

# The Cf-9 Disease Resistance Protein Is Present in an ~420-Kilodalton Heteromultimeric Membrane-Associated Complex at One Molecule per Complex

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The tomato *Cf-9* gene confers race-specific resistance to the fungal pathogen *Cladosporium fulvum* expressing the corresponding avirulence gene *Avr9*. In tobacco, *Cf-9* confers a hypersensitive response to the *Avr9* peptide. To investigate *Cf-9* protein function in initiating defense signaling, we engineered a functional C-terminal fusion of the *Cf-9* gene with the TAP (Tandem Affinity Purification) tag. In addition, we established a transient expression assay in *Nicotiana benthamiana* leaves for the production of functional *Cf-9:myc* and *Cf-9:TAP*. Transiently expressed *Cf-9:myc* and *Cf-9:TAP* proteins induced an *Avr9*-dependent hypersensitive response, consistent with previous results with stably transformed tobacco plants and derived cell suspension cultures expressing c-myc-tagged *Cf-9*. Gel filtration of microsomal fractions solubilized with octylglucoside revealed that the *Cf-9* protein, either as c-myc or TAP fusions, migrated at a molecular mass of 350 to 475 kD. By using blue native gel electrophoresis, the molecular size was confirmed to be ~420 kD. Our results suggest that only one *Cf-9* protein molecule is present in the *Cf-9* complex and that *Cf-9* is part of a membrane complex consisting of an additional glycoprotein partner(s). The high structural similarity between *Cf* proteins and *Clavata2 (CLV2)* of *Arabidopsis*, together with the similarity of molecular mass between *Cf-9* and *CLV* complexes (420 and 450 kD, respectively), led us to investigate whether *Cf-9* is integrated into membrane-associated protein complexes like those formed by *CLV1* and *CLV2*. Unlike *CLV2*, the *Cf-9* protein did not form disulfide-linked heterodimers, no ligand (*Avr9*)-dependent shift in the molecular mass of the *Cf-9* complex was detected, and no Rho-GTPase-related proteins were found associated with *Cf-9* under the conditions tested. Thus, *Cf-9*-dependent defense signaling and *CLV2*-dependent regulation of meristem development seem to be accomplished via distinct mechanisms, despite the structural similarity of their key components *Cf-9* and *CLV2*.

## INTRODUCTION

A major goal in plant pathology is to understand the molecular mechanism of disease resistance. In gene-for-gene interactions, a plant disease resistance gene (*R* gene) confers resistance to invading pathogens that carry the corresponding avirulence (*Avr*) gene. Despite the cloning of numerous *R* genes in recent years, progress in understanding *R* protein function in *Avr* perception and signal transmission has been comparatively slow. It has been postulated that *R* gene products are receptors for pathogen-encoded *Avr* components (Staskawicz et al., 1995; Ellis et al., 2000). However, direct physical interaction in an *R/Avr* combination has been reported only for the *Pto* and *Pi-ta* *R* gene products using the yeast two-

hybrid system (Scofield et al., 1996; Tang et al., 1996) and an overlay assay (Jia et al., 2000). This and several other lines of evidence suggest that *R* proteins are part of protein complexes, with additional protein partners participating in *Avr* perception and subsequent signal initiation (Dixon et al., 2000; Dangl and Jones, 2001). Whereas the isolation of *R* genes has been achieved largely by genetic approaches, protein biochemistry tools are now needed to facilitate the understanding of *R* protein function.

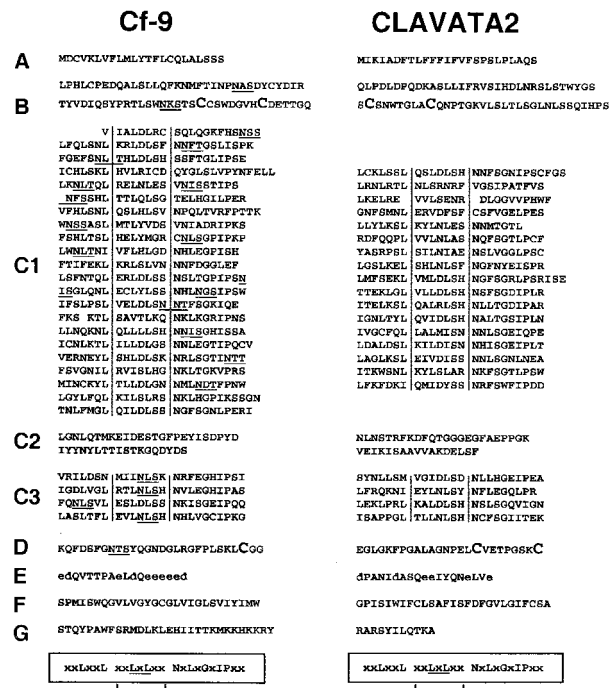
The *Cf-9* gene from tomato confers resistance to the biotrophic leaf mold pathogen *Cladosporium fulvum* through recognition of the fungus-encoded *Avr9* peptide. In this gene-for-gene model system, both components have been characterized in detail and the corresponding genes have been isolated. The *Cf-9* gene encodes a mainly extra-cytoplasmic, membrane-anchored glycoprotein composed predominantly of leucine-rich repeats (LRRs) and a short cytoplasmic domain that lacks an apparent signaling domain.

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This structure is consistent with a receptor function but provides no obvious mechanism for subsequent signal transduction. The *Avr9* gene product is a small, secreted protein carrying multiple internal disulfide bridges (Joosten and de Wit, 1999; van den Hooven et al., 2001). Recognition of this elicitor by tomato and tobacco genotypes carrying the *Cf-9* resistance gene results in the activation of defense responses, including a hypersensitive response (Hammond-Kosack and Jones, 1997). However, the molecular mechanism of *Avr9* perception has yet to be determined (Dixon et al., 2000).

*Cf-9* is highly glycosylated and located predominantly in the plasma membrane, despite the presence of the C-terminal endoplasmic reticulum (ER) retention/retrieval signal KKRY (Piedras et al., 2000). However, the KKRY conserved sequence also has been reported to confer ER localization of *Cf-9* (Bengezhal et al., 2000), although the activity of *Cf-9* was not measured. Thus, the precise location of the functional *Cf-9* protein remains controversial. The N-terminal region of the *Cf-9* gene delimits the domain that determines the recognitional specificity of *Avr9* recognition. The sequence variation within the central LRRs of domain C1 and variation in LRR copy number (Figure 1) play a major role in determining the recognition specificity of *Cf-9* (Van der Hoorn et al., 2001a; Wulff et al., 2001). Most of the variant residues in the C1 domain are predicted to form a solvent-exposed surface that can interact with the cognate ligand (Thomas et al., 1997). In addition, the *Avr9* peptide is secreted into the apoplasmic space, consistent with the idea that *Avr9* is the ligand and *Cf-9* is the receptor. However, previous studies performed in parallel in four different laboratories did not show a direct interaction between *Cf-9* and *Avr9* (Luderer et al., 2001). Additionally, a high-affinity binding site for *Avr9* was found in tomato lines regardless of whether they expressed the *Cf-9* gene (Kooman-Gersmann et al., 1996). These observations, together with the fact that the *Cf-9* protein lacks any obvious domain suitable for signal transduction, suggest that additional proteins may be involved in the *Cf-9/Avr9*-mediated perception event that triggers the downstream responses. A key question now is whether the *Cf-9* protein functions in a multiprotein complex.

The involvement of an LRR protein in functional protein complexes has been described recently for the Arabidopsis gene *Clavata2* (*CLV2*), and a model for its role in meristem and organ development has been presented (Jeong et al., 1999). Interestingly, the *CLV2* protein shares high structural similarity with *Cf* gene products and other LRR-containing receptor-like proteins in that it consists of predicted extracellular LRRs (Figure 1). These LRRs are flanked by Cys pairs separated by seven residues, a motif common to plant receptor-like proteins with predicted extracellular LRRs (Jones and Jones, 1997) (Figure 1, domains B and D). *CLV2* has been postulated to form an inactive disulfide-linked heterodimer of 185 kD with *CLV1*. *CLV1* is similar to the *Cf* family of genes in that it encodes a protein with predicted extracellular LRRs. Formation of the *CLV1-CLV2* heterodimer is likely to be mediated by disulfide linkage between the conserved Cys pairs flanking the



**Figure 1.** Comparison of Tobacco *Cf-9* and Arabidopsis *CLV2* Proteins.

Comparison of the amino acid sequences of *Cf-9* (left) and *CLV2* (right) (Jeong et al., 1999) reveals that both proteins can be subdivided into nine distinct domains (A to G) of similar structure. The amino acid sequence identity between *CLV2* and *Cf-9* is 21.5%. Potential NxS/T glycosylation sites are underlined in *Cf-9*. Domain A represents a putative signal peptide, and domain B is the mature N terminus. Cys pairs flanking the LRRs (domains C1 and C3) (Jones and Jones, 1997) are shown in larger type in domains B and D. Domains E (acidic domain with acidic residues shown in lowercase letters), F (transmembrane domain), and G (cytosolic domain) are predicted to anchor and orient *Cf-9* in the cell membrane (Jones et al., 1994). The consensus sequences for plant extracellular LRRs are shown boxed and aligned below the amino acid sequences, and consensus residues with a possible  $\beta$ -strand configuration are underlined. Residues corresponding to the  $\beta$ -strand/ $\beta$ -turn structural motif (Kobe and Deisenhofer, 1994; Jones and Jones, 1997) are indicated by brackets and delimited by vertical lines.

LRR regions (Jeong et al., 1999) (Figure 1, domains B and D). Upon recognition of the ligand, *CLV3*, and phosphorylation of the *CLV1* kinase domains, an active 450-kD complex is formed containing the protein phosphatase KAPP and a Rho GTPase-related (Rop) protein (Trotochaud et al., 1999). However, unlike *Cf* proteins, *CLV1* contains an intracellular protein kinase domain that probably is involved in signal transduction (Clark et al., 1997). Given the similar structure shared by *CLV2* and *Cf* resistance proteins, together with the fact that a small extracellular peptide ligand is required in both cases to trigger the appropriate responses, it has been speculated that *Cf-9/*

Avr9-dependent defense signaling is achieved via a similar mechanism (Joosten and de Wit, 1999; Luderer et al., 2001).

Heterologous expression of the wild-type *Cf-9* gene in tobacco and derived cell suspension cultures already has proven to be a valuable tool for studying physiological and biochemical changes in a gene-for-gene interaction after elicitation with Avr9 (Romeis et al., 2000a). Early and specific responses include changes in ion fluxes (Blatt et al., 1999), generation of reactive oxygen species (Piedras et al., 1998), activation of protein kinases (Romeis et al., 1999, 2000b), induction of gene expression (Durrant et al., 2000), and cell death (B. Belenghi, M. Smoker, and J.D.G. Jones, unpublished data). In addition, the production of functional Cf-9:myc transgenic tobacco lines and cell cultures allowed initial biochemical characterization and localization studies of the Cf-9 protein (Piedras et al., 2000).

Here, we establish transient assays as a useful tool for the expression of functional, tagged Cf-9 proteins in *Nicotiana benthamiana* leaves and describe the generation of a new Tandem Affinity Purification (TAP)-tagged Cf-9 construct. The TAP method (Rigaut et al., 1999) is a significant new tool for protein complex characterization and proteome exploration. Two tags (the calmodulin binding peptide and the IgG binding units of protein A from *Staphylococcus aureus*), fused in tandem and separated by a Tobacco Etch Virus (TEV) protease cleavage site, have sufficient affinity for the quantitative recovery of fusion protein present at low cellular levels under native conditions in a complex mixture. This provides an additional tool to study Cf-9 protein function in Avr9 perception and signal transmission, complementing the SLJ9161 and SLJ9171 lines that express Cf-9:myc (Piedras et al., 2000). By using c-myc-tagged Cf-9 plants and cell cultures and transiently expressed Cf-9:myc and Cf-9:TAP, the Cf-9 protein was shown, by both gel filtration and blue native non-denaturing gel electrophoresis, to be part of a protein complex with a molecular mass of ~420 kD. Importantly, this complex appeared to contain only one Cf-9 molecule, supporting the idea that additional protein partners participate with Cf-9 in the perception of the Avr9 protein. In addition, we showed that the Cf-9 complex shows different characteristics compared with those proposed for CLV function in Arabidopsis development, suggesting that different signaling mechanisms are involved in Cf-9-mediated disease resistance to *C. fulvum*.

## RESULTS

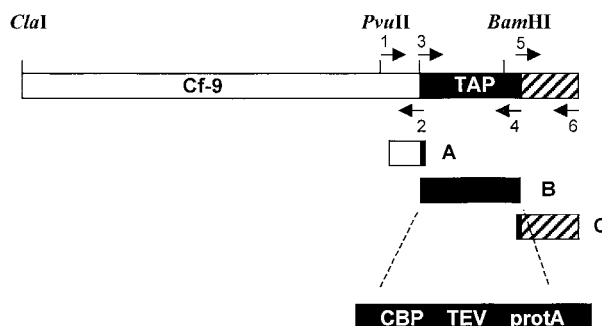
### Generation of a C-Terminal TAP-Tagged Cf-9 Construct

Transgenic tobacco plants and cell cultures that expressed triple c-myc-tagged Cf-9 from the 35S promoter were generated previously (Piedras et al., 2000). The resulting Cf-9:myc proteins were tagged either behind the putative signal peptide cleavage site (domain B) or in the putative cytoplasmic

tail (domain G) of the Cf-9 protein (Figure 1, domains B and G) (Cf-9:mycB and Cf-9:mycG, respectively). To generate an additional tool to study Cf-9 protein function, a C-terminal fusion of the Cf-9 gene with the TAP tag (Rigaut et al., 1999) was engineered by chimeric polymerase chain reaction (PCR) as detailed in Methods (Figure 2). In this construct, the TAP tag was inserted after the conserved KKRY sequence, which has been proposed to confer Cf-9 localization (Bengezhal et al., 2000). This construct was fused to either the 35S promoter or the Cf-9 native promoter and cloned into an *Agrobacterium tumefaciens* binary plasmid.

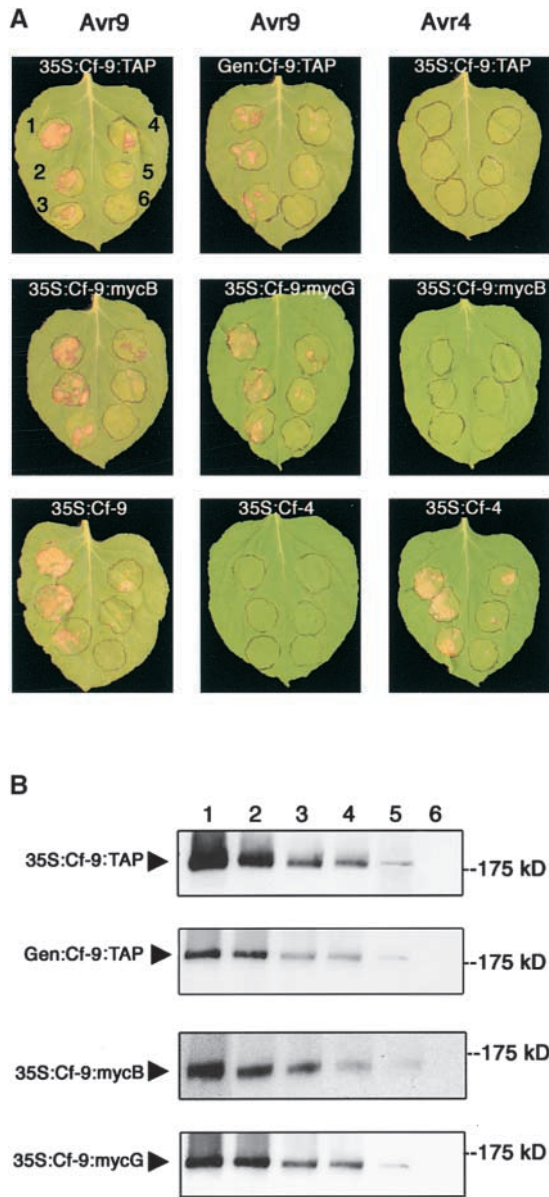
### Transient Expression and Functional Analysis of Tagged Cf-9 Proteins in *N. benthamiana* Leaves

An *A. tumefaciens*-mediated transient assay was designed to test the in vivo functionality of the Cf-9:TAP gene, under the control of the 35S or the Cf-9 native promoter, in *N. benthamiana* plants expressing Avr9 (Thomas et al., 2000). Additionally, previously generated 35S:Cf-9:myc (tagged either in the B or the G domain), 35S:Cf-9, and 35S:Cf-4 were tested. In control experiments, Avr4-expressing *N. benthamiana* leaves also were infiltrated with *Agrobacterium* (Thomas et al., 2000). The Cf-9/Avr9-dependent hypersensitive cell death reaction always was observed in the gene-for-gene combination. Strong necrosis in Avr9-expressing leaves was observed after inoculation with *A. tumefaciens* carrying either the wild-type Cf-9 or any of the Cf-9-tagged constructs, whereas no necrotic phenotype was detected when Avr4 plants were inoculated with the same constructs



**Figure 2.** Scheme of Cf-9:TAP.

The Cf-9 sequence is represented by a white bar. The TAP tag is shown in black, with the relative positions of the calmodulin binding peptide (CBP), the TEV protease cleavage site (TEV), and the IgG binding units of protein A from *S. aureus* (protA) indicated within the magnified block. The 3' untranslated sequence of Cf-9 is shown in the striped box. The TAP sequence was inserted by chimeric polymerase chain reaction at the 3' end of the Cf-9 gene (see Methods). The three overlapping fragments used for the chimeric reaction (A, B, and C) are shown. Positions of the primers and the restriction sites used for cloning are indicated.



**Figure 3.** Function and Detection of Cf-9:TAP and Cf-9:myc Transiently Expressed in *N. benthamiana* Leaves.

**(A)** Leaves of *N. benthamiana* transgenic for Avr9 (left and middle) or Avr4 (right) were infiltrated with *A. tumefaciens* carrying different Cf constructs, under 35S or Cf-9 endogenous promoter (Gen), at six different cell densities (1,  $OD_{600} = 0.1$ ; 2,  $OD_{600} = 0.05$ ; 3,  $OD_{600} = 0.02$ ; 4,  $OD_{600} = 0.01$ ; 5,  $OD_{600} = 0.005$ ; 6,  $OD_{600} = 0.002$ ), as indicated in the leaf at top left. After 5 days, the Cf-9/Avr9-dependent hypersensitive cell death reaction was observed in the Avr9 leaves infiltrated with Cf-9 constructs.

**(B)** *N. benthamiana* leaves were infiltrated as in **(A)**. Microsomal fractions were prepared 2 days after infiltration. Proteins (50  $\mu$ g) were separated on a SDS-PAGE gel and analyzed by immunoblotting using a PAP or an anti-c-myc antibody for the detection of TAP- and c-myc-tagged Cf-9, respectively. Positions of c-myc Cf-9 and

(Figure 3A). Likewise, the Avr9-dependent necrotic phenotype also was detected in nontransgenic *N. benthamiana* leaves inoculated with *A. tumefaciens* carrying the same constructs and reinjected with intracellular fluid containing Avr9 2 days after agro-infiltration (data not shown).

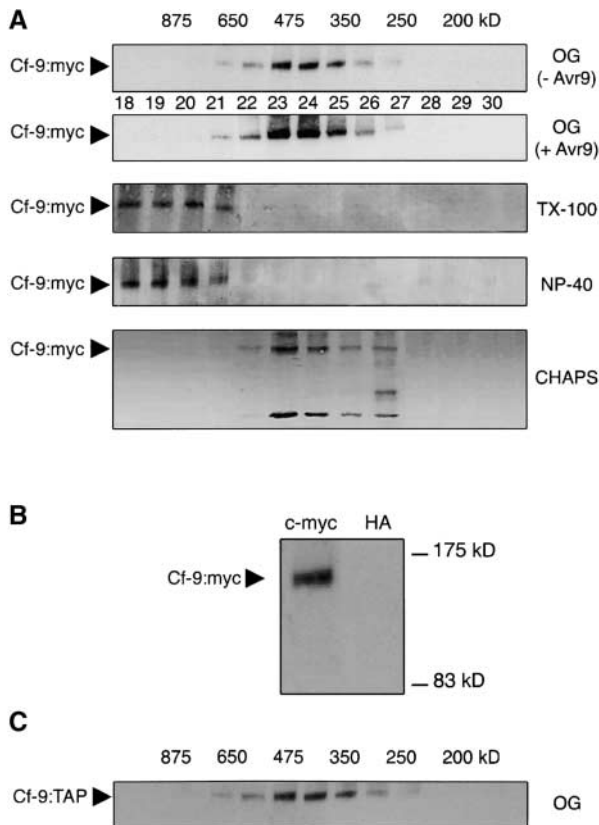
In parallel experiments, Cf-9 protein expression was tested in these agro-infiltrated, non-transgenic *N. benthamiana* leaves using antibodies against the engineered tags. Bands of the expected sizes ( $\sim 185$  and  $\sim 160$  kD for Cf-9:TAP-inoculated and Cf-9:myc-inoculated leaves, respectively) could be detected with peroxidase-antiperoxidase (PAP) or anti-c-myc antibodies (Figure 3B). These results provide clear evidence that *A. tumefaciens*-mediated transient expression can be used as an effective source of functional Cf-9 protein.

Further validation of the use of the transient assay was obtained by comparison of the necrosis observed in *N. benthamiana* leaves expressing Avr9 after infiltration with *A. tumefaciens* at different cell densities and leaves expressing either Cf-9:TAP (under the control of the 35S or the Cf-9 native promoter) or 35S:Cf-9:myc (with the c-myc tag in the B or G domain), and the expression of the Cf-9 protein as detected by protein gel blot analysis with the appropriate antibody. Importantly, correlation between the protein expression levels and the hypersensitive response was found consistently (Figures 3A and 3B). Together, these results demonstrate that Cf-9:TAP and Cf-9:myc, when expressed transiently in *N. benthamiana*, retain recognition of the Avr9 peptide and in vivo functionality. Furthermore, this newly developed transient expression system provides a valuable complement to the stable Cf-9:myc transgenic lines and should facilitate the biochemical elucidation of the Cf-9 protein.

#### Identification of a Membrane-Associated Cf-9-Containing Complex Using Gel Filtration Chromatography

In a previous study, we proposed that the perception of Avr9 by Cf-9 might be mediated by, at a minimum, a third interacting partner (Luderer et al., 2001). To investigate the potential association of Cf-9 with other protein(s), Cf-9:myc microsomal preparations from SLJ9161 tobacco plants were solubilized with octylglucoside (OG) and subjected to gel filtration chromatography. Two-milliliter fractions were collected, and aliquots were analyzed for the presence of Cf-9:myc by immunoblot analysis (Figure 4A, top). Identical elution profiles were obtained irrespective of whether the triple c-myc tag was integrated in either the B or the G domain of Cf-9, whether the 35S or the Cf-9 endogenous promoter

Cf-9:TAP are indicated by arrowheads. Numbers at top indicate the *A. tumefaciens* cell density used for infiltration.



**Figure 4.** Gel Filtration Analysis of the Cf-9:myc and Cf-9:TAP Microsomes.

**(A)** Microsomal proteins (0.5 mg) from SLJ9161 tobacco cell suspensions were solubilized with detergents TX-100 (0.1%), Nonidet P-40 (NP-40; 0.1%), CHAPS (0.5%), or OG (40 mM; before [–Avr9] and after [+Avr9] elicitation) for 30 min on ice, and the supernatants were subjected to gel filtration chromatography on a Sephacryl S-300 column. Two-milliliter fractions were collected, and aliquots were analyzed by SDS-PAGE and immunoblotting with an anti-myc antibody. Fraction numbers of the elution profile are indicated by the numbers between the gels. The molecular mass estimated for each fraction (in kD) is given at top. Cf-9:myc is indicated by arrowheads.

**(B)** Immunoprecipitation of Cf-9:myc from pooled fractions of the gel filtration. Microsomes were prepared from SLJ9161 homozygous tobacco plants, solubilized with OG, and subjected to gel filtration chromatography as described in **(A)**. Fractions containing Cf-9:myc were pooled and subjected to immunoprecipitation with a monoclonal anti-c-myc or anti-hemagglutinin (HA) antibody (control). Precipitated proteins were analyzed by SDS-PAGE and immunoblotting using a polyclonal anti-c-myc antibody. The position of Cf-9:myc is indicated by an arrowhead, and the sizes of molecular mass markers are shown at right.

**(C)** Two *N. benthamiana* leaves were infiltrated with *A. tumefaciens* carrying 35S:Cf-9:TAP. Two days after infiltration, microsomal proteins (0.5 mg) were solubilized with 40 mM OG and subjected to gel filtration chromatography as described in **(A)**. The molecular mass estimated for each fraction (in kD) is given at top. Cf-9:TAP is indicated by an arrowhead.

was used for the expression of Cf-9 (data not shown), or whether protein extracts were prepared from transiently infiltrated *N. benthamiana* leaves or from stable transgenic tobacco plants or cell cultures (data not shown).

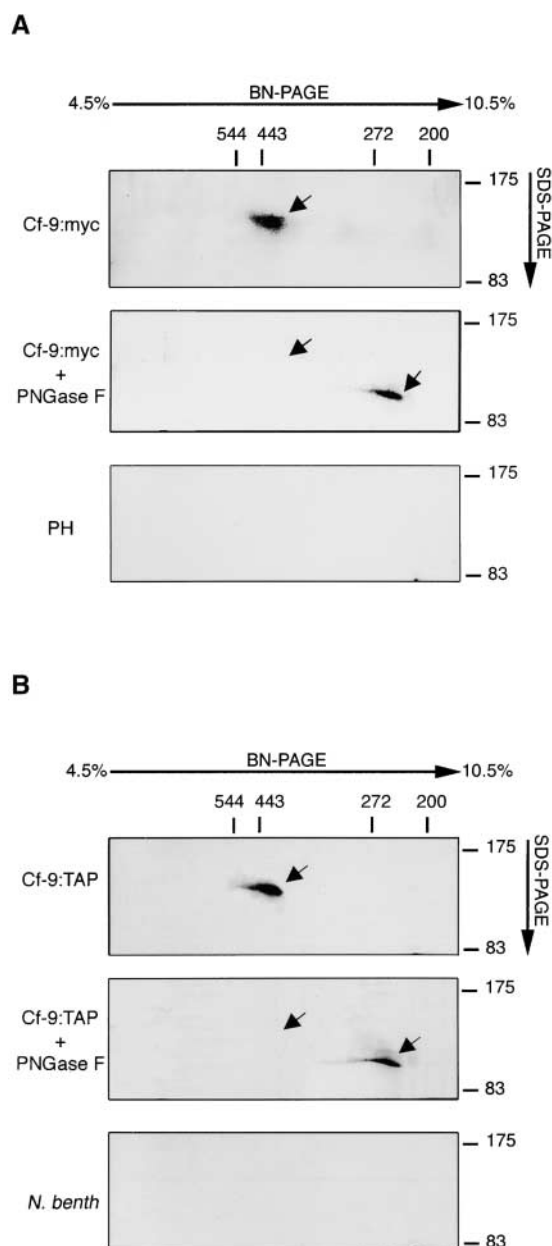
The estimated molecular mass of the Cf-9 protein from the eluted fractions ranged between 350 and 475 kD, peaking at ~410 kD (Figure 4A, top). This was higher than expected for the monomeric form of Cf-9:myc, strongly suggesting that Cf-9 is part of a protein complex in the membrane. No cross-reacting band was observed when extracts from untransformed Petit Havana plants were used (data not shown) (Luderer et al., 2001). To further confirm the specificity of the cross-reacting bands in Figure 4A, eluted fractions 23 to 25 from the gel filtration column containing Cf-9:myc were pooled and subjected to immunoprecipitation. A cross-reacting band was detected for Cf-9:myc when using a monoclonal anti-c-myc antibody but not a non-related hemagglutinin antibody (Figure 4B).

To further verify the significance of this observation, different solubilizing agents were used in gel filtration studies. The agents used were OG, Triton X-100 (TX-100), Nonidet P-40, and 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS). The use of different detergents greatly influenced the estimation of the molecular size of the proteins present in the Cf-9-containing fractions: 750 to 1000 kD when using TX-100 or Nonidet P-40, and 350 to 475 kD when using OG or CHAPS (Figure 4A). The high molecular mass observed with TX-100 and Nonidet P-40 (very close to the void volume value of the column) probably reflects the presence of non-specific protein aggregates (Simons et al., 1973) and the large micelle size of these detergents, whereas the 350- to 475-kD complex seen using CHAPS or OG most likely is the result of Cf-9 association with other protein(s) and/or with itself (Figure 4A). OG was chosen to extend the Cf complex characterization because it is a milder detergent than CHAPS and, in addition, protein gel blot analysis revealed a slight degradation of the Cf-9 protein after solubilization with CHAPS (Figure 4A).

Additionally, the stability of the Cf complex was studied under (1) different extraction buffer compositions, and (2) two different extraction procedures (frozen leaves or cells were ground in liquid nitrogen, or fresh leaves or cells were ground using a blender). The elution pattern of Cf-9 remained identical in the pH range 6.0 to 8.5 and with NaCl concentrations between 50 and 500 mM, regardless of the extraction procedure used (data not shown).

To further verify the significance of the Cf-9 complex, the *A. tumefaciens*-mediated transient assay was used to express Cf-9:TAP in *N. benthamiana* leaves. Microsomes were prepared, solubilized with OG, and subjected to gel filtration chromatography. Significantly, as for Cf-9:myc obtained from stable transformants, the estimated size of the eluting Cf-9 protein also ranged between 350 and 475 kD (Figure 4C), with a peak at ~410 kD.

In conclusion, we showed that Cf-9 is part of a 350- to 475-kD membrane-associated complex, regardless of (1)



**Figure 5.** BN-PAGE of Solubilized Cf-9:myc and Cf-9:TAP Microsomes.

**(A)** Microsomal proteins (0.5 mg) of SLJ9161 cell cultures (Cf-9:myc; top and middle) and Petit Havana (PH; bottom) were solubilized with 40 mM OG and 750 mM  $\epsilon$ -aminocaproic acid in 50 mM bis-Tris for 30 min on ice. Aliquots of supernatants (80  $\mu$ g of protein in 40  $\mu$ L) were either treated with PNGase F (middle; see legend to Figure 2A) or incubated directly with 5% Coomassie blue. Proteins were separated on a 4.5 to 10.5% BN-PAGE gradient gel. Cf complexes were resolved by two-dimensional 7.5% SDS-PAGE and blotted onto nitrocellulose. Cf-9:myc protein was finally detected by immunoblot analysis of the SDS gel using an anti-c-myc antibody. The sizes of the molecular mass markers are indicated at top (BN-PAGE)

the tissue used as a protein source (stable tobacco plants, cell cultures, or transiently infiltrated *N. benthamiana*), (2) the tag used (triple c-myc or TAP), (3) the position of the tag within the protein sequence (B or G domain of Cf-9), (4) the promoter used for Cf-9 protein expression (35S or Cf-9 endogenous promoter), or (5) the extraction conditions used (different extraction buffer compositions or extraction procedures).

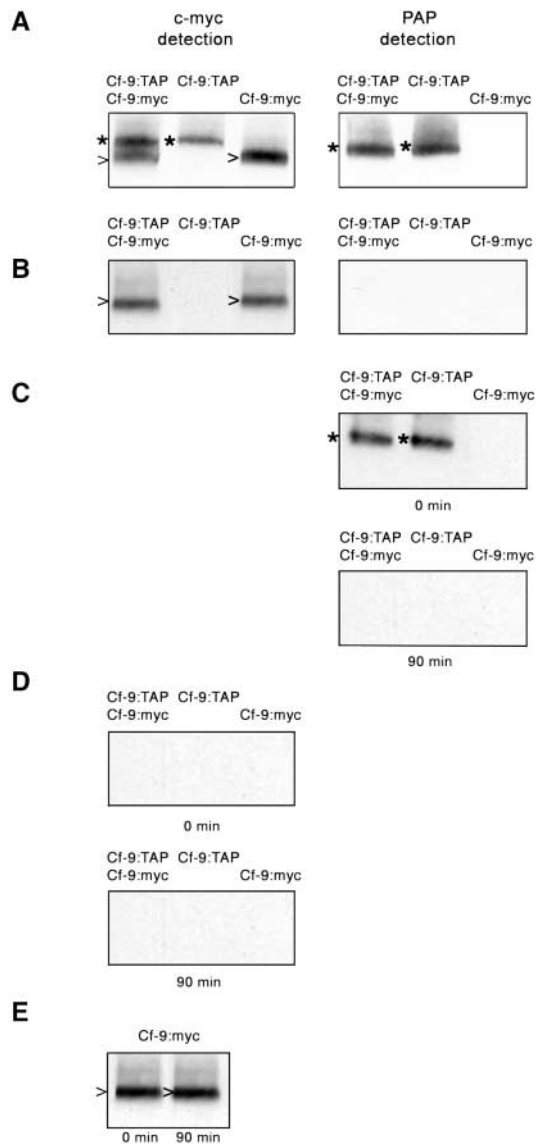
#### Confirmation of the Existence of an $\sim$ 420-kD Cf-9 Complex by Blue Native PAGE

In an independent approach to studying a Cf-9 protein complex, solubilized Cf-9 was analyzed by non-denaturing blue native PAGE (BN-PAGE). In this technique, Coomassie dyes and aminocaproic acid were introduced to induce a charge shift and improve the solubilization of membrane proteins. This methodology was developed for the isolation of membrane-associated protein complexes in an enzymatically active form (Schägger and von Jagow, 1991; Caliebe et al., 1997; Arnold et al., 1999). Solubilized Cf-9:myc microsomes from either plants or cell cultures were subjected to BN-PAGE (see Methods). To detect Cf-9:myc within potential protein complexes, single lanes of the BN gel were cut, mounted on a denaturing SDS gel in the second dimension, and subjected to immunoblot analysis using an anti-c-myc antibody. A strong cross-reacting band that, compared with protein standards from the BN-PAGE, corresponded to a molecular mass of  $\sim$ 420 kD, was detected (Figure 5A, top). No cross-reacting signal was detected at this position when microsomes from the untransformed tobacco line Petit Havana were subjected to the same experimental procedure (Figure 5A, bottom).

To confirm that the signal detected corresponded to a Cf-9-containing complex, before loading in BN-PAGE, microsomes were incubated with peptide-N-glycosidase F (PNGase F), a glycoamidase that liberates nearly all N-linked oligosaccharides from glycoproteins (Maley et al., 1989) and that has been used to demonstrate that Cf-9 is a glycoprotein (Piedras et al., 2000). PNGase F treatment of solubilized microsomes resulted in a shift of the cross-reacting band

and at right (SDS-PAGE). The positions of Cf-9:myc are indicated by arrows.

**(B)** *N. benthamiana* (*N. benth*) leaves were infiltrated or not with *A. tumefaciens* carrying 35S:Cf-9:TAP. After 2 days, microsomal proteins (0.5 mg) of infiltrated leaves (top and middle) and non-injected leaves (bottom) were solubilized as indicated in **(A)**. Cf-9:TAP preparations were treated or not with PNGase F (top and middle, respectively), and then BN-PAGE was conducted. Cf-9:TAP protein was detected by immunoblot analysis using the PAP antibody. The positions of Cf-9:TAP are indicated by arrows.



**Figure 6.** Stoichiometry of the Cf-9 Protein in the Cf-9 Complex.

*N. benthamiana* leaves were infiltrated with *A. tumefaciens* expressing Cf-9:TAP and Cf-9:myc, Cf-9:TAP, or Cf-9:myc. Leaves were harvested 2 days after infiltration, and microsomal proteins were prepared.

(A) Transient protein expression was analyzed by protein gel blotting with monoclonal anti-c-myc (left) and PAP antibodies (right).

(B) Protein extracts were subjected to immunoprecipitation with rabbit IgG-agarose beads, immunoprecipitates were collected by centrifugation, and the non-immunoprecipitated material was analyzed again by protein gel blotting with monoclonal anti-c-myc (left) and PAP antibodies (right).

(C) and (D) Immunoprecipitates were cleaved with the TEV protease, and aliquots of both IgG beads (C) and cleaved material (D) were immunoanalyzed with the PAP and the anti-c-myc antibody, respectively, before (0 min) and after (90 min) incubation with TEV enzyme.

(E) A control incubation of Cf-9:myc microsome with the TEV protease was also performed. Cf-9:myc before (0 min) and after (90 min)

from 420 to ~250 kD in BN-PAGE (and from 160 to ~105 kD in SDS-PAGE; Figure 4B). Assuming that ~55 kD of sugar is released from Cf-9 after glycosidase treatment, the extra 115 kD of sugar must be derived from other components of the complex. Alternatively, the complex might lose one or more components after glycosidase treatment.

Regarding gel filtration chromatography, identical results were obtained when transiently expressed Cf-9:TAP was used for the BN-PAGE analysis (Figure 5B). Therefore, by using two distinct, independent techniques, the Cf-9 protein was shown to constitute part of a membrane complex of ~420 kD.

### The Cf-9 Complex Does Not Contain Homomultimers of Cf-9

The ~420-kD molecular mass of the Cf-9 complex is consistent with more than one Cf-9 protein molecule present in the complex. To clearly establish the stoichiometry of the Cf-9 protein in the complex, both Cf-9:myc and Cf-9:TAP were expressed simultaneously in *N. benthamiana* using the *A. tumefaciens*-mediated transient assay. As a control, either Cf-9:myc or Cf-9:TAP was also expressed. Protein extracts were prepared, and expression of the proteins was checked by protein gel blot analysis. A single band of ~185 kD, corresponding to the expected size of Cf-9:TAP, was detected with the PAP antibody (Figure 6A, right). Two distinct bands of the expected sizes (~185 and ~160 kD for Cf-9:TAP and Cf-9:myc, respectively) were detected using the anti-c-myc antibody (Figure 6A, left), because the protein A moiety in the TAP tag is recognized by any antibody. After preparation of the extracts, proteins were subjected to immunoprecipitation with rabbit IgG-agarose beads, which are specifically aimed to immunoprecipitate TAP-tagged Cf-9.

After collecting the immunoprecipitates by centrifugation, supernatants were analyzed with anti-c-myc and PAP antibodies for the presence or absence of Cf-9:myc and Cf-9:TAP. No Cf-9:TAP band was detected with the PAP antibody in the supernatant (Figure 6B, right), demonstrating the effectiveness of the immunoprecipitation of the TAP-tagged protein. Likewise, no TAP-tagged Cf-9 was detected in the supernatant when using anti-c-myc, whereas the Cf-9:myc signal remained unaltered (Figure 6B, left), indicating no association between the TAP and the c-myc-tagged Cf-9 proteins and suggesting that only one Cf-9 molecule participates in the Cf-9 complex.

To obtain additional evidence for this finding, Cf-9:TAP was released from the IgG beads by incubation of the immunoprecipitates with the TEV protease. Protein gel blot

incubation with TEV was detected with a monoclonal anti-c-myc antibody.

\*, Cf-9:TAP; >, Cf-9:myc.

analysis of the IgG beads with the PAP antibody, before and after the TEV reaction, showed that Cf-9:TAP was cleaved completely from the beads, because no signal could be detected after TEV treatment (Figure 6C). The protein A moiety of the TAP tag could be detected in control experiments as an ~15-kD band that remained attached to the beads after TEV reaction (data not shown). Therefore, no signal corresponding to Cf-9 was detected when analyzing the cleaved material with the PAP antibody, because the protein A sequence was cleaved from the TAP tag (data not shown). Significantly, no Cf-9:myc signal was detected in the cleaved material either (Figure 6D). The possibility of protein degradation by the TEV enzyme was excluded in a parallel incubation of Cf-9:myc with TEV in which no effect of the protease on c-myc-tagged Cf-9 was detected after treatment (Figure 6E). Together, these results confirm that Cf-9:TAP and Cf-9:myc do not associate with each other and suggest that there is only one Cf-9 protein molecule in the Cf-9 complex.

#### **Cf-9 Is Not Disulfide Linked to Another Protein, and the Cf-9 Complex Does Not Change in Size or Recruit GTP Binding Proteins on Elicitation**

Cf proteins show a high structural similarity to Arabidopsis CLV2 (Figure 1). Furthermore, CLV2 was previously shown to form part of a 450-kD complex (Trotochaud et al., 1999), which is of similar size to the Cf-9 complex (~420 kD). Therefore, we investigated whether the Cf-9 protein complex initiating defense responses shares additional characteristics with that formed by CLV1 and CLV2. Conserved Cys pairs, immediately before and after the LRRs (Jones and Jones, 1997) (Figure 1, domains B and D), have been proposed to be involved in the formation of a disulfide-linked heterodimer between CLV1 and CLV2 (Jeong et al., 1999).

We tested the possibility that Cf-9 was associated with other proteins in the membrane by covalent disulfide bonds between Cys residues. Solubilized extracts from either cell cultures or leaves expressing Cf-9:myc were boiled in SDS-PAGE loading buffer in the presence or absence of the reducing agent  $\beta$ -mercaptoethanol. Samples then were analyzed by SDS-PAGE and immunoblotting with an anti-c-myc antibody. Figure 7A shows the protein gel blot obtained for the Cf-9:mycB and Cf-9:mycG expressed in tobacco plants. No difference in the molecular mass of the protein based on treatment was found (Figure 7A). The same result also was obtained consistently when either leaf material was used (regardless of the position of the c-myc tag) or Cf-9:myc and Cf-9:TAP were expressed transiently in *N. benthamiana*. This result indicates that the Cf-9 protein does not form disulfide-linked heterodimers in the membrane.

CLV1 (a receptor-like kinase) is proposed to be present as an inactive disulfide-linked heterodimer with CLV2, and CLV3 (a small, secreted peptide) functions to promote the

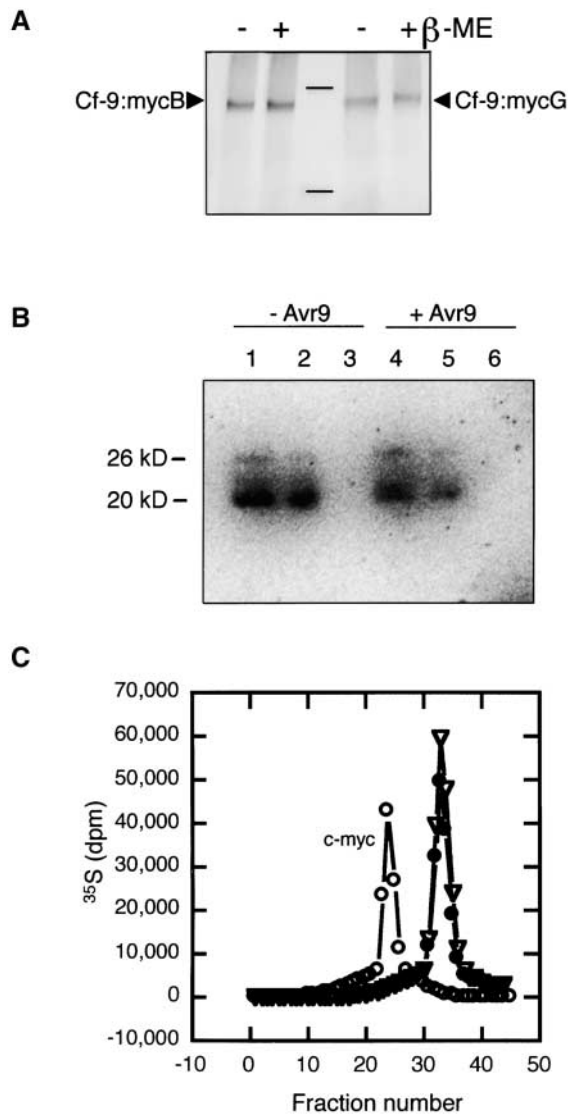
assembly of the active 450-kD complex (Trotochaud et al., 1999, 2000). To determine if the Cf-9 protein functions similarly, we investigated whether treatment with Avr9 induces changes in the gel filtration profile of the Cf-9-containing complexes. Cf-9:myc tobacco cell cultures were treated with the Avr9 peptide and harvested 20 min after elicitation. Microsomes were prepared, solubilized with OG, and subjected to gel filtration analysis. However, no change in the elution profile of the Cf-9 protein after Avr9 treatment was observed (Figure 4A), which is a second major difference between CLV and Cf-9 complexes.

Rop-like proteins identified in plants belong to the Rho protein subfamily of small GTPases (Li et al., 1998). Rop proteins were found to be recruited by the active CLV complex (Trotochaud et al., 1999). Therefore, we tested for the presence of small GTPases in the Cf-9 complex using the GTP analog  $\gamma$ -<sup>35</sup>S-GTP. Microsomes were prepared from cell cultures before and after elicitation with the corresponding Avr9 protein, solubilized, and immunoprecipitated with an anti-c-myc antibody. The presence of ~20- and ~26-kD Rho-GTPase-related proteins was detected in total extracts by SDS-PAGE analysis and autoradiography (Figure 7B, lanes 1 and 4), but no radioactivity was found in the immunoprecipitated material (Figure 7B, lanes 3 and 6). This observation suggests that small GTP binding proteins and Cf-9 are not likely to be associated, even after elicitation. In addition, solubilized Cf-9:myc microsomes were incubated with  $\gamma$ -<sup>35</sup>S-GTP and subjected to gel filtration chromatography. Fractions were collected, and incorporated radioactivity was determined. No radioactivity coeluted with Cf-9 in either the unelicited or the elicited state (Figure 7C). Identical results were obtained using wild-type Cf-9-expressing plants and cell cultures before and after elicitation with Avr9 (data not shown). This finding suggests that the ~420-kD Cf-9 complex does not contain small GTP binding proteins, either before or after elicitation.

Together, our data lead us to conclude that, despite the structural similarities between Cf-type proteins and CLV2, different molecular mechanisms probably govern CLV2 function in Arabidopsis and the Cf-9-mediated response to the Avr9 peptide in tomato and tobacco.

## **DISCUSSION**

In a previous study, heterologous expression of functional c-myc-tagged Cf-9 in tobacco allowed the preliminary biochemical characterization of Cf-9 (Piedras et al., 2000). However, the molecular mechanism of Cf-9-dependent Avr9 perception and subsequent defense signaling remained obscure. Advances in understanding require the development of additional protein biochemistry tools. Therefore, we engineered a new Cf-9:TAP construct (Rigaut et al., 1999) tagged at the C terminus of the mature protein, after the conserved KKRY domain, which was previously reported to



**Figure 7.** Characterization of the Cf-9:myc Complex.

**(A)** Disulfide linkage analysis of Cf-9:mycB and Cf-9:mycG. Solubilized microsomal fractions from SLJ9171 homozygous tobacco plants (Cf-9:mycB; lanes 1 and 2) and SLJ9161 homozygous tobacco plants (Cf-9:mycG; lanes 3 and 4) were boiled in sample buffer in the presence (+) or absence (-) of  $\beta$ -mercaptoethanol ( $\beta$ -ME). Proteins were analyzed subsequently by SDS-PAGE and immunoblotting using an anti-myc antibody. Positions of Cf-9:myc are indicated by arrowheads.

**(B)** Cf-9:myc cell cultures elicited (+) or not (-) with 15 nM synthetic Avr9 were immunoprecipitated with the anti-myc antibody. Aliquots of the incubation mixtures (lanes 1 and 4), non-immunoprecipitated proteins (lanes 2 and 5), and immunoprecipitated proteins (lanes 3 and 6) were analyzed by SDS-PAGE and blotted onto nitrocellulose. The membrane then was incubated with the GTP analog  $\gamma$ - $^{35}\text{S}$ -GTP and autoradiographed.

**(C)** Elution profile of gel filtration analysis of Cf-9:myc and small, GTP binding proteins. Total extracts of Cf-9:myc cell cultures har-

confer ER localization of Cf-9 (Bengezhal et al., 2000). In mammals and yeast, this cytosolic dilysine motif (KKXX) promotes the retrieval of type I membrane proteins from the Golgi apparatus to the ER, involving the coat protein I complex (Jackson et al., 1990; Cosson and Letourneur, 1994; Cosson et al., 1996). Here, we established an *A. tumefaciens*-mediated transient assay for the expression of functional Cf-9-tagged proteins and thus complemented the previously established Cf-9:mycB and Cf-9:mycG transgenic tobacco plants and cell cultures (Piedras et al., 2000). Using this newly developed transient assay, we showed that the TAP-tagged Cf-9 protein is functional in vivo (Figure 3A). This result is consistent with the observation made by Van der Hooft et al. (2001b) that the conserved C-terminal dilysine motif is not required for Cf-9 function and provides an additional argument against the ER location of functional Cf-9.

Significantly, the transient assay provided validation of the use of the 35S promoter in our constructs because consistent correlation was found between the protein expression levels and the observed Cf-9/Avr9-dependent hypersensitive response (Figure 3). These results provide strong evidence against the idea that the use of the 35S promoter might saturate the cellular ER retrieval-retention mechanism.

We used both the tobacco lines expressing Cf-9:myc and the transiently expressed Cf-9:myc and Cf-9:TAP proteins to test the hypothesis that the Cf-9 gene product participates in a membrane-associated protein complex. Detergents provide a means of disrupting the integrity of biological membranes and thus allow the characterization of membrane proteins. Proteins may be solubilized in a native-like state with retention of functional properties, provided that solubilization occurs in a gentle manner. Mild detergents often are unable to dissociate the protein-protein interactions that keep protein complexes together. The complexes therefore are solubilized, delipidated, and kept in solution as units. This often is accomplished by the use of nonionic detergents with polyoxyethylene (TX-100 or Nonidet P-40) or sugar head groups (OG) (Helenius et al., 1979). Gel filtration chromatography in the presence of TX-100 was used previously to reveal the CLV complex from *Arabidopsis* (Trotochaud et al., 1999).

In contrast, we showed here that the Cf-9 protein solubilized with either TX-100 or Nonidet P-40 probably forms nonspecific aggregates of high molecular mass (750 to 1000 kD) (Figure 4A), because this molecular mass is highly unlikely to be caused by specific protein-protein interactions

vested before (closed circles) or after (open inverted triangles) elicitation with 50 ng/mL synthetic Avr9 were incubated with the GTP analog  $\gamma$ - $^{35}\text{S}$ -GTP and subjected to gel filtration. Eluted fractions were collected and analyzed for the presence of Cf-9:myc by immunoblotting (open circles) and for the incorporation of  $\gamma$ - $^{35}\text{S}$ -GTP by scintillation counting before and after elicitation (closed circles and open inverted triangles, respectively).

and may reflect the aggregation of several Cf-9 complexes together. Artifactual aggregation of proteins in the presence of these detergents has been reported previously (Simons et al., 1973). This interpretation is consistent with previous data, because solubilization of Cf-9 microsomes with Nonidet P-40 has been reported to disturb the binding of Avr9 to the high-affinity binding site detected in tobacco by Kooman-Gersmann et al. (1996) (Luderer et al., 2001).

The micelle size of the detergent is a very important factor in the analysis of protein complexes by gel filtration. In the presence of excess detergent, most or all of the lipid around membrane proteins is exchanged for detergent, resulting in the formation of soluble protein-detergent complexes and mixed lipid/detergent micelles. Separation of different proteins according to size therefore will be easier in the presence of a detergent with a smaller micelle size. The micelle size of TX-100 and Nonidet P-40 is 90,000 D, whereas OG and CHAPS form smaller micelles of 8,000 and 6,150 D, respectively. Accordingly, gel filtration of solubilized Cf microsomes with either OG or CHAPS shifted the elution of Cf-9-containing fractions to a molecular size of 350 to 475 kD (Figure 4A).

In addition to the micelle size, the mode of interaction and the amount of bound detergent are important factors that contribute significantly to the shape and the molecular mass of the detergent-solubilized membrane protein complex (Møller and le Marie, 1993). Previous studies on the binding of various detergents to different integral membrane proteins showed that the number of detergent molecules bound to such proteins ranged between 75 and 300, depending on the detergent and the protein of choice (Møller and le Marie, 1993). Because the molecular mass of an OG monomer is 292.4 D, even in the extreme case of 300 molecules of OG bound to Cf-9 the contribution of the detergent to the molecular size of the complex would be just 88 kD. However, because the Cf-9 protein is a type I membrane protein, with just a short stretch of the protein structure forming a single transmembrane domain, it is very unlikely that OG interaction with Cf-9 makes a large contribution to the size of the Cf-9 complex. In conclusion, unlike with TX-100 or Nonidet P-40, the molecular mass of 350 to 475 kD observed with both OG and CHAPS most likely reflects the specific association of Cf-9 gene products with other proteins and/or with themselves.

We provided independent confirmation of the presence of the Cf-9 gene product in an ~420-kD protein complex by means of BN-PAGE (Figure 5). This technique avoids the problem of detergent interference by introducing the use of Coomassie blue dyes and aminocaproic acid (Schägger and von Jagow, 1991). The specificity of the signal detected was demonstrated by both the shift of the cross-reacting signal after endoglucanase treatment and the lack of signal when using microsomes from untransformed tobacco.

To clearly establish the stoichiometry of the Cf-9 protein in the complex, coimmunoprecipitation experiments with Cf-9 using two independent tags, c-myc and TAP, were per-

formed. Interestingly, our data are inconsistent with the presence of more than one Cf-9 protein molecule in the Cf-9 complex (Figure 6), indicating that additional protein partners participate with Cf-9 in initiating defense signaling. It also could be argued that the complex is dissociated after immunoprecipitation. However, we consider this unlikely, because the complex size is unaltered after using different extraction procedures, or extraction buffers with different ionic strengths (50 to 500 mM NaCl), pH values (6.5 to 8.5), or detergent used for solubilization (OG [nonionic] or CHAPS [zwitterionic]). Therefore, it is difficult to imagine that immunoprecipitation in the presence of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 40 mM OG could be more disruptive to the stability of the complex than all of the assayed conditions. Because glycosidase treatment shifts the mass of the complex from ~420 to ~250 kD, additional partners must include at least one glycoprotein and thus at least one other protein with an extracellular domain. Alternatively, the complex might lose one or more components after glycosidase treatment.

Cf proteins and CLV2 are highly similar in structure, and triggering the appropriate responses requires the mediation of a small, secreted peptide in both cases. Furthermore, the molecular mass of the Cf-9 complex (~420 kD) is very similar to that reported for the CLV complex (450 kD) (Trotochaud et al., 1999). Therefore, we investigated whether the Cf-9/Avr9 recognition event might be explained by the formation of a CLV-like complex in the membrane. In this article, three major points were addressed to test this hypothesis. First, like the large majority of plant receptor-like proteins with predicted extracellular LRRs, Cf and CLV2 gene products contain a pair of conservatively spaced Cys residues both immediately before and after the LRRs (Jones and Jones, 1997) (Figure 1, domains B and D). These Cys pairs have been proposed to be involved in the formation of a disulfide-linked heterodimer between CLV1 and CLV2 (Jeong et al., 1999). In contrast, we found that the Cf-9 protein is not associated with other proteins by disulfide bonds between Cys residues (Figure 7A). This observation may be associated with the absence of one Cys residue in the Cys pair immediately after the LRRs in the Cf-9 protein compared with CLV2 (Figure 1, domain D). However, we cannot exclude structural differences between the two proteins to explain this result or an interaction with a different kind of protein.

Second, it has been proposed that CLV3 acts as the ligand to promote the assembly of the active 450-kD complex from the inactive disulfide-linked heterodimer CLV1-CLV2 (Fletcher et al., 1999; Trotochaud et al., 1999). However, no Avr9-dependent change in the elution pattern of the Cf-9 protein was detected upon elicitation (Figure 4). It might be argued that this is the result of (1) the failure to find the appropriate experimental conditions to detect such changes, and/or (2) the fact that the identified Cf-9 complex of ~420 kD has no role in Avr9 perception and the induction of defense. However, because the addition of the Avr9 peptide still induced active oxygen species accumulation and

mitogen-activated protein kinase activation in parallel control experiments (data not shown), we consider that at least three other more likely reasons can be presented. (1) The gel filtration technique may not be sensitive enough to differentiate between close molecular masses. Therefore, the mass of the protein(s) recruited to the Cf-9 complex upon elicitation might be below the threshold of differentiation by gel filtration. In this situation, we would not expect to detect any difference in the elution profile from the size fractionation column between the unelicited and the elicited state. (2) The triggering of signaling events leading to defense might depend on a conformational change of one or more proteins forming part of the Cf-9 complex rather than a change in its precise composition. This conformational change might activate downstream signaling components, with no recruitment of additional proteins required to trigger defense. (3) A transient association of Cf-9 with its protein partners would make it more difficult to detect such an interaction.

Finally, a Rop protein was found to form part of the active 450-kD CLV complex (Trotochaud et al., 1999). We tested whether members of this plant protein subfamily of small GTP binding proteins were recruited to the Cf-9 complex upon elicitation. If this was the case, the low molecular mass (~20 to 25 kD) of Rop-like proteins might explain the absence of a detectable shift in the elution profile of Cf-9 by gel filtration. Rop-like proteins were detected in total plant and cell culture extracts, but no association with the Cf-9 protein could be found by either immunoprecipitation or gel filtration analysis (Figures 7B and 7C), strongly suggesting that the recruitment of GTP binding proteins to the Cf-9 complex does not take place *in vivo* on elicitation by Avr9.

Further verification of the significance of our data was obtained when similar experiments were conducted with the tomato Cf-4 protein, which is highly homologous with Cf-9 and responds specifically to the *C. fulvum*-encoded avirulence determinant Avr4 (Thomas et al., 1997; Wulff et al., 2001). Significantly, we identified an ~400-kD heteromultimeric Cf-4 membrane-associated complex with identical characteristics to the Cf-9 complex (Rivas et al., 2002). Therefore, the Cf-4 data provide independent confirmation that members of the Cf family of R proteins are present in the type of complex described in this study. This is particularly significant in light of the widespread discussion of the "guard" hypothesis (Dixon et al., 2000; Dangl and Jones, 2001). For example, different Cf proteins could guard different pathogen targets, possibly by constitutive association with these host components. We believe that the apparent size similarity between the Cf-9 and Cf-4 protein complexes has profound implications for the mechanism of action of these proteins.

In summary, we have shown by two independent experimental approaches that the epitope-tagged *R* gene products Cf-9:myc and Cf-9:TAP are present in an ~420-kD membrane-associated complex. Despite the high structural similarity between CLV2 and Cf proteins, the nature of the Cf-9 complex appears to be different from that of the CLV

complex, strongly suggesting a major difference between the molecular mechanisms of regulation of meristem development in *Arabidopsis* and resistance to *C. fulvum* in tomato. Because we showed that only one Cf-9 protein molecule participates in the Cf-9 complex, future work needs to focus on the identification of a protein partner(s) of the Cf-9 gene product in the membrane and their role in Avr9 perception. At this stage, we can only speculate about the biological function of the Cf-9 complex. Conceivably, it contains transmembrane or associated protein kinases or a calcium channel that is gated by the action of Avr9 on Cf-9. In the future, it may be helpful to investigate how multiple loss-of-function mutants affect the Cf-9 complex. The Cf-9:myc tobacco lines and the transient expression assays described here have proved to be excellent tools to facilitate the investigation of the protein(s) associated with Cf-9 to achieve disease resistance.

## METHODS

### Generation of the Cf-9:TAP Construct

The TAP sequence was inserted at the 3' end of the Cf-9 gene as an overlapping polymerase chain reaction (PCR) product. Three individual PCRs, rendering fragments A, B, and C (Figure 2), were conducted before the chimeric reaction: (A) primers 1 (5'-GTGACAACTCCAGCTGAGCTAGATCAA-3') and 2 (5'-ATATCTTTTCTGTGCTTTTCATTTT-3') were used to amplify the 3' end of the Cf genes using p129P6A-5 (Thomas et al., 1997) as a template; (B) the TAP sequence was amplified from pBS1479 (<http://www.embl-heidelberg.de/ExternalInfo/seraphin/TAP.html>) using primers 3 (5'-AAAATGAAAAGCACAAAGAAAAGATATATGGAAAAGAGAAGATGGAAAA-3') and 4 (5'-CTGGAGGTATAGCTACTCACTATCAGGTTGACTTCCCGC-3'); and (C) primers 5 (5'-ATAGTGAGTAGCTATACCTCCAG-3') and 6 (5'-GTAAAACGACGCCAGT-3') were used to amplify the 3' untranslated sequence of Cf-9 using p129P6A-5 as a template (Thomas et al., 1997).

The three PCR products were purified and used in an overlapping PCR with primers 1 and 6. The 1.3-kb chimeric PCR product was purified and digested with *PvuII* and *BamHI*. The 5' end of Cf-9 was obtained from *Clal*-*PvuII* digestion of SLJ9161 (Piedras et al., 2000) and ligated to the 3' end containing the TAP sequence into a *Clal*-*BamHI*-digested pBluescript SK- vector, rendering SLJ13950. The *Clal*-*BamHI* fragment from SLJ13950 was ligated with either the *EcoRI*-*Clal* fragment from SLJ4K1, containing the 35S promoter (Jones et al., 1992), or the *XbaI*-*Clal* fragment from p129P6A-8, containing the native Cf-9 promoter (Thomas et al., 1997), into a pBin19 vector (Frisch et al., 1995). Therefore, the resulting plasmids, SLJ14070 and SLJ14190, contained the TAP-tagged Cf-9 gene under the control of the 35S or the native Cf-9 promoter, respectively.

### Transient Expression of Proteins in *Nicotiana benthamiana*

Overnight bacterial cultures of *Agrobacterium tumefaciens* strain GV3101 expressing the protein of interest were harvested by centrifugation. Cells were resuspended in induction buffer (10 mM MgCl<sub>2</sub>,

10 mM Mes, pH 5.6, and 150  $\mu$ M acetosyringone) to an OD of 0.1 unless indicated otherwise. After 2 hr at 22°C, cells were infiltrated into leaves of 4-week-old *N. benthamiana* plants. Two days after *A. tumefaciens* infiltration, leaf discs used for experiments were harvested, frozen immediately in liquid nitrogen, and stored at -70°C.

### Elicitation of Suspension Cultures

For elicitation, cell cultures expressing Cf-9:myc (Piedras et al., 2000) were challenged with synthetic Avr9 (Piedras et al., 1998) at a concentration of 15 nM. At the time indicated in the text, cells were harvested by filtration, frozen in liquid nitrogen, and stored at -70°C.

### Preparation of Protein Extracts

Both transgenic tobacco (*Nicotiana tabacum*) plants and cell suspension cultures expressing Cf-9:myc and transiently expressed Cf-9:myc and Cf-9:TAP were used as Cf-9 protein sources. Leaf or cell samples were ground in liquid nitrogen, thawed in 2 volumes of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride, 2  $\mu$ g/mL antipain, 2  $\mu$ g/mL leupeptin, and 2  $\mu$ g/mL aprotinin), filtered through two layers of Miracloth, and centrifuged at 1000g for 10 min at 4°C. Alternatively, fresh leaves or cells were ground in extraction buffer using a pre-chilled blender. The supernatant was ultracentrifuged subsequently at 100,000g for 1 hr at 4°C, and the microsomal membranes in the pellet were resuspended in solubilization buffer (extraction buffer supplemented with different detergents (0.1% Triton X-100 [TX-100], 0.1% Nonidet P-40, 40 mM octylglucoside [OG], or 0.5% 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid [CHAPS]) and ultracentrifuged at 100,000g for 1 hr at 4°C. The supernatant was stored at -70°C in aliquots. The protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Chester, UK) using BSA as a standard.

### Cf-9 Homomultimerization Assays

Cf-9:myc and Cf-9:TAP, either separately or together, were expressed transiently in *N. benthamiana* leaves as described above. Leaf discs (1 cm diameter) were harvested 2 days after inoculation, and microsomal extracts were prepared as described above. Immunoprecipitation of the microsomal preparations was performed as described by Luderer et al. (2001) with the exception that rabbit IgG-agarose beads (Sigma) were used instead of anti-c-myc antibodies. Cleavage at the Tobacco Etch Virus (TEV) site was performed as described (<http://www.embl-heidelberg.de/ExternalInfo/seraphin/TAP.html>). Aliquots were taken at every step and analyzed by protein gel blotting with either the peroxidase-antiperoxidase (PAP) or the anti-myc antibody.

### Disulfide Bond Detection

Protein extracts from both Cf-9:mycB-expressing and Cf-9:mycG-expressing tobacco plants and cell cultures were prepared. Two times loading buffer (20% glycerol, 4% SDS, 30 mM Tris-HCl, pH 6.8, and 1% bromophenol blue), either supplemented or not with 2% (v/v)  $\beta$ -mercaptoethanol, was added to an equal volume of protein extract. The samples were boiled for 5 min and separated by SDS-

PAGE as described above. The proteins were transferred to nitrocellulose, and the membrane was probed with the anti-myc antibody.

### Gel Filtration Analysis

Gel filtration was performed using a fast protein liquid chromatography system (Pharmacia) with High Prep 26/60 Sephacryl S-300 high-resolution columns (Pharmacia). All gel filtration assays were performed at 4°C. Column equilibration and chromatography were performed in solubilization buffer (see above). Fractions were collected every 2 mL and probed with the c-myc antibody by either protein gel blot or dot blot analysis.

### SDS-PAGE and Immunoblotting

Proteins were separated on a 7.5% SDS gel (Laemmli, 1970) and transferred onto nitrocellulose (Amersham Pharmacia Biotech, Little Chalfont, UK) by wet electroblotting (Mini-Protean II system; Bio-Rad, Hemel Hempstead, UK). For detection of Cf-9:myc, the blots were incubated with c-myc polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a final dilution of 1:2000 for 1 hr, and subsequently, antigen-antibody complexes were visualized using alkaline phosphatase-conjugated goat anti-rabbit IgG under standard conditions. For Cf-9:TAP detection, rabbit PAP soluble complex (Sigma) was used at a final dilution of 1:5000 for 1 hr. Bands were visualized using the ECL Plus kit (Amersham Pharmacia Biotech) under standard conditions.

### Blue Native Gel Electrophoresis

Cf-9:myc and Cf-9:TAP microsomes from stable plants or cell cultures, or from transiently infiltrated leaves, were solubilized in solubilization buffer supplemented with 750 mM aminocaproic acid and 50 mM bis-Tris, pH 7.0. Aliquots of the supernatants were either treated with peptide:N-glycosidase F (PNGase F) (see above) or incubated directly with 5% Coomassie Brilliant Blue G 250. Blue native PAGE then was performed following the method of Schagger and von Jagow (1991) on a 4.5 to 10.5% gradient polyacrylamide gel (12 mm  $\times$  180 mm  $\times$  2 mm). Marker proteins ( $\beta$ -amylase [200 kD], apoferritin [443 kD], urease [540 kD], and thyroglobulin [669 kD]) were run alongside Cf samples. Stripes of lanes corresponding to molecular masses between 150 and 800 kD were cut and mounted on a 7.5% denaturing SDS gel as a second dimension. Finally, Cf-9:myc and Cf-9:TAP were detected by immunoblot analysis of the SDS gel using an anti-myc or PAP antibody as described above.

### Deglycosylation Assays

Cf-9:myc-containing microsomes were solubilized and treated with PNGase F as described previously (Piedras et al., 2000).

### Immunoprecipitations

Immunoprecipitation with the anti-c-myc antibodies was performed as described previously (Luderer et al., 2001). Cf-9:TAP was immunoprecipitated as described (<http://www.embl-heidelberg.de/ExternalInfo/seraphin/TAP.html>).

### $\gamma$ -<sup>35</sup>S-GTP Overlays

Microsomal preparations were obtained from c-myc-tagged Cf-9-expressing tobacco plants and cell cultures. The presence of Rop-like proteins in Cf microsomes was tested first as described by Drøbak et al. (1995). Briefly, Cf-9:myc microsomes were immunoprecipitated with the anti-myc antibody. Aliquots of the total extracts, nonimmunoprecipitated proteins, and immunoprecipitated proteins were separated by SDS-PAGE and transferred onto nitrocellulose. For  $\gamma$ -<sup>35</sup>S-GTP overlay assays, membranes were washed with three changes of buffer B (50 mM Tris-HCl, pH 7.5, 12  $\mu$ M MgCl<sub>2</sub>, 1 mM DTT, and 0.3% [v/v] Tween-20) and incubated with  $\gamma$ -<sup>35</sup>S-GTP (75 kBq/mL) and 10  $\mu$ M ATP for 30 min at room temperature under gentle agitation. After three washes with buffer B, the membranes were air dried and radioactive bands were visualized by autoradiography.

### Detection of Rop-Like Proteins by Gel Filtration

Microsomal preparations from tobacco plants and cell cultures containing Cf-9:myc were incubated with 1 mM MgCl<sub>2</sub>, 10  $\mu$ Ci of  $\gamma$ -<sup>35</sup>S-GTP, and 10  $\mu$ M ATP for 1 hr at room temperature. The incubation mixture was loaded on the gel filtration column (see above), and 2-mL fractions were collected. Radioactivity was determined after the addition of 3 mL of scintillation cocktail (OptiPhase HiSafe 2; Wallac, Perkin-Elmer Life Sciences Ltd., Cambridge, UK) in a Wallac 1410 liquid scintillation counter.

### Accession Number

The GenBank accession number for CLV2 is AF177672.

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