Cloning vector pCB301-I1M

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Introduction

pCB301-I1M is a binary plasmid derived from mini-binary vector pCB301 (Xiang et al. Plant Molecular Biology 40: 711–717, 1999; accession AF139061), pAvr9 (Van der Hoorn et al. MPMI Vol. 13, No. 4, pp. 439–446, 2000), and the chimeric PR1-INF1 ORF (Kamoun at al. MPMI, 12: 459-462, 1999). This vector is useful for cloning ORFs in a 35S cassette to use in agroinfiltration and related assays. The ORF of interest can be directly cloned in pCB301-I1M as a *NcoI-SacI* fragment. The resulting plasmid can be electroporated into *Agrobacterium* and is ready for agroinfiltration.

Overall description

The PR1-INF1 ORF was cloned into the 35S-TPII expression cassette of pAvr9 as a *NcoI-SacI*. The 35S-INF1-TPII cassette was then transferred to pCB301 as a EcoRI-HindIII fragment resulting in pCB301-INF1. To eliminate the *SacI* site present in the multiple cloning site of pCB301, pCB301-INF1 was digested with *EcoRI* and *BglII*, blunt ended by filling the protruding ends with pfu DNA polymerase, and self ligated. The resulting plasmid is pCB301-I1M. Both *EcoRI* and *BglII* sites were confirmed to be missing in pCB301-I1M. pCB301-I1M was confirmed to function by agroinfiltration of *Nicotiana* leaves (W. Hamada).

Appendix

1. Map of pCB301 (Fig. 1, Xiang et al. Plant Molecular Biology 40: 711–717)
2. Map of pAvr9 (Fig. 1, Van der Hoorn et al. MPMI Vol. 13, No. 4, pp. 439–446)

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A mini binary vector series for plant transformation

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Abstract

A streamlined mini binary vector was constructed that is less than 1/2 the size of the pBIN19 backbone (3.5 kb). This was accomplished by eliminating over 5 kb of non-T-DNA sequences from the pBIN19 vector. The vector still retains all the essential elements required for a binary vector. These include a RK2 replication origin, the *nptIII* gene conferring kanamycin resistance in bacteria, both the right and left T-DNA borders, and a multiple cloning site (MCS) in between the T-DNA borders to facilitate cloning. Due to the reduced size, more unique restriction sites are available in the MCS, thus allowing more versatile cloning. Since the *traF* region was not included, it is not possible to mobilize this binary vector into *Agrobacterium* by triparental mating. This problem can be easily resolved by direct transformation. The mini binary vector has been demonstrated to successfully transform *Arabidopsis* plants. Based on this mini binary vector, a series of binary vectors were constructed for plant transformation.

Introduction

Since the birth of the concept and the first generation of binary vectors for plant transformation (Hoekema *et al.*, 1983; Bevan, 1984), many useful and versatile vectors have been constructed (An *et al.*, 1985, 1986; Klee *et al.*, 1985; Simoens *et al.*, 1986; Schardl *et al.*, 1987; Becker *et al.*, 1992; Gleave, 1992; Ma *et al.*, 1992). These vectors have greatly facilitated transformation technology and have become the popular choice to generate transgenic plants for both basic research and biotechnology. In general, these binary vectors are fairly large (usually more than 10 kb), making *in vitro* manipulation inconvenient. In addition, large plasmids offer fewer unique restriction sites for cloning. For these reasons, smaller binary vectors have obvious advantages (Hajdukiewicz *et al.*, 1994). Moreover, gene targeting in higher plants is an important emerging technology (Miao *et al.*, 1995; Kempin *et al.*, 1997) and demands vectors which can accept large genomic flanking sequences. Small vectors would certainly ease the difficulties in constructing these targeting plasmids which require multiple cloning steps with large DNA fragments.

Frisch *et al.* (1995) completely sequenced the binary vector pBIN19 and predicted that more than 1/2 of its backbone was nonessential. Using the complete pBIN19 sequence, we designed and tested a streamlined backbone vector that is less than 1/2 the size of the parent vector. The mini binary vector was fully functional in transforming *Arabidopsis* plants. Based on this mini vector, a series of binary vectors have been constructed for various plant transformation tasks.

Materials and methods

Bacterial strains, plasmids, in vitro *DNA manipulation, and cloning*

All *in vitro* manipulation and cloning were conducted using standard techniques (Sambrook *et al.*, 1989). The plasmids pBlueScript II (Stratagene, La Jolla,

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF 139061 (pCB301 plasmid).

CA), pBI101 and pBI221 (Clontech, Palo Alto, CA), pGPTV-BAR (Becker *et al.*, 1992), pJIT117 (Guerineau *et al.*, 1988), pBIN-mgfp5-ER (Haseloff *et al.*, 1997), pEL103 (E. Lam, personal communication) and pAHC 25 (Christensen and Quail, 1996) were used in study.

The bacterial strain DH5α was used for cloning and *Agrobacterium tumefaciens* strain C58 (pMP90) was used to transform *Arabidopsis*.

Vector construction

To streamline the backbone sequence of the pBI101 binary vector, we first PCR-amplified the RK2 replication origin and *nptIII* using 5′-GCTCTAGAGACGCT CACCGGGCTGGTT-3′ and 5′-GCTCTAGAGTAAA GCGCTGGCTGCTGAAC-3′ primers with *Xba*I sites included in their 5′ ends. The PCR product was digested with *Xba*I and recircularized to produce plasmid pCB298.

The vector backbone was further streamlined by removing the non-essential *is1* sequence, two primers 5′-CGCTCGAGCGGGCCGGGAGGTTC-3′ and 5′-TCCTCGAGTTGCAGCATCACCCATAAT-3′ were designed to amplify the minimal RK2 replication origin and the *nptIII* gene. The amplified DNA fragment was digested with *XhoI* and recircularized to produce pCB299.

The T-DNA border sequences were next introduced into pCB299, the whole T-DNA region of pBI101 was PCR-amplified with primers 5′-GGCCACTAGTTCTGGGGAACCCTGT-3′ and 5′-GGACTAGTGGACTGATGGGCTGCCTGTAT-3′. The PCR product was digested with *Spe*I and inserted into pCB299 at the *Xba*I site to produce pCB300. Both *Spe*I and *Xba*I sites were lost upon ligation.

The mini binary backbone plasmid pCB301 was produced by replacing the DNA region between the T-DNA border sequences with a multiple cloning sites (MCS) polylinker. Two primers (5′-TGGGTACCAAAACCACCCCAGTACTA-3′ and 5′-TCGAGCTCAGATTGTCGTTTCCC-3′) were designed to amplify only the T-DNA borders of the T-DNA region and the whole non-T-DNA sequence of pCB300. The resultant PCR fragment was digested with *Sst*I and *Kpn*I, and ligated with the *Sst*I/*Kpn*I-digested polylinker of pBlueScriptII to produce pCB301.

The Pnos-*bar*-Tnos expression cassette in pCB302 was constructed by replacing the *Hin*dIII/*Sst*I fragment (35S-*uidA*) of pBI221 with a *Hin*dIII/*Bam*HI fragment (Pnos-*bar*) isolated from the pGPTV-BAR plasmid (Becker *et al.*, 1992). The ligation was made possible by a *Bam*HI/*Sst*I adapter (5′-GATCAGCT-3′) so that both *Bam*HI and *Sst*I sites were destroyed upon ligation. The resultant Pnos-*bar*-Tnos expression cassette was isolated as a *Hin*dIII/*Eco*RI fragment and inserted into pCB301 at *Hin*dIII and *Eco*RI sites to give pCB302.

The vector pCB302-1 was constructed by inserting the *Sst*I/*Xho*I fragment of the pJIT117 plasmid (Guerineau *et al.*, 1988) into pCB302 at the *Sst*I and *Eco*RI sites by using a *Xho*I/*Eco*RI adaptor. The vector pCB302-2 was constructed by replacing the 160 bp *rbcS* transit peptide sequence with a 180 bp *Hin*dIII/*Bam*HI fragment containing the mitochondrial targeting sequence of the ATPase β subunit. The plasmid pCB302-3 was constructed by inserting the 35S promoter-driven expression cassette isolated as a *Hin*dIII/*Eco*RI fragment from pEL103 into pCB302 at the *Sst*I and *Eco*RI sites by using a *Sst*I/*Hin*dIII adapter during ligation. The vector pCB306 was made by inserting the *Hin*dIII/*Eco*RI fragment of the *bar* expression cassette isolated from pACH25 (Christensen and Quail, 1996) into pCB302 at the *Hin*dIII and *Eco*RI sites. The plasmid pCB308 was made by inserting the *Bam*HI/*Eco*RI fragment (promoterless *uidA*) of pBI101 into pCB302 at the *Bam*HI and *Eco*RI sites. The plasmid pCB307 was constructed by replacing the GUS coding region in pCB308 with the *gfp5* coding region at the *Bam*HI and *Sst*I sites. The *gfp5* coding region was PCR-amplified using primers with restriction sites *Bam*HI (5′ end) and *Sst*I (3′ end). The *uidA* region was excised by a complete digestion by *Bam*HI and a partial digestion by *Sst*I.

Arabidopsis *transformation and DNA gel blot analysis*

Agrobacterium tumefaciens C58 was transformed with vector pCB302 by electroporation (Cangelosi *et al.*, 1991). The pCB302-transformed *Agrobacterium* was grown in LB medium containing 50 mg/l, gentamycin and kanamycin. *Arabidopsis* transformation via the vacuum infiltration method was performed as previously described (Bechtold *et al.*, 1993; Bent and Clough, 1998). Total DNA was isolated from transformed *Arabidopsis* plants using the Nucleon Phytopure kit (Vector Laboratories, Burlingame, CA). DNA gel blot analysis was performed as previously described (Xiang *et al.*, 1997).

Results and discussion

Construction and features of the streamlined mini binary vector pCB301

The availability of the complete 11 777 bp sequence of pBIN19 made it possible to streamline this binary vector to a much reduced size. A PCR-based approach was taken to eliminate the nonessential sequences. Primers corresponding to sequences 11770–11788 (19 bp) and 4566–4547 (GenBank accession number U09365; Frisch *et al.*, 1995) were designed to amplify the minimal replication origin and the *nptIII* gene using the pBIN19 derivative, pBI101, as the template. The amplified DNA segment was circularized to produce plasmid pCB298. This plasmid conferred kanamycin resistance in *Escherichia coli* after transformation indicating that both the replication origin and *nptIII* gene were intact.

The pBIN19 sequence (Frisch *et al.*, 1995) revealed the presence of a short unknown sequence (619–692), a nonfunctional fragment of the *KlaC* gene (693–964), and a transposable element, *isl* (sequence 1316–2085). This *isl* sequence separates the distal (965–1315) 5′ end of the *nptIII* gene from its coding region. The *nptIII* gene is still active, suggesting that the *isl* sequence does not disrupt its function. This distal 5′ region of the *nptIII* gene is not, therefore, required for the expression of kanamycin resistance. In order to eliminate the nonessential sequence 606–2069 from pCB298, primers corresponding to sequences 624–607 and 2070–2091 were synthesized to amplify the rest of the pCB298 plasmid. The resulting PCR product (about 3.0 kb) was circularized to give plasmid pCB299. As expected, the plasmid pCB299 still conferred kanamycin resistance without the sequence 606–2069. The level of kanamycin resistance was apparently not altered as pCB299-transformed DH5α cells grew normally on LB plates containing various concentrations of kanamycin ranging from 25 to 200 mg/l with no obvious differences in colony morphology compared to bacteria containing the parent plasmid. The growth curves in liquid culture with various concentrations of kanamycin did not show significant difference from those of the parent plasmid (data not shown). This confirms that this region is not essential for plasmid replication in *E. coli* and does not affect expression of the *nptIII* gene.

Thus, a streamlined backbone vector, pCB299, was constructed. This plasmid is small (ca. 3.0 kb), but still contains the broad-host-range RK2 replication origin and *nptIII* for kanamycin resistance. The exclusion of nonessential sequences eliminated many restriction endonuclease sites present in the parent plasmid, and made it possible to include more unique restriction sites in the binary vectors derived from pCB299.

In order to insert the T-DNA border repeats (Wang *et al.*, 1984), the whole T-DNA region was amplified from pBI101 using primers corresponding to sequences 9421–9401 and 6040–6060 (based on pBIN19). This DNA fragment was cloned into pCB299 such that the T-DNA right border was linked to the 3′ end of RK2 *trfA* and the left border was linked to the 5′ end of RK2 *oriV*. The resulting plasmid was named pCB300. The region between the T-DNA border repeats was removed from pCB300 by PCR using primers corresponding to sequences 9260–9282 and 6201–6183 (based on pBIN19). These primers amplified 160 bp of the right and 140 bp of the left T-DNA border sequences and all of the CB299 sequence. A polylinker from pBlueScript II was inserted between the T-DNA borders while circularizing the PCR product producing the plasmid pCB301.

The plasmid pCB301 has been completely sequenced. This mini binary vector is the smallest (3.5 kb) of its kind so far reported, and is ready for accepting DNA fragments to be transferred into the plant genome (Figure 1). This plasmid retains all the backbone features of pCB299. In addition, it contains the T-DNA border repeats enclosing a MCS. The unique restriction sites in the MCS are *Sst*I, *Xba*I, *Spe*I, *Bam*HI, *Sma*I, *Pst*I, *Eco*RI, *Hin*dIII, *Sal*I, *Apa*I, and *Kpn*I. Restriction enzymes that cut twice within pCB301 include *Sst*II, *Bst*XI, *Not*I, *Eco*RV, *Cla*I, *Acc*I, and *Xho*I. The following common restriction enzymes do not cut within pCB301 and can be engineered into the MCS in future modifications: *Aat*I, *Aat*II, *Acc*III, *Apa*LI, *Asc*I, *Ava*III, *Avr*II, *Bcl*I, *Fse*I, *Hpa*I, *Mlu*I, *Nar*I, *Nco*I, *Nhe*I, *Nru*I, *Nsi*I, *Pac*I, *Pvu*I, *Pvu*II, *Sca*I, and *Stu*I. Among these sites, only *Apa*LI, *Avr*II and *Hpa*I are unique in pBIN19. The plasmid pCB301 is incapable of conjugal transfer due to the removal of *traF* region containing the *oriT* required for triparental mating. The transfer of pCB301 to *Agrobacterium* can be readily achieved by direct transformation using electroporation (Cangelosi *et al.*, 1991) or the freeze-thaw method (Holsters *et al.*, 1987).

This mini binary vector is stable in both *E. coli* and *Agrobacterium tumefaciens* We never noticed any problem of plasmid instability during the course of the study of about three years. The copy number of

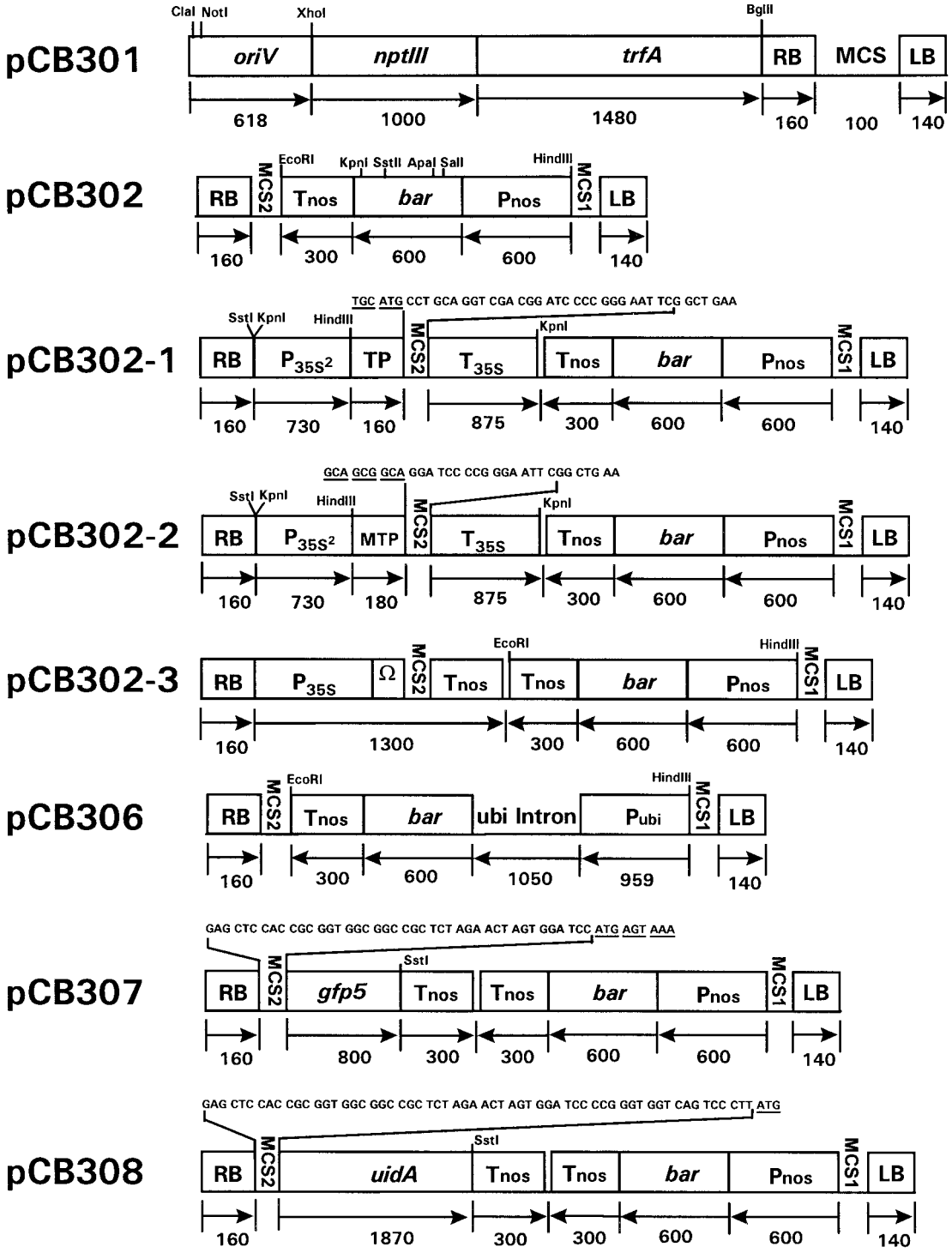
pCB301
ClaI NotI
XhoI
BglII
oriV
nptIII
trfA
RB
MCS
LB
618
1000
1480
160
100
140
pCB302
EcoRI KpnI SstII ApaI SalI
HindIII
MCS2
MCS1
RB
Tnos
bar
Pnos
LB
160
300
600
600
140
pCB302-1
TGC ATG CCT GCA GGT CGA CGG ATC CCC GGG AAT TCG GCT GAA
SstI KpnI
HindIII
KpnI
RB
P35S2
TP
T35S
Tnos
bar
Pnos
LB
160
730
160
875
300
600
600
140
pCB302-2
GCA GCG GCA GGA TCC CCG GGA ATT CGG CTG AA
SstI KpnI
HindIII
KpnI
RB
P35S2
MTP
T35S
Tnos
bar
Pnos
LB
160
730
180
875
300
600
600
140
pCB302-3
EcoRI
HindIII
RB
P35S
Ω
Tnos
Tnos
bar
Pnos
LB
160
1300
300
600
600
140
pCB306
EcoRI
HindIII
RB
Tnos
bar
ubi Intron
Pubi
LB
160
300
600
1050
959
140
pCB307
GAG CTC CAC CGC GGT GGC GGC CGC TCT AGA ACT AGT GGA TCC ATG AGT AAA
SstI
RB
gfp5
Tnos
Tnos
bar
Pnos
LB
160
800
300
300
600
600
140
pCB308
GAG CTC CAC CGC GGT GGC GGC CGC TCT AGA ACT AGT GGA TCC CCG GGT GGT CAG TCC CTT ATG
SstI
RB
uidA
Tnos
Tnos
bar
Pnos
LB
160
1870
300
300
600
600
140

Figure 1. Features of the mini binary vector pCB301 and plant transforming vectors derived from pCB301. The schematic representation of pCB301 contains all of the DNA fragments assembled to make this mini binary vector. The circular plasmid is linearized in this representation. The other plasmids are identical to pCB301 except for the sequences contained between the RB and LB of T-DNA and that is the only region illustrated. The numbers under each DNA region indicate the approximate size of that region in base pairs and the arrow indicates the orientation. The restriction sites and the number of times each site occurs in each plasmid is shown in Table 1. *bar*, gene for phosphinothricin acetyltransferase; *gfp5*, gene for green fluorescent protein (from pBIN-mgfp5-ER); LB, left border of T-DNA; MCB, multiple cloning site (from pBluescript II); MTP, mitochondrial targeting sequence from tobacco β-ATPase subunit; *nptIII*, gene for neomycin phosphotransferase for kanamycin resistance (from pBIN19); *oriV*, part of RK2 origin of replication (from pBIN19); P_{35S}, 35S promoter of cauliflower mosaic virus; $P_{35S}{}^2$, 35S promoter with double enhancers; Pnos, promoter of *nos* (nopaline synthase) gene; Pubi, maize ubiquitin-1 promoter; RB, right border of T-DNA; Tnos, terminator of *nos* (nopaline synthase) gene; TP, plastid targeting sequence of Rubisco small subunit; *trfA*, part of RK2 origin of replication; ubi intron, intron-1 from maize ubiquitin-1 gene; *uidA*, gene for β-glucuronidase (GUS); Ω, the translational enhancer of TMV.

Table 1. Identification of the multiple cloning sites in pCB302 and its derivatives. The locations of the individual MCS regions are shown in Figure 1. The restriction sites in each of the MCS is shown in order. The superscript number on each restriction site indicates how many times that restriction site occurs in the indicated plasmid.

Vector	MCS1	MCS2
pCB302	*Hin*dIII^1, *Cla*I^2, *Sal*I^2, *Xho*I^2, *Apa*I^2, *Kpn*I^2	*Sst*I^1, *Sst*II^2, *Not*I^2, *Bst*XI^2, *Xba*I^1, *Spe*I^1, *Bam*HI^1, *Sma*I^1, *Pst*I^1, *Eco*RI^1
pCB302-1	*Hin*dIII^1, *Cla*I^2, *Sal*I^3, *Xho*I^3, *Apa*I^2, *Kpn*I^4	*Sph*I^3, *Pst*I^1, *Sal*I^3, *Bam*HI^1, *Sma*I^1, *Eco*RI^1
pCB302-2	*Hin*dIII^2, *Cla*I^2, *Sal*I^2, *Xho*I^2, *Apa*I^2, *Kpn*I^4	*Bam*HI^1, *Sma*I^1, *Eco*RI^1
pCB302-3	*Hin*dIII^1, *Cla*I^2, *Sal*I^2, *Xho*I^2, *Apa*I^2, *Kpn*I^2	*Sst*I^2, *Sst*II^2, *Not*I^2, *Xba*I^1, *Spe*I^1, *Bam*HI^1
pCB306	*Hin*dIII^1, *Cla*I^2, *Sal*I^3, *Xho*I^2, *Apa*I^2, *Kpn*I^2	*Sst*I^1, *Sst*II^2, *Not*I^2, *Bst*XI^2, *Xba*I^1, *Spe*I^1, *Bam*HI^1, *Sma*I^1, *Pst*I^3, *Eco*RI^2
pCB307	*Hin*dIII^1, *Cla*I^2, *Sal*I^2, *Xho*I^2, *Apa*I^2, *Kpn*I^2	*Sst*I^2, *Not*I^2, *Bst*XI^2, *Xba*I^1, *Spe*I^1, *Bam*HI^1
pCB308	*Hin*dIII^1, *Cla*I^2, *Sal*I^2, *Xho*I^2, *Apa*I^2, *Kpn*I^2	*Sst*I^2, *Sst*II^2, *Not*I^2, *Bst*XI^2, *Xba*I^1, *Spe*I^1, *Bam*HI^1, *Sma*I^1

this plasmid in *E. coli* was similar to that of its parent as estimated by plasmid yield and Southern blot hybridization (data not shown).

Functional testing of the mini binary vector in transforming Arabidopsis

Although the mini binary vector pCB301 is stable and functional in *E. coli*, the ultimate test of its utility would be to demonstrate that it is stable and functional in *Agrobacterium* and that its T-DNA can be transferred to plants and will integrate into the plant genome. For this test of functionality, the dominant selectable marker *bar* was chosen for easy selection of transformants. The *bar* expression cassette (without the terminator) was excised from the pGPTV-BAR plasmid (Becker *et al.*, 1992) to replace the CaMV 35S promoter-driven *uidA* coding region in pBI221. The resulting *nos* promoter-*bar*-*nos* terminator expression cassette was inserted between the *Eco*RI and *Hin*dIII sites of the MCS of pCB301. The resultant plasmid pCB302 was introduced into *A. tumefaciens* strain C58 (pMP90). We chose *Arabidopsis* for the transformation test because of its well-established non-tissue culture transformation method (Bechtold *et al.*, 1993; Bent and Clough, 1998). Three independent transformation experiments were performed at different times using different batches of bacteria and plants. For each transformation, a flat of 18 10 cm × 10 cm pots of healthy plants were vacuum-infiltrated. Seeds were harvested in bulk for each transformation experiment in order to examine the overall transformation frequency, viz. (number of transformants survived/number of seeds sown) × 100. A transformation frequency of about 2% was consistently obtained for all three transformation experiments. The transformation frequency obtained with this mini binary vector,

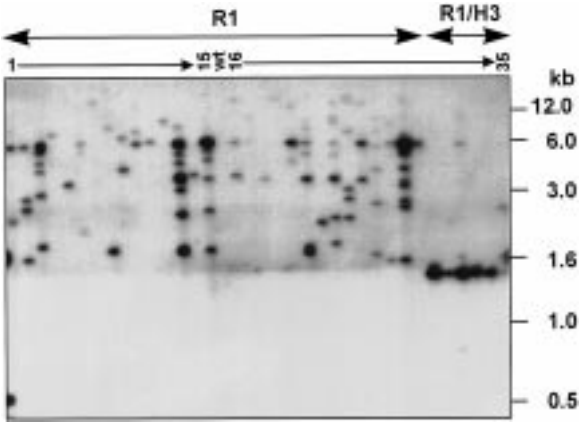

Figure 2. Southern blot analysis of *Arabidopsis* transformants generated with pCB302. Thirty-five randomly chosen herbicide-resistant plants and a wild-type Col-O plant (wt) were subjected to DNA blot analysis. About 0.5 μg total DNA of each sample was digested either with *Eco*RI (R1) or *Eco*RI plus *Hin*dIII (R1/H3). After transfer, the filter was hybridized with the ^{32}P-labeled *bar* coding sequence as described in Materials and methods. Molecular size markers in kb are indicated on the right.

pCB302, was comparable to or better than those obtained with several pBI101-based constructs using the same *Agrobacterium* strain and transformation method (data not shown).

A total of 35 randomly chosen herbicide-resistant transformants were subjected to genomic DNA blot analysis. The results in Figure 2 show that all transformants analyzed were positive for the presence of the *bar* gene. The predicted 1.5 kb DNA fragment was observed for the samples double-digested with *Hin*dIII and *Eco*RI (Figure 2). Among the transformants analyzed, 37% contained one insert, 27% contained 2 inserts, and 36% contained 3 or more inserts. Compared to other transformations we have done in the laboratory with several pBI101-based constructs, this is an average or higher than average frequency for single-copy transformation events.

We have demonstrated that our mini binary vector, pCB302, worked as well as, if not better than, its parent binary vector. The successful transformation of *Arabidopsis* by pCB302 also confirmed that the *traF* and *isl* regions present in the parent plasmid were not essential for either replication in bacteria or T-DNA transfer and integration.

Construction of a series of plant transforming vectors

A series of plant transforming vectors have been constructed based on our mini vector (Figure 1). The herbicide-resistance gene, *bar*, was chosen as a dominant selectable marker for these vectors because it works effectively on both monocots and dicots and because of the ease in selecting transformants. In addition, we have constructed kanamycin and hygromycin resistance cassettes in case a different selectable marker has to be used. These two cassettes use the 35S promoter and are designed to be cloned into the *Eco*RI/*Hin*dIII sites of pCB301.

Vectors pCB302 and pCB306 are mainly for constructing gene replacement plasmids for gene targeting experiments (Miao and Lam, 1995; Kempin *et al.*, 1997); pCB302 is for dicots and pCB306 is for monocots. Vector pCB306 was constructed by inserting the maize *ubi*-1 promoter-*bar*-*nos* terminator expression cassette from pAHC25 (Christensen and Quail, 1996) into pCB301 between the *Hin*dIII and *Eco*RI sites. Both vectors can also be used as backbone vectors for inserting other gene expression cassettes. The multiple cloning sites flanking the dominant selectable marker should prove useful for these purposes.

Vectors pCB302-1, pCB302-2, and pCB302-3 were designed for expressing genes whose products are targeted to chloroplasts, mitochondria and the cytosol, respectively. Vector pCB302-1 was constructed by inserting the expression cassette from pJIT117 (Guerineau *et al.*, 1988) into pCB302. pCB302-2 was produced by replacing the chloroplast transit peptide sequence in pCB302-1 with the mitochondrial targeting sequence of the β subunit of the F1-ATPase from tobacco (Boutry *et al.*, 1987). pCB302-3 was constructed by inserting the 35S promoter overexpression cassette from pEL103 (E. Lam, personal communication) into pCB302.

Vectors pCB307 and pCB308 were constructed for promoter analysis using the *uidA* and *gfp* genes as reporters. The *gfp5* gene in pCB307 was PCR-amplified from pBIN-mgfp5-ER (Haseloff *et al.*, 1997) without its N-terminal basic chitinase signal sequence and C-terminal HDEL sequence. The *uidA* gene in pCB308 is from pBI101. All these vectors have been confirmed for their MCS by sequencing and are currently undergoing testing for transformation efficiency (Table 1).

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Agroinfiltration Is a Versatile Tool That Facilitates Comparative Analyses of *Avr*9/*Cf-9*-Induced and *Avr*4/*Cf-4*-Induced Necrosis

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The avirulence genes *Avr*9 and *Avr*4 from the fungal tomato pathogen *Cladosporium fulvum* encode extracellular proteins that elicit a hypersensitive response when injected into leaves of tomato plants carrying the matching resistance genes, *Cf-9* and *Cf-4,* respectively. We successfully expressed both *Avr*9 and *Avr*4 genes in tobacco with the *Agrobacterium tumefaciens* transient transformation assay (agroinfiltration). In addition, we expressed the matching resistance genes, *Cf-9* and *Cf-4,* through agroinfiltration. By combining transient *Cf* gene expression with either transgenic plants expressing one of the gene partners, *Potato virus X* (PVX)-mediated *Avr* gene expression, or elicitor injections, we demonstrated that agroinfiltration is a reliable and versatile tool to study *Avr/Cf*-mediated recognition. Significantly, agroinfiltration can be used to quantify and compare *Avr/Cf*-induced responses. Comparison of different *Avr/Cf*-interactions within one tobacco leaf showed that *Avr*9/*Cf-9*-induced necrosis developed slower than necrosis induced by *Avr*4/*Cf-4*. Quantitative analysis demonstrated that this temporal difference was due to a difference in *Avr* gene activities. Transient expression of matching *Avr/Cf* gene pairs in a number of plant families indicated that the signal transduction pathway required for *Avr/Cf*-induced responses is conserved within solanaceous species. Most non-solanaceous species did not develop specific *Avr/Cf*-induced responses. However, coexpression of the *Avr*4/*Cf-4* gene pair in lettuce resulted in necrosis, providing the first proof that a resistance (*R*) gene can function in a different plant family.

Co-evolution between plants and pathogens has enabled plants to develop effective surveillance systems to recognize pathogens and mount defense responses. Defense responses are diverse and usually include a hypersensitive response (HR) where tissue surrounding the infection site becomes necrotic (Hammond-Kosack and Jones 1996). The plant surveillance system has a genetic basis, involving dominant resistance (*R*) genes that confer the ability to recognize invading pathogens carrying matching avirulence (*Avr*) genes. Tremendous efforts in the past decade have resulted in the cloning of many *R* and *Avr* genes.

R gene products can be broadly classified into two groups based on their predicted cellular location (De Wit 1997; Jones and Jones 1997; Parker and Coleman 1997). The first and largest group of R proteins is cytoplasmic and the members often contain leucine-rich repeats (LRRs) and nucleotide binding sites (NBSs). Members of this group have been cloned from flax (e.g., *L* genes), lettuce (*Dm* genes), *Arabidopsis thaliana* (e.g., *RPP* genes), several solanaceous species (e.g., *N*, *Mi*, *Gpa*, *Bs2*), and monocots (*Xa1* and *Cre3*) (reviewed by Van der Biezen and Jones 1998). The *Pto* gene, which encodes a serine-threonine kinase, is the only cytoplasmic R protein within this group that lacks LRRs and NBSs. The second and smaller group of *R* genes encodes putative plasma membrane-anchored proteins. They all carry extracellular LRR domains and members of this group have been cloned from rice (*Xa21*), sugar beet (HS^{Pro-1}), and tomato (e.g., *Cf-9* and *Cf-4*) (Cai et al. 1997; Jones and Jones 1997).

Proteins that are encoded by *Avr* genes share less common features (Culver et al. 1991; Laugé and De Wit 1998; Van den Ackerveken and Bonas 1997). Their predicted cellular location often fits with that of their matching *R* gene product. For example, the *Avr*9 and *Avr*4 genes from the biotrophic leaf mold fungus *Cladosporium fulvum* encode elicitor proteins that are secreted into the tomato leaf apoplast. Injection of these elicitor proteins into extracellular leaf spaces of tomato plants that carry a matching *Cf* gene is sufficient to trigger an HR (Joosten and De Wit 1999). In contrast, viral and bacterial AVR proteins only elicit an HR when produced in the host cytoplasm and not when injected into leaves (Bonas and Van den Ackerveken 1997). The latter proteins possibly interact with *R* gene products in the host cytoplasm, as was shown for the AvrPto and Pto proteins (Scofield et al. 1996; Tang et al. 1996).

To improve our understanding of *Avr/Cf* interactions at the molecular level, transient expression with *Potato virus X* (PVX; Chapman et al. 1992) has been employed to study the effects of mutations in *Avr*9 and *Avr*4 genes (Joosten et al. 1997; Kooman-Gersmann et al. 1997). However, transient expression of *Cf* genes through PVX is constrained by the size of the inserted gene that is allowed in the recombinant virus. In contrast to PVX, *Agrobacterium tumefaciens* can accom-

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modate large genes and has a broad host range (Bundock and Hooykaas 1998). Transient expression of genes through infiltration of *A. tumefaciens* cultures into leaf tissue (agroinfiltration) is a quick and easy method to study genes of interest (Kapila et al. 1997; Rossi et al. 1993).

In this report, we demonstrate that *Avr*9 and *Avr*4, as well as their large matching *R* genes *Cf-9* and *Cf-4*, respectively, can be successfully expressed by agroinfiltration. We show that agroinfiltration can be combined with either transgenic plants expressing one of the matching gene partners, PVX-mediated *Avr* gene expression, or injection of elicitor protein. In addition, we used agroinfiltration for quantitative analysis and comparison of different *Avr/Cf*-induced responses in tobacco and other plant species. Although initial results indicated that *Avr*9/*Cf-9*-induced necrosis developed slower than that induced by *Avr*4/*Cf-4*, we demonstrate that this temporal difference is due to differences in *Avr* gene activities. Transient expression of matching gene partners in a number of plant species revealed that the signal transduction pathway required for *Avr/Cf*-induced responses is conserved within solanaceous species. Most non-solanaceous species did not show specific *Avr/Cf*-induced responses, with the exception of lettuce, in which necrosis was induced by co-expression of the *Avr*4/*Cf-4* gene pair.

RESULTS

Transient expression of *Avr* and *Cf* genes in tobacco.

As tobacco can be transformed easily and *Cf-9* transgenic tobacco was found to respond with an HR upon injection with AVR9 protein (Hammond-Kosack et al. 1998), we used *Cf-9*-transgenic tobacco to transiently express the *Avr*9 gene through agroinfiltration. When young, fully expanded leaves were infiltrated with *A. tumefaciens* carrying pAvr9 (Fig. 1), the entire infiltrated area became necrotic (Fig. 2A). Leaf tissue started to collapse at 1 day post infiltration (dpi) and had developed into a yellow-brown sector by 7 dpi. In wild-type tobacco no necrosis occurred upon transient *Avr*9 expression (Fig. 2B). Similarly, transient expression of *Avr*4 resulted in necrotic sectors in *Cf-4* transgenic tobacco but not in wild-type tobacco (data not shown). A major advantage of agroinfiltration is that the T-DNA can accommodate large genes such as the 2.6-kb open reading frame (ORF) of the *Cf-9* resistance gene. Transient expression of *Cf-9* in *Avr*9 transgenic tobacco (Hammond-Kosack et al. 1994) resulted in necrosis (Fig. 2C), while no necrotic responses were induced in wild-type tobacco (Fig. 2B). Together, these results demonstrate that genes that encode the extracellular elicitors AVR9 or AVR4 and the large, extracellular, membrane-anchored Cf9 protein can be successfully expressed in tobacco through agroinfiltration.

Both *Avr*9 and *Avr*4 have been transiently expressed through the PVX expression system (Hammond-Kosack et al. 1995; Joosten et al. 1997). To test whether transient *Cf* gene expression through agroinfiltration can be combined with PVX-mediated *Avr* gene expression, wild-type tobacco plants were inoculated with PVX::*Avr*9, PVX::*Avr*4, or wild-type PVX. Two weeks after PVX inoculation, *A. tumefaciens* carrying pCf9 or pCf4 (Fig. 1) was infiltrated into leaves that showed clear mosaic symptoms. Necrosis only appeared in sectors where matching gene pairs were expressed (Fig. 2D). This indicates that agroinfiltration of both *Cf-9* and *Cf-4* genes can successfully be combined with PVX-mediated expression of the matching *Avr* gene.

Elicitor peptides AVR9 and AVR4 were originally purified from apoplastic fluids (AFs) isolated from compatible *C. fulvum*-tomato interactions (Scholtens-Toma and De Wit 1988; Joosten et al. 1994). In addition to these proteins, AFs contain many other fungal elicitor proteins. To test the specificity of tobacco leaves that transiently express *Cf-9* or *Cf-4* for AVR9 and AVR4 detection, respectively, crude AFs were injected 1 day after agroinfiltration of wild-type tobacco with pCf9 or pCf4. Necrotic responses were only detected in leaf sectors that were injected with AFs containing a matching elicitor (Fig. 2E), indicating that tobacco tissue that transiently expresses *Cf* genes has the same specificity for recognition of AVR proteins as tomato genotypes containing the native *Cf* genes.

Moneymaker-Cf9 (MM-Cf9) tomato leaves are very sensitive to injection of AVR9 elicitor protein, as concentrations of AVR9 as low as 300 nM result in a clear necrotic response (Kooman-Gersmann et al. 1998). When tobacco leaves that transiently express the *Cf-9* gene were injected with a concentration series of AVR9 peptide, concentrations as low as 10 nM resulted in a clear necrotic response above background (Fig. 2F). As expected, only background responses developed in tissue that transiently expressed *Cf-4* (Fig. 2F). This suggests that transient *Cf-9* expression levels in tobacco are high enough to detect low concentrations of AVR9.

Comparison of necrotic responses induced by transient expression of *Avr*9/*Cf-9* and *Avr*4/*Cf-4* gene pairs.

To date, comparisons between *Avr*9/*Cf-9*- and *Avr*4/*Cf-4*-induced responses have not been conclusive, due to developmental and/or genetic differences between plants that harbor

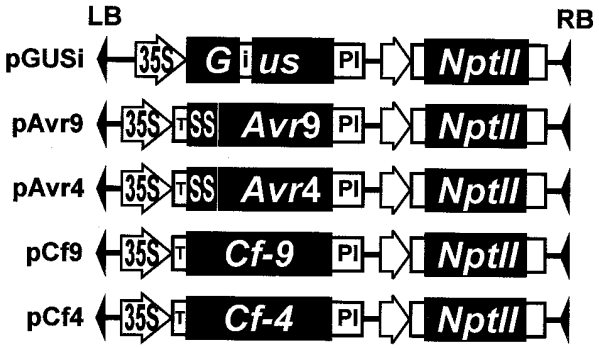

Fig. 1. Schematic representation of the T-DNAs present on the five binary plasmids used in this study. Each binary plasmid is named after the gene that is present on the T-DNA. The β-glucuronidase (*gus*) gene is interrupted by an intron, which excludes *A. tumefaciens*-derived *gus* expression (Vancanneyt et al. 1990). *Avr*9 and *Avr*4 are fused to the tobacco pathogenesis-related *PR1a* signal sequence to ensure extracellular targeting. Symbols: black boxes, ORFs; open boxes, untranslated regions; open arrows, promoters; black triangles, T-DNA borders. Abbreviations: *Gus*, gene encoding β-glucuronidase; i, intron; *Avr*9, ORF encoding 28 amino acid mature AVR9 protein; *Avr*4, ORF encoding 86 amino acid mature AVR4 protein; SS, signal sequence from tobacco pathogenesis-related *PR1a* gene; *Cf-9*, ORF encoding wild-type Cf9; *Cf-4*, ORF encoding wild-type Cf4; 35S, *Cauliflower mosaic virus* (CaMV) 35S promoter; T, omega *Tobacco mosaic virus* (TMV) leader; PI, potato proteinase inhibitor-II polyadenylation region; RB, right border of T-DNA; LB, left border of T-DNA; *Npt*II, neomycin-phosphotransferase II.

the different *Cf* genes. The ability to express *Cf* genes simultaneously in the same leaf tissue enabled us for the first time to compare induced responses within the same leaf. Therefore, *A. tumefaciens* cultures carrying the pAvr and pCf plasmids were mixed in a 1:1 ratio and infiltrated into wild-type tobacco leaves. Necrosis only developed in leaf sectors that expressed matching gene pairs (Fig. 3A). Clear differences between *Avr*9/*Cf-9*- and *Avr*4/*Cf-4*-induced responses were observed. Tissue collapse induced by expression of the *Avr*9/*Cf-9* gene pair occurred 1 day later than that induced by the *Avr*4/*Cf-4* gene pair (Fig. 3B). Typically, *Avr*9/*Cf-9*-induced necrosis only started to develop after the entire *Avr*4/*Cf-4*-expressing area had collapsed. Although the pattern of tissue collapse was identical, the *Avr*9/*Cf-9*-induced collapse was preceded by weak chlorosis. The color of the necrotic sector resulting from *Avr*9/*Cf-9* co-expression gradually turned dark brown (Fig. 3A), suggesting that there was time for the accumulation of phenolic compounds.

Although the binary constructs used in this study were comparable, differences observed in the speed of necrotic responses could be caused by differences in activities of the pAvr or pCf plasmids upon agroinfiltration. We, therefore, quantified responses induced upon infiltration of a dilution series of *A. tumefaciens* carrying pAvr and pCf (Fig. 3C and D). To exclude differences between culture densities, cultures of equal density that carry matching pAvr and pCf plasmids were mixed in different ratios. The percentage of infiltrated leaf area that had become necrotic at 7 dpi was measured and plotted against the percentage of *A. tumefaciens* cultures that carry pAvr and pCf. The percentage of the culture containing

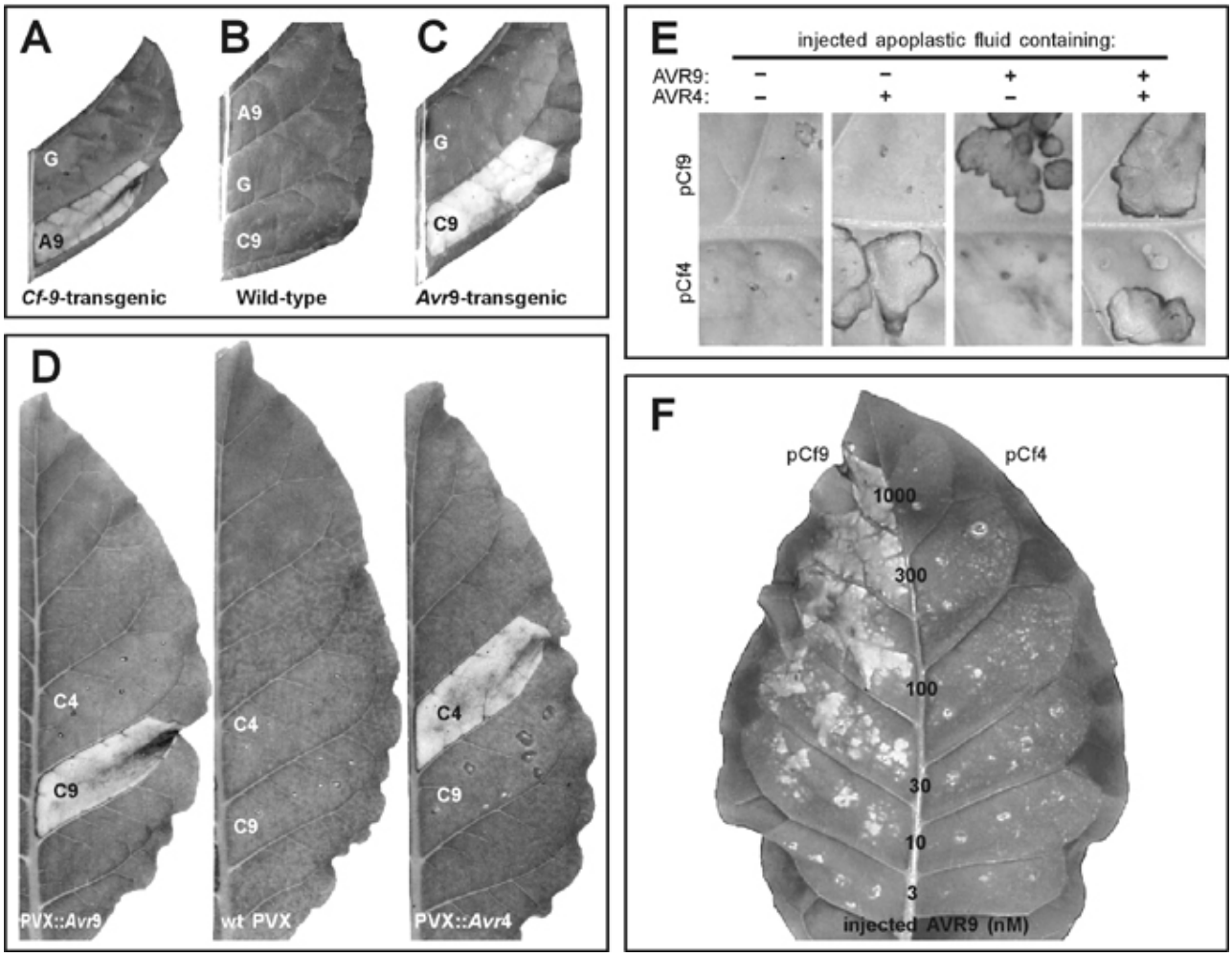

Fig. 2. Transient expression of *Avr* and *Cf* genes in tobacco leaves. *Agrobacterium tumefaciens* carrying pGUSi (G), pAvr9 (A9), pCf9 (C9), or pCf4 (C4) was infiltrated into young, fully expanded leaves of 6-week-old tobacco plants. Photographs were taken at 7 days post infiltration (dpi). **A,** Transient expression of the β-glucuronidase (*gus*) gene and *Avr*9 in *Cf-9* transgenic tobacco. **B,** Transient expression of *Avr*9, *gus*, and *Cf-9* in wild-type tobacco. **C,** Transient expression of *gus* and *Cf-9* in *Avr*9 transgenic tobacco. **D,** Transient *Cf* gene expression in *Potato virus X* (PVX)-inoculated, wild-type tobacco plants. Two weeks post inoculation, cultures of *A. tumefaciens* carrying pCf9 or pCf4 were infiltrated into young, fully expanded leaves that showed mosaic symptoms caused by PVX. **E,** Injection of apoplastic fluids (AFs), isolated from different compatible *C. fulvum*-tomato interactions into tobacco leaves at 1 dpi with *A. tumefaciens* containing pCf9 or pCf4. AFs were isolated from tomato cultivar Moneymaker Cf0 inoculated with *C. fulvum* race 2.4.5.9.11 (MM-Cf0/race 2.4.5.9.11) (lacks AVR9 and AVR4, first panel), MM-Cf5/race 2.5.9 (lacks AVR9, second panel), MM-Cf4/race 2.4.8.11 (lacks AVR4, third panel), and MM-Cf0/race 5 (contains both AVR9 and AVR4, fourth panel). **F,** Injection of a concentration series of AVR9 protein, performed at 1 dpi with *A. tumefaciens* containing pCf9 (left leaf half) or pCf4 (right leaf half) into wild-type tobacco leaves. Photograph was taken at 7 dpi.

pCf that induced 50% necrosis (NC^{50}) of the infiltrated leaf area was calculated from two independent experiments. NC^{50} values for pCf9 were 1.86 and 3.74%, respectively, whereas NC^{50} values for pCf4 were calculated as 1.38 and 4.92%, respectively (Fig. 3C). This indicates that pCf9 and pCf4 have comparable activities. NC^{50} values for pAvr9 and pAvr4 were calculated as 2.56 ± 0.88% and 0.27 ± 0.12%, respectively (n = 4, Fig. 3D), indicating that pAvr9 has a 10-fold lower activity, compared with pAvr4. Significantly, at concentrations corresponding to these NC^{50} values no difference in timing between *Avr*9/*Cf-9*- and *Avr*4/*Cf-4*-induced necrosis was observed (data not shown). These data indicate that the temporal differences in necrotic responses induced by *Avr*9/*Cf-9* and *Avr*4/*Cf-4* gene pairs when cultures were mixed in a 1:1 ratio are caused by differences in activities between pAvr plasmids upon agroinfiltration.

Transient expression of matching *Avr/Cf* gene pairs in different plant families.

The extensive homology between *R* gene products suggests that signal transduction cascades that lead to disease resistance are highly conserved between plant families. To examine whether species other than tobacco and tomato have the signal transduction components that are required for *Avr/Cf*-induced responses, we transiently co-expressed matching gene pairs in a number of different plant species. Transient expression of the β-glucuronidase (*gus*) gene served as an indication of the transformation efficiency and the level of gene expression. Specific responses that were induced by co-expressing matching gene pairs were compared with aspecific responses induced by *gus* expression and by co-expressing non-matching gene pairs.

Some plant species were difficult to infiltrate (e.g., soybean, rice, and maize) whereas others showed very low levels of GUS staining (e.g., sugar beet, broad bean, and Brussels sprouts) (data not shown). Plant species that showed severe background responses included tomato, potato, cucumber, and pepper (data not shown). Therefore, agroinfiltration in these plant species remains to be optimized.

Plant species that showed significant GUS staining and low background responses are shown in Table 1. All tobacco cultivars tested showed a strong necrotic response within the entire infiltrated area upon co-expression of matching gene pairs, while aspecific responses remained negligible. As with tobacco cv. Petite Havana SR1, transient co-expression of the *Avr*9/*Cf-9* gene pair in other tobacco cultivars always resulted

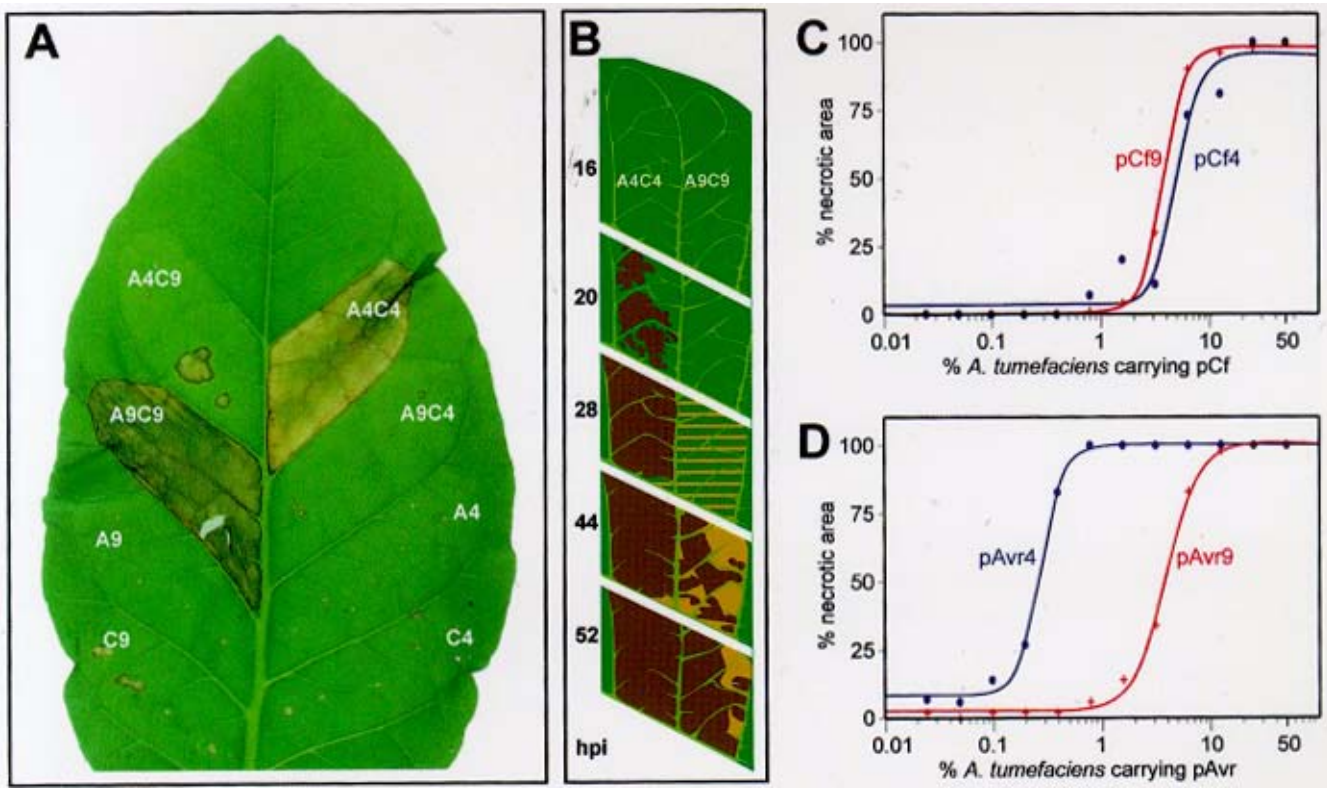

Fig. 3. Comparison necrosis induced by different matching *Avr/Cf* gene pairs. **A,** Transient expression and co-expression of *Avr*9 (A9), *Cf-9* (C9), *Avr*4 (A4), and *Cf-4* (C4) in wild-type tobacco. For transient co-expression, *Agrobacterium tumefaciens* cultures were mixed in a 1:1 ratio and infiltrated. Photograph was taken at 7 days post infiltration (dpi). **B,** Drawing representing development of responses induced by co-expression of *Avr*9/*Cf-9* or *Avr*4/*Cf-4* gene pairs in wild-type tobacco. Drawings were made of the same leaf at different hours post infiltration (hpi) and show intact tissue (green), strong chlorotic areas (yellow), weak chlorotic areas (yellow stripes), and areas with collapsed tissue (brown). **C,** Quantification of necrosis induced by transient *Cf* gene expression. *A. tumefaciens* carrying pCf was diluted with *A. tumefaciens* carrying the matching pAvr and infiltrated into wild-type tobacco leaves. pCf9 (+) and pCf4 (•) dilution series were infiltrated into opposite leaf halves. Percentage of infiltrated leaf area that had become necrotic at 7 dpi was measured and plotted against concentration of *A. tumefaciens* that carries pCf. **D,** Quantification of necrosis induced by transient *Avr* gene expression. *A. tumefaciens* carrying pAvr was diluted with *A. tumefaciens* carrying matching pCf and infiltrated into wild-type tobacco leaves. pAvr9 (+) and pAvr4 (•) dilution series were infiltrated into opposite leaf halves. Percentage of infiltrated leaf area that had become necrotic at 7 dpi was measured and plotted against concentration of *A. tumefaciens* that carries pAvr. **C** and **D,** One representative experiment is shown in each.

in a more dark brown necrotic sector than co-expression of the *Avr4*/*Cf-4* gene pair. *Nicotiana benthamiana* and *N. plumbaginifolia* showed specific chlorotic responses, often with a necrotic center. In *N. clevelandii*, specific necrotic responses were only visible at the site of infiltration, whereas GUS staining was also present at more distal sites. In *N. glutinosa*, specific necrosis developed within 7 days, whereas aspecific necrosis developed later. In *Petunia hybrida*, a specific chlorotic response developed with both gene pairs. The observation that all solanaceous species that were tested showed necrotic or chlorotic responses upon co-expression of *Avr*9/*Cf-9* and *Avr*4/*Cf-4* suggests that components that are required for *Avr/Cf*-induced responses are conserved within this family.

Transient expression of *Avr/Cf* gene pairs in the non-solanaceous species *A. thaliana*, radish, lupine, pea, and flax did not induce any chlorotic or necrotic responses (Table 1), even though significant GUS staining and low aspecific responses were observed (Fig. 4A). In lettuce, which showed clear GUS staining and low aspecific responses, a necrotic response was induced upon co-expression of the *Avr*4/*Cf-4* gene pair, whereas expression of *Avr*4 and *Cf-4* in nonmatching combinations with *Cf-9* and *Avr*9, respectively, did not induce necrosis (Fig. 4B). Surprisingly, co-expression of the *Avr*9/*Cf-9* gene pair in lettuce did not result in necrosis under the conditions tested.

DISCUSSION

Transient expression of *Avr/Cf* gene pairs.

The gene pairs investigated in this study are derived from the fungal pathogen *C. fulvum* and its only host, tomato. As the fungus grows extracellularly, it is expected that secreted AVR proteins are perceived on the tomato plasma membrane via *R* gene products (Joosten and De Wit. 1999). By demonstrating that specific necrosis occurs upon transient expression of matching *Avr/Cf* gene pairs, we have shown for the first time that agroinfiltration can be used to study extracellular perception. Prior to this study, transient expression through agroinfiltration was only used to express the small cytoplasmic R protein Pto (Frederick et al. 1998; Rathjen et al. 1999) and to demonstrate that perception of AvrBs3, AvrPto, *Tobacco mosaic virus* (TMV)-helicase, and PVX coat protein

Table 1. Transient expression of β-glucuronidase *(gus)* and *Avr/Cf* gene pairs in different plant species

		Induced responses[b]			
Plant species	**Family**	**GUS[a]**	**Aspecific**	***Avr*9/*Cf-9***	***Avr*4/*Cf-4***
Nicotiana tabacum cv. Petit Havana	Solanaceae	+++	–	+++	+++
N. tabacum cv. Samsun NN	Solanaceae	+++	–	+++	+++
N. tabacum cv. Xanthi	Solanaceae	+++	–	+++	+++
N. tabacum cv. White Burley	Solanaceae	+++	+	+++	+++
N. benthamiana	Solanaceae	+++	+	++	++
N. clevelandii	Solanaceae	+++	–	+	+
N. glutinosa	Solanaceae	+++	++	+++	+++
N. rustica	Solanaceae	++	+	++	++
N. plumbaginifolia	Solanaceae	ND	–	+	+
Petunia hybrida W115	Solanaceae	ND	+	++	++
Lactuca sativa (lettuce)	Compositae	+++	–	–	+++
Arabidopsis thaliana cv. Col-0	Cruciferae	+++	–	–	–
Raphanus sativus (radish)	Cruciferae	+	–	–	–
Lupinus albus (lupine)	Leguminosae	+	–	–	–
Pisum sativum (pea)	Leguminosae	++	–	–	–
Linum usitatissimum (flax)	Linaceae	+++	–	–	–

[a] *gus* gene expression, as estimated by GUS staining at 7 days post infiltration (dpi); + = low, ++ = moderate, and +++ = high *gus* expression; ND = not determined.

[b] Induced responses at 7 dpi by transient expression of *Avr*4/*Cf-9*, *Avr*9/*Cf-4*, or *gus* (aspecific responses), *Avr*9/*Cf-9* and *Avr*4/*Cf-4* (specific responses). – = no response, compared with non-infiltrated area; + = weak chlorosis/necrosis; ++ = moderate chlorosis/necrosis; +++ = severe necrosis of entire infiltrated area. Co-expression was done by infiltrating cultures that were mixed in a 1:1 ratio.

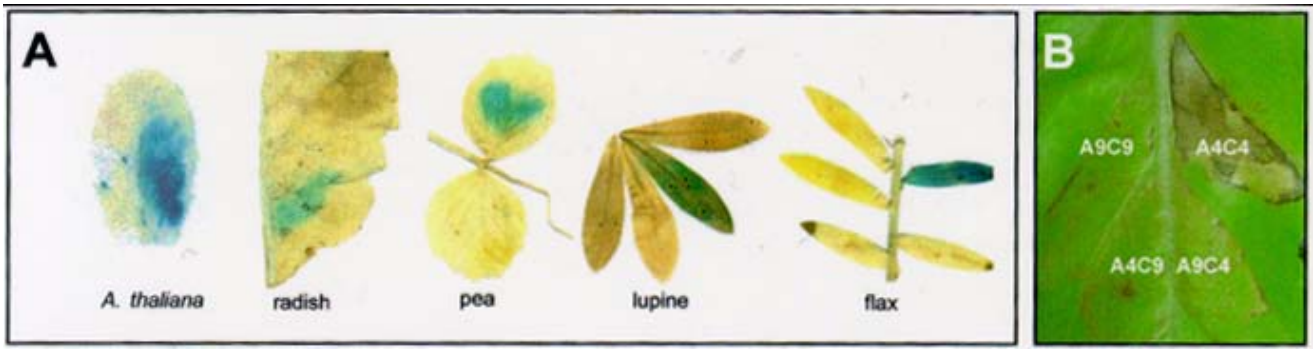

Fig. 4. Expression of β-glucuronidase (*gus*) and *Avr/Cf* gene pairs in different plant species. **A,** Transient *gus* expression in different plant species following agroinfiltration with pGUSi. GUS staining was performed at 7 days post infiltration (dpi). **B,** Transient co-expression of *Avr*9 (A9), *Cf-9* (C9), *Avr*4 (A4), and *Cf-4* (C4) in lettuce by infiltration of cultures that were mixed in a 1:1 ratio. Photograph was taken at 7 dpi.

occurs in the cytosol (Abbink et al. 1998; Bendahmane et al. 1999; Erickson et al. 1999; Scofield et al. 1996; Tang et al. 1996; Van den Ackerveken et al. 1996).

Our results demonstrate that *Cf*-mediated recognition of *Avr* gene products is retained when agroinfiltration is combined with established assays, such as transgenic tobacco plants expressing a matching gene partner, PVX-mediated *Avr* gene expression, or elicitor protein injections. Injection of a dilution series of AVR9 protein into leaves that transiently express *Cf-9* shows that agroinfiltration is sufficiently sensitive to study *Avr/Cf* interactions. Furthermore, necrosis induced by agroinfiltration of *Avr/Cf* gene pairs can be quantified and can also be used to study *Avr/Cf*-induced responses in a large number of plant species. Together, these results indicate that agroinfiltration is a powerful and versatile tool for further studies on *Avr* and *Cf* gene function.

R gene products are key components in the signal transduction pathway leading to induction of programmed cell death and defense responses that eventually stop invasion of the pathogen. Therefore, overexpression of *R* genes in the absence of matching elicitors may result in auto-necrosis. For example, overexpression of the *Pto* gene induced necrosis in the absence of AvrPto (Tang et al. 1999). The pCf constructs used in this study were all driven by a 35S promoter to ensure sufficient *Cf* gene expression. Indeed, dilution experiments showed that a culture containing only 2% pCf-carrying *A. tumefaciens* is sufficient to induce specific necrosis in the presence of a matching *Avr* gene. Nevertheless, agroinfiltration of 35S-driven *Cf* genes in the absence of their matching *Avr* genes did not result in necrosis. This may suggest that overproduction of Cf proteins is not toxic to the plant or that mechanisms exist that keep Cf protein levels sufficiently low. pCf dilution experiments also suggest that agroinfiltration of *A. tumefaciens* cultures that carry *Cf* genes with weak promoters should result in necrosis when expressed with their matching *Avr* genes. Indeed, agroinfiltration of the *Cf-9* gene, driven by its native promoter, was found to induce necrosis when co-expressed with *Avr*9 (data not shown).

Comparison of *Avr*9/*Cf-9*- and *Avr*4/*Cf-4*-induced responses.

Transient *Cf* gene expression allowed us for the first time to compare responses induced by different *Avr/Cf* gene pairs within the same leaf. We found that *Avr*9/*Cf-9*-induced necrosis developed slower than *Avr*4/*Cf-4*-induced necrosis when pAvr- and pCf-containing cultures were combined in a 1:1 ratio. However, at concentrations of pAvr-containing cultures that correspond to NC^{50} values, no temporal differences between the two gene pairs were observed. This indicates that the temporal differences between *Avr*9/*Cf-9*- and *Avr*4/*Cf-4*-induced responses are correlated with the activities of the pAvr constructs. These different pAvr activities can for example be due to differences in transcriptional or post-transcriptional modification, AVR protein stability, or perception. Comparison of different *Avr/Cf*-induced responses in other systems has also indicated that the *Avr*9/*Cf-9* gene pair is less active than the *Avr*4/*Cf-4* gene pair (M. H. A. J. Joosten, *personal communication*). For example, tomato seedlings die quicker when derived from seeds of a cross between *Avr*4 transgenic tomato and MM-Cf4 than when derived from seeds of a cross between *Avr*9 transgenic tomato and MM-Cf9. Similarly, inoculation of PVX::*Avr*4 on MM-Cf4 results in a more severe systemic necrosis than inoculation of PVX::*Avr*9 on MM-Cf9 plants. Our results suggest that these temporal differences are due to differences between the *Avr*9 and *Avr*4 gene activities when expressed in planta, rather than to intrinsic differences between the function of *Cf* gene products.

Presence of *Avr/Cf* signal transduction pathways in other plant families.

The striking similarities between proteins encoded by *R* genes cloned from different plant species suggest that different *R* genes would function in other plant species. Indeed, the tomato *Pto* gene has been shown to function in *Nicotiana* spp. (Thilmony et al. 1995), the tobacco *N* gene is active in tomato (Witham et al. 1996), and the tomato *Cf-9* gene is functional in potato and tobacco (Hammond-Kosack et al. 1998). We could extend this analysis by transient expression studies and show that *Cf-9* and *Cf-4* genes from tomato function in all *Nicotiana* spp. tested, as well as in *Petunia hybrida*. This indicates that the signal transduction pathway required for *Avr/Cf*-mediated necrosis is conserved within the Solanaceae. It also suggests that these plant species may recruit the same signal transduction pathway to activate defense responses against their pathogens.

In contrast, *A. thaliana*, radish, lupine, pea, and flax did not show necrotic responses upon transient co-expression of matching *Avr/Cf* gene pairs, even though *gus* expression was detected and background responses were sufficiently low. This indicates that these plant species lack components that are required for *Avr/Cf*-induced responses, implying that functional transfer of an *R* gene from one plant family to another has its limitations.

An exception to the above is lettuce, a composite that seems to contain all components required for *Avr*4/*Cf-4*-induced necrosis. This is the first report of an *R* gene that can function in a different plant family. Surprisingly, *Avr*9/*Cf-9*-induced necrosis was not observed in lettuce, suggesting that the level of expression of *Avr*9 in lettuce is below the threshold level that is required for activity. Alternatively, lettuce may lack one or more components of the signal transduction pathway required for *Avr*9/*Cf-9*-induced necrosis.

MATERIAL AND METHODS

Plant material, GUS staining, PVX inoculation, and protein preparations.

Plants were grown under standard greenhouse conditions except *for A. thaliana*, which was grown under short day conditions. For most assays, 4- to 8-week-old *N. tabacum* cv. Petite Havana (SR1) plants were used, unless stated otherwise. 35S::*Avr*9-transgenic tobacco line SLJ6201A (Hammond-Kosack et al. 1994) and transgenic tobacco line 6A3 carrying a genomic clone of *Cf-9* were used (Kamoun et al. 1999). GUS staining was performed as described by Jefferson (1987). PVX inoculations with wild-type PVX, PVX::*Avr*9, and PVX::*Avr*4 were performed as described before (Joosten et al. 1997; Hammond-Kosack et al. 1995). Synthetic AVR9 was prepared as described previously (Kooman-Gersmann et al. 1998). Apoplastic fluids were isolated from compatible *C. fulvum*-tomato interactions at 14 to 20 days after inoculation, as described by De Wit and Spikman (1982).

DNA manipulations and plasmids.

All DNA manipulations were performed by standard protocols (Sambrook et al. 1989). Polymerase chain reaction (PCR) was performed with *Pfu* polymerase (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Restriction enzymes, T4 ligase, and *Escherichia coli* DH5α cells were from Life Technologies (Breda, The Netherlands). Primers were synthesized by Amersham-Pharmacia (Buckinghamshire, UK). Authenticity of all cloned PCR fragments was confirmed by sequencing.

The following plasmids were used in our studies: pFM4 and pMOG800 (Honée et al. 1998), pCf9.5 (*prp1::Cf-9*, pMOG1048; Honée et al. 1998), pGUSi (Fig. 1; pMOG410; Hood et al. 1993), PVX::*Avr*4 (Thomas et al. 1997), and pAvr9 (Fig. 1; pMOG978; Honée et al. 1998). pFT43, containing a *Cf-4* genomic clone, was kindly provided by Frank Takken (Department of Genetics, BioCentrum Amsterdam; Takken 1999).

pCf9 and pCf4 were constructed as follows: with *Xba*I and *Nco*I restriction sites, the 35S promoter from pFM4 was cloned into pCf9.5, thereby replacing the *prp1* promoter and creating pRH1. The 5′ part of the *Cf-4* gene was amplified from pFT43, with primers ttagtgcagccatgggttgtg and catgcaacttatttgatctcaagc (*Nco*I site is underlined). The latter primer anneals 3′ of the *Hin*dIII site, which is present in both *Cf-9* and *Cf-4*. With *Nco*I and *Hin*dIII restriction sites, the PCR product was cloned into pRH1, thereby replacing the 5′-terminal part of *Cf-9* with that of *Cf-4*, generating pRH46. The 3′ region of the *Hin*dIII restriction site of *Cf-9* and *Cf-4* genes encodes identical amino acids. The promoter-ORF-terminator cassettes of pRH1 and pRH46 were subsequently transferred to pMOG800 with *Bam*HI and *Kpn*I restriction sites, creating binary plasmids pCf9 (pRH21) and pCf4 (pRH48).

For the construction of pAvr4, the 35S promoter was amplified from pRH1 with primers gatctctagaggtcaacatggtggagcacg and aaaactgcagctcgaggtcgacaccatggtattgtaaatagtaattgtaatgttg (*Xba*I, *Pst*I, and *Nco*I sites are underlined, respectively) and cloned into pRH1 with *Xba*I and *Pst*I. This construct (pRH80) carries the 35S promoter and the PI-II terminator (An et al. 1989) flanking a multiple cloning site (*Nco*I-*Sal*I-*Xho*I-*Pst*I). The ORF encoding the mature AVR4 protein fused to the signal peptide of the pathogenesis-related gene *PR1a* was amplified from PVX::*Avr*4 with primers cgttccactggagtccttttgg, ccaaaaggactccagtggaacg, ttagtgcagccatgggttgtg, and aaaactgcagtcattgcggcgtctttaccggacacg (*Nco*I and *Pst*I sites are underlined, respectively). The first two primers were designed to remove the *Pst*I site from *Avr*4 by PCR overlap-extension. The PCR product was cloned into pRH80 with *Nco*I and *Pst*I, thereby creating pRH85. The promoter-ORF-terminator cassette of pRH85 was cloned into pMOG800 with *Xba*I and *Eco*RI, creating the binary plasmid pAvr4 (pRH87).

***A. tumefaciens*-mediated transient expression.**

The *A. tumefaciens* strain MOG101 (Hood et al. 1993) was transformed by electroporation. Recombinant *A. tumefaciens* containing the different binary plasmids was grown overnight (28°C, 200 rpm; LABOTECH RS500; Labotec, Belgium) in tubes containing 3 ml of YEB medium (per liter: 5 g of beef extract [Sigma, St. Louis, MO], 1 g of yeast extract [Oxoid, Hampshire, UK], 5 g of bacteriological peptone [Oxoid], 5 g of sucrose, and 2 ml of 1 M $MgSO_4$) containing 50 μg of kanamycin (Duchefa, Haarlem, The Netherlands) per ml and 25 μg of rifampicin (Sigma) per ml. These cultures were used to inoculate a 300-ml conical flask containing 100 ml of YEB medium supplemented with 1 ml of 1 M *N*-morpholinoethanesulfonic acid (MES; Sigma), 50 μg of kanamycin per ml, and 2 mM acetosyringone (Aldrich, Steinheim, Germany). After overnight incubation (28°C, 200 rpm; LABOTECH RS500; Labotec, Belgium), cells were harvested at an OD_{600} of 0.6 to 1.2 by centrifugation (8′, 4,000 × *g*) and resuspended in MMA to a final OD of 2 (1 liter of MMA: 5 g of MS salts [Duchefa], 1.95 g of MES, 20 g of sucrose, pH adjusted to 5.6 with 1 M NaOH], containing 200 μM acetosyringone. At this stage, cultures were mixed as described in the figure legends. Cultures were infiltrated into leaves with a 2-ml disposable syringe without a needle. Leaves were superficially wounded with a needle to improve infiltration.

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