

# Rapid migration in gel filtration of the Cf-4 and Cf-9 resistance proteins is an intrinsic property of Cf proteins and not because of their association with high-molecular-weight proteins

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## Summary

Gel filtration is frequently used to study the behaviour and composition of protein complexes. In previous studies, gel filtration analysis of solubilised membranes containing the tomato Cf-4 and Cf-9 resistance proteins indicated that these Cf proteins are present in an approximately 400- and 420-kDa protein complex, respectively, which contains only one Cf molecule per complex, does not contain Rho-related proteins, and does not alter in size upon elicitation. Here, we show that inactive Cf-4 and Cf-9 mutant proteins have a similar large apparent size upon gel filtration analysis. The size remains unaltered after pre-treating the samples under harsh conditions, such as boiling with SDS and incubation in 6 M urea. A similar large apparent size was found for Cf-4 and Cf-9 isolated from SDS gel and for Cf-9 expressed by insect cells. Therefore, the large apparent size observed in our studies appears to be an intrinsic property of the Cf proteins, rather than being caused by association with high-molecular-weight protein(s). Taken together, these results suggest that caution should be taken when interpreting data obtained from gel filtration of LRR-containing proteins.

**Keywords:** leucine-rich repeat proteins, receptor-like kinase, protein complex, receptor-like protein, gel filtration, resistance gene.

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## Introduction

Plants are equipped with sensitive, specific recognition systems that guide developmental programmes or perceive changes in the environment. An important recognition system confers perception of extracellular signals, such as hormones or pathogen-derived elicitors, which is often mediated by members of the large class of receptor-like kinases (RLKs) and receptor-like proteins (RLPs) of which most carry extracellular leucine-rich repeats (LRRs) (The Arabidopsis Genome Initiative, 2000). Another important class of LRR proteins consists of cytoplasmic resistance proteins, which act inside the cell to confer recognition of invading pathogens. The current challenge is to unravel how these proteins function.

As RLKs and RLPs are type I membrane proteins, biochemical studies on the signalling complexes involving such receptor proteins require special attention. To analyse such complexes biochemically, they are often solubilised

with mild detergents and subsequently analysed using non-denaturing biochemical methods. A well-established method to characterise protein complexes is gel filtration, a technique which has successfully been used to study the receptor complexes of the RLKs CLAVATA-1 (CLV1, Trotochaud *et al.*, 1999), which regulates meristem fate in *Arabidopsis*, and Wak1 (Park *et al.*, 2001), an *Arabidopsis* wall-associated receptor with yet unknown function. Furthermore, gel filtration analysis has been applied to the tomato RLPs Cf-4 and Cf-9, which mediate recognition of the elicitor proteins AVR4 and AVR9, respectively, of the fungal pathogen *Cladosporium fulvum* (Rivas *et al.*, 2002a,b). These studies revealed that CLV1, in the presence of the CLV3 ligand, assembles into a protein complex of 450 kDa that also includes the kinase-associated protein phosphatase (KAPP), a Rho-related GTPase (Trotochaud *et al.*, 1999) and most likely also the RLP CLV2 (Jeong *et al.*,

1999). Similarly, Wak1 assembles into a 500-kDa signalling complex, which includes AtGRP-3, a glycine-rich protein (Park *et al.*, 2001). Furthermore, resistance protein Cf-9 appeared to be part of a large complex of approximately 420 kDa. However, unlike the CLV complex, the Cf-9 complex did not change in size upon elicitation and contained only one molecule of Cf-9 per complex, whereas Rho-related proteins were not detected (Rivas *et al.*, 2002a). Similar results were obtained for the slightly smaller Cf-4 protein, which appeared to be part of an approximately 400 kDa complex (Rivas *et al.*, 2002b).

Here, we focus on the biological relevance of the previously identified Cf complexes. We show that inactive Cf-4 and Cf-9 mutants still have a large apparent size upon gel filtration analysis. Further biochemical characterisation revealed that their large apparent size cannot be ascribed to association of Cf proteins with high-molecular-weight proteins, but rather to an intrinsic property of the Cf proteins themselves. This study indicates that the conclusions drawn from gel filtration studies with LRR-proteins should be interpreted with great caution.

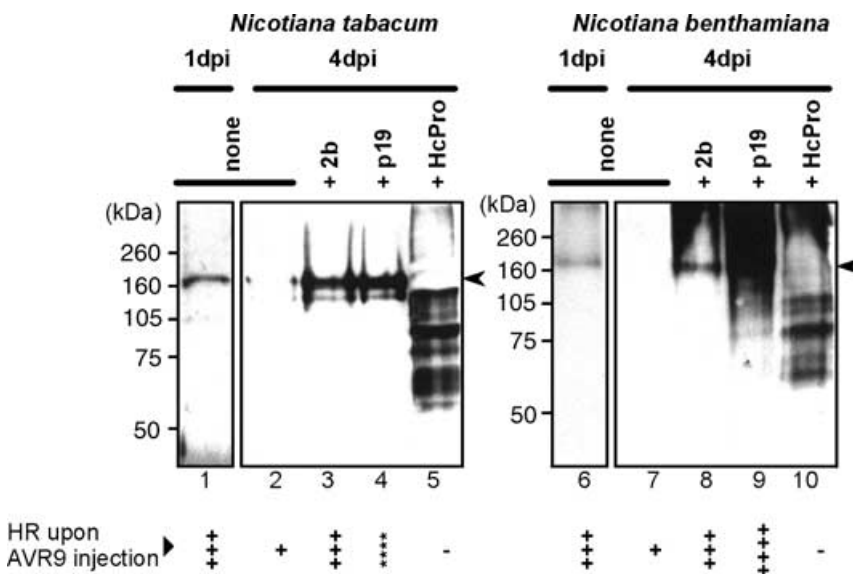
**Results**

*Correlation between accumulation and activity of agroinfiltrated myc-Cf-9*

As this study deals with the characteristics of epitope-tagged Cf proteins, experiments proving that these forms represent the Cf in their active states are crucial. In previous studies, a clear correlation between Cf-9 accumulation and Cf-9 activity was found by *Agrobacterium* dilution experiments (Rivas *et al.*, 2002a) and co-expression of the Cf-9

gene with the silencing inhibitor p19 (Voinnet *et al.*, 2003). We now supplement these data with AVR9 injections that show the sensitivity of tissue at certain time points after agroinfiltration, and the use of silencing inhibitors 2b and HcPro in *Nicotiana tabacum* and *N. benthamiana*.

Agroinfiltration of myc-Cf-9 without silencing inhibitor, in both *N. tabacum* and *N. benthamiana*, results in an accumulation of myc-Cf-9 at 1 day post infiltration (dpi) (Figure 1, lanes 1 and 6), followed by a quick decrease to very low levels at later time points (Figure 1, lanes 2 and 7, and data not shown). The development of a hypersensitive response upon injection of AVR9 into these tissues clearly shows that the tissue is less sensitive at 4 than at 1 dpi (Figure 1). In the presence of silencing inhibitors 2b or p19, myc-Cf-9 accumulates to much higher levels at 4 dpi, and the sensitivity of the corresponding tissue for AVR9 injection demonstrates a clear correlation between myc-Cf-9 accumulation and myc-Cf-9 activity both in *N. tabacum* and in *N. benthamiana* (Figure 1, lanes 3, 4, 8 and 9). Interestingly, co-expression of myc-Cf-9 with HcPro results in the accumulation of truncated myc-Cf-9 products, and consistent with the absence of a full-size 170-kDa glycosylated myc-Cf-9 protein, the corresponding tissue is not AVR9-sensitive (Figure 1, lanes 5 and 10). Co-expression of myc-Cf-9 with p19 results in the highest accumulation in *N. benthamiana*, as was shown previously by Voinnet *et al.* (2003). In *N. tabacum*, however, agroinfiltration of p19 alone already induces a strong necrotic response (indicated with ★★★★★ in Figure 1). The high sensitivity for AVR9 injection of leaves of *N. benthamiana*, agroinfiltrated with myc-Cf-9 and p19, indicates that this myc-Cf-9 represents functional Cf protein, and we therefore followed this approach to transiently express Cf proteins for our analysis.



**Figure 1.** Direct correlation between accumulation and activity of myc-Cf-9 in agroinfiltrated leaves.

Cf-9 was co-expressed either without or with silencing inhibitors 2b, p19 or HcPro in *Nicotiana tabacum* or *N. benthamiana*. At 1 or 4 days post infiltration (dpi), proteins were extracted from the tissue and subjected to immunoblot analysis to determine myc-Cf-9 accumulation (the arrowhead indicates 170-kDa glycosylated myc-Cf-9) or AVR9 was injected to determine the sensitivity of the tissue (fully sensitive (++++) to insensitive (-)). Expression of p19 alone in *N. tabacum* results in autonecrosis (indicated by ★★★★★).

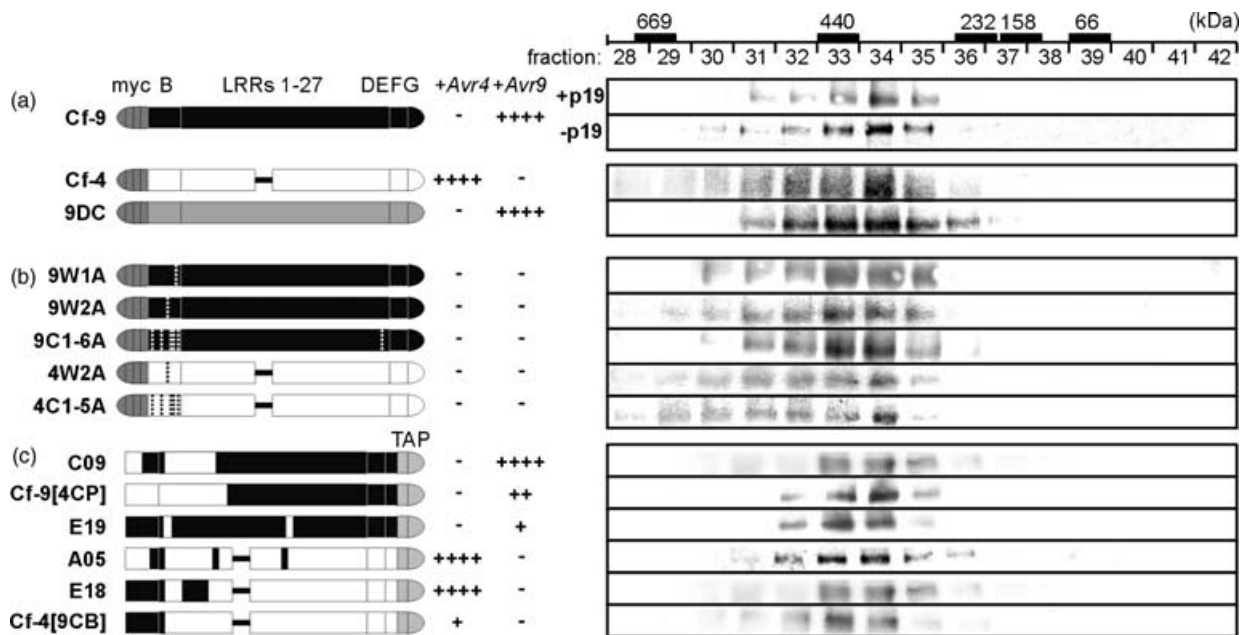
### Active and inactive Cf proteins show similar gel filtration profiles

Previously, gel filtration of solubilised membranes containing tagged Cf proteins was used to show that Cf-4 and Cf-9 are present in complexes of approximately 400 and 420 kDa, respectively (Rivas *et al.*, 2002a,b). We now used this technique to test whether inactive Cf-4 and Cf-9 mutants are still present in a protein complex of similar size. To assay large numbers of different Cf-4 and Cf-9 mutants, we often co-expressed myc-Cf proteins and mutant derivatives with p19 in *N. benthamiana* and we used a Superose 6HR column for our analysis. This column that allows quick fractionation was also used to analyse the CLV1 and Wak1 complexes (Park *et al.*, 2001; Trotochaud *et al.*, 1999).

Gel filtration of proteins present in solubilised membranes, obtained from agroinfiltrated leaves co-expressing myc-Cf-9 and p19, showed that myc-Cf-9 elutes as a complex that is slightly smaller than 440 kDa (Cf-9 + p19, Figure 2a). This elution profile is similar to that of myc-Cf-9 produced without p19 (Cf-9-p19, Figure 2a), and the size of the complex is consistent with data presented previously by Rivas *et al.* (2002a). A similar profile was found for the myc-Cf-4 protein (Cf-4, Figure 2a), again consistent with the data previously described by Rivas

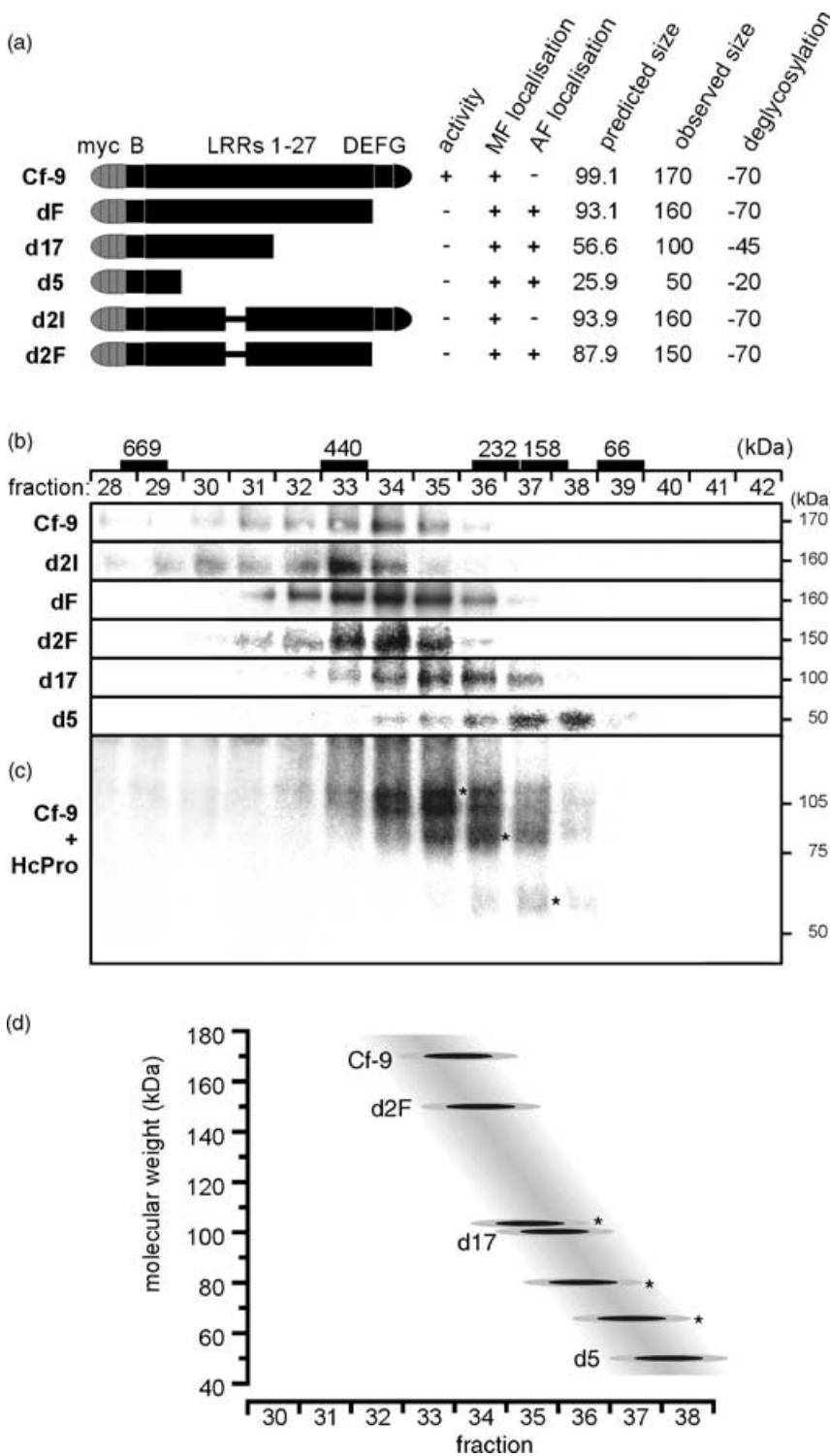
*et al.* (2002b). In addition, myc-tagged 9DC, a Cf protein that also confers AVR9 recognition and has a similar size to Cf-9 (Van der Hoorn *et al.*, 2001a), elutes similar to myc-Cf-9 (9DC, Figure 2a), suggesting that this protein also participates in a similar complex.

With the established assays of agroinfiltration, p19-mediated Cf protein production and Superose 6HR gel filtration, we now examined whether inactive Cf-4 and Cf-9 mutants also elute as complexes with similar large sizes, or whether their elution profile is significantly altered. Inactive mutants that were analysed were the 9W1A and 9W2A mutants of Cf-9, and 4W2A mutant of Cf-4, carrying tryptophan to alanine substitutions in the B-domain, and the 9C1-6A mutant of Cf-9 and 4C1-5A mutant of Cf-4 in which cysteines in the LRR-flanking domains have been substituted by alanine residues (Van der Hoorn *et al.*, in preparation). Additionally, we analysed the elution profiles of TAP-tagged versions of domain swap mutants Cf-4[9CB] and Cf-9[4CP] and Cf-4/Cf-9-shuffled clones A05, C09, E18 and E19 (Wulff *et al.*, 2001; Van der Hoorn *et al.*, in preparation). These mutants vary in necrosis-inducing activity, as indicated in Figure 2(b,c). Unexpectedly, both active and inactive Cf-4 and Cf-9 proteins show a similar elution profile (Figure 2b,c). The analysis of the mutants shown in Figure 2(c) was also performed using a Sephacryl S-300 column, with similar results (data not shown).



**Figure 2.** Gel filtration profiles of various active and inactive epitope-tagged Cf proteins.

From left to right: name of mutant; schematic representation of (mutant) Cf protein; activity upon co-expression with *Avr4* and *Avr9*, respectively (fully active (++++), to inactive (-)), and elution profile upon gel filtration. Myc-tagged (a, b) or TAP-tagged (c) Cf proteins were co-expressed with p19 by agroinfiltration of *Nicotiana benthamiana*, and microsomal fractions were prepared at 4 days post agroinfiltration. When p19 was not co-infiltrated, proteins were extracted at 1 dpi. Microsomal fractions were solubilised with octylglucoside, and the proteins were fractionated on Superose 6HR. Fractions were analysed by immunoblotting with anti-c-myc (a, b) or PAP antibody (c). The gel filtration column was calibrated with large-molecular-weight calibration markers (indicated as horizontal bars at the top). Shown elution profiles are representative for at least two independent experiments.



**Figure 3.** Characterisation and gel filtration of Cf-9 deletion mutants.

(a) Features of wild-type myc-Cf-9 and myc-Cf-9 deletion mutants. From left to right: name of mutant; schematic representation of the deletion mutants (F, transmembrane domain); activity upon co-expression with *Avr9*; presence of mutant protein in microsomal fraction (MF) and/or apoplastic fluid (AF); predicted size of the unglycosylated protein in kilodaltons (kDa); observed size of Cf-9 protein during SDS-PAGE in kDa; and observed size difference after deglycosylation with PGNaseF in kDa.

(b, c) Gel filtration profiles of Cf-9 deletion mutants (b), and HcPro-induced Cf-9 breakdown products (c). Expression, fractionation and detection were performed as described in Figure 2.

(d) Linear relationship between molecular weight and elution time of Cf-9 deletion mutants. Data for this graph are derived from (c) (indicated by ★) and from a gel filtration experiment with a mixture of Cf-9, d2F, d17 and d5.

*Cf-9 deletion mutants also elute with large apparent sizes*

As all inactive tagged Cf mutants described above elute as large complexes similar to active tagged Cf proteins, a different strategy is required to identify regions in tagged Cf proteins that are essential for the formation and/or

stability of such a complex. To this aim, we focussed on the Cf-9 protein and generated a number of myc-tagged Cf-9 deletion mutants to study their characteristics (Figure 3).

Cf-9 mutants dF, d17 and d5 lack the C-terminal transmembrane domain, and are trimmed from the F-domain (dF), LRR17 (d17), and LRR5 (d5) (Figure 3a). Cf-9 mutant d2I

lacks the two LRRs that are also absent in Cf-4, whereas mutant d2F both lacks these two LRRs and the C-terminal F- and G-domains. As indicated in Figure 3(a), none of these mutants is active when co-expressed with *Avr9*, whereas the proteins are detected in all cases. Mutants carrying a C-terminal deletion (dF, d17, d5 and d2F) were both detected in apoplastic washing fluid (AF) as well as in membrane fractions (MF) (Figure 3a), indicating that these proteins accumulate extracellularly but also still associate with membranes.

Comparison of the predicted size and the observed size of the deletion mutants suggests that all proteins are heavily glycosylated, similar to the wild-type Cf-9 protein itself (Figure 3a). Indeed, deglycosylation with PGNaseF and subsequent analysis by SDS-PAGE revealed sizes that corresponded to the predicted molecular weight for the unglycosylated mutant proteins (summarised in Figure 3a). Deglycosylation assays also showed that dF, d17 and d5 carry approximately 70, 45 and 20 kDa of glycosylation, respectively. This suggests that Cf-9 is equally glycosylated over three regions: domain B to LRR 5 (seven putative glycosylation sites and *c.* 20 kDa of glycosylation), LRRs 6–17 (eight sites with *c.* 25 kDa of glycosylation), and LRR 18 to domain E (seven sites with *c.* 25 kDa of glycosylation).

Surprisingly, when the deletion mutants present in solubilised microsomal fractions were subjected to gel filtration, mutants lacking the two LRRs (d2I), or the F- and G-domains (dF), or both the two LRRs and the F- and G-domains (d2F), still eluted with a large apparent size, which is only slightly smaller than myc-Cf-9 (Figure 3b). Moreover, mutants trimmed from LRR 17 or LRR 5 (d17 and d5, respectively), elute later (Figure 3b), but not as late as predicted for monomeric proteins of 100 and 50 kDa, respectively. Similar results were obtained when these deletion mutants were analysed using a Sephacryl S-300 column (data not shown). Furthermore, similar observations were made upon gel filtration of HcPro-mediated Cf-9 truncation products (Figure 3c), as also these fragments elute in later fractions but still not as late as predicted for monomeric proteins.

A more detailed gel filtration analysis was performed with a mixture of myc-Cf-9, d2F, d17 and d5 deletion mutants, and their molecular weight was plotted against the peak fraction of their elution profile (Figure 3d). We also included the 65-, 80- and 110-kDa HcPro-induced Cf-9 breakdown products of Figure 3(c) (indicated with an asterisk in Figure 3c,d). This graph indicates the presence of a linear relationship between the molecular weight and the elution times of myc-Cf-9 and its deletion mutants (Figure 3d).

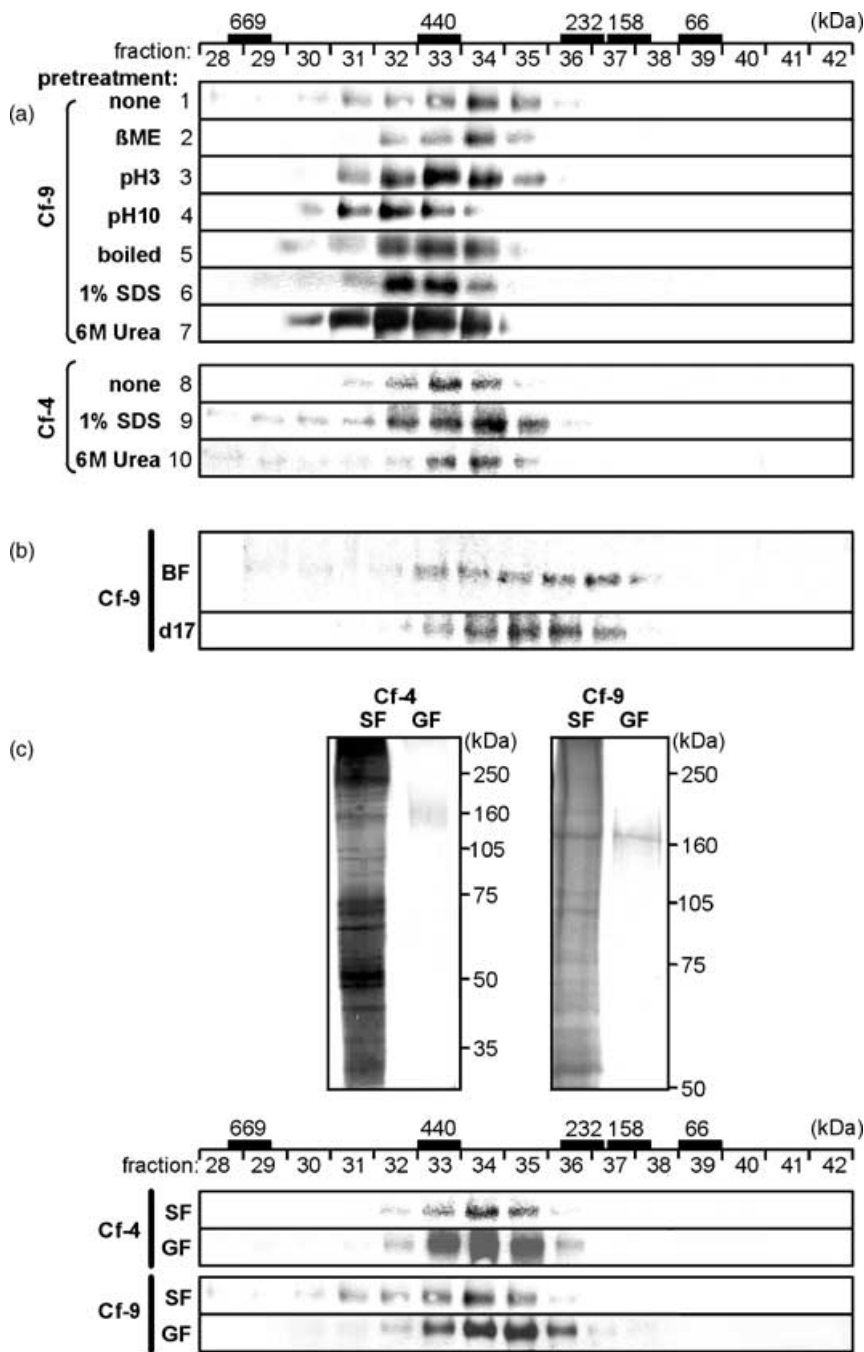
#### *Harsh treatments of myc-Cf-9 do not alter its elution profile*

The observation that all mutant Cf proteins analysed so far elute as large apparent protein complexes prompted us to

attempt to disrupt the myc-Cf complex using harsh treatments, followed by centrifugation to remove aggregates, and subsequent standard gel filtration analysis. In all cases, hardly any signals were detected in the void volume of the gel filtration column (fractions 0–22, data not shown), indicating that hardly any insoluble aggregates were formed during gel filtration. Treatment of solubilised fractions containing myc-Cf-9 with reducing agents such as  $\beta$ -mercaptoethanol, or incubation at low or high pH did not significantly change the elution profile (compare profiles 1–4, Figure 4a). Surprisingly, the solubilised fraction could even be boiled with or without SDS, or treated with denaturing agents (6 M urea), without changing the elution profiles of myc-Cf-4 or myc-Cf-9 (compare profiles 1 and 8 with 5–7 and 9–10, respectively, Figure 4a). There are several explanations for these observations. First, the Cf protein complexes may be extremely stable. However, this is unlikely as myc-Cf proteins migrate as monomeric proteins on an SDS gel. Second, the Cf protein complexes may re-associate on the column. Although this cannot be excluded at this stage, it seems unlikely as it requires a quick re-naturation of the proteins and complex assembly before the components are separated on the column. Third, there is no protein complex associated with Cf proteins, and their migration is an intrinsic property of the Cf proteins under these conditions.

#### *Cf proteins produced in insect cells or isolated from SDS gel have similar elution profiles*

The unaltered apparent mass for myc-Cf-9 deletion mutants and myc-Cf proteins treated under harsh conditions suggests that the behaviour of myc-Cf proteins upon gel filtration is perhaps an intrinsic property of the Cf proteins themselves, rather than being caused by high-molecular-weight proteins associated with the Cf proteins. To test this hypothesis, we performed gel filtration analysis with C-terminally truncated, His-tagged Cf-9 protein, which was produced by insect cells and purified under denaturing conditions (Cf-9<sup>H</sup>; Luderer *et al.*, 2001). Despite its source, this 116-kDa glycosylated form of Cf-9 still elutes as a large complex and has an elution profile very similar to the 100-kDa d17 Cf-9 deletion mutant (Figure 4b). Furthermore, we separated solubilised microsomal membrane proteins containing myc-Cf-4 or myc-Cf-9 on an SDS gel (lanes SF in top panels of Figure 4c), and the region containing the 160–180-kDa proteins was excised from the gel. Proteins were eluted from the gel fragment and were re-run on an SDS gel (lanes GF in top panels of Figure 4c), or subjected to gel filtration analysis (profiles GF in lower panels of Figure 4c). Significantly, both myc-Cf-4 and myc-Cf-9 purified from the gel have an elution profile very similar to that of myc-Cf-4 or myc-Cf-9 present in the original solubilised membranes (lower panels of Figure 4c, compare profiles SF and GF).



**Figure 4.** Gel filtration analysis of Cf proteins treated under harsh conditions, produced by insect cells or isolated from SDS gel.

(a) Elution profiles of solubilised myc-Cf proteins after harsh treatments. Solubilised microsomal fractions containing myc-Cf-4 or myc-Cf-9 were treated with various agents or heated (see Experimental procedures) and centrifuged, and proteins present in the supernatant were immediately fractionated by gel filtration and analysed by immunoblotting. Gel filtration was carried out as described in Figure 2.

(b) Elution profile of Cf-9 produced in baculovirus-infected insect cells (BF). Samples containing purified His-tagged Cf-9 were solubilised and fractionated by gel filtration. Fractions were analysed by immunoblotting using a Cf-9 antibody. Gel filtration was carried out as described in Figure 2. The d17 elution profile is the same as in Figure 3(b).

(c) Elution profile of myc-Cf-4 and myc-Cf-9 isolated from SDS gel. Proteins present in solubilised membranes containing myc-Cf-4 or myc-Cf-9 were separated by SDS-PAGE, and the 160–180-kDa region was excised from the gel. Proteins were eluted, freeze-dried and dissolved in solubilisation buffer. Both the gel-isolated Cf protein (GF) and the solubilised membranes from which the Cf protein was isolated (SF) were visualised on a Coomassie-stained SDS-PAGE (top) and fractionated by gel filtration, followed by SDS-PAGE and immunoblot analysis of the collected fractions using myc antibody (bottom). Gel filtration was carried out as described in Figure 2.

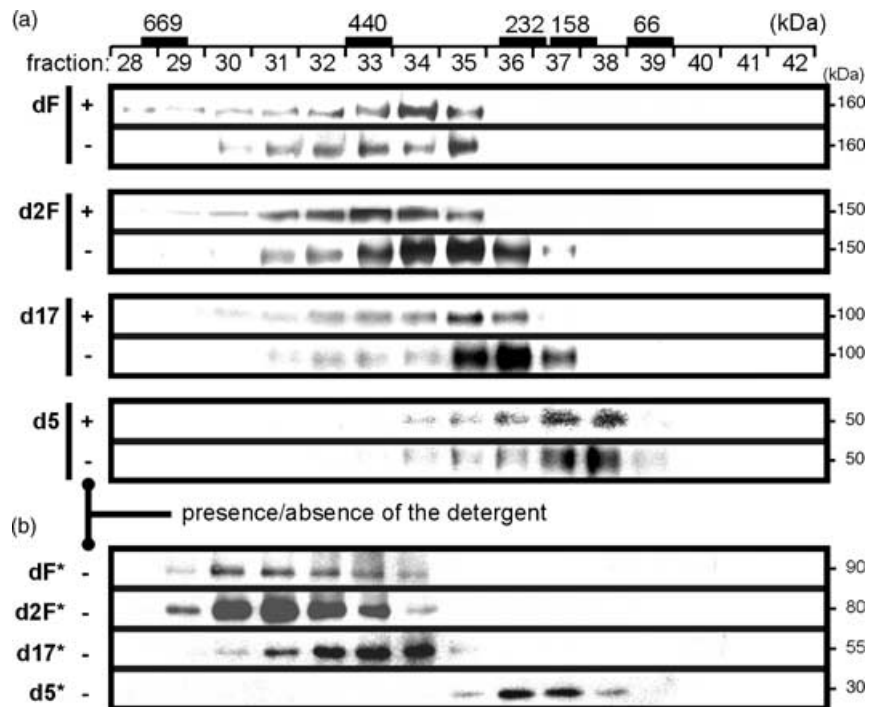
*Effect of detergent and deglycosylation on the elution profile of the Cf-9 protein*

The large apparent size of Cf proteins during gel filtration can have different causes. To investigate the contribution of the detergent to the apparent size, we performed gel filtration analysis of myc-Cf-9 in the presence or absence of octyl glucoside. Calibration of the gel filtration column with high-molecular-weight marker proteins showed that the presence or absence of detergent does not alter the

elution profile of these proteins (Figure 5a, top). To obtain soluble myc-Cf-9 protein without the use of detergent, soluble extracts (see Experimental procedures) were prepared from tissues agroinfiltrated with myc-Cf-9 deletion mutants lacking the transmembrane domain (dF, d17, d5 and d2F, Figure 3a). When fractionated in the presence of detergent, deletion mutants present in these soluble extracts show elution profiles similar to those obtained for solubilised membranes (compare Figure 5a, profiles marked with '+' with the profiles shown in Figure 3b).

**Figure 5.** Effect of detergent and deglycosylation on the behaviour of soluble myc-Cf-9 deletion mutants.

(a, b) Soluble extracts were obtained from agroinfiltrated tissue at 4 dpi without using detergent. These extracts were fractionated on Superose 6HR (a) with (+) or without (-) detergent, or (b) first deglycosylated with PGNaseF (★) and then fractionated without detergent (-). Elution profiles of high molecular weight markers were the same in the presence or absence of detergent (top).



However, when fractionated in the absence of detergent, the apparent sizes of the larger deletion mutants dF, d2F and d17 decrease with approximately 100 kDa, whereas the apparent size of mutant d5 remains unaltered (Figure 5a, profiles marked with '-'). Thus, the detergent contributes approximately 100 kDa to the apparent size of the large deletion mutants lacking the transmembrane domain. However, also in the absence of detergent, the Cf-9 mutant proteins still elute with a much larger apparent size than expected.

To further investigate the contribution of glycosylation to the apparent size of myc-Cf-9, soluble extracts containing myc-Cf-9 deletion mutants were deglycosylated in the absence of any detergents and fractionated by gel filtration in the absence of detergent. In five independent runs, deglycosylated deletion mutants dF, d2F, d17 and d5 eluted in fractions 29–35, 29–35, 30–36 and 35–38, respectively (examples are shown in Figure 5b). Surprisingly, deglycosylation, resulting in a smaller molecular weight on SDS gel (Figure 5b, indicated at the right), appears to increase the apparent size, as the proteins elute earlier from the column (compare Figure 5a,b). Thus, even without detergent and without glycosylation, deletion mutants dF, d2F, d17 and d5 have an apparent size (350–550, 350–550, 300–500 and 100–250 kDa, respectively) that is still about sixfold larger than expected from their actual molecular weight (approximately 90, 80, 55 and 30 kDa, respectively). This observation indicates that the large apparent size is an intrinsic property of the (unglycosylated) Cf protein itself.

## Discussion

### Indications for the existence of a Cf complex

Several earlier observations suggest that Cf proteins probably act in a complex. First, functional Cf proteins lack an obvious cytoplasmic domain for downstream signalling, and therefore they probably associate with other proteins that can provide this function (Joosten and De Wit, 1999; Rivas and Thomas, 2002). Second, no direct interaction was found between Cf-9 and its proposed ligand, the AVR9 elicitor (Luderer *et al.*, 2001), which suggests the involvement of, for example, the high-affinity binding site for AVR9, which has been detected in membranes of solanaceous plants (Kooman-Gersmann *et al.*, 1996). Third, many RLKs and RLPs appear to act in a complex with other proteins (Becraft, 2002; Torii, 2000). A recent example is the interaction between the RLKs BRI1 and BAK1, which act in brassinosteroid signalling (Li *et al.*, 2002; Nam and Li, 2002). Finally, resistance proteins like Cf-4 and Cf-9 probably act by 'guarding' cellular components, a mechanism that seems to be favoured by balancing selection in nature (Van der Hoorn *et al.*, 2002).

The structural similarity of Cf proteins with CLV2 suggests that they might participate in a complex that also includes a RLK-like CLV1 and perhaps a KAPP and a Rho-related protein (Joosten and De Wit, 1999; Rivas *et al.*, 2002a). Examination of the Cf-9 complex indeed revealed that Cf-9 migrates at an apparent size of approximately 420 kDa, which is similar to the approximately 450-kDa

CLV complex. However, unlike the CLV complex, the Cf-9 complex did not change in size upon elicitation, appeared not to contain Rho-related proteins and contained only one molecule of Cf-9 per complex (Rivas *et al.*, 2002a). Similar results were obtained for Cf-4 (Rivas *et al.*, 2002b). Although these experiments suggested the existence of a protein complex associated with Cf proteins, the composition of this complex remained unclear.

#### *Direct correlation between accumulation and activity of myc-Cf protein*

Here, we further investigated the biological relevance of the identified Cf complexes. For these experiments, it is essential that the detected, epitope-tagged Cf protein represents the protein in its active state. For Cf-9, several experiments indicate that there is a strict correlation between the accumulation level of myc-Cf-9 and the sensitivity of the corresponding tissue for AVR9. These observations were made by *Agrobacterium* dilution experiments (Rivas *et al.*, 2002a), time-course experiments (Figure 1; Voinnet *et al.*, 2003), and co-expression with silencing inhibitors p19 (Voinnet *et al.*, 2003), 2b and HcPro (Figure 1). Together, these data strongly suggest that the detected myc-Cf protein represents the active Cf protein, and that myc-tagged Cf proteins can be used to study the Cf protein in its active state.

Furthermore, a few additional intriguing observations were made from the experiments described here. First, the rapid decline in Cf-9 accumulation after agroinfiltration (Figure 1; Voinnet *et al.*, 2003) indicates that Cf proteins have a relatively high turn-over as compared to other proteins, such as GFP (Johansen and Carrington, 2001). Second, we observed a HcPro-induced breakdown of the extracytoplasmic domains of N-terminally tagged myc-Cf-9 (Figure 1), which is probably not a result of the action of HcPro itself, as this cysteine protease is most likely localised in the cytoplasm (Carrington *et al.*, 1989). Third, the increased AVR9 sensitivity of the tissue with a highly elevated accumulation of myc-Cf9 (co-expression with p19, Figure 1; Voinnet *et al.*, 2003) suggests that the amount of Cf protein is the limiting factor in the response of the plant upon elicitor perception, and that all other components required for induction of an HR are present in excess. Although these topics are not the focus of this study, these observations warrant future studies.

#### *Elution profile of Cf proteins during gel filtration*

In this study, further examination of the behaviour of Cf-4 and Cf-9 and derived mutants during gel filtration revealed that the apparent size of Cf proteins is an intrinsic property of the Cf protein itself, rather than being caused by association with high-molecular-weight proteins. The first

indications in this direction were provided by the analysis of inactive mutants of Cf-4 and Cf-9 made by mutagenesis, or Cf-4/Cf-9 domain swap and shuffling. Both active and inactive Cf mutants still migrate with similar large apparent sizes (Figure 2b,c). In addition, a series of inactive deletion mutants of Cf-9 and HcPro-induced breakdown products of Cf-9 also migrate with large apparent sizes (Figure 3b,c), and there is a linear relationship between their molecular weight and their elution times (Figure 3d). More significantly, the large apparent size of Cf-4 and Cf-9 is not altered by harsh treatments, such as boiling with SDS, or treatment with 6 M urea (Figure 4a). Finally, similar large apparent sizes were also observed for Cf-9 produced in insect cells (Figure 4b) and for Cf-4 and Cf-9 isolated from SDS gel (Figure 4c).

Several possible causes for the large apparent size of Cf proteins during gel filtration under native conditions can be considered. First, the presence of a hydrophobic transmembrane domain can be ruled out as one of the causes, as deletion mutants of Cf-9 lacking this domain (dF and d2F) have similar apparent sizes as Cf-9 itself and its mutant d2I, respectively (Figure 3b). Second, homodimerisation of Cf proteins can also be excluded as a cause, as previously reported immunoprecipitation assays with solubilised membranes containing both myc-Cf and TAP-Cf proteins showed that homodimerisation of Cf proteins did not occur (Rivas *et al.*, 2002a,b). Third, glycosylation seems to contribute only marginally to the large apparent size, as deglycosylation increases, rather than decreases, the apparent size of the Cf protein (Figure 5b). This observation is unexpected and perhaps results from interactions of the sugar moieties that are attached to the proteins with the column material, which is composed of cross-linked agarose. Abolishing this interaction may result in a relatively fast elution. Alternatively, an altered overall structure of the deglycosylated Cf protein itself could result in a relatively fast elution. Fourth, in addition to possible adhesive interactions of myc-Cf proteins with the column material, repulsive interactions, which may accelerate the migration through the column, could also explain the observed large apparent size. However, this is unlikely as a similar large apparent size was also found for myc-Cf proteins separated on blue native gel, which is composed of a polyacrylamide matrix and Sephacryl S-300, which is composed of cross-linked acrylamide and allyl dextran (Rivas *et al.*, 2002a,b). Fifth, the contribution of the detergent to the large apparent size was found to be significant, as we demonstrated that the presence of octylglucoside in the elution buffer adds approximately 100 kDa to the apparent size of mutants lacking the transmembrane domain (Figure 5a). This contribution may be significantly more for full-length Cf proteins. Sixth, Cf proteins may not have a globular structure like the standard marker proteins that are used to calibrate the gel filtration column. Even without detergent and

glycosylation, Cf-9 deletion mutants have a sixfold larger apparent size than expected (Figure 5b). This could be because of the fact that Cf proteins predominantly consist of LRRs, which give the proteins a rod-like, curved or coiled structure (Kajava and Kobe, 2002). Denaturing treatments should disrupt these structures, but may still yield an extended protein that behaves similarly during gel filtration. In conclusion, both the detergent and the non-globular structure of Cf proteins contribute to the large apparent size of Cf-4 and Cf-9 during gel filtration.

Our observation that the major cause of the large apparent size of Cf-4 and Cf-9 during gel filtration is probably a result of an intrinsic property of the Cf proteins does not exclude the possibility that Cf proteins participate in a complex with additional protein partners *in vivo*. It is still possible that Cf proteins interact with small proteins of a size below the resolution of gel filtration (<70 kDa). In addition, active Cf-containing signalling complexes may be transient in nature or existing complexes might have been disrupted during protein extraction and/or solubilisation. However, our observations show that extreme caution should be taken with gel filtration techniques to study sizes of complexes containing LRR proteins. The presence of proteins associated with LRR proteins cannot be concluded from gel filtration studies alone, without the identification of these additional proteins, followed by additional experiments such as immunoprecipitations. However, gel filtration analysis is a versatile technique if used properly, and it is worth to explore the behaviour of Cf protein complexes under different conditions. We are currently adopting alternative approaches, such as yeast two-hybrid screening and tandem-affinity purification, to understand how Cf proteins interact with other proteins to mediate recognition of the matching AVR protein and initiate subsequent downstream signalling.

## Experimental procedures

### Cloning procedures

Binary vectors encoding silencing inhibitors 2b, HcPro and p19 were kindly provided by Professor David Baulcombe. Binary vectors pRH385, pRH395 and pRH511, carrying a 35S-driven gene encoding myc-tagged Cf-9, Cf-4, and 9DC, respectively, and derived vectors pRH477, pRH468, pRH461, pRH520 and pRH462, encoding mutants 9W1A, 9W2A, 4W2A, 9C1-6 and 4C1-5A, respectively, will be described elsewhere (Van der Hoorn *et al.*, in preparation). The construction of TAP-tagged versions of Cf-4/Cf-9 domain swap constructs and shuffled clones (Wulff *et al.*, 2001) will also be described elsewhere (Van der Hoorn *et al.*, in preparation). Plasmids pRH1, pRH5 and pRH18 and primers CF1, SR2 and BR1 have been described previously by Van der Hoorn *et al.* (2001b). PCR was performed with Pfu polymerase (Stratagene, La Jolla, CA, USA), and the authenticity of all cloned PCR fragments was confirmed by sequencing. All DNA manipulations were performed using standard protocols (Sambrook *et al.*, 1989).

Binary vector pRH397 (encoding mutant dF, having C-terminal sequence ...EEEDISWQ) was generated as follows. The 3' part of Cf-9, encoding LRRs 25–27 and domains D and E, was amplified with primers 5'-gcattattggagatcttggg-3' and 5'-aaaactgcagc-tactgccaactgatcattggg-3' and cloned with *Bgl*II and *Pst*I (underlined) into pRH143, a pRH18 derivative, resulting in pRH150. The *Bam*HI–*Eco*RI fragment containing the 5' part of the expression cassette was subsequently cloned from pRH150 into pRH385 (myc-Cf-9), resulting in pRH397. Binary vector pRH509 (encoding mutant d17, having C-terminal sequence ...LILLDLGS) was constructed by replacing the *Bam*HI–*Eco*RI fragment of pRH385 (myc-Cf-9), containing the 5' part of the expression cassette starting in LRR 17, by a PCR fragment amplified from the polyadenylation region of pRH1 with primers 5'-gatctctgagtagggatcctagagcttggacc-3' and 5'-tctagaattccagctcgatatg-3', using *Bam*HI and *Eco*RI restriction sites (underlined). Binary vector pRH517 (encoding mutant d5, having C-terminal sequence ...ISSTIPGS) was constructed by replacing the *Clal*–*Bam*HI fragment encoding LRRs 1–17 of pRH509 (mutant d17) with a fragment encoding LRRs 1–5, obtained by amplifying Cf-9 with primers CF1 and SR2. Binary vector pRH458 (encoding mutant d2I) was constructed by replacing a *Clal*–*Bam*HI fragment encoding LRRs 1–17 of pRH385 (myc-Cf-9) by a fragment encoding LRRs 1–17 lacking LRRs 11 and 12, amplified from a pRH5 derivative using primers CF1 and BR1. Binary vector pRH479 (encoding mutant d2F) was constructed by replacing the *Bam*HI–*Eco*RI fragment containing the 5' end of Cf-9 and polyadenylation region of pRH458 (mutant d2I) with a fragment obtained from pRH150, which carries the C-terminal deletion of the F- and G-domains.

### Protein expression and extraction

Agroinfiltration of tobacco (*N. tabacum*) and *N. benthamiana* plants was performed as described previously by Van der Hoorn *et al.* (2000). Co-expression of multiple constructs was carried out by mixing *Agrobacterium* cultures. Tissues were harvested at 1 dpi (tobacco) or 4 dpi (*N. benthamiana*), unless indicated otherwise. Microsomal fractions were isolated and solubilised as described previously by Rivas *et al.* (2002a). Soluble extract was obtained during isolation of microsomal fractions by taking the supernatant after the last centrifugation step. Extraction buffer contained 50 mM Tris, pH 7.5, 150 mM NaCl, and protease inhibitor cocktail (Complete tablet, Roche, Mannheim, Germany). Solubilisation buffer consisted of the extraction buffer supplemented with 1.2 w/v octyl glucoside (Roche). AVR9 injections of agroinfiltrated sectors was administered with 10 µg ml<sup>-1</sup> synthetic AVR9 (Kooman-Gersmann *et al.*, 1998).

### Other biochemical procedures

Protein concentration was measured using the BCA protein assay (Perbio, Bonn, Germany). Deglycosylation using PGNaseF (New England Biolabs, Frankfurt, Germany) was performed according to the manufacturer's instructions. Harsh treatments of the myc-Cf complexes were carried out as follows: solubilised membranes containing myc-Cf-4 or myc-Cf-9 were supplemented with 10% β-mercaptoethanol (βME), 100 mM acetic acid (pH 3), 100 mM Na<sub>2</sub>CO<sub>3</sub> (pH 10) or 6 M urea and incubated for 1 h at room temperature. Other samples were boiled for 20 min, with or without 1% w/v SDS. The samples were centrifuged for 5 min at 13 000 g, and the supernatant was subjected to gel filtration analysis.

C-terminally His-tagged, extracellular Cf-9, produced by insect cells was kindly provided by Dr Rianne Luderer. This sample was purified under denaturing conditions (Luderer *et al.*, 2001), solubilised with 1.2% w/v octyl glycoside and centrifuged at 13 000 g, and the supernatant was subjected to gel filtration analysis.

Gel-isolated myc-Cf-4 and myc-Cf-9 were obtained by excising the 160–180-kDa region from an SDS–PAGE gel loaded with microsomal fractions containing myc-Cf-9. Proteins were eluted overnight in water, and the eluate was freeze-dried, dissolved in solubilisation buffer and subjected to gel filtration analysis.

#### Gel filtration analysis

Gel filtration was performed at 4°C using a Superose 6HR 10/30 column (Amersham Pharmacia Biotech, Bucks, UK) and the solubilisation buffer as eluent at a flow rate of 0.2 ml min<sup>-1</sup>, according to the instructions of the manufacturer. Fractions of 0.4 ml were collected and analysed by immunoblotting. The column was calibrated with bovine serum albumin (66 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) (HMW gel filtration calibration kit, Amersham Pharmacia Biotech). Where indicated, a Sephacryll S-300 column was used as previously described by Rivas *et al.* (2002a).

#### SDS–PAGE and immunoblotting

Proteins were separated on 7.5% SDS gel (Laemmli, 1970) and transferred onto nitrocellulose (Protran, Schleicher and Schuell, Germany). Antibodies used were antimyc (rabbit polyclonal, Santa Cruz, CA, USA), anti-Cf-9 (Luderer *et al.*, 2001), rabbit PAP soluble complex (Sigma, Dorset, UK) and antirabbit Ig conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). All antibodies were used at a 1 : 1000 dilution. Bands were visualised using Super Signal Chemiluminescent Substrate (Pierce) or Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

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#### References

- Becraft, P.W.** (2002) Receptor kinase signaling in plant development. *Annu. Rev. Cell Dev. Biol.* **18**, 163–192.
- Carrington, J.C., Cary, S.M., Parks, T.D. and Dougherty, W.G.** (1989) A second proteinase encoded by a plant potyvirus genome. *EMBO J.* **8**, 365–370.
- Jeong, S., Trotochaud, A.E. and Clark, S.E.** (1999) The *Arabidopsis* CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell*, **11**, 1925–1933.
- Johansen, L.K. and Carrington, J.C.** (2001) Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiol.* **126**, 930–938.
- Joosten, M.H.A.J. and De Wit, P.J.G.M.** (1999) The tomato–*Cladosporium fulvum* interaction: a versatile experimental system to study plant–pathogen interactions. *Annu. Rev. Phytopathol.* **37**, 335–367.
- Kajava, A.V. and Kobe, B.** (2002) Assessment of the ability to model proteins with leucine-rich repeats in light of the latest structural information. *Protein Sci.* **11**, 1082–1090.
- Kooman-Gersmann, M., Honée, G., Bonnema, G. and De Wit, P.J.G.M.** (1996) A high-affinity binding site for the AVR9 peptide elicitor of *Cladosporium fulvum* is present on plasma membranes of tomato and other solanaceous plants. *Plant Cell*, **8**, 929–938.
- Kooman-Gersmann, M., Vogelsang, R., Vossen, P., Van den Hooven, H.W., Mahé, E., Honée, G. and De Wit, P.J.G.M.** (1998) Correlation between binding affinity and necrosis-inducing activity of mutant AVR9 peptide elicitors. *Plant Physiol.* **117**, 609–618.
- Laemmli, U.K.** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E. and Walker, J.C.** (2002) BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell*, **110**, 213–222.
- Luderer, R., Rivas, S., Nürnberger, T. et al.** (2001) No evidence for binding between resistance gene product Cf-9 of tomato and avirulence gene product AVR9 of *Cladosporium fulvum*. *Mol. Plant Microbe Interact.* **14**, 867–876.
- Nam, K.H. and Li, J.** (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell*, **110**, 203–212.
- Park, A.R., Cha, S.K., Yun, U.J., Jin, M.Y., Lee, S.H., Sachetto-Martins, G. and Park, O.K.** (2001) Interaction of the *Arabidopsis* receptor protein kinase Wak1 with a glycine-rich protein, AtGRP-3. *J. Biol. Chem.* **276**, 26688–26693.
- Rivas, S. and Thomas, C.M.** (2002) Recent advances in the study of tomato Cf-resistance genes. *Mol. Plant Pathol.* **3**, 277–282.
- Rivas, S., Romeis, T. and Jones, J.D.G.** (2002a) The Cf-9 disease resistance protein is present in an ~420-kilodalton heteromultimeric membrane-associated complex at one molecule per complex. *Plant Cell*, **14**, 689–702.
- Rivas, S., Mucyn, T., Van den Burg, H.A., Vervoort, J. and Jones, J.D.G.** (2002b) An ~400 kDa membrane-associated complex that contains one molecule of the resistance protein Cf-4. *Plant J.* **29**, 783–796.
- Sambrook, J., Fritsch, E.F. and Maniatis, T.T.** (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- The Arabidopsis Genome Initiative** (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796–815.
- Torii, K.U.** (2000) Receptor kinase activation and signal transduction in plants: an emerging picture. *Curr. Opin. Plant Biol.* **3**, 361–367.
- Trotochaud, A.E., Hao, T., Wu, G., Yang, Z. and Clark, S.E.** (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *Plant Cell*, **11**, 393–405.
- Van der Hoorn, R.A.L., Laurent, F., Roth, R. and De Wit, P.J.G.M.** (2000) Agroinfiltration is a versatile tool that facilitates comparative analysis of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis. *Mol. Plant Microbe Interact.* **13**, 439–446.
- Van der Hoorn, R.A.L., Kruijt, M., Roth, R., Brandwagt, B.F., Joosten, M.H.A.J. and De Wit, P.J.G.M.** (2001a) Intragenic recombination generated two distinct Cf genes that mediate AVR9 recognition in the natural population of *Lycopersicon pimpinellifolium*. *Proc. Natl. Acad. Sci. USA*, **98**, 10493–10498.
- Van der Hoorn, R.A.L., Roth, R. and De Wit, P.J.G.M.** (2001b) Identification of distinct specificity determinants in resistance protein Cf-4 allows construction of a Cf-9 mutant that confers recognition of avirulence protein AVR4. *Plant Cell*, **13**, 273–285.
- Van der Hoorn, R.A.L., De Wit, P.J.G.M. and Joosten, M.H.A.J.** (2002) Balancing selection favors guarding resistance proteins. *Trends Plant Sci.* **6**, 67–71.

**Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D.** (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* **33**, 949–956.

**Wulff, B.B.H., Thomas, C.M., Smoker, M., Grant, M. and Jones, J.D.G.** (2001) Domain swapping and gene shuffling identify sequences required for induction of an Avr-dependent hypersensitive response by the tomato Cf-4 and Cf-9 proteins. *Plant Cell*, **13**, 255–272.