no seedling resistance because it is expressed only in mature plants. The 1.6-kb regions between tomato *loxC* gene remnants and the start codons of *Cf-9* and *Cf-9B*, which contain the *Cf-9* and *Cf-9B* promoters, are very similar, except for several small insertions and deletions and a central region of low similarity (61% nucleotide identity) approximately –900 to –660 bp upstream of the *Cf-9* start codon and –910 to –620 bp upstream of the *Cf-9B* start codon. Both regions share putative binding sites for transcription factors involved in a number of stress and phytohormone responses (Abe et al. 1997; Baumann et al. 1999; Logemann et al. 1995;) as well as TATA boxes, which support basal transcription by forming recognition sites for RNA polymerase II (Voet and Voet 1990). The *Cf-9B* region also contains putative binding sites for a bZIP transcription factor involved in abscisic acid response (Kim et al. 1997) and a WRKY box transcription factor involved in plant defense signaling (Eulgem et al. 1999). These sequence differences could perhaps confer differences in promoter activity.

The purpose of this study was to test, using three independent approaches, whether promoter activity accounts for the late onset of *Cf-9B*-mediated resistance. The methods used were: inoculation of tomato plants containing reciprocal *Cf-9/Cf-9B* promoter-coding region swaps with different races of *C. fulvum* and crossing of these plants to a tomato line expressing Avr9; analysis of tomato plants containing promoter-*gusA* fusions; and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Cf-9* and *Cf-9B* expression in tomato plants of different ages.

RESULTS

Transgenic tomato lines.

The tomato cv. Moneymaker, which lacks any known genes for resistance to *C. fulvum*, was used for all tomato transformations. Kanamycin-resistant seedlings from 11 independent *Cf-9B* transformants (SLJ9642) were selfed and four plants from each of six T3 lines lacking kanamycin-sensitive seedlings were screened at the early flowering stage for resistance to *C. fulvum* race 5,9 (Marmeisse et al. 1993). This race expresses Avr9B, but not Avr9, allowing *Cf-9B*-mediated resistance to be tested. Flowering SLJ9642Y1 T3 plants and flowering Cf9 plants had a comparable level of resistance to *C. fulvum* race 5,9 (data not shown). Therefore, SLJ9642Y1 plants were used for further studies of *Cf-9B*-mediated resistance and as a positive transgenic control for *Cf-9B* resistance in inoculation experiments.

Kanamycin-resistant T2 seedlings from seven independent *Cf-9* transformants (SLJ9572) showed necrosis after injection with intercellular fluids from SLJ6201A tobacco plants that express Avr9 (data not shown). These lines were selfed and four T3 lines lacking kanamycin-sensitive seedlings were inoculated with *C. fulvum* race 5 (Marmeisse et al. 1993). This

race expresses both Avr9 and Avr9B, allowing *Cf-9*-mediated resistance to be tested in tomato seedlings, where *Cf-9B* resistance is not expressed. Of the T3 lines, SLJ9572G3 showed the strongest resistance and was used as a positive transgenic control for *Cf-9* resistance experiments.

Kanamycin-resistant T2 seedlings of four *Cf-9B* promoter:*Cf-9* coding region transformants (CBJ140) were inoculated with *C. fulvum* race 5 to test whether the activity of the *Cf-9B* promoter affected the onset of *Cf-9*-mediated fungus resistance. Kanamycin-resistant T2 seedlings of five *Cf-9* promoter:*Cf-9B* coding region transformants (CBJ179) were also tested to determine whether the activity of the *Cf-9* promoter affected the onset of *Cf-9B*-mediated resistance. Kanamycin-resistant T2 seedlings of transformants containing a *Cf-9* promoter:*gusA* fusion (CBJ145) or a *Cf-9B* promoter:*gusA* fusion (CBJ146) were used for further comparison of promoter activities.

Cf-9B encodes mature-plant resistance to *C. fulvum*.

The results of five separate inoculation experiments showed that SLJ9642Y1 (*Cf-9B*) plants at the early fruiting stage were consistently more resistant to *C. fulvum* race 5,9 than seedlings

Fig. 1. *Cf-9B*-mediated resistance to *Cladosporium fulvum*. Plants of the Moneymaker and SLJ9642Y1 (*Cf-9B*) lines were inoculated with *C. fulvum* race 5,9 at the vegetative (five- to seven true leaves) and early fruiting stages of development in order to characterize *Cf-9B*-mediated resistance. Leaflets of corresponding position from infected plants were scored for macroscopic symptoms of fungal infection. Leaves from vegetative plants of both genotypes showed extensive growth of aerial hyphae (white coloration) as well as sporulation (brown coloration). The leaf from a fruiting Moneymaker plant shows small patches of aerial hyphae, while only a single small chlorotic patch (white arrow) is visible on a corresponding leaf from a fruiting *Cf-9B* plant. Samples taken from the infected leaves of fruiting plants of both lines were stained with trypan blue and photomicrographs of the abaxial epidermis and the mesophyll layer show extensive growth of fungal hyphae in Moneymaker and sparse growth in a *Cf-9B* plant (black arrows). Scale bars are equal to 0.5 cm in photographs of whole leaflets and 25 μ m in photomicrographs.

Fig. 2. Contribution of the *Cf-9* and *Cf-9B* promoters to disease resistance and elicitor recognition. **A**, Seedlings of CBJ140 (*Cf-9* promoter:*Cf-9B* coding region) and CBJ179 (*Cf-9B* promoter:*Cf-9* coding region) lines were inoculated with *Cladosporium fulvum* race 5 when they had one to four true leaves in order to test for *Cf-9*-mediated seedling resistance. Leaves were scored for signs of fungal growth 21 days after inoculation and sections were stained with trypan blue and checked microscopically for aerial hyphae and spores on the abaxial surfaces of leaflets. Leaves of CBJ140 (*Cf-9B:Cf-9*) seedlings showed macroscopic chlorosis and necrosis and microscopic analysis revealed occasional infection sites with sparse hyphae and some sporulation (black arrow). Leaves of CBJ179 (*Cf-9:Cf-9B*) seedlings showed continuous regions of aerial hyphae and microscopic examination revealed widespread aerial hyphae and spore formation. Fruiting plants containing these constructs also were inoculated with *C. fulvum* race 5,9 in order to test for *Cf-9B*-mediated mature-plant resistance. Leaves of CBJ179 (*Cf-9:Cf-9B*) plants showed no symptoms of disease but small patches of aerial hyphae were seen on leaves of CBJ140 (*Cf-9B:Cf-9*) plants (white arrows), similar to those observed on Moneymaker plants that had been inoculated with *C. fulvum* race 5,9 at the same stage of growth. **B**, SLJ9572G3 (*Cf-9*) and Moneymaker control leaflets showed chlorosis and sporulation, respectively, after infection with *C. fulvum* race 5 at the seedling stage. **C**, Seedlings from a cross between a CBJ140 (*Cf-9B:Cf-9*) line and the SLJ6201B (*Avr9*) line segregated for normal plants and those showing lesion development on cotyledons and subsequent seedling death. In contrast, no lesions were seen among the progeny of a cross between the SLJ6201B (*Avr9*) line and Moneymaker, which lacks *Cf-9*. Scale bars are equal to 0.5 cm in photographs of whole leaflets or seedlings and 25 μ m in photomicrographs.

and vegetative plants. Fruiting *Cf-9B* plants showed no macroscopic symptoms of infection apart from barely visible chlorotic areas of approximately 5-mm diameter on the abaxial surface of leaves (Fig. 1). Microscopic examination of trypan blue-stained leaf material showed that these chlorotic areas contained restricted patches of aerial hyphae at the leaf surface and a low density of hyphae in the mesophyll (Fig. 1). Vegetative *Cf-9B* plants with one to four true leaves (data not shown) or with five to seven true leaves (one experiment, Fig. 1) were highly susceptible to *C. fulvum* race 5,9. These leaves showed macroscopic aerial hyphae and sporulation that was verified by microscopic examination of leaf tissue stained with trypan blue (Fig. 1). Hence, *Cf-9B* provided resistance that protected fruiting plants but not vegetative plants from *C. fulvum* race 5,9.

Susceptible Moneymaker tomato plants showed aerial hyphae on abaxial leaf surfaces after inoculation with *C. fulvum* race 5,9 at all three stages of development (Fig. 1). However, fruiting plants showed less extensive fungal growth on the abaxial leaf surfaces than vegetative plants with one to four or five to seven true leaves (Fig. 1). However, in contrast to fruiting *Cf-9B* plants, microscopic examination of trypan blue-stained leaf samples from fruiting Moneymaker plants revealed that white patches on the abaxial surfaces of leaves corresponded to large regions of profuse aerial hyphae and a dense network of hyphae among mesophyll cells. These results showed that fruiting Moneymaker plants have a nonspecific form of mature-plant resistance to *C. fulvum* race 5,9 that is weaker than resistance provided by *Cf-9B*.

The promoters of *Cf-9* and *Cf-9B* do not have a major role in the onset of disease resistance and elicitor recognition.

Kanamycin-resistant T2 seedlings of two CBJ140 (*Cf-9B:Cf-9*) transformants were inoculated with *C. fulvum* race 5 when they had two to four true leaves and showed chlorosis

and necrosis, but no macroscopic signs of infection (Fig. 2). However, microscopic examination revealed localized, sparse patches of aerial hyphae and conidia in leaf samples stained with trypan blue. However, kanamycin-resistant seedlings of two other *Cf-9B:Cf-9* transformants were fully susceptible to *C. fulvum* race 5 (data not shown), presumably because of transgene position effect or incomplete T-DNA transfer. In comparison, hardly any hyphal growth was seen in mesophyll tissue of SLJ9572G3 (*Cf-9*) T3 seedlings (Fig. 2). More *Cf-9B:Cf-9* tomato lines would need to be tested in order to determine whether the weakness of the seedling resistance in the two resistant lines, compared with the *Cf-9* line, was a consequence of transgene position effect or reflected a real difference in the relative strengths of the *Cf-9* and *Cf-9B* promoters. Nevertheless, these results showed that the *Cf-9B* promoter was active in seedlings.

Progeny from crosses between a Moneymaker tomato line, expressing Avr9 as a transgene (SLJ6201B), and each of the two resistant *Cf-9B:Cf-9* lines segregated for both normal seedlings and those that showed sunken, necrotic lesions approximately 10 days after germination and did not survive to maturity (Fig. 2). This was comparable to the necrosis and seedling death seen in lines containing both *Cf-9* and Avr9 (Hammond-Kosack et al. 1994), and confirmed that expression of *Cf-9* under the control of the *Cf-9B* promoter was sufficient to allow recognition of Avr9 in tomato seedlings.

Aerial hyphae and spores were seen in profusion on the leaves of kanamycin-resistant T2 seedlings from three CBJ179 (*Cf-9:Cf-9B*) lines after inoculation with *C. fulvum* race 5 (Fig. 2). In contrast, kanamycin-resistant mature plants from four *Cf-9:Cf-9B* lines showed resistance to *C. fulvum* race 5,9 that was comparable to that of SLJ9642Y1 (*Cf-9B*) plants (Fig. 2). These results suggested that the *Cf-9* promoter, despite its known activity in seedlings, could not confer seedling resistance when used to drive the expression of *Cf-9B*.

The promoters of *Cf-9* and *Cf-9B* are both active in tomato seedlings.

Kanamycin-resistant T2 tomato seedlings with one to two true leaves from seven CBJ145 (*Cf-9* promoter:*gusA*) lines and eight CBJ146 (*Cf-9B* promoter:*gusA*) lines showed a similar level of staining for glucuronidase activity (Fig. 3). Some seedlings were uniformly stained and, in others, staining appeared to be more concentrated in vascular tissue. However, the appearance of both staining patterns in individual plants of the same transgenic line suggested that the variation was caused by differences in the efficiency of leaf infiltration with the glucuronidase assay solution. These results suggested that both the *Cf-9* and *Cf-9B* promoters were active in tomato seedlings.

Both *Cf-9* and *Cf-9B* are transcribed in tomato seedlings.

Qualitative RT-PCR was used to test for the presence of *Cf-9* and *Cf-9B* transcripts in total RNA from Moneymaker or Cf9 tomato plants (Fig. 4). Moneymaker plants contain a pseudogene at the Milky Way locus (Parniske et al. 1997), but Cf9 plants contain both *Cf-9* and *Cf-9B*. An RT-PCR product of approximately 0.9 kb was amplified from the RNA of Cf9 cotyledons using *Cf-9B* primers. This product was not seen when reverse transcriptase was omitted from the reaction or when RNA from Moneymaker cotyledons was tested. The same 0.9-kb product, together with a 1-kb product, was seen when RNA from the leaves of mature Cf9 plants were analyzed. A less-abundant PCR product of approximately 1 kb was seen when reverse transcriptase was omitted from a reaction with the same template, and no product was seen when RNA from the leaves of mature Moneymaker plants was tested. Probably, the 1-kb product was amplified from

Fig. 3. Activity of the *Cf-9* and *Cf-9B* promoters in tomato seedlings containing promoter-*gusA* fusions. Fusions were made between the *gusA* reporter gene and the *Cf-9* and *Cf-9B* promoters in order to compare the activity of these promoters in tomato seedlings. Seedlings containing a *Cf-9B* promoter:*gusA* fusion and a *Cf-9* promoter:*gusA* fusion showed similar levels of glucuronidase activity (blue staining). No activity was detected in control seedlings lacking the *gusA* gene. The scale bar is equal to 1 cm.

contaminating genomic DNA and presumably contained an intron at the 3' end of *Cf-9B*. The 0.9-kb product was also amplified from RNA extracted from the cotyledons of seedlings from a CBJ179 (*Cf-9:Cf-9B*) line when reverse transcriptase was included.

An RT-PCR product of approximately 0.9 kb was amplified from the total RNA of Cf9 cotyledons and the leaves of mature Cf9 tomato plants using *Cf-9* primers (Fig. 4), but not in reactions lacking reverse transcriptase or in reactions where the RNA of MoneyMaker cotyledons was used as the template. Another product of approximately 1.05 kb was also amplified from the RNA of Cf9 seedlings and mature plants whether reverse transcriptase was omitted or not. Probably, the smaller product represented the *Cf-9* cDNA and the larger product was amplified from contaminating genomic DNA. The latter presumably contained the 114-bp intron at the 3' end of *Cf-9*. Neither product was amplified using the *Cf-9* primers when RNA from leaves of mature MoneyMaker plants was used as the template. Both products were seen when RNA from the cotyledons of kanamycin-resistant CBJ140 (*Cf-9B:Cf-9*) T2 seedlings was used as the template, but only the larger product was seen in the absence of reverse transcriptase. These results showed that both *Cf-9* and *Cf-9B* transcripts were present in seedlings.

An intron is removed from the *Cf-9B* transcript at an alternative splice donor site.

RT-PCR products that had been amplified using *Cf-9B* primers on RNA from a mature Cf9 plant and from the cotyledons of kanamycin-resistant T2 seedlings from a CBJ179 (*Cf-9:Cf-9B*) line were cloned. The 3' ends of three cloned RT-PCR products from mature Cf9 plants and one from a *Cf-9:Cf-9B* seedling were sequenced. The cloned RT-PCR product from the *Cf-9:Cf-9B* line had the same sequence as the other clones (GenBank accession number AY082885), with the exception of a presumed PCR-induced point mutation upstream of the stop codon. Otherwise, all four of the cloned RT-PCR products showed 100% nucleotide identity with *Cf-9B* upstream of the stop codon. This confirmed that RT-PCR had been specific for *Cf-9B* in RNA extracted from tomato plants containing homologous genes at the Milky Way, Southern Cross, and Northern Lights loci (Parniske et al. 1997, 1999).

The 3' untranslated region of *Cf-9B* within the cloned RT-PCR products was analyzed to find out why the *Cf-9B* transcript was spliced despite having a point mutation in a critical position in the conserved splice donor site that allows splicing of the *Cf-9* intron. Alignment of the sequences obtained from the cloned RT-PCR products and the genomic sequence of *Cf-9B* revealed that the transcript had been spliced at an alternative donor site overlapping the stop codon. This site begins at the terminal G residue of the stop codon and therefore has the potential to destroy the stop codon. However, the G residue immediately following the intron restores the stop codon (Fig. 5). The 87-bp *Cf-9B* intron ends at the same conserved splice acceptor site as the *Cf-9* intron.

DISCUSSION

The purpose of this study was to describe *Cf-9B*-mediated resistance to *C. fulvum* in tomato plants and to investigate the possibility that transcriptional regulation of *Cf-9B* might control the onset of this resistance. *Cf-9B* mediated a weaker form of resistance than *Cf-9* and became active between the vegetative and fruiting stages of development. The analysis of tomato lines containing *Cf-9B* promoter:*gusA* fusions, the fungal inoculation of tomato plants expressing reciprocal *Cf-9/Cf-9B* promoter:coding region swaps, and an RT-PCR approach sug-

gested that the promoter of *Cf-9B* was active in seedlings and therefore did not control the late onset of *Cf-9B* resistance.

Inhibition of fungal growth by *Cf-9B*-mediated resistance.

Cf-9B-mediated resistance inhibited growth of *C. fulvum* race 5,9 in mature tomato plants, but not in seedlings or vegetative plants. Fruiting SLJ9642Y1 (*Cf-9B*) plants had a lower density of hyphae in mesophyll tissues and fewer emergent aerial hyphae and spores after fungal infection than fruiting MoneyMaker plants (Fig. 1). However, *Cf-9B* allowed more extensive hyphal growth between mesophyll cells than *Cf-9*,

Fig. 4. Analysis of *Cf-9* and *Cf-9B* expression at susceptible and resistant stages of development by reverse transcriptase-polymerase chain reaction (RT-PCR). **A**, The diagrams show the relative positions of gene-specific, intron-spanning RT-PCR primers relative to the stop codons (TAG), introns and the polyadenylation signals (AATAAA) at the 3' end of *Cf-9* and *Cf-9B* (not to scale). **B** and **C**, *Cf-9* protects both tomato seedlings and mature plants but *Cf-9B* protects only mature plants. RNA was extracted from the cotyledons of seedlings or from tissues pooled from leaves three, six, and nine of mature plants at the early fruiting stage and RT-PCR was performed on RNA using the intron-spanning primer pairs specific to the 3' end of **B**, *Cf-9B* or **C**, *Cf-9*. Cf9 plants contained *Hcr9-9A*, *Cf-9B*, *Cf-9*, *Hcr9-9D*, and *Hcr9-9E* from *Lycopersicon pimpinellifolium*, whereas MoneyMaker (MM) plants lacked these genes. CBJ179 (179) tomato seedlings contained a fusion between the *Cf-9* promoter and the *Cf-9B* coding region, and CBJ140 (140) seedlings contained the reciprocal fusion. The presence (+) or absence (-) of RT in each reaction is indicated. Approximate molecular sizes are indicated in kilobases (kb).

which generally restricts hyphal growth to within 1 to 2 epidermal cell lengths of the site of penetration (Hammond-Kosack and Jones 1993). Death of plant cells was not a feature of *Cf-9B*-mediated resistance in this study. These data are consistent with observations of weak resistance conferred by genes carried on the *Cf9* introgression segment other than *Cf-9* itself (Lauge et al. 1998; Parniske et al. 1997), suggesting that *Cf-9B* may have been the main contributor to that resistance. Only one other gene on the *Cf9* introgression segment has been shown to confer any resistance to *C. fulvum*. The *Hcr9-9E* "reduced susceptibility" gene delays sporulation by a week compared with fully susceptible Moneymaker plants (Parniske et al. 1997). The differences between the timing and strength of resistance encoded by *Cf-9*, *Cf-9B*, and *Hcr9-9E* need to be addressed at the molecular level.

Differential elicitor stability.

A comparison of the biochemical properties of Avr4 and Avr9 suggests that relative stability of fungal elicitors in planta could form the basis for weak and strong resistances to *C. fulvum* (Joosten et al. 1994, 1997). Mature Avr9 is a protease- and heat-resistant, 28-amino acid (aa) polypeptide, held together by three disulfide bonds (Joosten et al. 1994; Van den Ackerveken et al. 1992, 1993; Veervoort et al. 1997). In contrast, Avr4 is a heat-labile, 86-aa polypeptide for which the structure has not yet been determined (Joosten et al. 1994). *Cf-9*-mediated resistance is more effective at inhibiting growth of *C. fulvum* in tomato plants than *Cf-4*-mediated resistance (Hammond-Kosack and Jones 1993). However, Avr4 and *Cf-4* were at least as effective at eliciting a specific necrotic response as *Cf-9* and Avr9 when the corresponding pairs of genes were coexpressed in plants (Thomas et al. 1999; Van der Hoorn et al. 1999). These studies suggest that *Cf-9* probably encodes stronger resistance to *C. fulvum* than *Cf-4* because Avr9 is more resistant to fungal or plant proteases than Avr4. Hence, lability of Avr9B could explain why *Cf-9B*-mediated resistance is less effective at inhibiting growth of *C. fulvum* than *Cf-9*-mediated resistance. Moreover, the late onset of *Cf-9B*-mediated resistance might be due to developmental control of plant processes that affect the stability of Avr9B, and consequently its effectiveness as an elicitor.

Alternative splicing of *Cf-9B*.

The donor and acceptor sites used in splicing of the *Cf-9* intron are highly conserved among *Cf-9* homologues (Fig. 6). The *Cf-9B* intron is spliced at a second donor site that occurs in a region also conserved among *Cf-9* homologues, but is not

known to be used in splicing of the *Cf-9* intron. This raises the possibility that the presence of the intron at the 3' end of *Cf* gene transcripts has been subject to strong stabilizing selection to the point where a "contingency" splice donor site is used when the primary donor site fails. The presence of an intron in the 3' untranslated region could affect mRNA turnover and may play an important role in regulating *Cf*- gene expression.

The use of different splice donor sites in *Cf-9* and *Cf-9B* could have different effects on transcript stability, with consequences for the levels of protein expression and the degree of resistance conferred. A possible explanation for the mature onset of *Cf-9B*-mediated resistance might be that developmental control of alternative splicing could cause differential transcript stability.

Promoter activity and *Cf-9B*-mediated resistance.

This study has shown that the onset of *Cf-9B*-mediated resistance during plant development does not correlate with *Cf-9B* promoter activity. This is consistent with a molecular study of *Xa21*, which mediates mature-plant resistance in rice. The onset of *Xa21*-mediated resistance during plant development did not correlate with abundance of *Xa21* transcripts, which were expressed constitutively at a low level (Century et al. 1999). To date, there is no evidence to show that mature-plant resistance is caused by the induced expression of a resistance gene. However, the results of this study did not exclude the possibility that *Cf-9* provides a higher level of resistance to *C. fulvum* than *Cf-9B* because of quantitative differences in the levels of transcription from their respective promoters.

There are a number of alternative explanations for the protection of mature plants but not seedlings by a resistance gene. Mature-plant resistance could be caused by post-transcriptional or post-translational regulation of a resistance gene (Century et al. 1999). The approaches used in this work would not have detected quantitative differences in the stability of *Cf-9B* messenger RNA or in turnover of the *Cf-9B* protein associated with development. Epitope-tagging has allowed the subcellular localization of *Cf-9* to be studied (Benghezal et al. 2000; Piedras et al. 2000). Expression of epitope-tagged versions of *Cf-9* and *Cf-9B* in tomato could allow the levels of these proteins to be compared in seedlings and in mature plants.

The possible contribution of nonspecific resistance to *Cf-9B*-mediated resistance.

Mature-plant resistance provided by *Cf-9B* could be the additive effect of a weak, constitutive form of race-specific resistance provided by *Cf-9B* and a weak, nonspecific form of resistance that is under developmental control. Fruiting plants of a susceptible tomato genotype are more resistant to *C. fulvum* than vegetative plants (Bond 1938 and this work), suggesting that tomato plants have weak nonspecific resistance to *C. fulvum* that is under developmental control. In tobacco plants, the vegetative-floral transition marks the onset of nonspecific resistance to blue mold and black shank disease (Hugot et al. 1999; Wyatt et al. 1991). The onset of this resistance correlates with the upregulation of salicylic acid production and at least three pathogenesis-related proteins throughout the plant, suggesting that it may be similar to systemic acquired resistance (Fraser 1981; Wyatt et al. 1991; Yalpani et al. 1993). The same mechanism might provide the weak nonspecific resistance of mature tomato plants to *C. fulvum* and contribute quantitatively to the stronger resistance observed in SLJ9642Y1 (*Cf-9B*) plants. Alternatively, the drawing of mineral salts, sugars, and amino acids to ripening fruits and their depletion from leaves (Salisbury and Ross 1978) could reduce their availability to fungal pathogens in fruiting plants. The effect of this change in nutrient availability on fungal growth in combina-

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Cf-9B TATTAGTGAGTAGCTATACCTCCCG 2617
cDNA TATTA-----

Cf-9B GTATTCAACTTTTTATATCAATG 2642
cDNA -----

Cf-9B TATTTTCATCCTCAAAGCTCTTACC 2667
cDNA -----

Cf-9B TTTAATTCTTATTGCAGGATTC 2689
cDNA -----GATTC

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Fig. 5. The intron at the 3' untranslated region of *Cf-9B*. A partial cDNA sequence was obtained from a reverse transcriptase-PCR product that was amplified using *Cf-9B*-specific primers and RNA from mature *Cf9* tomato plants, containing *Cf-9B*. The partial cDNA sequence was aligned with the genomic sequence of *Cf-9B* in order to determine the position of the intron in the 3' untranslated region of the gene. The stop codon interrupted by the intron is underlined in the cDNA sequence.

tion with weak resistance provided by *Cf-9B* might protect fruiting tomato plants but not vegetative plants from infection by *C. fulvum*.

Developmentally controlled expression of a protein “guarded” by *Cf-9B*.

Some resistance proteins are thought to recognize complexes formed between specific plant proteins and Avr proteins that would otherwise contribute to virulence (Dixon et al. 2000). This idea, known as the “guard hypothesis”, could explain why Avr9 binds to a proteinaceous high-affinity binding site on the plasma membrane of tomato plants, regardless of the presence or absence of *Cf-9* (Kooman-Gersmann et al. 1996). The late onset of *Cf-9B*-mediated resistance could be caused by *Cf-9B* “guarding” a protein that is a target for Avr9B and whose expression is under developmental control. The fungal elicitor recognized by *Cf-9B* has not been identified and a reliable assay for the presence of Avr9B in the intercellular fluids of infected tomato leaves was not found in this study. Advances in these areas could allow the potential role of Avr9B in pathogenicity to be understood and could contribute to further development of the guard hypothesis.

MATERIALS AND METHODS

Preparation of plasmid constructs for plant transformation.

The *Cf-9* gene was cloned as a 7.8-kb *Pst*I fragment from cosmid 2/9-34 (Thomas et al. 1997) into dark Bluescript KS+ (Jones et al. 1992) to generate pSLJ7584. After several intermediate steps, *Cf-9* was subcloned as a 5.3-kb *Xba*I/*Sal*I fragment ligated into *Xba*I/*Xho*I cut pSLJ7291 (Thomas et al., 1997) to generate pSLJ9572 used for tomato transformation. The *Cf-9* gene in pSLJ9572 differs from wild-type *Cf-9* only by the presence of an engineered *Cla*I site at the start codon. The *Cf-9B* gene was cloned as a 7.9-kb *Pst*I fragment from cosmid 2/9-34 into dark Bluescript KS+ to generate pSLJ7582. It then was subcloned as a 6.4-kb *Cla*I fragment into the binary vector pSLJ7291 to generate pSLJ9642 used for tomato transformation.

The *Cf-9* and *Cf-9B* genes were modified by the removal and addition of restriction sites after they had been cloned into dark Bluescript KS+ in order to facilitate domain swapping between *Cf-9* and *Cf-9B* in this and other studies. The *Cf-9* gene in pSLJ9595 and pSLJ9597, whose construction was described by Benghezal and associates (2000), differs from wild-type *Cf-9* by a GAA to TCG mutation before the start codon that introduces a *Cla*I site, a silent A2067G mutation in the coding sequence that removes a *Bgl*III site, and a C to A mutation that removes a *Cla*I site in the 3' untranslated region. These plasmids carry the same version of the *Cf-9* gene, but cloned in opposite orientations.

The 6.4-kb *Cla*I fragment carrying *Cf-9B* from pSLJ7582 also was subcloned into dark Bluescript KS+ to generate pSLJ9651. Three mutations were introduced simultaneously into pSLJ9651 by site-directed mutagenesis (Kunkel et al. 1985) to generate pSLJ10601. A *Cla*I site was introduced at the start codon by a GAA to TCG mutation immediately preceding the ATG. A *Sac*I site homologous to that of *Cf-9* was introduced by an A586G mutation leading to the replacement of a lysine in *Cf-9B* by a glutamate residue homologous to that of *Cf-9*. An *Eco*RI site was removed by a silent A732G mutation. Only the *Cla*I sites at the start codons of *Cf-9* and *Cf-9B* were utilized to make the constructs described here.

A promoterless *gusA* reporter gene (Jefferson et al. 1985) was cloned into pSLJ7291 as a *Hind*III-*Cla*I fragment from pSLJ4K (Jones et al. 1992) to generate pSLJ10621. The *Cf-9*

and *Cf-9B* promoters were excised as 2.35- and 3.09-kb *Cla*I fragments from pSLJ9597 and pSLJ10601. Fusions between the *Cf-9* promoter (pCBJ145) or the *Cf-9B* promoter (pCBJ146) and the *gusA* coding region were made by cloning the promoter fragments into the *Cla*I site of pSLJ10621, which precedes the start codon of the *gusA* gene.

The *Cf-9B* promoter:*Cf-9* coding region swap was made in Bluescript by ligating the 2.93-kb *Bam*HI-*Hind*III fragment of pBluescript KS- to the 2.36-kb *Hind*III-*Cla*I *Cf-9B* promoter fragment from pSLJ10601 and the 3.01-kb *Cla*I-*Bam*HI *Cf-9* coding fragment from pSLJ9597. The 5.4-kb *Bam*HI-*Sal*I fragment containing the swap was excised from pCBJ139 and cloned into pSLJ7291, which had been cut with *Bam*HI and *Xho*I, to give pCBJ140.

The intermediate construct pCBJ141 was made by cloning the 2.32-kb *Bam*HI-*Cla*I *Cf-9* promoter fragment from pSLJ9595 into the multiple cloning site (MCS) of pSLJ7292, which is identical to pSLJ7291, apart from the orientation of the *lacZ* gene carrying the MCS. The 3.31-kb *Cla*I coding region fragment of *Cf-9B* from pSLJ10601 was then cloned downstream of the *Cf-9* promoter fragment within pCBJ141 to give pCBJ179, which contains the *Cf-9* promoter:*Cf-9B* coding region swap.

All DNA manipulations were performed using standard methods (Sambrook et al. 1989) and constructs were verified by restriction analysis. The pCBJ plasmids described here

Fig. 6. Homology in the sequences flanking the intron in the 3' untranslated region of *Cf* resistance genes. **A**, The conserved *Cf-9* splice donor site (underlined) could potentially be used in all the sequences shown except for that of *Cf-9B*. **B**, The splice donor site of *Cf-9B* (underlined) could potentially be used in the alternative splicing of *Cf-9*, *Cf-4*, and *Hcr9-4E* but is not present in members of the *Cf-2/5* gene family. **C**, The underlined splice acceptor site shared by *Cf-9* and *Cf-9B* is also conserved among other *Cf* resistance genes. Vertical lines indicate experimentally-confirmed splice donor or acceptor sites.

were also checked by PCR. Primer pairs were designed to match sites within the sequences of *Cf-9*, *Cf-9B*, and the vectors used in construct-making in order to test for the presence and orientation of these sites in constructs. PCR was performed on purified plasmids or on boiled bacterial cells using an MJ Research (Watertown, MA) PTC-200 thermal cycler and REDTaq DNA polymerase (Sigma-Aldrich Co., St Louis) according to the manufacturer's instructions.

Generation and screening of tomato transformants.

The Moneymaker cultivar of *L. esculentum*, lacking known genes for resistance to *C. fulvum*, was used for *Agrobacterium*-mediated plant transformation as previously described (Jones et al. 1994). The term T1 refers to primary transformants and the terms T2 and T3 refer to subsequent generations of plants. Seed of transgenic plants were screened for kanamycin resistance on Murashige and Skoog (MS) media containing kanamycin sulfate (100 µg/ml), which provided selection for plants expressing the *nptII* gene contained within the binary vectors (Jones et al. 1992). This analysis suggested that the SLJ9642Y1 (*Cf-9B*) T3 line contained copies of the transgene at two loci and that the SLJ9572G3 (*Cf-9*) line contained a single locus insertion.

Inoculation of tomato plants with *C. fulvum*.

C. fulvum cultures were prepared as described by Hammond-Kosack and Jones (1993) and were used to make spore suspensions containing Silwet L-77 (Osi Specialties, Sistersville, WV, U.S.A.) at 0.5 ml/L for plant inoculations. The apices of plants used for inoculation at the fruiting stage were removed to leave one to three leaves above the first flowering truss. Plants with two to four true leaves, five to seven true leaves, or at the early fruiting stages of development were treated with paclobutrazol and inoculated as previously described (Balint-Kurti et al. 1994). After inoculation, mature plants were placed on a saturated capillary mat within a sealed plastic-covered frame (90 by 90 by 80 cm) designed to maximize humidity. A similar frame (90 by 90 by 50 cm) was used for seedling inoculations. The plastic covering these frames was perforated after 3 days to reduce the humidity slightly and to allow gas exchange for the remainder of the incubation period. Plants were scored for macroscopic signs of infection 21 to 24 days after inoculation and leaf samples were stained with lactophenol trypan blue for microscopic examination (Hammond-Kosack and Jones 1993).

Histochemical analysis

of seedlings expressing promoter-*gusA* fusions.

Kanamycin-resistant seedlings of CBJ145 (*Cf-9* promoter-*gusA*) and CBJ146 (*Cf-9B* promoter-*gusA*) tomato transformants were screened on MS media containing kanamycin sulfate, transferred to the glasshouse, and harvested when two to three true leaves had appeared. Glucuronidase staining was carried out using the method of Jefferson and Wilson (1991).

Total RNA extraction from tomato plants and RT-PCR.

Total RNA was extracted from tomato plants using the RNeasy Plant Kit (Qiagen, Venlo, The Netherlands) and used to make first-strand complementary DNA (cDNA) with the Gibco BRL (Grand Island, NY, U.S.A.) cDNA Synthesis System. Thirty cycles of PCR were performed in triplicate on first-strand cDNA using REDTaq DNA polymerase. The 3' end of the *Cf-9* cDNA was amplified using the oligonucleotides 5'-AACTGGTGGGATACCTATTTCAA-3' and 5'-TTTATCTGCATCCCAAGCAACTGG-3' as gene-specific forward and reverse primers, respectively. The 3' end of the *Cf-9B* cDNA was amplified with 5'-AACTGGTGGGATACCTATCTCAT-3' as

the forward primer and 5'-CACAGACTATGAAAGATAA-AGGA-3' as the reverse primer. RT-PCR products generated using the *Cf-9B*-specific primers from RNA extracted from leaves of mature Cf9 tomato plants and CBJ179E (*Cf-9*: *Cf-9B*) cotyledons were cloned into the pCR2.1 vector, using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA, U.S.A.). Plasmid DNAs from four clones containing the partial cDNA were purified with the QIAprep Spin miniprep kit (Qiagen) for sequencing.

Bioinformatic methods.

The *Cf-9* and *Cf-9B* promoter sequences between the *loxC* homology region and the translation start codons (GenBank accession number AJ002236) were aligned using the Stretcher global alignment program (Myers and Miller CABIOS 1989, version 2.0u, modified for EMBOSS May 1999). Putative cis-acting promoter elements from plants were identified using the PLACE database (Higo et al. 1999). DNA Strider Version 1.2 (Christian Marck, Centre National de la Recherche Scientifique, Gif Sur Yvette, France) was used to design primers and plasmid constructs. Untranslated sequences downstream of the coding region of *Cf-9* (GenBank accession number AJ002236), *Cf-4* (GenBank accession number AJ002235), *Cf-2.1* (GenBank accession number U42444), and *Cf-5* (GenBank accession number AFO53993), and the *Cf-9B* genomic DNA (GenBank accession number AJ002236) and cDNA sequences were aligned with the corresponding region of the *Cf-9* cDNA (GenBank accession number U15936) using ClustalW version 1.5 (Thompson et al. 1994).

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