

An ~400 kDa membrane-associated complex that contains one molecule of the resistance protein Cf-4

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Summary

Despite sharing more than 91% sequence identity, the tomato Cf-4 and Cf-9 proteins discriminate between two *Cladosporium*-encoded avirulence determinants, Avr4 and Avr9. Comparative studies between Cf-4 and Cf-9 are thus of particular interest. To investigate Cf-4 protein function in initiating defence signalling, we established transgenic tobacco lines and derived cell suspension cultures expressing c-myc-tagged Cf-4. Cf-4:myc encodes a membrane-localized glycoprotein of approximately 145 kDa, which confers recognition of Avr4. Elicitation of Cf-4:myc and Cf-9:myc tobacco cell cultures with Avr4 and Avr9, respectively, triggered the synthesis of active oxygen species and MAP kinase activation. Additionally, an *Agrobacterium*-mediated transient assay was used to express Cf-4:myc and a newly engineered fusion protein Cf-4:TAP. Both transiently expressed proteins were found to be functional in an *in vivo* assay, conferring a hypersensitive response (HR) to Avr4. Consistent with previous observations that Cf-9 is present in a protein complex, gel filtration analysis of microsomal fractions solubilized with octylglucoside revealed that epitope-tagged Cf-4 proteins migrated at a molecular mass of 350–475 kDa. Using blue native gel electrophoresis, the molecular size was confirmed to be approximately 400 kDa. Significantly, this complex appeared to contain only one Cf-4 molecule, supporting the idea that, as previously described for Cf-9, additional glycoprotein partners participate with Cf-4 in the perception of the Avr4 protein. Intriguingly, Cf proteins and Clavata2 (CLV2) of *Arabidopsis* are highly similar in structure, and the molecular mass of Cf-4 and CLV complexes is also very similar (400 and 450 kDa, respectively). However, extensive characterization of the Cf-4 complex revealed essentially identical characteristics to the Cf-9 complex and significant differences from the CLV2 complex.

Keywords: resistance gene, protein complex, glycoprotein, *Cladosporium fulvum*, tomato, plant defence

Introduction

In plants, specific recognition of invading pathogens is frequently mediated by resistance (*R*) genes. Surprisingly, *R* genes that confer resistance to different types of pathogens, with distinct colonization strategies, encode very similar proteins with a limited number of motifs, suggesting that the mechanisms controlling pathogen recognition and initiation of signal transduction are broadly conserved. The different combinations of these motifs define five major structural classes of *R* proteins (Dangl and Jones, 2001). The vast majority of *R* genes encode cytoplasmic proteins containing a nucleotide-binding site (NB) and leucine-rich repeats (LRR). Members of this NB-LRR class have been cloned from

various plant species and confer resistance against bacterial, fungal, aphid, oomycete, nematode and viral pathogens (Dangl and Jones, 2001).

There are four other classes of *R* genes. *Pto* from tomato confers resistance to *Pseudomonas syringae* strains carrying *avrPto*, and encodes a functional cytoplasmic serine/threonine protein kinase with no LRRs (Martin *et al.*, 1993), which may trigger a phosphorylation cascade upon interaction with AvrPto (Scofield *et al.*, 1996; Tang *et al.*, 1996). An NB-LRR protein, Prf, is required for *Pto* function (Salmeron *et al.*, 1996). *Xa21* from rice, which confers resistance to a range of *Xanthomonas oryzae* pv *oryzae* strains, represents a third class of *R* genes and encodes a

transmembrane protein, with predicted extra-cytoplasmic LRRs and a functional cytoplasmic serine/threonine protein kinase domain (Song *et al.*, 1995). *RPW8* from *Arabidopsis* encodes a small, probable transmembrane protein with a coiled-coil domain and essentially no homology to known proteins (Xiao *et al.*, 2001). Finally, the tomato *Cf* genes confer resistance to infection by the biotrophic leaf mould pathogen *Cladosporium fulvum* expressing the matching avirulence determinants (Avr), and encode type I transmembrane proteins with extracellular LRRs and a short cytoplasmic region with no similarity to known signalling domains.

When expressed in tomato or tobacco, Cf-4 and Cf-9 induce a hypersensitive response (HR), dependent on the presence of the cognate Avr protein (Hammond-Kosack and Jones, 1997). A comparative analysis between Cf-9 and Cf-4 is thus of particular interest because they discriminate between two *Cladosporium*-encoded avirulence determinants. Both Cf-4 and Cf-9 share more than 91% sequence identity and are distinguished by sequences in their N-terminal domains A and B, their N-terminal LRRs in domain C1 and their LRR copy number (25 and 27 LRRs, respectively). In contrast, the Cf-9 and Cf-4 C-termini are highly conserved, with the last 351 amino acids being identical (Thomas *et al.*, 1997). Sequence variation within the central LRRs of domain C1 and variation in LRR copy number play a major role in determining recognition specificity in Cf-9 and Cf-4 (Van der Hoorn *et al.*, 2001a; Wulff *et al.*, 2001).

In contrast to the overall similarities among isolated R genes, sequence analysis of bacterial and fungal Avr genes revealed few similarities and few clues to their functions for the pathogen. In particular, Avr4 shows no sequence homology to Avr9 or to other known proteins (Joosten *et al.*, 1994; Van Kan *et al.*, 1991). Both genes encode cysteine-rich pre-pro-proteins that are processed by fungal and plant proteases to yield cystine knot peptides of 86–88 amino acids (Avr4; Joosten *et al.*, 1997), and 28 amino acids (Avr9; Van der Ackerveken *et al.*, 1993; Van der Hooven *et al.*, 2001).

At present, elucidation of the role of LRR-containing R proteins in pathogen recognition and initiation of defence signalling is the subject of active research. Annotation has revealed approximately 170 LRR receptor-like kinases in the *Arabidopsis* genome. Some, such as FLS2, are implicated in defence (Gómez-Gómez and Boller, 2000). Others play a role in various plant developmental processes. For instance, ERECTA is required for proper organ elongation, Clavata1 (CLV1) determines cell fate in shoot and floral meristems, and BRI1 encodes a putative brassinosteroid receptor (Clark *et al.*, 1997; Li and Chory, 1997; Torii *et al.*, 1996). Intriguingly, the CLV2 protein, which associates with CLV1, shares high structural similarity with *Cf* gene products, in that it carries extracellular LRRs and a

transmembrane region, but no obvious signalling domain (Jeong *et al.*, 1999; Rivas *et al.*, 2002; Thomas *et al.*, 1997). Additionally, small extracellular peptide ligands (CLV3 and Avr9, respectively) are required in both cases to trigger the appropriate responses, and it has been shown that both CLV1/CLV2 and Cf-9 participate in membrane-associated protein complexes of similar size (approximately 450 and 420 kDa, respectively) (Rivas *et al.*, 2002; Trotochaud *et al.*, 1999). However, extensive analysis of the Cf-9 complex revealed significant differences between the CLV1/CLV2 and the Cf-9 complexes (Rivas *et al.*, 2002). Therefore, Cf-9-dependent defence signalling and CLV1/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.

We set out to investigate whether the type of protein complex previously described for Cf-9 constitutes an example of a more general case for other members of the Cf family of R proteins, in particular Cf-4. Despite the identification of a high-affinity binding site (HABS) for Avr9 in plasma membranes of solanaceous plants (Kooman-Gersmann *et al.*, 1996), the molecular mechanism of Avr perception remains unclear (Dixon *et al.*, 2000). Various models have been proposed to address this question (Joosten and De Wit, 1999). Among them, the 'guard' hypothesis (Dangl and Jones, 2001; Dixon *et al.*, 2000) predicts that the HABS is the pathogenicity target of Avr9. In this model, Cf-9 would 'guard' the Avr9 HABS, which is also found in plants that do not express Cf-9, and detect the association between the HABS and Avr9. According to this model, Cf-9 and Cf-4 might 'guard' different pathogen targets and Avr4 might thus interact with different 'guarded' proteins. Therefore, one might expect that Cf-9 and Cf-4 could participate in different types of complexes. We set out to investigate this possibility.

A better understanding of the molecular basis of recognitional specificity in *Cf* genes requires the generation of additional protein biochemistry tools. In this paper, we describe the generation of Cf-4:myc tobacco plants and cell cultures that retain recognition specificity and functionality towards Avr4. This enables studies on Cf-4 protein function in Avr4 perception and signal transmission, complementing the SLJ9161 and SLJ9171 lines that express Cf-9:myc (Piedras *et al.*, 2000). In addition, we describe a new TAP-tagged Cf-4 construct. In an earlier study, the TAP sequence (Tandem Affinity Purification) was used to epitope-tag the Cf-9 gene, and, using an *Agrobacterium*-mediated transient assay for expression of functional tagged Cf-9 proteins in *Nicotiana benthamiana* leaves, facilitated additional characterization of the Cf-9 complex (Rivas *et al.*, 2002). Using the c-myc-tagged Cf-4 plants and cell cultures and the transiently expressed Cf-4:myc and Cf-4:TAP, we showed, by both gel filtration and blue native non-denaturing gel electrophoresis, that the

Cf-4 protein is part of a protein complex with a molecular mass of approximately 400 kDa. Significantly, this complex appeared to contain only one Cf-4 molecule, supporting the idea that, as previously described for Cf-9, additional protein partners participate with Cf-4 in the perception of the Avr4 protein. Finally, we showed that the Cf-4 complex, being essentially identical to the Cf-9 complex, appears to have different characteristics compared to that proposed for CLV function in *Arabidopsis* development. In conclusion, taken together, these data suggest that a common mechanism of pathogen recognition and signalling initiation may be used by Cf-9 and Cf-4 to confer disease resistance to *Cladosporium fulvum*.

Results

Characterization of functional c-myc-tagged Cf-4 tobacco lines

Transgenic tobacco plants and cell cultures were previously established that expressed triple c-myc-tagged Cf-9, in which a triple c-myc sequence was inserted in-frame either behind the putative signal peptide cleavage site or in the putative cytoplasmic tail of the Cf-9 protein (Cf-9:mycB and Cf-9:mycG, respectively) (Piedras *et al.*, 2000). We engineered Cf-4:mycB and Cf-4:mycG inserting a triple c-myc sequence (EQKLISEEDL) at positions analogous to Cf-9:mycB and Cf-9:mycG. These constructs were fused to the 35S promoter and cloned into an *Agrobacterium* binary plasmid. Tobacco plants were transformed and 10 independent kanamycin-resistant plants were analysed for each construct. The analysis of the Cf-4:mycG lines is described below. Similar methodology was followed for Cf-4:mycB.

Primary transformants were analysed for the expression of Cf-4:myc by immunoblot with anti-c-myc antibodies. After fractionation of the total extracts, a strong cross-reacting band of approximately 145 kDa was detected in the microsomal fraction of three lines (G981, G986 and G990), whereas no band of that size was detected in the soluble fraction in any case (not shown). No signal was observed in the protein samples obtained from control untransformed plants, confirming the specificity of the anti-c-myc antibody.

In order to determine whether Cf-4:myc-expressing plants responded to Avr4, two different *in vivo* assays based on the induction of a Cf-4/Avr4-dependent HR were conducted. Firstly, all Cf-4:myc tobacco primary transformants were infiltrated with *A. tumefaciens* expressing either PVX:Avr4 (Thomas *et al.*, 1997) or PVX:Avr9 (Hammond-Kosack *et al.*, 1995). As in the wild-type Cf-4 transgenic tobacco plants (Figure 1a, middle panel), strong necrosis was observed in the Cf-4:myc leaf panels 5 days after infiltration with PVX:Avr4 but not PVX:Avr9

(Figure 1a, right panel). Importantly, this phenotype was only observed in the three lines expressing Cf-4:myc (G981, G986 and G990), while the other seven primary transformants showed very slight or no necrosis (not shown). In addition, the Petit Havana control line showed no symptoms after infiltration with either PVX:Avr4 or PVX:Avr9 (Figure 1a, left panel). Secondly, Cf-4:myc transgenic tobacco plants were crossed to homozygous 35S:Avr4 transgenic tobacco lines. A stunted seedling phenotype (Thomas *et al.*, 2000) was observed in 50% of the progeny from the G981, G986 and G990 lines (not shown), consistent with a T-DNA insertion in a single locus. The specificity of this phenotype was demonstrated by the normal growth shown by seedlings obtained from crosses to Avr9 control plants. Therefore, c-myc-tagged Cf-4 heterologously expressed in tobacco specifically responds to the Avr4 peptide and is functional *in vivo*.

Tobacco lines G981, G986 and G990 were selected for further analysis and six independent T2 lines were produced from each primary transformant. Based on Cf-4:myc protein expression levels and the induction of the Avr4-dependent necrotic response, one homozygous (G990F) and three heterozygous (G986A, G986F and G990A) T2 lines were selected for generation of stable transformants, and transgenic tobacco cell suspension cultures were produced. In contrast to the Cf-9:mycG line, which exhibited slightly weaker Avr9 responsiveness (Piedras *et al.*, 2000) compared to Cf-9:mycB and wild-type Cf-9 tobacco, Cf-4:mycB and Cf-4:mycG showed a similar response to Avr4 challenge compared with each other and wild-type Cf-4. This is consistent with the observation that Cf-4/Avr4 interaction elicits a stronger response compared to Cf-9/Avr9.

Cf-4:myc protein could also be detected in the cell suspension cultures (Figure 1b). Although the predicted molecular mass of Cf-4 is approximately 88 kDa, the protein cross-reacted with the anti-c-myc antibodies as a band of approximately 145 kDa, slightly smaller than Cf-9:myc. The insertion of the triple c-myc sequence (approximately 5 kDa) cannot account for the difference between the observed and the estimated molecular masses. Because the Cf-4 protein contains 20 putative glycosylation sites, we investigated whether, as in Cf-9, glycosylation of Cf-4 protein occurred. Solubilized microsomal fractions obtained from c-myc-tagged Cf-4 tobacco plants were treated with PNGase F, a glycoamidase that liberates nearly all N-linked oligosaccharides from glycoproteins (Maley *et al.*, 1989). Incubation of solubilized microsomes with PNGase F induced a shift in the electrophoretic mobility of the Cf-4:myc protein (Figure 1c). Within 5 min, two smaller bands, of approximately 137 and 97 kDa, were detected. As the endoglycosidase reaction proceeded, the intensity of the 97 kDa band, which is close to the predicted molecular mass of the Cf-4 amino

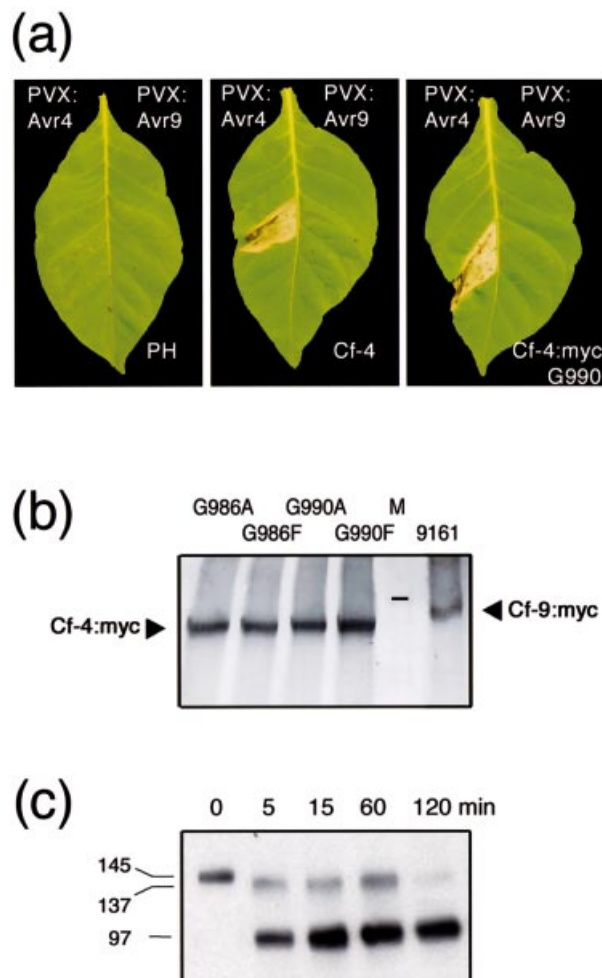


Figure 1. Functional analysis of c-myc-tagged Cf-4 tobacco primary transformants, and expression analysis and characterization of c-myc-tagged Cf-4 cell suspension cultures.

(a) Tobacco lines Petit Havana (wild-type), transgenic for *Cf-4* or *Cf-4:myc* (G990) were infiltrated with *Agrobacterium* expressing PVX:Avr4 (left leaf half; Thomas *et al.*, 1997) and PVX:Avr9 (right leaf half; Hammond-Kosack, 1995). After 5 days, the Cf-4/Avr4-dependent hypersensitive cell death reaction was observed in both Cf-4-expressing lines (middle and right panel). (b) Microsomal fractions were prepared from tobacco cell cultures that are heterozygous (G986A, G986F, G990A) and homozygous (G990F) for *Cf-4:myc*, or homozygous for *Cf-9:myc* (9161, carrying a triple c-myc tag in the G domain; Piedras *et al.*, 2000). Proteins (50 µg) were separated in an SDS-PAGE gel and analysed by immunoblot using an anti-c-myc antibody. The positions of Cf-4:myc and Cf-9:myc are indicated by arrowheads. (c) Deglycosylation of Cf-4:myc protein. Aliquots (50 µg) of Cf-4:myc microsomes from homozygous G990F tobacco plants were incubated with 5 units of glycosidase PNGase F for the times indicated. Reactions were subsequently analysed by SDS gel and immunoblot. The positions of the molecular mass markers in kDa are indicated on the left.

acid sequence, increased, while the 137 kDa band became fainter. This indicates that Cf-4, like Cf-9, is highly glycosylated.

The *in vivo* functionality of Cf-4:myc- and Cf-9:myc-expressing cell cultures was studied by analysing the

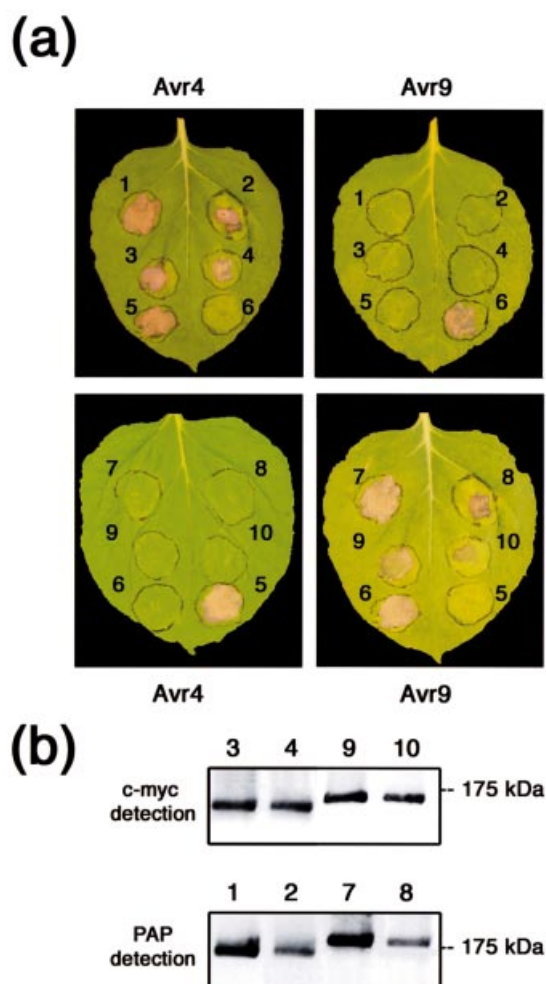


Figure 3. Function and detection of TAP and c-myc-tagged Cf proteins transiently expressed in *N. benthamiana* leaves.

(a) Leaves of *N. benthamiana* transgenic for Avr4 (left) or Avr9 (right) were infiltrated with *Agrobacterium* carrying different Cf constructs, as indicated: (1) 35S:*Cf-4:TAP*; (2) Gen:*Cf-4:TAP*; (3) 35S:*Cf-4:mycB*; (4) 35S:*Cf-4:mycG*; (5) 35S:*Cf-4*; (6) 35S:*Cf-9*; (7) 35S:*Cf-9:TAP*; (8) Gen:*Cf-9:TAP*; (9) 35S:*Cf-9:mycB*; (10) 35S:*Cf-9:mycG*. After 5 days, the Cf/Avr-dependent hypersensitive cell death reaction was observed. (b) *N. benthamiana* leaves were infiltrated as in (a). Microsomal fractions were prepared 2 days after infiltration. Proteins (50 µg) were separated in an SDS-PAGE gel and analysed by immunoblot using a PAP or anti-c-myc antibody for detection of TAP- and c-myc-tagged Cf-4 and Cf-9, respectively. The numbers above the columns indicate the construct used for agro-infiltration, see (a). Sizes of molecular mass markers are shown on the right.

induction of Avr-dependent early defence responses. Upon specific elicitation, a rapid production of active oxygen species (AOS) occurred, with the highest H₂O₂ levels being detected in Cf-4:myc lines, particularly in the homozygous G990F line (Figure 2a). No effect was observed after treatment of the Cf-9:myc lines with Avr4, or of Cf-4:myc lines with Avr9 (not shown). Untransformed tobacco cultures did not respond to Avr treatment, underlining the specificity of the observed response. In addition,

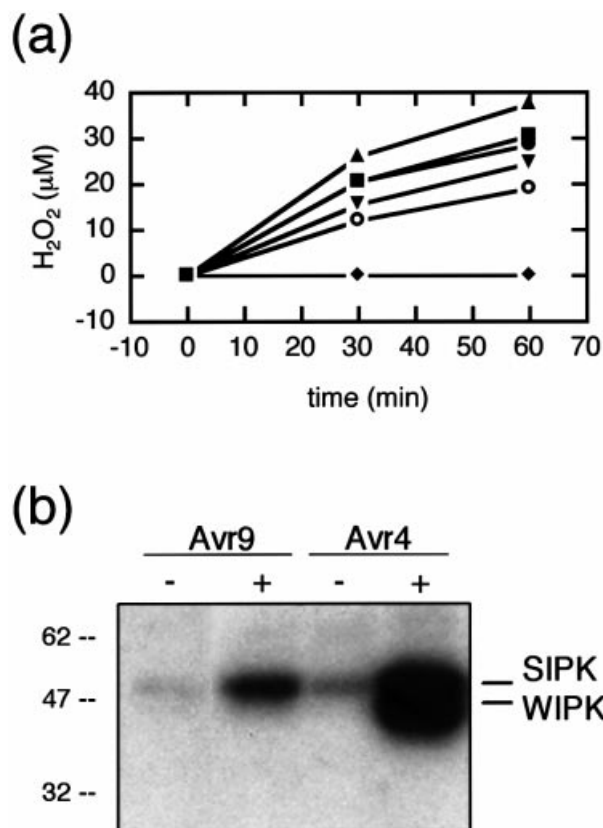


Figure 2. c-myc-tagged Cf proteins trigger early Avr-dependent defence responses in tobacco cell cultures.

(a) Synthesis of active oxygen species (AOS). Cell cultures, heterozygous (G986A (●), G986F (▼) and G990A (■)) and homozygous (G990F (▲)) for *Cf-4:myc*, homozygous for *Cf-9:myc* (9161 (○)) and Petit Havana (control (◆)) were elicited and the accumulation of H₂O₂ was determined by the ferricyanide-catalysed oxidation of luminol at the times indicated. (b) Activation of MAP kinases. Cell cultures homozygous for *Cf-9:myc* and *Cf-4:myc* were elicited with 15 nM Avr9 and Avr4 (+) or water (-), and harvested after 15 min. Total protein extracts were prepared and 50 μg of protein were analysed by an in-gel kinase assay with myelin binding protein as a substrate. The positions of WIPK and SIPK are indicated, and the sizes of molecular markers are shown on the left.

two protein kinases of 46 and 48 kDa were found to be specifically activated in an in-gel kinase assay, using myelin basic protein as a substrate, upon elicitation of c-myc-tagged Cf cell cultures with the corresponding Avr protein (Figure 2b; the 46 kDa band was detected in the *Cf-9:myc* cells after a longer film exposure). These kinases were previously identified as wounding-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK), respectively (Romeis *et al.*, 1999). Again, kinase activation was stronger in *Cf-4:myc*/Avr4 interaction compared with *Cf-9:myc*/Avr9. Taken together, these results demonstrate that *Cf-4:myc* and *Cf-9:myc*, when heterologously expressed in both tobacco plants and cell cultures, retain responsiveness to Avr peptides and *in vivo* functionality, and should facilitate the biochemical dissection of Cf proteins.

Transient expression and functional analysis of TAP-tagged and c-myc-tagged Cf-4 proteins in *N. benthamiana* leaves

The Tandem Affinity Purification (TAP) method, in which two tags (the calmodulin-binding peptide and the IgG-binding units of protein A from *Staphylococcus aureus*) are fused in tandem and separated by a TEV protease cleavage site (Rigaut *et al.*, 1999), was previously shown to be a valuable tool in the identification and characterization of the Cf-9 membrane-associated complex of approximately 420 kDa (Rivas *et al.*, 2002). As previously described for Cf-9, we engineered a C-terminal fusion of the *Cf-4* gene with the TAP tag after the C-terminal KKRY sequence (see Experimental procedures). This construct was fused to either the 35S promoter or the *Cf-4* native promoter and cloned into an *Agrobacterium* binary plasmid.

We used the *Agrobacterium*-mediated transient assay, previously established for the production of epitope-tagged Cf-9 (Rivas *et al.*, 2002), to test the *in vivo* functionality of the *Cf-4:TAP* gene, under the 35S or *Cf-4* native promoter, in *N. benthamiana* plants expressing Avr4 (Thomas *et al.*, 2000). The transient assay was used as an additional confirmation of the *in vivo* function of the 35S:*Cf-4:myc* constructs (tagged either in the B or in the G domain). 35S:*Cf-9:mycB*, 35S:*Cf-9:mycG* and *Cf-9:TAP* (under 35S or native promoter), together with 35S:*Cf-4* and 35S:*Cf-9*, were also included in the assay. In control experiments, Avr9-expressing *N. benthamiana* leaves were also agro-infiltrated (Thomas *et al.*, 2000). The Cf/Avr-dependent hypersensitive cell death reaction was always observed in the gene-for-gene combinations. Strong necrosis in Avr4-expressing leaves was observed after inoculation with *Agrobacterium* carrying either the wild-type *Cf-4* or any of the *Cf-4*-tagged constructs (Figure 3a, top left), whereas no necrotic phenotype was detected when Avr9 plants were inoculated with the same constructs (Figure 3a, top right).

Using antibodies against the engineered tags, expression of *Cf-4:TAP* and *Cf-4:myc* was detected in non-transgenic *N. benthamiana* leaves after infiltration with *Agrobacterium* carrying the various constructs. Bands of the expected sizes (approximately 165 and 145 kDa for *Cf-4:TAP*- and *Cf-4:myc*-inoculated leaves, respectively) could be detected with PAP or anti-c-myc antibodies (Figure 3b). In the control experiments, *Cf-9:TAP* and *Cf-9:myc* (approximately 185 and 160 kDa, respectively) were also detected.

Identification of a Cf-4 complex by gel filtration chromatography

In a previous study, gel filtration was used to identify a membrane-associated complex containing Cf-9 (Rivas

et al., 2002). The high structural similarity and amino acid sequence homology between Cf-9 and Cf-4 led us to investigate whether Cf-4 participates in a similar protein complex. Cf-4 microsomes were thus prepared from tobacco plants (G990F), solubilized with octylglucoside (OG) and subjected to gel filtration chromatography. Fractions (2 ml) were collected and analysed for the presence of Cf-4:myc by immunoblot with the anti-c-myc antibody (Figure 4a). Identical elution profiles were obtained irrespective of whether the triple c-myc tag was integrated in the B or the G domain of Cf-4, or whether leaf material or cell cultures were used to prepare protein extracts (not shown). The estimated molecular mass of Cf-4 from the eluted fractions ranged between 475 and 350 kDa, with a peak at the fraction corresponding to approximately 410 kDa (Figure 4a). This estimated molecular mass, higher than expected for the monomeric form of Cf-4:myc, strongly suggests that Cf-4 participates in a protein complex in the membrane.

To confirm the specificity of the cross-reacting bands in Figure 4(a), eluted fractions 23–25 from the gel filtration column containing Cf-4:myc were pooled and subjected to immunoprecipitation. A cross-reacting band was detected for Cf-4:myc when using a monoclonal anti-c-myc, but not with a non-related HA antibody (Figure 4b).

Analysis of the stability of the Cf-4 complex

Firstly, the stability of the Cf-4 complex was studied under (a) different extraction buffer compositions, and (b) two different extraction procedures (frozen leaves or cells were ground in liquid nitrogen, or fresh leaves or cells were ground using a blender). As previously shown for Cf-9, the Cf-4 complex proved to be very stable, as the elution pattern of Cf-4 proteins remained identical in the pH range 6.0–8.5 and for NaCl concentrations between 50 and 500 mM, regardless of the extraction procedure employed (not shown).

Secondly, an extensive analysis of the elution profile of the Cf-4 complex was performed using different detergents (namely OG, Triton X-100 (TX-100), Nonidet P-40 (NP-40) and CHAPS) for solubilization. As previously observed for Cf-9, the use of different detergents greatly influenced the estimation of the molecular size of the Cf-4-containing fractions: 1000–750 kDa after solubilization with TX-100 or NP-40, and 475–350 kDa in the presence of OG or CHAPS. Figure 4(a) shows examples of the elution profiles after solubilization with OG and TX-100. OG was chosen to extend the Cf-4 complex characterization as (a) it is a milder detergent than CHAPS, (b) Western blot analysis revealed slight degradation of the Cf-4 protein after solubilization with CHAPS (not shown), and (c) previous studies on the Cf-9 complex were performed in the presence of OG (Rivas et al., 2002).

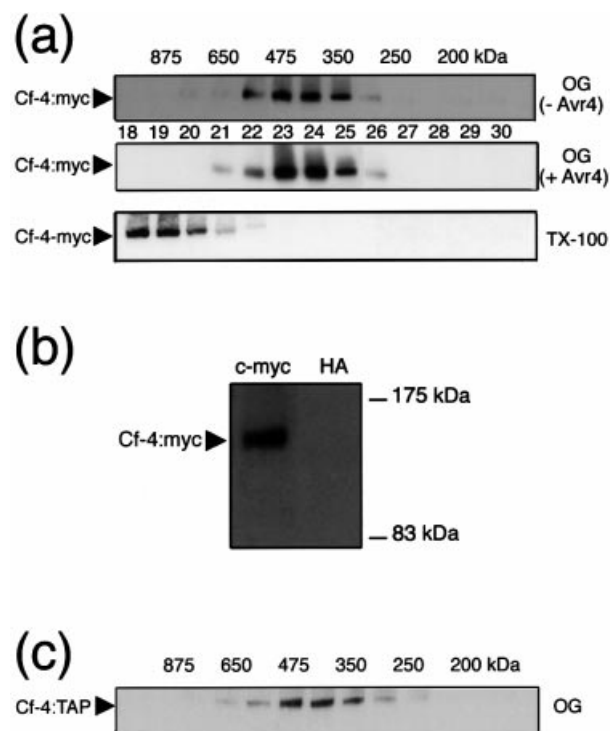


Figure 4. Gel filtration analysis of the Cf-4:myc and Cf-4:TAP microsomes.

(a) Microsomal proteins (0.5 mg) from G990F tobacco cell suspensions were solubilized with the detergents octylglucoside (40 mM) (before (-Avr4) and after elicitation (+Avr4)) or TX-100 (0.1%) for 30 min on ice, and the supernatants were subjected to gel filtration chromatography in a Sephacryl S-300 column. Fractions (2 ml) were collected and aliquots were analysed by SDS gel and immunoblot with an anti-c-myc antibody. Fraction numbers of the elution profile are indicated by the numbers between the panels. The molecular size estimated for each fraction in kDa is given above. Cf-4:myc is indicated by arrowheads. (b) Immunoprecipitation of Cf-4:myc from pooled fractions of the gel filtration. Microsomes were prepared from homozygous G990F tobacco plants, solubilized with OG and subjected to gel filtration chromatography as described in (a). Fractions containing Cf-4:myc were pooled and subjected to immunoprecipitation with a monoclonal anti-c-myc or anti-HA antibody (control). Precipitated proteins were analysed by SDS-PAGE and immunoblot using a polyclonal anti-c-myc antibody. The position of Cf-4:myc is indicated by an arrowhead, and the sizes of molecular mass markers are shown on the right. (c) *N. benthamiana* leaves were infiltrated with *Agrobacterium* carrying 35S:Cf-4:TAP. Two days after infiltration, microsomal proteins (0.5 mg) were solubilized with 40 mM octylglucoside and subjected to gel filtration chromatography as described in (a). The molecular size estimated for each fraction in kDa is given above. Cf-4:TAP is indicated by an arrowhead.

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

In conclusion, we have shown that Cf-4 is part of a 475–350 kDa membrane-associated complex, regardless of (a) the tissue used as protein source (stably transformed tobacco plants, cell cultures or transiently transformed *N. benthamiana*), (b) the tag used (triple c-myc or TAP), (c) the position of the tag within the protein sequence (B or G domain of Cf-4), and (d) the extraction conditions (different buffer compositions or extraction procedures).

Blue native PAGE analysis of the Cf-4 complex

The most important problem in native electrophoresis of membrane proteins is that membrane protein aggregation should be minimized. Blue native polyacrylamide gel electrophoresis (BN-PAGE) has been developed for the isolation of membrane-associated protein complexes in enzymatically active form (Arnold *et al.*, 1999; Caliebe *et al.*, 1997; Schägger and von Jagow, 1991). In this technique, Coomassie dyes and aminocaproic acid are introduced to induce a charge shift and improve the solubilization of membrane proteins, thereby avoiding the problem of detergent interference observed in gel filtration analysis (see below). In an earlier study, use of this methodology allowed a more accurate estimation of the molecular size of the Cf-9 complex (Rivas *et al.*, 2002). To detect Cf-4:myc within potential protein complexes, OG-solubilized Cf4:TAP microsomes from agro-infiltrated *N. benthamiana* leaves were subjected to BN-PAGE (Figure 5a). Similarly, Cf-9:TAP microsomes were used in a control experiment (Figure 5b). Single lanes of the BN gel were cut, mounted on a denaturing SDS gel in the second dimension, and subjected to immunoblot analysis using a PAP antibody (see Experimental procedures). Strong cross-reacting bands, which, by comparison with protein standards from the BN-PAGE, corresponded to a molecular mass of approximately 400 and 420 kDa were detected for the Cf-4:TAP and the Cf-9:TAP microsomes, respectively. (Figure 5a,b, top panel). The same result was obtained

when TX-100 was used for solubilization of microsomal preparations (not shown). No cross-reacting signal was detected at this position when microsomes from non-infiltrated *N. benthamiana* leaves were subjected to the same experimental procedure (Figure 5a, bottom panel). To confirm that the detected signal corresponded to a Cf-4-containing complex, microsomes were incubated with PNGase F prior to loading in BN-PAGE. This treatment resulted in a shift of the cross-reacting band from 400 kDa

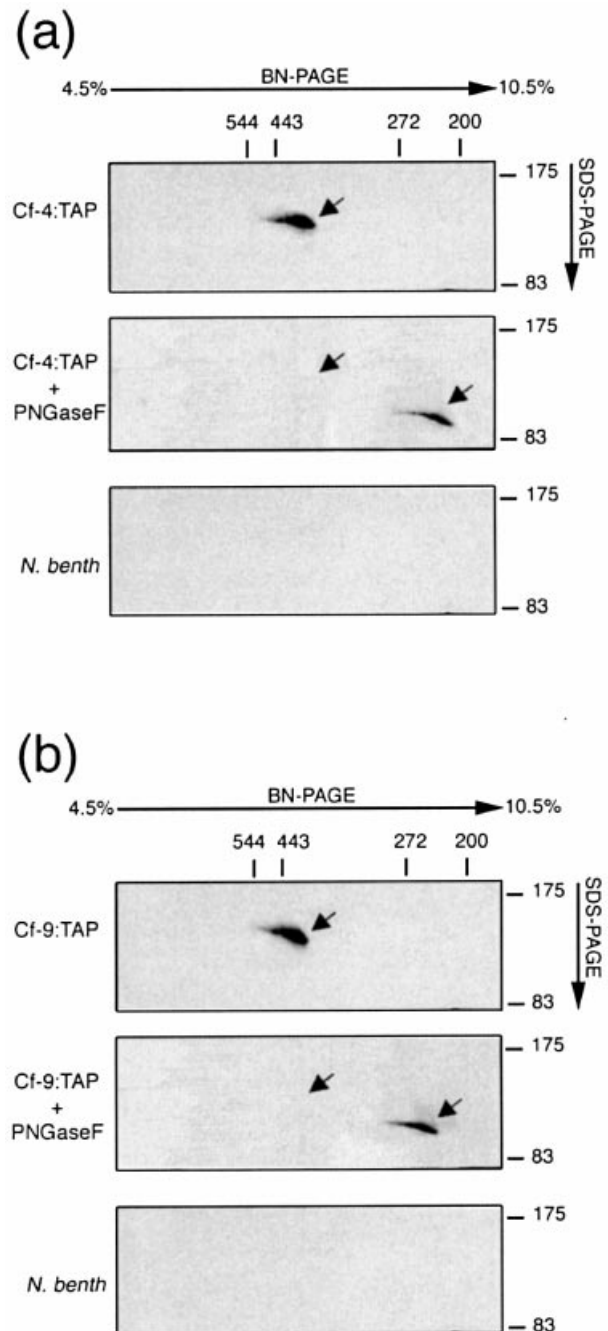


Figure 5. Blue native polyacrylamide gel electrophoresis of solubilized Cf-4:TAP microsomes.

N. benthamiana leaves were infiltrated or not with *Agrobacterium* carrying 35S:Cf-4:TAP (a) or 35S:Cf-9:TAP (b). After two days, microsomal proteins (0.5 mg) of infiltrated leaves (upper and middle panel) and non-injected leaves (lower panel) were solubilized with 40 mM octylglucoside and 750 mM aminocaproic acid in 50 mM Bis-Tris for 30 min on ice. Aliquots of supernatants (80 µg of protein in 40 µl) were either treated with PNGase F (middle panel; see legend to Figure 1c) or directly incubated with 5% Coomassie brilliant blue G (upper panel). Proteins were separated on a 4.5–10.5% BN-polyacrylamide gradient gel. Cf complexes were resolved by second dimension 7.5% SDS gel and blotted onto nitrocellulose. Cf-4:TAP and Cf-9:TAP were finally detected by immunoblot of the SDS gel using the PAP antibody. The sizes of the molecular mass markers are indicated on top (BN-PAGE) and on the right (SDS-PAGE). The positions of Cf-4:TAP and Cf-9:TAP are indicated by arrowheads.

to approximately 230 kDa in the BN-PAGE (and from 145 kDa to about 97 kDa in the SDS-PAGE, see Figure 1c) (Figure 5a, middle panel). Assuming that approximately 48 kDa of sugar groups are released from Cf-4 after glycosidase treatment, the extra 122 kDa due to glycosylation might be derived from other components of the complex.

Finally, Cf-4:myc from either tobacco plants or cell cultures was also subjected to BN-PAGE analysis, using an anti-c-myc antibody for the immunoblot analysis. Identical results were obtained for Cf-4:myc (not shown), confirming that Cf-4 is part of a membrane complex of approximately 400 kDa.

The Cf-4 complex does not contain homo-multimers of Cf-4

The approximately 400 kDa molecular size of the Cf-4 complex is consistent with more than one Cf-4 protein molecule being present in the complex. To address this question, both Cf-4:myc and Cf-4:TAP were simultaneously expressed in *N. benthamiana* using the *Agrobacterium*-mediated transient assay. In parallel infiltrations, either Cf-4:myc or Cf-4:TAP were also expressed. Additionally, as a control, the same experiment was performed with Cf-9. Protein extracts were prepared and expression of the proteins checked by Western blot. Bands of the expected sizes were detected with the PAP antibody (approximately 185 and 165 kDa for Cf-9:TAP and Cf-4:TAP, respectively) (Figure 6a, right). Two distinct bands of the expected size (approximately 185 and 160 kDa for Cf-9:TAP and Cf-9:myc, and approximately 165 and 145 kDa for Cf-4:TAP and Cf-4:myc, respectively) were detected when using the anti-c-myc antibody (Figure 6a, left), as the protein A moiety in the TAP tag is recognized by any antibody. Following preparation of the extracts, proteins were subjected to immunoprecipitation with rabbit IgG-agarose beads, specifically aimed at immunoprecipitating the TAP-tagged Cf proteins. After collecting the immunoprecipitates by centrifugation, supernatants were analysed with PAP and anti-c-myc antibodies for the presence or absence of TAP- or c-myc-tagged Cf-9 and Cf-4. Neither Cf-9:TAP nor Cf-4:TAP could be detected with the PAP antibody in the supernatant (Figure 6b, right), demonstrating the effectiveness of the immunoprecipitation of the TAP-tagged proteins. Likewise, no TAP-tagged Cf proteins could be detected in the supernatant when using anti-c-myc, whereas the c-myc signal remained unaltered (Figure 6b, left), indicating no association between the TAP and the c-myc-tagged Cf proteins and suggesting that, as previously shown for Cf-9, only one Cf-4 molecule participates in the Cf-4 complex. To further prove this finding, TAP-tagged Cf proteins were released from the IgG beads by incubation of the immunoprecipitates with the

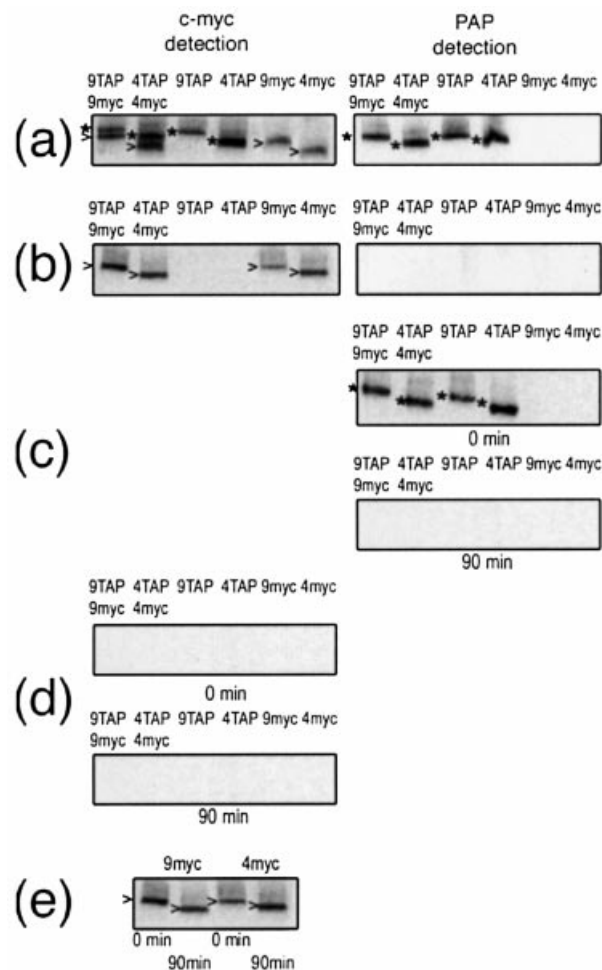


Figure 6. Stoichiometry of the Cf proteins in the Cf complex. *N. benthamiana* leaves were infiltrated with *Agrobacterium* expressing either Cf-4:TAP and Cf-4:myc, or Cf-4:TAP or Cf-4:myc. As a control, either Cf-9:TAP and Cf-9:myc, or Cf-9:TAP or Cf-9:myc were also expressed. Leaves were harvested 2 days after infiltration and microsomal proteins were prepared. Transient protein expression was analysed by Western blot (a) with monoclonal anti-c-myc (left) and PAP antibodies (right). TAP and c-myc-tagged proteins are indicated by the symbols * and >, respectively. Protein extracts were subjected to immunoprecipitation with rabbit IgG agarose beads, immunoprecipitates were collected by centrifugation and the non-immunoprecipitated material again analysed by Western blot (b) with monoclonal anti-c-myc (left) and PAP antibodies (right). Immunoprecipitates were cleaved with the TEV protease and aliquots of both IgG beads (c) and cleaved material (d) were immunoanalysed with the PAP and anti-c-myc antibodies, respectively, before (0 min) and after (90 min) incubation with TEV enzyme. A control incubation of Cf-9:myc and Cf-4:myc microsomes with the TEV protease was also performed. Cf-4:myc and Cf-9:myc before (0 min) and after (90 min) incubation with TEV were detected with a monoclonal anti-c-myc antibody (e).

TEV protease. Western blot analysis of the IgG beads with the PAP antibody, before and after the TEV reaction, showed that Cf-9:TAP and Cf-4:TAP were completely cleaved from the beads, as 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 approximately 15 kDa band that remained attached to the

beads after TEV proteolysis (not shown). Therefore, no signal corresponding to Cf protein was detected when analysing the cleaved material with the PAP antibody, as the protein A sequence was cleaved off from the TAP tag (not shown). Significantly, no Cf-4:myc signal was detected in the released material either (Figure 6d). The possibility of protein degradation by the TEV enzyme was ruled out in a parallel incubation of Cf-9:myc and Cf-4:myc with TEV in which no effect of the protease on c-myc-tagged Cf proteins could be detected after treatment (Figure 6e). Taken together, these results confirm that Cf-4:TAP and Cf-4:myc do not associate with each other, and suggest that, as previously shown for Cf-9, there is only one Cf-4 protein molecule in the Cf-4 complex.

Cf-4 is not disulphide-linked to another protein and the Cf-4 complex does not change in size or recruit GTP-binding proteins on elicitation

In an attempt to further characterize the Cf-4 complex, we investigated whether the Cf-4 complex shares further similarities with the CLV or the Cf-9 complex, beyond the similarity in protein structure and complex sizes (Rivas *et al.*, 2002; Trotochaud *et al.*, 1999). In particular, we were interested in testing three major properties of the CLV complex, previously tested for the Cf-9 complex (Rivas *et al.*, 2002). Conserved cysteine pairs, immediately before and after the LRRs (Jones and Jones, 1997) were proposed to be involved in the formation of a disulphide-linked heterodimer between CLV1 and CLV2 (Jeong *et al.*, 1999). In contrast, we showed that Cf-9 is not associated with other proteins in the membrane by the formation of covalent disulphide bonds between cysteine residues. Thus, solubilized extracts from either cell cultures or leaves expressing Cf-4:myc were boiled in SDS-PAGE loading buffer in the presence or absence of the reducing agent β -mercaptoethanol. Samples were then analysed by SDS-PAGE and immunoblot with an anti-c-myc antibody. No difference in the molecular mass of the protein dependent on the treatment was found (Figure 7a, left). The same result was also consistently obtained when either leaf material was used (regardless of the position of the c-myc tag), or Cf-4:myc and Cf-4:TAP were transiently expressed in *N. benthamiana*. Figure 7(a) shows the Western blot obtained for Cf-4:mycG expressed in cell cultures (left) and Cf-4:TAP transiently expressed in *N. benthamiana* (right). This result indicates that the Cf-4 protein does not form disulphide-linked heterodimers in the membrane.

CLV1 (a receptor-like kinase) is present as an inactive disulphide-linked heterodimer with CLV2, and CLV3 (a small secreted peptide) functions to promote the assembly of the active 450 kDa complex (Trotochaud *et al.*, 1999; Trotochaud *et al.*, 2000). Because no Avr9-dependent shift

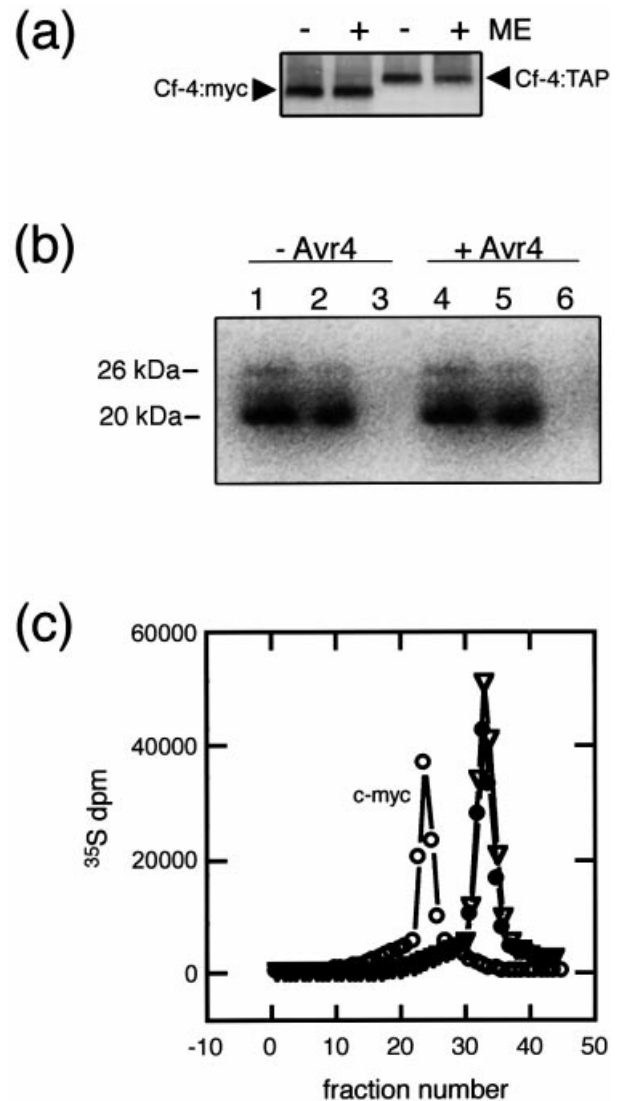


Figure 7. Characterization of the Cf-4:myc complex.

(a) Disulphide linkage analysis of Cf-4:mycG and Cf-4:TAP. Solubilized microsomal fractions from homozygous G990F tobacco cell cultures (left) or transiently expressed Cf-4TAP (right) were boiled in sample buffer in the presence (+) or absence (-) of β -mercaptoethanol (ME). Proteins were subsequently analysed by SDS-PAGE and immunoblot using an anti-c-myc antibody. The positions of Cf-4:myc and Cf-4:TAP are indicated by arrowheads. (b) Cf-4:myc cell cultures elicited (+) or not (-) with 15 nM synthetic Avr4 were immunoprecipitated with the anti-c-myc antibody. Aliquots of the incubation mixtures (lanes 1 and 4), non-immunoprecipitated (lanes 2 and 5) and immunoprecipitated proteins (lanes 3 and 6) were run in SDS-PAGE and blotted onto nitrocellulose. The membrane was then incubated with the GTP analogue GTP[γ ³⁵S] and autoradiographed. Lanes 3 and 6 correspond to 40 times more protein (compared with lanes 1, 2, 4 and 5) after immunoprecipitation. (c) Elution profile of gel filtration analysis of Cf-4:myc and small GTP-binding Rop proteins. Total extracts of Cf-4:myc cell cultures harvested before (●) or after (▽) elicitation with 15 nM synthetic Avr4 were incubated with the GTP analogue GTP[γ ³⁵S] and subjected to gel filtration. Eluted fractions were collected and analysed for the presence of Cf-4:myc by immunoblot (○) and for the incorporation of GTP[γ ³⁵S] by scintillation counting (●, ▽).

in the gel filtration profile of the Cf-9 complex was previously found, we investigated whether treatment with Avr4 induces changes in the elution of the Cf-4-containing complexes. Cf-4:myc tobacco cell cultures were therefore treated with the Avr4 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-4 protein after Avr4 treatment was observed (Figure 4a).

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), but not by the Cf-9 complex either before or after elicitation (Rivas *et al.*, 2002). We therefore tested for the presence of small GTPases in the Cf-4 complex using the GTP analogue GTP[γ - 35 S]. Microsomes were prepared from cell cultures, before and after elicitation with Avr4, solubilized and immunoprecipitated with an anti-c-myc antibody. The presence of Rho-GTPase-related proteins of approximately 20 and 26 kDa 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-4 are not likely to be associated, even after elicitation. In addition, solubilized Cf-4:myc microsomes were incubated with GTP[γ - 35 S] and subjected to gel filtration chromatography. Fractions were collected and incorporated radioactivity was determined. No radioactivity co-eluted with Cf-4 either in the unelicited or the elicited state (Figure 7c). Identical results were obtained using wild-type Cf-4-expressing plants and cell cultures before and after elicitation with Avr4 (not shown). This suggests that the approximately 400 kDa Cf-4 complex does not contain small GTP-binding proteins, either before or after elicitation.

Taken together, our data lead us to conclude that, as for Cf-9, and despite the structural similarities between Cf type proteins and CLV2, different molecular mechanisms are involved in CLV2 function in *Arabidopsis* and Cf-mediated responses to Avr proteins in tomato and tobacco.

Discussion

Using epitope-tagged Cf-9, expressed transiently or stably, we previously identified and characterized a membrane-associated complex that contains Cf-9 (Rivas *et al.*, 2002). Here we show that c-myc:Cf-4 is a membrane-associated glycoprotein that confers specific recognition of Avr4. Interestingly, the Avr-dependent responses were stronger in the c-myc-tagged Cf-4 lines than in the c-myc-Cf-9 lines (Figure 2), consistent with the observation that Cf-4-expressing tobacco plants exhibit a faster and stronger

necrotic response to Avr treatment compared to Cf-9 plants (Thomas *et al.*, 2000; Van der Hoorn *et al.*, 2001a; Wulff *et al.*, 2001). Alternatively, these results could reflect higher protein expression in the Cf-4 transgenic lines compared to the Cf-9 transformants (Figure 1b). In summary, these functional c-myc-tagged Cf-4 tobacco lines constitute a valuable new tool to study the earliest events in Avr-dependent Cf-mediated defence responses.

In addition, we engineered a Cf-4:TAP construct (Rigaut *et al.*, 1999), tagged after the conserved C-terminal KKRY domain which was previously reported to confer ER localization of Cf-9 (Bengezhal *et al.*, 2000). However, data presented in other studies argued against the ER location of functional Cf-9 (Piedras *et al.*, 2000; Rivas *et al.*, 2002; Van der Hoorn *et al.*, 2001b). Here, using an *Agrobacterium*-mediated transient assay (Rivas *et al.*, 2002), we show that the TAP-tagged Cf-4 protein is functional *in vivo* (Figure 3a), indicating that a free conserved C-terminal di-lysine motif, required for retrieval of type I membrane proteins from the Golgi apparatus to the ER (Cosson and Letourneur, 1994; Cosson *et al.*, 1996; Jackson *et al.*, 1990), is not required for Cf-4 function. Based on these and other results, it is tempting to speculate about a similar localization of Cf-9 and Cf-4, although no data regarding the cellular localization of Cf-4 have been reported to date and the Cf-9 location remains controversial (Bengezhal *et al.*, 2000; Piedras *et al.*, 2000).

Gel filtration chromatography in the presence of OG was previously used to identify the Cf-9 complex (Rivas *et al.*, 2002). Likewise, we showed that the Cf-4 protein solubilized with OG participates in an essentially identical kind of complex ranging in size between 475 and 350 kDa, with a peak at approximately 410 kDa (Figure 4). Extensive analysis of the Cf-4 complex in the presence of different detergents revealed very similar characteristics to the previously described Cf-9 complex (Rivas *et al.*, 2002). The high molecular size observed with TX-100 and NP-40 (1000–750 kDa), very close to the exclusion volume of the column, could reflect artefactual protein aggregation (Simons *et al.*, 1973). As Cf proteins are type I membrane proteins with just a short stretch of the protein structure forming a single transmembrane domain, it is very unlikely that interaction with OG makes a large contribution to the size of the Cf complexes (Møller and le Marie, 1993). Therefore, the 475–350 kDa complex observed using CHAPS or OG most likely reflects Cf-4 association with other protein(s) and/or with itself.

Independent confirmation of the presence of the Cf-4 protein in an approximately 400 kDa protein complex was provided by means of BN-PAGE (Figure 5a). The specificity of the detected signal was demonstrated by both the shift of the cross-reacting signal after endoglucanase treatment and the lack of signal when using microsomes from non-infiltrated *N. benthamiana* leaves.

The estimated molecular size of the Cf-4-containing fractions is higher than that expected for the monomeric form of Cf-4. This suggests that Cf-4 associates with additional protein partner(s) and/or with itself. In order to establish the stoichiometry of the Cf-4 protein in the complex, co-immunoprecipitation experiments with Cf-4 using two independent tags, namely c-myc and TAP, were performed. Interestingly, our data are inconsistent with the presence of more than one Cf-4 protein molecule in the Cf-4 complex (Figure 6), indicating that additional protein partners participate with Cf-4 in initiating defence signalling. As glycosidase treatment shifts the size of the complex from approximately 400 to 230 kDa, these additional partners might include at least one glycoprotein and thus at least one other protein with an extracellular domain.

Here we show that, despite the high structural similarity between CLV2 and Cf proteins, the nature of the Cf-4 complex appears to be different to that of the CLV complex, consistent with the significant differences previously found between the Cf-9 and CLV complexes (Rivas *et al.*, 2002). Indeed, unlike CLV2 and similarly to Cf-9, the Cf-4 protein did not form disulphide-linked heterodimers, no ligand (Avr4)-dependent shift in the molecular mass of the Cf-4 complex was detected, and no GTP-binding proteins were found to be associated with Cf-4 under the conditions tested. Therefore, the data presented in this study further validate our inference that different molecular mechanisms probably govern regulation of meristem development in *Arabidopsis* and resistance to *Cladosporium fulvum* in tomato. Furthermore, except for the minor difference in the molecular weight of the complex, consistent with the different sizes of Cf-4 and Cf-9, both Cf complexes appeared to be indistinguishable, containing only one Cf protein molecule per complex.

To what extent do our data on the Cf-9 and Cf-4 complexes test the idea that Cf proteins 'guard' host targets for the Avr9 and Avr4 presumed pathogenicity factors? The guard model is consistent with at least two possible molecular mechanisms. The association of R proteins with their 'guarded' protein might be induced upon Avr binding to a pathogen target. Alternatively, R proteins might be constitutively associated with the 'guarded' protein, and, upon Avr binding, a conformational change is produced that activates downstream signalling components (Dangl and Jones, 2001). If the 'guard' model is valid, data from this study would not be consistent with the first alternative, as we did not observe an Avr-dependent shift in the size of the Cf complex. Furthermore, if Cf-9 and Cf-4 function by detecting a conformational change in host proteins that are targets for pathogenicity factors, they must thus either 'guard' two molecules of about the same size or the same molecule. Alternatively, Cf-4 and Cf-9 proteins might provide distinct

recognition specificities to a 400/420 kDa receptor complex. Although no evidence for direct binding between Cf-9 and Avr9 was found in previous studies (Luderer *et al.*, 2001), the possibility of Cf-9 being part of an Avr9-binding complex was not ruled out, as such a complex might have been unstable under the conditions tested.

Future studies thus need to focus on the identification of protein partner(s) of the Cf gene products in the membrane and their role in Avr perception. It is also crucial to investigate whether the similarities between Cf-9 and Cf-4 complexes extend further, and, importantly, to other members of the Cf family such as Cf-2 and Cf-5. The c-myc-tagged Cf tobacco lines, and transiently expressed Cf9:TAP and Cf-4:TAP, have proved to be excellent tools to facilitate the investigation of the similarities/differences between the protein(s) associated with Cf-9 and/or Cf-4 to achieve disease resistance.

Experimental procedures

Generation of c-myc-tagged Cf-4:myc transgenic tobacco lines.

Two constructs, SLJ11932 and SLJ133902, were engineered for Cf-4:mycG and Cf-4:mycB expression, respectively. To generate Cf-4mycG, a 2 kb *XhoI*-*BglII* fragment from p129P6A-4 (Wulff *et al.*, 2001) was isolated and subcloned into *XhoI*-*BglII*-digested SLJ8951, which contained a triple c-myc sequence coding for a C-terminal tag fused in-frame to Cf-9 (Piedras *et al.*, 2000), yielding SLJ11902. Both *Clal*-*NdeI* and *NdeI*-*BamHI* fragments from SLJ11902 were cloned into *Clal*-*BamHI*-digested SLJ4K1 (Jones *et al.*, 1992) to produce plasmid SLJ11922, which codes for the Cf-4 gene with a triple c-myc tag in the G domain of the protein, under the control of the 35S promoter. The *EcoRI*-*XhoI* fragment from SLJ4K1, containing the 35S promoter, and the *XhoI*-*BamHI* fragment of SLJ11922, containing the Cf-4:mycG gene, were ligated into the *EcoRI*-*BamHI*-digested SLJ7292 binary plasmid to produce SLJ11932.

To generate Cf-4mycB, *EcoRI* and *HindIII* restriction sites were created in the B domain of Cf-4 by oligomutagenesis. Two different DNA fragments were amplified by PCR using 35SCf4 (Thomas *et al.*, 1997) as a template. 35S1 primer (Wulff *et al.*, 2001) with 5'-CAA ATG GAA TTC AGG TAA GGA TGA CGA GGA AAC-3' and 5'-TTA CCT GAA TTC ATG AAG CTT CAT TTG TGC CCC GAA GAT CAA 3' with C_{Tom7} primer (Wulff *et al.*, 2001) were used to insert the underlined *EcoRI* and *HindIII* sites, respectively. The resulting PCR fragments were digested with *Clal*-*EcoRI* and *EcoRI*-*BglII*, respectively, and purified. A 3.2 kb *Clal*-*HindIII* fragment from Cf4DS (Wulff *et al.*, 2001) plasmid was isolated and self-ligated after blunt-end reaction with T4 DNA polymerase. The resulting plasmid was digested with *Clal*-*BglII* and ligated with the digested PCR fragments to produce SLJ133898. The triple c-myc coding sequence was isolated from a KS pBluescript vector after *EcoRI*-*HindIII* digestion (Piedras *et al.*, 2000) and inserted within the created *EcoRI* and *HindIII* sites from SLJ133898 generating SLJ133899. The *Clal*-*BglII* fragment from this latter plasmid was inserted in *Clal*-*BglII*-digested Cf4DS to produce SLJ133900. Finally, the 35S promoter (as an *EcoRI*-*Clal* fragment from SLJ4K1) and the final Cf-4 with the triple c-myc epitope in the B domain (as a *Clal*-*BamHI* fragment from SLJ133900) were

cloned in the binary vector SLJ7291 digested with *EcoRI* and *BamHI* to generate the vector SLJ133902.

Generation of the *Cf-4:TAP* construct

The *TAP* sequence was inserted at the 3' end of the *Cf-4* gene as an overlapping PCR product of 1.3 kb which was purified and digested with *PvuII* and *BamHI* (Rivas *et al.*, 2002). The 5' end of *Cf-4* was obtained from *Clal*-*PvuII* digestion of SLJ11922 (see above) and ligated with the 3' end containing the *TAP* sequence into a *Clal*-*BamHI*-digested pBluescript vector, generating SLJ13920. The *Clal*-*BamHI* fragment from SLJ13920 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-6, containing the native *Cf-4* promoter (Thomas *et al.*, 1997), into a pBin19 vector (Frisch *et al.*, 1995). Therefore, the resulting plasmids SLJ14040 and SLJ14180 contained the *TAP*-tagged *Cf-4* gene under the control of the 35S or the native *Cf-4* promoter, respectively.

Plant transformation and growth conditions

The binary clones SLJ11932 and SLJ133902 were mobilized into *Agrobacterium tumefaciens* LBA4404 and introduced into tobacco cultivar Petit Havana (*Nicotiana tabacum*) as described by Piedras *et al.* (2000). Plants were grown as described by Hammond-Kosack *et al.* (1998).

Generation of suspension cultures and elicitation

Cultures were generated from leaf pieces taken from 11932 and 133902 tobacco plants, as described by Piedras *et al.* (1998). For elicitation, cells were challenged with either Avr9 or Avr4, as indicated. Synthetic Avr9 (Piedras *et al.*, 1998) was used at a concentration of 15 nM. Avr4 was produced using the methylotrophic yeast *Pichia pastoris* in a fermentor. The protein accumulated to up to 125 mg l⁻¹ in a 300 ml fermentor, which is at least 24 times greater than in a shaken flask culture. The methanol-utilizing strain (Mut⁺) was used, with methanol as the only carbon source. Purification of Avr4 from the cell-free culture filtrate was achieved using a phenyl-Sepharose fast-flow column followed by a Q-Sepharose fast-flow column. The purified protein was checked on SDS-PAGE as well as with MALDI-TOF MS (matrix assisted laser Desorption/Ionization-Time of flight spectrometry) and LC-MS (liquid chromatography-Mass spectrometry). Avr4 was used at a concentration of 15 nM. At the time indicated, cells were harvested by filtration, immediately frozen in liquid nitrogen and stored at -70°C.

Agrobacterium infiltration

Stationary-phase bacterial cultures of *A. tumefaciens* GV3101 strain expressing either PVX:Avr4 (Thomas *et al.*, 1997) or PVX:Avr9 (Hammond-Kosack *et al.*, 1995) were infiltrated in *Nicotiana tabacum* leaves following the protocol described by Wulff *et al.* (2001).

Transient expression of proteins in *Nicotiana benthamiana*

Overnight bacterial cultures of *A. tumefaciens* GV3101 strain expressing the protein of interest were harvested by centrifuga-

tion (1000 g). Cells were resuspended in induction buffer (10 mM MgCl₂, 10 mM MES, pH 5.6, 150 μM acetosyringone) to an OD of 0.1, unless otherwise indicated. After 2 h at 22°C, cells were infiltrated into leaves of 4-week-old *N. benthamiana* plants. At the indicated times after *Agrobacterium*-infiltration, leaf discs used for experiments were harvested, immediately frozen in liquid nitrogen and stored at -70°C.

Determination of AOS

The production of AOS was measured by chemiluminescence from the ferricyanide-catalysed oxidation of luminol as previously described (Piedras *et al.*, 1998).

MAP kinase assays

Preparation of protein extracts and in-gel kinase assays with myelin basic protein (MBP) as a kinase substrate were performed as previously described (Romeis *et al.*, 1999).

Protein techniques

All manipulations of Cf proteins (preparation of protein extracts, deglycosylation assays, Cf homo-multimerization assays, disulphide bond detection, gel filtration, blue native gel electrophoresis, immunoprecipitations, SDS-PAGE and immunoblotting, GTP-[γ-³⁵S] overlays, and detection of Rop-like proteins by gel filtration) were performed as described previously (Rivas *et al.*, 2002).

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