Structure–Function Analysis of Cf-9, a Receptor-Like Protein with Extracytoplasmic Leucine-Rich Repeats

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The tomato (Lycopersicon pimpinellifolium) resistance protein Cf-9 belongs to a large class of plant proteins with extracytoplasmic Leu-rich repeats (eLRRs). eLRR proteins play key roles in plant defense and development, mainly as receptor-like proteins or receptor-like kinases, conferring recognition of various pathogen molecules and plant hormones. We report here a large-scale structure–function analysis of an eLRR protein. A total of 66 site-directed mutants of Cf-9 were analyzed for activity in Avr9 recognition and for protein stability and the results interpreted with the help of a homology model of the Cf-9 structure. Conserved Trp and Cys pairs in the N-terminal LRR-flanking domain appear to be important for Cf-9 activity and are probably exposed at the putative concave inner surface of the Cf-9 protein, where recognition specificity also resides. Removal of each of the 22 putative N-linked glycosylation sites (PGS) revealed that many PGSs contribute to Cf-9 activity and that the PGSs in the putative α-helices of the LRR modules are essential. Immunoblot analysis and mass spectrometry showed that all but one of the PGSs are N-glycosylated. Introduction of glycosylation at the putative concave β-sheet surface blocks Cf-9 activity, in some cases probably by disturbing specific recognition, and in another case by steric hindrance with existing N-glycans. The glycosylation pattern and several other features are conserved in other eLRR proteins, where similar mutations show similar phenotypes.

INTRODUCTION

Proteins with extracytoplasmic Leu-rich repeats (eLRRs) play a crucial role in plant defense and development by perceiving extracellular signals that can be pathogen-derived molecules or plant hormones, respectively. The LRR domain is proposed to act as a versatile recognition surface where specific protein–protein interactions occur. This LRR domain is usually flanked by small domains containing Cys residues and fused to other domains, through which the eLRR proteins can be classified (Figure 1).

A function has been assigned to many eLRR proteins (references in Table 1). PGIP is a polygalacturonase inhibiting protein, AFP inhibits ice crystallization, and LRX1 is required for root hair morphogenesis. Many receptor-like proteins (RLPs) with eLRRs confer disease resistance through recognition of pathogen-derived molecules (e.g., Cf-9, RPP27, and EIX1), whereas other RLPs function in meristem development (FEA2 and CLV2) or distribution of stomata (TMM). Some receptor-like kinases (RLKs) with eLRRs also confer pathogen recognition (e.g., FLS2 and Xa21), whereas others act in perception of steroid or peptide hormones (e.g., BRI1, PSKR, and SR160) or play a role in other developmental processes, such as meristem development, stem elongation, abscission, pollination, and nodulation (e.g., CLV1, ER, HAESA, LePRK, and SYMRK). It can be expected that additional functions will be assigned to eLRR proteins, given the fact that the Arabidopsis thaliana genome encodes two PGIP-like proteins, 11 LRX-like proteins, 59 eLRR RLPs, and 216 eLRR RLKs (Arabidopsis Genome Initiative, 2000; Shiu and Bleecker, 2001; Baumberger et al., 2003; Ferrari et al., 2003; Tör et al., 2004).

Several interactors of eLRR proteins have been identified. Ligands can be proteins (e.g., polygalacturonase, CLV3, LAT52, and xylanase) or peptides (e.g., systemin, flagellin, and phyto-sulfokine) (references in Table 1). Many eLRR proteins are also found to interact with other components of signaling complexes. BRI1, for example, interacts with BAK1 (Li et al., 2002; Nam and Li, 2002), CLV2 is expected to interact with CLV2 (Jeong et al., 1999), and LePRK2 associates with LePRK1 (Wengier et al., 2003).

Specific protein–protein interactions can be mediated by the LRR domain. Much information on the LRR structure and
function is derived from studies with nonplant LRR proteins, such as the porcine ribonuclease inhibitor (RI). The crystal structure of RI revealed a horseshoe-like structure, where multiple LRRs are stacked to form a parallel β-sheet at the concave (inner) side of the protein and a series of α-helices on the convex (outer) side of the protein (Kobe and Deisenhofer, 1993). Binding of RI to ribonuclease occurs through solvent-exposed residues (x in xuxLxxLxx) at the concave β-sheet surface (Kobe and Deisenhofer, 1996). The variation of displayed residues at the β-sheets contributes to the diverse recognition specificities of LRR proteins.

Plant eLRR domains are typified by a distinct LRR consensus sequence, Cys-containing LRR-flanking domains, and a large number of putative glycosylation sites (Kajava, 1998). In addition, the crystal structure of PGIP revealed, when compared with RI, the presence of a second parallel β-sheet on one side of the LRR domain and a twisted, decreased curvature of the β-sheet surface (Di Matteo et al., 2003). Domain-swap experiments showed that the concave β-sheet surface of PGIP contains the residues that determine specificity for different polygalacturonases (Leckie et al., 1999). Similar domain-swap experiments revealed that specificity determinants in Cf-4 and Cf-9 reside in only a few residues exposed at the putative β-sheet of the central LRRs (Van der Hoorn et al., 2001; Wulff et al., 2001).

In summary, there is a large class of plant-specific proteins with eLRRs for which diverse functions in defense and development have been described, but little is known about how they function. To provide more information on eLRR mechanisms, we undertook a large scale structure–function analysis on conserved features of eLRR proteins. Cf-9 was chosen as a prototype for the analysis because this RLP contains conserved features of eLRR proteins, and a reliable, convenient quantitative agroinfiltration assay is available to analyze Cf-9 function (Van der Hoorn et al., 2000). This quick assay was shown to represent the phenotypes for resistance in tomato (Lycopersicon esculentum) plants (Wulff et al., 2001). We monitored expressed mutant proteins, which provided additional knowledge of the glycosylation pattern. The phenotypes arising from mutagenesis are discussed in the light of a homology model for the Cf-9 structure. These data will provide useful insights for our understanding of how proteins with eLRRs function.

RESULTS

Conserved Trp and Cys Residues Are Essential for Cf-9 Activity

To identify amino acid residues important for function of proteins with eLRRs, we compared LRR-flanking domains of eLR proteins with known functions (Figure 1, Table 1) and used directed mutagenesis of Cf-9 to investigate the importance of these residues for Cf-9 activity.

Several conserved motifs could be distinguished in the LRR-flanking domains. The N-terminal LRR-flanking domain (B-domain) contains the conserved motifs LLxxK, LssW, and CxWxGVxC, whereas the C-terminal LRR-flanking domain (D-domain) contains the conserved motif GNxGLCGxPLxxxC (Figure 2A). Cf-9 also carries these motifs, except that Cf-9 carries an Arg instead of the first Cys in the D-domain (Figure 2A).

Furthermore, Cf-9 carries three additional Cys residues in the B-domain, some of which can also be found in other eLRR proteins (Figure 2A). Cys residues are of particular interest because it has been proposed that they can be involved in the formation of intramolecular and/or intermolecular disulfide bridges (Diévat and Clark, 2003).

Nine conserved residues in the Cf-9 LRR-flanking domains were selected for mutagenesis, namely the Lys in the LLxxK motif (designated K1), the Trp in the LssW and CxWxGVxC motifs (W1 and W2, respectively), and the six Cys residues (called C1 to C6, of which C4 and C5 are in the conserved CxWxGVxC motif and C6 is in the D-domain). For each of these residues, an Ala substitution mutant of Cf-9 was generated and its activity determined by coexpression with Avr9 in at least three independent agroinfiltration assays (Van der Hoorn et al., 2000). These experiments show that W1A and W2A mutations completely abolish Cf-9 activity (Figure 2C). This severe phenotype was also observed for the additional mutant W2T (Figure 2C). By contrast, mutations K1A, C1A, and C6A have no effect on Cf-9 activity (Figures 2B and 2C, curves a, b, and g). Furthermore, mutants C2A and C3A show only slightly reduced activity (Figures 2B and 2C, curves c and d), whereas mutants C4A and C5A are nearly inactive (Figures 2B and 2C, curves e and f). Thus, substitution of the conserved Trp (W1 and W2) and the two most conserved Cys residues (C4 and C5) abolish or severely reduce Cf-9 activity, respectively. None of the active mutants induce necrosis in the absence of Avr9 (data not shown), indicating that the mutants are not autoactivating.

To verify that the expressed mutant proteins are still stable, cMyc-epitope-tagged versions of the mutant proteins were constructed using a new agroinfiltration vector (Figure 2D). Significantly, all myc-tagged Cf-9 mutant proteins accumulate...
to similar levels as myc-Cf-9 wild-type protein (Figure 2E), indicating that none of these residues are essential for Cf-9 protein stability. The activities of the myc-tagged Cf-9 mutants, however, are lower than those without myc-tag (Figure 2C). This is probably due to the fact that the N-terminal myc-tag slightly reduces the overall Cf-9 activity, compared with that of the wild-type Cf-9 (Figure 2B, curve h). As a result, phenotypes for myc-tagged C2A, C3A, C4A, and C5A mutants are even more pronounced than without a myc-tag. Significantly, myc-K1A, myc-C1A, and myc-C6A mutants remain as active as myc-Cf-9, indicating that these mutations have not even a weak effect on Cf-9 activity. Taken together, these data indicate that the order of reduction of Cf-9 activity is as follows: (W1A/C25/W2A/T/C29/C5A/C4A) > (C3A/C2A) > (C1A/C25/C6A/K1A/Cf-9).

The reduced activities of the Cf-9 Cys mutants also suggest that intramolecular disulfide bridges may exist between Cys C2 and C3 and between C4 and C5 because substitutions of these Cys result in similar phenotypes within each couple. To investigate this further, double mutants C2,3A and C4,5A were constructed and their activities determined. Mutant C2,3A is less active than C2A or C3A, suggesting that C2A and C3A phenotypes do not result from a broken putative disulfide bridge alone, but from additive effects. Double mutant C4,5A has a weak activity that is too low to compare with the weak activities of the C4A and C5A mutants.

We also constructed mutants C1-5A and C1-6A, in which five or all six of the Cys were substituted. These mutants are all inactive, indicating that the phenotypes of the single Cys mutations are additive (Figure 2C). Myc-tagged versions of these mutants, including the C1-6A mutant accumulate to similar levels as wild-type myc-Cf-9 (Figure 2E), demonstrating that these proteins are stable, despite being unable to form disulfide bridges through these Cys.

Putative Glycosylation Sites Are Important for Cf-9 Activity

Another distinct feature of plant eLRR proteins is their large number of putative N-linked glycosylation sites (PGSs; N in NxT/S motifs). Cf-9 contains 22 PGSs (Figure 3A) in its extracellular domain, of which 19 reside in the LRR domain. Previous N-deglycosylation studies using PNGase F showed that Cf-9 is heavily N-glycosylated (Piedras et al., 2000) and that this glycosylation is evenly distributed over three parts of Cf-9 (Van der Hoorn et al., 2003). The relevance of PGSs in Cf-9 became

### Table 1. Plant eLRR Proteins with Known Function

<table>
<thead>
<tr>
<th>Name</th>
<th>Typea</th>
<th>Species</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
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<td>PGIP-like</td>
<td>Carrot</td>
<td>Antifreeze</td>
<td>Worrall et al. (1998)</td>
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<tr>
<td>PGIP</td>
<td>PGIP-like</td>
<td>Bean</td>
<td>Polygalacturonase inhibition</td>
<td>Toubart et al. (1992)</td>
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<tr>
<td>LRF1</td>
<td>LRF-like</td>
<td>Tomato</td>
<td>Root hair morphogenesis</td>
<td>Baumberger et al. (2001)</td>
</tr>
<tr>
<td>CI-2</td>
<td>RLP</td>
<td>Tomato</td>
<td>Leaf mold resistance</td>
<td>Dixon et al. (1996)</td>
</tr>
<tr>
<td>CI-9</td>
<td>RLP</td>
<td>Tomato</td>
<td>Leaf mold resistance</td>
<td>Jones et al. (1994)</td>
</tr>
<tr>
<td>CLV2</td>
<td>RLP</td>
<td>Arabidopsis</td>
<td>Meristem development</td>
<td>Jeong et al. (1999)</td>
</tr>
<tr>
<td>FEA2</td>
<td>RLP</td>
<td>Maize</td>
<td>Meristem development</td>
<td>Taguchi-Shiobara et al. (2001)</td>
</tr>
<tr>
<td>LeEin2</td>
<td>RLP</td>
<td>Tomato</td>
<td>Xylanase elicitor perception</td>
<td>Ron and Avni (2004)</td>
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<tr>
<td>RPP27</td>
<td>RLP</td>
<td>Arabidopsis</td>
<td>Downy mildew resistance</td>
<td>Tör et al. (2004)</td>
</tr>
<tr>
<td>Ve1</td>
<td>RLP</td>
<td>Tomato</td>
<td>Fungal resistance</td>
<td>Kawchuk et al. (2001)</td>
</tr>
<tr>
<td>V12</td>
<td>RLP</td>
<td>Apple</td>
<td>Apple scab resistance</td>
<td>Vinatzer et al. (2001); Belfanti et al. (2004)</td>
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<tr>
<td>BAK1</td>
<td>RLP</td>
<td>Arabidopsis</td>
<td>Brassinosteroid signaling</td>
<td>Li et al. (2002); Nam and Li (2002)</td>
</tr>
<tr>
<td>BRI1</td>
<td>RLP</td>
<td>Arabidopsis</td>
<td>Brassinosteroid perception</td>
<td>Li and Chory (1997); Wang et al. (2001)</td>
</tr>
<tr>
<td>CLV1</td>
<td>RLP</td>
<td>Arabidopsis</td>
<td>Meristem development</td>
<td>Clark et al. (1997)</td>
</tr>
<tr>
<td>EMS1/EXS</td>
<td>RLP</td>
<td>Arabidopsis</td>
<td>Anther cell development</td>
<td>Zhao et al. (2002); Canales et al. (2002)</td>
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<tr>
<td>ER</td>
<td>RLP</td>
<td>Arabidopsis</td>
<td>Stem elongation</td>
<td>Torii et al. (1996)</td>
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<td>FLS2</td>
<td>RLP</td>
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<td>Flagellin perception</td>
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<td>Leaf abscission</td>
<td>Walker (1993); Jinn et al. (2000)</td>
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<td>Lotus</td>
<td>Root development and nodulation</td>
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<td>RLP</td>
<td>Tomato</td>
<td>Pollen LAT52 perception</td>
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<td>RLP</td>
<td>Pea</td>
<td>Brassinosteroid perception</td>
<td>Nomura et al. (2003)</td>
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<td>NARK</td>
<td>RLP</td>
<td>Soybean</td>
<td>Nodule autoregulation</td>
<td>Searle et al. (2003)</td>
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<tr>
<td>OsBRI1</td>
<td>RLP</td>
<td>Rice</td>
<td>Internode elongation</td>
<td>Yamamura et al. (2000)</td>
</tr>
<tr>
<td>PSKR</td>
<td>RLP</td>
<td>Carrot</td>
<td>PSK perception</td>
<td>Matsubayashi et al. (2002)</td>
</tr>
<tr>
<td>SR160</td>
<td>RLP</td>
<td>Tomato</td>
<td>Systemin perception</td>
<td>Scheer and Ryan (2002)</td>
</tr>
<tr>
<td>SYMRK</td>
<td>RLP</td>
<td>Lotus</td>
<td>Bacterial and fungal symbiosis</td>
<td>Endre et al. (2002); Stracke et al. (2002)</td>
</tr>
<tr>
<td>Xa21</td>
<td>RLP</td>
<td>Rice</td>
<td>Rice blast resistance</td>
<td>Song et al. (1995)</td>
</tr>
<tr>
<td>Xa26</td>
<td>RLP</td>
<td>Rice</td>
<td>Rice blast resistance</td>
<td>Sun et al. (2004)</td>
</tr>
</tbody>
</table>

*a Classification as represented in Figure 1.
**Figure 2.** Targeted Mutagenesis of LRR-Flanking Domains of Cf-9.

(A) Alignment of LRR-flanking domains. The B-domain is the N-terminal LRR-flanking domain and starts after the predicted signal peptide and

<table>
<thead>
<tr>
<th>Domain</th>
<th>CF-9</th>
<th>B-domain</th>
<th>LRRs</th>
<th>D-domain</th>
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<tbody>
<tr>
<td>Consensus</td>
<td>LLxK</td>
<td>LIAxK</td>
<td>2</td>
<td>3</td>
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<td>APD</td>
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<td>FGIP</td>
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<tr>
<td>SHY</td>
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<td>LUX</td>
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<td>EAATLARKATKHKNDNLSMPSNAQGTVGCFN</td>
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<td>CI-3</td>
<td>LPQEDLPRKATKHKNDNLSMPSNAQGTVGCFN</td>
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<td>PIA2</td>
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<td>BAR1</td>
<td>AECDNLARKSLDAPKNVYDLMVTCNF</td>
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<td>Ka26</td>
<td>Ka26VRKPPKPSDVSLMPSPDASQSSCDOD</td>
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(B) Activity of Cf-9 mutant proteins.

<table>
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<th>Mutant</th>
<th>Activity</th>
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<tbody>
<tr>
<td>K1A</td>
<td>+++</td>
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<td>-</td>
</tr>
<tr>
<td>W2T</td>
<td>-</td>
</tr>
<tr>
<td>C1A</td>
<td>+++b</td>
</tr>
<tr>
<td>C2A</td>
<td>+++c</td>
</tr>
<tr>
<td>C3A</td>
<td>+++d</td>
</tr>
<tr>
<td>C4A</td>
<td>+++e</td>
</tr>
<tr>
<td>C5A</td>
<td>+++f</td>
</tr>
<tr>
<td>C6A</td>
<td>+++g</td>
</tr>
<tr>
<td>C2,3A</td>
<td>+</td>
</tr>
<tr>
<td>C4,5A</td>
<td>+</td>
</tr>
<tr>
<td>C1-5A</td>
<td>-</td>
</tr>
<tr>
<td>C1-6A</td>
<td>-</td>
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</tbody>
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(C) Schematic representation of the B-domain region in Cf-9 and myc-Cf-9.

(D) Schematic representation of the intron-exon structure of Cf-9.

(E) Western blot analysis showing the expression of myc-Cf-9.
clear when three out of four inactive Cf-9 ethyl methanesulfonate (EMS) point mutants appeared to carry a mutation that likely affects N-glycosylation (Wulf et al., 2004). In mutant M2, PGS 18 in LRR 24 was removed (here called mutant 18SL), whereas in EMS mutants F965 and M140, one additional PGS was introduced in LRRs 12 and 18, respectively (here called mutants DN12 and DN18) (Wulf et al., 2004).

To investigate the relevance of these and other PGSs in Cf-9, an extensive series of mutant TAP-tagged Cf-9 proteins was generated (Figure 3B). For nearly all PGSs, Asn residues were substituted with Asp (N into D), which are common at similar positions in Cf-9 and other eLRR proteins (Figure 3A; data not shown). For similar reasons, PGS1 and 2 in the B-domain were mutated into NxA. Accumulation of the mutant Cf-9-TAP proteins was investigated by immunoblot analysis of agroinfiltrated tobacco tissues. All mutant Cf-9-TAP proteins accumulated to similar levels as wild-type Cf-9-TAP (Figure 3B), except for mutant 13ND, which we could not detect even when expressed to similar levels as wild-type Cf-9-TAP (Figure 3B), except for tobacco tissues. All mutant Cf-9-TAP proteins was investigated by immunoblot analysis of agroinfiltrated tobacco tissues. All mutant Cf-9-TAP proteins accumulated to similar levels as wild-type Cf-9-TAP (Figure 3B), except for mutant 13ND, which we could not detect even when expressed in the presence of P19 silencing inhibitor (Voinnet et al., 2003) in Nicotiana benthamiana (data not shown).

Agroinfiltration into Avr9-transgenic tobacco in at least three independent experiments revealed that most PGS mutants have reduced activities (Figure 3B). In addition to EMS mutant 18SL, mutants 6ND, 9ND, and 11ND also appeared inactive. Mutant 13ND is also inactive, which correlates with the absence of protein of this mutant. Activity of mutants 12ND and 19ND could just be detected, whereas all other PGS removal mutants are active, although at different levels (Figure 3B). The activity depends on the presence of Avr9 because no necrosis was induced in wild-type tobacco (data not shown).

To verify the most severely reduced activities, PGSs 6, 9, 11, 12, 13, and 19 were also mutated by changing NxS/T into NxA and PGS18 by changing NxA into DxA. All these mutant proteins accumulate to levels similar to wild-type Cf-9-TAP (Figure 3B). Mutant 13SA also accumulates, indicating that the instability of the 13ND mutant is not caused by the removal of glycosylation alone and that PGS13 is not required for Cf-9 activity. Significantly, the activities of the PGS removal mutants caused by S-into-A and T-into-A substitutions confirm the activities of the PGS removal mutants caused by N-into-D substitutions. Thus, mutants 8SA and 11TA are inactive, like mutants 6ND and 11ND. Mutant 12SA is inactive, whereas 12ND already had low activity, and mutant 9SA has low activity, whereas 9ND was inactive. Exceptions are the full activities of 18ND and 19SA, which contrast with the inactivity of EMS mutant 18SL and weak activity of mutant 19ND (Figure 3B).

Thus, for four out of seven PGS inactive mutants, the low activity of the PGS removal mutation was confirmed with an independent mutation. When summarized by plotting the activities onto the Cf-9 sequence, it becomes obvious that these four PGSs (6, 9, 11, and 12) all cluster in the putative α-helices of LRRs 4 to 12 (Figure 3C).

**Nearly all PGSs of Cf-9 Are Glycosylated**

Subsequently, we analyzed the PGS removal mutants on high-resolution SDS-PAGE gels to determine their size, when compared with wild-type Cf-9-TAP protein. Previously, N-deglycosylation of Cf-9 with PNGase F resulted in an increased mobility corresponding to a loss in apparent molecular mass of 60 kD (Piedras et al., 2000; Van der Hoorn et al., 2003). Theoretically, if each of the 22 PGSs is N-deglycosylated, then on average, each N-glycosylation site could contribute 3 kD to this apparent molecular mass. To detect such small size differences, we separated mutant proteins, interspaced by wild-type Cf-9-TAP protein, on 7% protein gels run overnight at low voltage. This revealed a small, but significantly increased mobility for most PGS mutants, when compared with wild-type Cf-9-TAP (Figures 3B and 3D). Exceptions are mutants 18SL and 18ND, which both migrate as wild-type Cf-9-TAP, and 2SA, for which the increased mobility was not always detected.

Two pieces of data show that the observed increased mobilities resulted from elimination of N-glycans and not from the amino acid substitutions themselves. First, the increased mobility was observed for both N-into-D and S/T-into-A substitutions. Second, N-deglycosylation of these mutant proteins by PNGase

---

**Figure 2.** (continued).

terminates two amino acids beyond the conserved Cys pair. The D-domain is the C-terminal LRR-flanking domain (only the conserved region is shown). Conserved residues are indicated in bold, and mutagenized residues are indicated below the Cf-9 sequence. Underlined numbers indicate the number of amino acid residues omitted to simplify the alignment.

**B** Comparative quantitative analysis of activities of a selection of nontagged Cf-9 mutants (indicated with superscript a to h in [C]). Agrobacterium cultures carrying a plasmid that encodes a (mutant) Cf-9 protein were mixed in different ratios with a culture of equal density carrying an Avr9-encoding plasmid and infiltrated into opposite tobacco leaf halves. At 7 d post-infiltration (dpi), the percentage of infiltrated area that had become necrotic was measured and plotted against the percentage of culture carrying (mutant) Cf-9. Agrobacterium cultures carrying a plasmid that encodes a (mutant) Cf-9 protein were mixed in different ratios with a culture of equal density carrying an Avr9-encoding plasmid and infiltrated into opposite tobacco leaf halves. At 7 d post-infiltration (dpi), the percentage of infiltrated area that had become necrotic was measured and plotted against the percentage of culture carrying (mutant) Cf-9. 

**C** Summary of hypersensitive response–inducing activities of the all nontagged or myc-tagged Cf-9 (mutant) proteins when coexpressed with Avr9. Leaves of tobacco plants were infiltrated with Agrobacterium carrying a binary plasmid encoding (mutant) Cf proteins. Avr9 was coexpressed by mixing Agrobacterium cultures with strains carrying Avr9 on binary plasmids. Necrosis was scored at 7 dpi. +++, necrosis of entire infiltrated area; +++, >50% necrosis of infiltrated area; +, 10 to 50% necrotic area; ++, 0 to 10% necrotic area; --, no necrosis. Superscripts a to h refer to (B). The data represent an average of at least three agroinfiltration assays.

**D** Myc-tagged Cf-9 construct designed for agroinfiltration. The four N-terminal myc-tags (top) are interrupted by an intron in the expression cassette (bottom) to exclude bacterial expression. 35S, constitutive Cauliflower mosaic virus promoter; SP, signal peptide of tobacco PR1a gene to ensure extracellular targeting; term, polyadenylation signal of Pi-II gene.

**E** Protein accumulation of (mutant) myc-tagged Cf-9 proteins at 1 dpi in tobacco with Agrobacterium cultures. Proteins were extracted from infiltrated leaves and analyzed on immunoblots using anti-myc antibody.
Figure 3. Effects of Removal and Addition of Putative Glycosylation Sites in Cf-9.

(A) Position of the putative glycosylation sites (N in NxS/T) in the Cf-9 protein. The consensus LRR sequence is shown on top, with the putative solvent-exposed residues of the putative β-sheet underlined.

(B) PGS removal mutants

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Additional PGS removal mutants

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PGS addition mutants

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F treatment resulted in identical electrophoretic mobility for all tested mutants (data not shown).

As an independent confirmation for the presence or absence of N-glycosylated sites, we analyzed purified Cf-9-TAP proteins by mass spectrometry (MS). Cf-9-TAP was produced by large scale agroinfiltration of N. benthamiana and purified from solubilized membranes by immunoprecipitation (see Methods). Cf-9-TAP was isolated from an SDS-PAGE gel, digested with trypsin, and analyzed three times (MS1-3) with matrix-associated laser desorption and ionization time-of-flight (MALDI-TOF) to maximize coverage of the Cf-9-TAP protein.

In total, 15 peptides were detected in independent experiments (Figure 4). Although much of the Cf-9-TAP protein sequence was covered with identified peptides, no peptide (except one, see below) containing a PGS was detected (Figure 4B). This is consistent with N-glycosylation of all PGSs (except one) as indicated by analysis of the PGS removal mutants. The additional, unassigned molecular masses that were detected (data not shown) could not, however, be assigned to certain glycosylated peptides without knowing the exact structure of the N-glycan. Significantly, a peptide carrying PGS18 was detected twice (peptide i, Figure 4). This peptide was also detected using quadrupole-time-of-flight (Q-TOF), which also confirmed the sequence of this peptide (Figure 4B). These data confirm that PGS18 is not N-glycosylated.

In conclusion, mutant protein electrophoresis and MS analysis showed that, except for PGS18, all PGSs of Cf-9 are N-glycosylated. This confirms earlier observations that glycosylation is evenly distributed over the different LRRs of Cf-9 (Van der Hoorn et al., 2003) and is consistent with the slightly increased mobility for each of the PGS removal mutants.

Adding Glycosylation at the Putative Concave β-Sheet Surface Affects Cf-9 Activity

In addition to PGS removal mutants, we also created and studied mutants with introduced PGSs at putative solvent-exposed positions of the putative concave β-sheet in LRRs 6, 12, 18, and 26 of Cf-9 (mutants QN6, DN12, DN18, and DN26, respectively; Figure 3B). The rationale for this was given by the inactivity of the EMS mutants where a PGS was introduced at the putative solvent-exposed β-sheet surface of LRRs 12 and 18 (mutants F965 and M140, here called mutants DN12 and DN18, respectively; Wulff et al., 2004). Furthermore, we introduced a PGS in LRR12, outside the putative solvent-exposed region, by introducing amino acids RSW, an insert found in nearly all homologs of Cf-9 (Parniske et al., 1997).

Agroinfiltration of Avr9-transgenic tobacco confirmed the inactivity of EMS mutants DN12 and DN18 and showed that mutant DN26 is also inactive, whereas mutants DN6 and RSW have only slightly reduced activity (Figure 3B). The mutant proteins accumulate to similar levels as wild-type Cf-9-TAP (Figure 3D), indicating that the reduced activities are not due to protein instability.

The inactivity of the D-into-N mutants can result from an introduced N-linked glycan or from the intrinsic property of the amino acid substitution itself. To discriminate between these two possibilities, we made D-into-Q mutants DQ12, DQ18, and DQ26. These mutants have a similarly changed charge as D-into-N mutants but do not carry an additional PGS. Agroinfiltration demonstrated that these D-into-Q mutants are fully active (Figure 3B), indicating that the phenotype of the D-into-N mutants results from an introduced N-glycan, rather than from the amino acid substitution itself. Expression of the mutant in the absence of Avr9 did not trigger necrosis (data not shown).

On immunoblots, PGS addition mutants QN6, DN12, DN26, and RSW have a significantly lower mobility than wild-type Cf-9-TAP (Figures 3B and 3D), consistent with additional glycosylation. As expected, control mutants DQ12 and DQ26 have a similar mobility as Cf-9-TAP (Figure 3B). The molecular mass of the DN18 and DQ18 mutants could not consistently be measured relative to wild-type Cf-9-TAP, but DN18 always had a decreased mobility when compared with DQ18, indicating that DN18 also carries additional glycosylation. The phenotypes of the PGS addition mutants are summarized in Figure 3C.

Glycosylation Patterns Are Conserved in Other Proteins with eLRRs

The glycosylation pattern and its role for Cf-9 activity may also hold for other eLRR proteins. We therefore examined the pattern of PGSs in other eLRR proteins (Table 1). The number of PGSs in LRR domains of eLRR proteins differs significantly, ranging from 0 for LePRK2 to 29 for Cf-2 (Figure 5A). Despite this variation, the overall distribution pattern of PGSs is similar for all these proteins (Figure 5A). PGSs dominate at three major and four minor

Figure 3. (continued).

(B) Table showing activities and sizes of all Cf-9 PGS removal and addition mutants. First column: acronym of Cf-9 mutant (*, originally identified as EMS mutant). Second column: hypersensitive response–inducing activity when agroinfiltrated into Avr9-transgenic tobacco (+, activity similar to wild-type Cf-9-TAP; +, activity less than Cf-9-TAP; $, threshold activity; $, no detectable activity). These data represent an average of at least three agroinfiltration assays. Third column: protein levels of agroinfiltrated PGS mutants analyzed by immunoblots compared with wild-type Cf-9-TAP (wt). All TAP-tagged mutant proteins except mutant 13ND accumulate to similar levels as wild-type Cf-9-TAP. Fourth column: size of PGS mutants on immunoblots when compared with wild-type Cf-9-TAP. Samples from at least two independent agroinfiltrations were analyzed multiple times on immunoblots next to Cf-9-TAP samples. Size differences compared with wild-type Cf-9-TAP were scored as $, $, $, or $ wild-type Cf-9-TAP and averaged in the right column. #, shown in immunoblots in (D).

(C) Summary of the positions and activities of the Cf-9 PGS removal (rectangles) and addition (circles) mutants. The gray scale indicates the effect of the mutations on Cf-9 activity as indicated in the accompanying legend.

(D) Representative immunoblots for some of the Cf-9 PGS mutants. Both PGS removal and PGS addition mutants are shown. Lanes marked with a C were loaded with the Cf-9-TAP control.
positions in the LRR consensus (Figure 5B). Asn in the conserved Asn ladder (N in xxLxLxxN) are never part of NxS/T signatures, presumably because these amino acids are buried in the structure. PGsS in the \( \alpha \)-helix region of LRRs are common in many eLRR proteins containing PGSS (Figure 5A) and may be essential for eLRR function, as we found for Cf-9. When plotted on the LRR module, it is evident that a PGSS at the middle position of the putative \( \beta \)-sheet of adjacent LRRs does not occur, with Cf-9 being the exception (Figure 5C). In the case of Cf-9, however, we found that PGSS18 in LRR24 is not glycosylated, whereas PGSS19 in LRR25 is. By contrast, PGSS in adjacent putative \( \alpha \)-helices are common among eLRR proteins (Figure 5D). This difference can be explained because glycosylation of adjacent putative \( \beta \)-sheets may cause steric hindrance, as this is the concave side of the curved LRR structure, whereas glycosylation at adjacent putative \( \alpha \)-helices is at the convex, more spacious side of the LRR curvature. Another interesting feature to note is that, as in Cf-9, most eLRR proteins have at least 8 to 12 adjacent LRRs without a PGSS at the putative \( \beta \)-sheet (Figure 5C), suggesting that these nonglycosylated regions provide a recognition surface for specific protein–protein interactions (Kobe and Deisenhofer, 1996). PGSS at the putative \( \beta \)-sheet are confined to the four most C-terminal LRRs.

**DISCUSSION**

Cf-9 carries several amino acid motifs that could contribute to its structure and function. In this investigation, we focused our attention on conserved amino acids in LRR-flanking domains and on putative glycosylation sites. The N-terminal LRR flanking B-domain contains two Trp that are essential for Cf-9 activity and four Cys that synergistically contribute to Cf-9 activity. We also found that most of the putative glycosylation sites contribute to Cf-9 activity and that those in putative \( \alpha \)-helices are essential.
Cf-9 is glycosylated at all PGSs, except for PGS18, and adding glycosylation sites at the putative concave \(\beta\)-sheet surface can abolish Cf-9 activity.

**A Model for the Cf-9 Structure**

To understand the various effects of the mutations, a homology model was generated for the structure of Cf-9, based on the crystal structures of bean (Phaseolus vulgaris) PGIP (Di Matteo et al., 2003) and bacterial LRR protein internalin A (InlA) (Schubert et al., 2002). Although Cf-9 displays only 34 and 28% sequence identity to PGIP and InlA, respectively, the repetitive nature of the LRR motif was of considerable help in generating a sequence alignment for homology modeling of the N-terminal LRR flanking B-domain and LRRs of Cf-9. However, the first 50 amino acids (including 27 amino acids from the B-domain) and the last 68 amino acids of Cf-9 (domains E to G) were omitted from the model because no suitable template structures could be found. Also, because neither of the templates contains a feature comparable to the loop-out region, we explored several alternative conformations for this region, generated by loop searching techniques, before selecting a model consisting of two \(\alpha\)-helices joined by a short loop, based on criteria of chemical feasibility.

The structural model of Cf-9 shows a typical nonglobular LRR fold (Figures 6A and 6B). At the concave (inner) side, the parallel putative \(\beta\)-sheet surface is curved and twisted in a right-handed superhelix, like the template structures from which it is derived. It is at this putative solvent-exposed positions, as explained by the key (top). Proteins are ordered as in (C).
by solvent-exposed residues (Van der Hoorn et al., 2001; Wulff et al., 2001). The putative $\alpha$-helices of the LRRs provide the outer surface of the structure and act as wedges that cause curvature of the putative $\beta$-sheet surface. The B-domain is compact and caps the hydrophobic core of the LRR.

The proposed nonglobular fold of Cf-9 is supported by experimental data. In gel filtration experiments, purified 160-kD myc-tagged Cf-9 protein was found to migrate with an apparent molecular mass of 420 kD (Rivas et al., 2002; Van der Hoorn et al., 2003). Deglycosylated Cf-9 still migrates with a large apparent molecular mass, indicating that this effect is due to the non-globular shape of the Cf-9 protein itself, rather than to its glycosylation (Van der Hoorn et al., 2003). The apparent molecular mass of Cf-9 is similar to the 440-kD ferritin that was used as
a calibration marker in these gel filtration assays. Ferritin is a globular protein complex with a physical radius of 74 Å (Granier, 1997). The Cf-9 model would fit in a sphere with a radius of 51 Å, but the model lacks the N and C termini, the N-glycans, and detergent molecules. However, although these predictions may match the experimental data, extreme caution should be taken when interpreting gel filtration experiments (Van der Hoorn et al., 2003).

The extended spring-like structure of Cf-9 is probably quite flexible, and the curvature of the LRR domain and exposure of residues at the concave inner surface may change upon binding to other proteins. These changes were detected in LRR protein RI upon binding with ribonuclease A (Kobe and Deisenhofer, 1996). Even more flexibility is expected to reside in the loop-out region, which may take different conformations and perhaps act as a hinge between the two LRR domains. Many mutations in this region were found to inactivate the BRI1 eLRR protein (Li and Chory, 1997; Friedrichsen et al., 2000). The inactivity of Cf-9 mutant 18SL may also result from interference with function of the loop-out region.

Role of Conserved Residues in the N-Terminal LRR-Flanking B-Domain

The N-terminal B-domain of Cf-9 preceding the LRRs contains two conserved Trp (W1 and W2) that are essential for Cf-9 activity and four Cys (C2 to C5) that synergistically contribute to Cf-9 activity. In the PGIP crystal structure, the Trp side chains are adjacent and coplanar, lying in the core of the B-domain, whereas the four Cys are linked into two Cys pairs by disulfide bonds. These features are preserved in our model. Thus, four Cys of Cf-9 are connected by two putative disulfide bridges, C2-C3 and C4-C5 (Figure 6C). These putative disulfide connections comply with our phenotypic data because C2A and C3A mutants have a similar reduced activity, and C4A and C5A mutants also have a similar weak activity. The weak activity of the double mutant C2,3A indicates that these Cys have additive roles besides holding the putative disulfide bridge. Intramolecular disulfide bridges in LRR-flanking domains are common, as they are also present in structures of PGIP (Mattei et al., 2001) and glycoprotein Ibα (Huizinga et al., 2002). The Trp, however, are only conserved among plant eLRR proteins. It is likely that both the coplanar Trp and the two clustered Cys bridges render this domain unusually rigid. Disturbance of this rigidity in the substitution mutants probably causes the severe phenotypes.

Alternatively, the LRR-flanking Cf-9 could also be involved in intermolecular disulfide bridges, connecting to other interacting proteins. This has been suggested for CLV1, which migrates at a higher molecular weight in the absence of reducing agent (Trotchaud et al., 1999). Notably, the conserved and important C4-C5 pair is probably exposed at the B-domain of Cf-9, at the putative concave inner surface of the protein (Figure 6D). This disulfide bridge is less exposed in PGIP (Figure 6E) but clearly exposed in a model for the B-domain of FLS2 that we generated (Figure 6F).

However, although the putative C4-C5 Cys bridge is probably exposed, in contrast with CLV1 studies, no disulfide-linked complexes have been observed for Cf-9 (Rivas et al., 2002) nor has it been reported for any of the other well-characterized eLRR proteins. Nevertheless, disulfide bridges may exist transiently when complexes are assembled. Transient assembly of signaling complexes occurs for the TGFβ receptor, resulting in a (TβRII)2(TβRII)2 heterotetrameric complex (Schlessinger, 2000). A similar transient assembly of signaling complexes appears to occur for CLV1 and possibly also for BRI1 and BAK1 (Trotchaud et al., 1999; Li et al., 2002; Nam and Li, 2002). LRR-flanking Cys may contribute to the stabilization of these complexes through transient intermolecular disulfide bridges. Redox-induced disulfide rearrangements play essential roles in the function of NPR1 (Mou et al., 2003) and integrins (Yan and Smith, 2001) and may be common in regulation of proteins (Hogg, 2003).

Distribution and Role of N-Glycosylation

We also showed that nearly all of the 22 PGSs are glycosylated in Cf-9. These PGSs are evenly distributed on the Cf-9 protein (Figures 6A and 6B, red and orange residues). The shift in molecular mass of the PGS removal mutants indicates that each Cf-9 molecule is fully glycosylated at each of these PGSs. Little is known about the identity of the N-glycans in Cf-9, but it has been shown that PGIP is N-glycosylated with a typical plant N-glycan composed of three N-acetylgalactosamines, three mannoses, one xylose, and one fucose (Mattei et al., 2001). Because Cf-9 and PGIP were both produced in Nicotiana species, it is conceivable that Cf-9 carries similar N-glycans for each PGS.

Immunoblot experiments and MS of Cf-9 tryptic digests showed that PGS18 is not glycosylated. In PGIP, one PGS at the β-sheet surface of the LRR domain also lacks glycosylation (Mattei et al., 2001). It is unclear why specifically these sites in PGIP and Cf-9 are not glycosylated. In the Cf-9 model, PGS18 is not exposed (Figure 6G, blue residue), but this does not explain the absence of an N-glycan because N-glycosylation would occur cotranslationally (Glabe et al., 1980). Studies in mammals showed that glycosylation can be inhibited by some amino acid residues, especially by Pro, at positions x and y in NxS/Ty (Shakim-Eshelman et al., 1996; Melliquist et al., 1998), but the two PGS sequences of Cf-9 and PGIP do not show this signature.

Remarkably, most of the 22 PGSs contribute to Cf-9 activity, and we identified four PGSs where the presence of N-glycans is essential for Cf-9 activity. These four are all in putative α-helices, exposed at the putative convex surface of the LRR domain (Figures 6A and 6B, red residues). Many other eLRR proteins also carry PGSs at these positions (Figure 5), presumably because these residues are exposed at the surface of the protein. There are several ways to explain the loss of activity of the many PGS removal mutants of Cf-9. Glycosylation may protect proteins from degradation (Gahring et al., 2001), but the Cf-9 PGS removal mutants are all stable proteins. N-glycans may also force a conformation of the LRR domain, facilitate interactions with the cell wall, serve as a quality control checkpoint for extracellular targeting, or play other roles (Leconte et al., 1994).

Glycosylation at the Putative Concave β-Sheet Surface

The putative concave LRR β-sheet is thought to provide a recognition surface where specific protein–protein interactions
occur (Kobe and Deisenhofer, 1996). Consistent with this, it was found that specificity determinants of PGIP, Cf-9, and Cf-4 reside in amino acids at putative solvent-exposed positions at the putative concave inner side of the eLRR proteins (Leckie et al., 1999; Van der Hoorn et al., 2001; Wulff et al., 2001).

Remarkably, we found that the C-terminal four LRRs of Cf-9 carry two N-glycans at the middle position of the putative concave β-sheet surface, indicating that putative solvent-exposed amino acids at this surface are unable to mediate specific protein–protein interactions. The presence of a PGS at these middle positions in C-terminal LRRs occurs often in other eLRR proteins (Figure 5), although removal of these N-glycans in Cf-9 does not affect Cf-9 activity (Figures 3B and 3C).

The middle position of the putative β-sheet surface is not glycosylated in adjacent LRRs. Thus, PGSs 19 and 21 are at the putative β-sheet surface of LRRs 25 and 27. PGS18 at the putative β-sheet surface of LRR24 is not glycosylated. Adding glycosylation at the putative β-sheet surface of LRR26 disturbs Cf-9 function (mutant DN26), possibly because of steric hindrance with the adjacent N-glycans in LRRs 25 and 27 (Figure 6G), which may cause a significantly disturbed LRR structure in this region.

In the first 22 LRRs before the loop-out domain, Cf-9 carries only one N-glycan at the β-sheet surface, but this is at the edge of the β-sheet surface of LRR14 (Figure 6B, orange residue). The activities of PGS addition mutants correlate with the conclusion that specificity of Cf-9 resides in LRRs 13 to 17 (Wulff et al., 2001). Introducing N-glycans in the adjacent LRR12 or LRR18 likely disturbs specific interactions, which renders the mutants inactive. By contrast, glycosylation is tolerated when more distant in LRR6.

**Other Mutant eLRR Proteins Have Similar Phenotypes**

The investigated Trp and Cys in the B-domain, as well as the glycosylation pattern, are well conserved amongst other eLRR proteins. This suggests that similar phenotypes would result if similar mutations are introduced in RLPS and RLKLs with eLRRs. Indeed, this prediction already holds for two mutants that resulted from EMS screens.

In the bri1-5 mutant, the second Cys of the conserved Cys pair in the B-domain of BR1 has been replaced by Tyr (C69Y). This mutation has a weak phenotype, resembling the weak activity of the corresponding C5A mutant of Cf-9. The weak phenotype of the bri1-5 allele proved useful for activation tagging experiments that resulted in the identification of BAK1, an RLK that interacts with BR11 and acts in BR1 signaling (Li et al., 2002), and of BRS1, a Ser protease that probably processes a protein involved in an early event in BR1 signaling (Li et al., 2001). Overexpression of each of these genes can complement the bri1-5 phenotype.

In the clv1-8 mutant, the D295N mutation results in the introduction of a PGS at the middle position of the putative β-sheet (LRR9 in CLV1) in the middle of a region of 15 LRRs that do not contain a PGS in the putative β-sheet (Clark et al., 1997). The inactivity of this mutant resembles that of Cf-9 mutants DN12 and DN18 that also contain an additional N-glycan on the putative β-sheets of LRRs 12 and 18, respectively. Interestingly, the clv1-8 mutation is dominant negative, probably because it interferes with other eLRR proteins that have a functional overlap with CLV1 (Diévart et al., 2003). This suggests that PGS addition mutants might still assemble in signaling complexes that are not active.

Taken together, this extensive structure–function analysis of Cf-9 has provided valuable details on eLRR protein function and creates important leads for further investigation of this functionally diverse protein family.

**METHODS**

**Cloning Procedures**

All DNA manipulations were performed using standard protocols (Sambrook et al., 1989). PCR was performed with Pfu polymerase (Stratagene, La Jolla, CA), and the authenticity of all cloned PCR fragments was confirmed by sequencing. pMOG410 is described elsewhere (Hood et al., 1993) and carries the 35S-gene interrupted by the second intron of the potato (Solanum tuberosum) ST-LS1 gene (Vancanneyt et al., 1990). Binary vector pRH109 (Cf-9) was described previously (Van der Hoorn et al., 2001). Oligonucleotide primers were synthesized by Amersham-Pharmacia Biotech (Buckinghamshire, UK) or Sigma-Aldrich (St. Louis, MO) and are summarized in Supplemental Table P online.

**Construction of (Mutant) myc-Tagged Cf-9 Vectors**

Binary vector pRH385 (encoding myc-tagged Cf-9) was generated as follows. The fragment containing the 3SS promoter and part of the tobacco (Nicotiana tabacum) Pr1a gene encoding the signal peptide was amplified from pRH87 (containing 3SS-SP-Avr4; Van der Hoorn et al., 2000) with primers XF5 and SR1 and cloned into the pBluescript ST-LS1 vector (pBS; Stratagene) using XbaI and SalI restriction sites, resulting in pRHx1. The fragment encoding four myc epitope tags interrupted by the intron of the potato ST-LS1 gene (Eckes et al., 1986) was amplified from pMOG410 using primers M4F and M4R (both encoding a double CMyC epitope tag) and cloned into pRHx1 using SalI and XhoI restriction sites, resulting in pRHx2. The fragment carrying the 3SS promoter and encoding the A- and B-domains of Cf-9 protein from pRH109 was replaced by a PCR fragment encoding only the B-domain, using primers XF and CR1 and XbaI and Clal restriction sites. This resulted in pRHx3, containing an introduced XhoI site encoding two Ser, followed by the N terminus of the mature Cf-9 protein. pRH385 was subsequently generated from this construct by cloning the 3SS-SP-2myc-intron-2myc fragment from pRHx2 into pRHx3, using XbaI and XhoI restriction sites.

Fragments containing the mutations C1A, C3A, C4A, W1A, and W2A were generated by amplification with primer pairs C1F + CR1, XFX + XCR, XFX + C4R, XFX + W1R, and XFX + W2R, respectively. Fragments containing the mutations K1A and C2A were generated by PCR overlap extension using border primer pair XFX + CR1 and overlap primer pairs K1f + K1r and C2R + C2r, respectively. These mutant fragments were generated using Cf-9 as a template and cloned into pBS using XhoI and Clal restriction sites. These fragments were subsequently used to replace the fragments encoding the B-domain in plasmid pRH385, resulting in mutated myc-Cf-9. Plasmid names for the myc-Cf-9 mutants are as follows: pRH406 (C1A), -407 (C2A), -408 (C3A), -409 (C4A), -445 (C23A), -494 (K1A), -477 (W1A), -468 (W2A), and -459 (W2T).

Fragments carrying the C5A mutations were generated by PCR on Cf-9 templates, using primer pair C5F + BR1, cloned into pBS, and used to clone the BCIamHII fragment encoding LRRs 1 to 17 in pRH385, resulting in C5A-mutated myc-Cf-9 (pRH410). The fragment carrying the C6A mutation was amplified by PCR overlap extension from Cf-9 using border primers BP2 + PR1 and overlap primers C6f + C6r. This fragment
was cloned and used to replace the fragment encoding LRRs 17-domain G of pRH385, resulting in C6A-mutated myc-Cf-9 (pRH451).

For construction of the C1-5A and C1-6A mutants, the C1-4A mutant B-domain was amplified using primers CF1 and C34R on template pRH407, which already contains the C2A mutation in Cf-9. This C1-4A mutated B-domain was used to replace the B-domain of the C5A mutant (pRH410), using Xhol and Clal restriction sites, generating C1-5A mutants of myc-Cf-9 (pRH483). The C6A mutation was subsequently introduced into pRH483 by replacing the fragment encoding the C-terminal half of the Cf proteins, resulting in a C1-6A mutated version of myc-Cf-9 (pRH520). The C4,5A mutant of myc-Cf-9 was constructed by replacing the Clal-BamHI fragment pRH409 (C4A), encoding the C-terminal part of the B-domain and 17 LRRs, with that of pRH410 (C5A), thereby creating pRH527 (C4,5A).

Cf-9 mutants lacking the myc-epitope tag were generated by replacing the 35S-SP-2myc-intron-2myc fragment from the vectors mentioned above by a cloned PCR fragment of the 35S promoter followed by a fragment encoding the signal peptide of Cf-9, amplified from pRH109 using primers XF5 and XR1, using Xbal and Xhol restriction sites. Plasmid names for the non-tagged Cf-9 mutants are pRH440(C1A), -441(C2A), -442(C3A), -443(C4A), -444(C5A), -468(C6A), -500(K1A), -480(W1A), and -473(W2A).

**Mutagenesis of Putative Glycosylation Sites**

The mutagenesis of the putative glycosylation sites in Cf-9 was performed in pBlueScript toolkit constructs containing Cf-9 cassettes using the Stratagene Quick Change kit according to the manufacturer’s instructions. The pBS construct SLJ13950 (Rivas et al., 2002) contains the full-length Cf-9 open reading frame fused to the TAP tag. SLJ20781 (pBS 5’ Cf-9) was made by digesting SLJ13950 with HincII and Scal and cloning the 2101-bp 5’ Cf-9 fragment in the EcoRV site of pBS KS+. SLJ20784 (pBS 3’ Cf-9) was made by digesting SLJ13950 with Clal and BstBI and self-ligating to remove the 1992-bp 5’ Cf-9 fragment. In the case of mutants 19ND, 21ND, and DN26, the toolkit construct pSLJ9595 (Benghezal et al., 1999) was used as a template for mutagenesis, and in the case of 18SL, the mutation was amplified from genomic DNA of the mutant M2 (Wulff et al., 2004). Mutated fragments were cloned into derivatives of the binary expression vector pSLJ14070 (Rivas et al., 2002) behind the 35S promoter of Cauliflower mosaic virus using unique restriction sites: In pSLJ20734, the full-length Cf-9:TAP fragment has been replaced by a Clal-BamHI cassette encoding part of the jellyfish green fluorescent protein. In pSLJ20808, the 5’ coding region of Cf-9 extending from the SnaBI site to the BsWI site has been replaced by a linker derived from oligos SnaBsiTOP and SnaBsiBOT (see Supplemental Table P online). In SLJ20808, the Cf-9 3’ coding sequence fused to TAP and the 3’ untranslated region have been replaced by a linker derived from oligos BisBamTop and BisBamBot. All mutants were sequenced from the binary constructs to exclude secondary PCR- or cloning-derived mutations.

**Agroinfiltration and Biochemical Procedures**

Agroinfiltration of tobacco (Nicotiana tabacum) and N. benthamiana plants was done as described previously (Van der Hoorn et al., 2000; Wulff et al., 2001). Hypersensitive response-inducing activity was scored at 7 dpi from at least three independent experiments by comparing activities with that of the positive control (Cf-9, myc-Cf-9, or Cf-9-TAP). Activities are summarized in the figures (with ++, +, −, or −−) as explained in the legends. A more extensive quantitative analysis was performed for the Cf-9 B-domain mutants in Figure 2B as described earlier (Van der Hoorn et al., 2000).

For detection of myc- and TAP-tagged Cf proteins, infiltrated leaf sectors were harvested at 1 dpi and ground in liquid nitrogen. Proteins were dissolved in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.5% polyvinyl polypyrrolidone, and protease inhibitors [PI Complete tablet; Roche, Indianapolis, IN]) and centrifuged for 15 min at 10,000g at 4°C, and SDS sample buffer was added to the supernatant. Protein gel blotting using anti-myc and peroxidase antiperoxidase and ECL detection were done as described previously (Van der Hoorn et al., 2003).

**Mass Spectrometric Analysis of Cf-9-TAP Protein**

Agrobacterium tumefaciens GV3101 pMP90 containing plasmid SLJ14190 was used to transiently express TAP-tagged Cf-9 under the control of its genomic promoter in N. benthamiana (Rivas et al., 2002). Two days after agroinfiltration, 72 leaves were harvested and protein extracts prepared as described (Rivas et al., 2002). Cf-9:TAP was subjected to immunoprecipitation with IgG beads (Sigma-Aldrich) for 2 h at 4°C, after a preclearing step with agarose beads for 1 h at 4°C. After incubation, beads were washed three times with extraction buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefablock; Sigma-Aldrich), 2 μg mL−1 of antipain, 2 μg mL−1 of leupeptin, and 2 μg mL−1 of aprotinin] supplemented with 40 mM octylglucoside (Rivas et al., 2002). Immunoprecipitated proteins were finally resuspended in SDS-PAGE sample buffer and separated on a 7.5% SDS-polyacrylamide gel (Laemmli, 1970). The gel was fixed in 50% ethanol and 2% phosphoric acid for at least 3 h and washed three times with water. Staining was performed in a solution of 34% methanol, 17% (NH4)2SO4, 3% phosphoric acid, and 0.66% Coomassie Brilliant Blue G-250 for at least 1 d. A band of ~185 kD, corresponding to Cf-9:TAP, was excised from the Coomassie-stained gel and subjected to in-gel tryptic digestion (Shevchenko et al., 1996). Tryptic peptides were eluted and analyzed by MALDI-TOF-MS and Q-TOF-MS/MS (John Innes Centre Proteomics Facility, Norwich, UK). Data were analyzed with Mascot and MS-Fit from Protein Prospector (http://prospector.ucsf.edu) and subjected to database searches against the Cf-9-TAP protein sequence.

**Homology Modeling**

Homology modeling was performed using the Homology module of Insight II (Insight II, Release 2000.1; Accelrys, Cambridge, UK). The x-ray crystal structures of PGIP (accession code 1OGQ) and Ina1 (16V) were retrieved from the Protein Data Bank (Bernstein et al., 2000) and used as templates to build the individual structures. Modeling was restricted to residues 51-795 of Cf-9 and 24-115 of FLS2. The B-domains were constructed using 1OGQ as a template, ensuring that the conserved Trp and Cys residues common to both 1OGQ and Cf-9 were matched in the sequence alignment. Disulfide linkages were constructed between residue pairs 53-77 and 78-85 of Cf-9 and 61-88 of FLS2. To model the large number of LRRs, a composite template was assembled using overlapping copies of 1OGQ and the LRR section of 16V by performing head- to-tail local structural alignments on the first and last few LRRs of each template as appropriate. The sequence alignment of Cf-9 with its template is included in the supplemental data online. Loops were generated using loop searching facility within Insight II and a database of loops taken from a parsed subset of release 103 of the Protein Data Bank. The model structures were energy minimized to a derivative of 1.0 using the consistent valence force field within the Discover module and validated using Procheck plus SwissPDBeViewer (http://www.expasy.ch/spdbv/mainpage.html) for inspection of the Ramachandran plots. Figures were generated using Molscript (Kraulis, 1991) and Raster 3D (Merritt and Bacon, 1997). The Cf-9 and FLS2 models are provided in the supplemental data online.
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REFERENCES


Kawchuk, L.M., Hachey, J., Lynch, D.R., Kulcsar, F., van Rooijen, G., Waterer, D.R., Robertson, A., Kokko, E., Byers, R., Howard, R.J.,


fasciated ear2 gene encodes a leucine-rich repeat receptor-like 
protein that regulates shoot meristem proliferation in maize. Genes 
Dev. 15, 2755–2766.

cysteine-rich extracellular protein, LAT52, interacts with the extracellular 
domain of the pollen receptor kinase LePRK2. Plant Cell 14, 
2277–2287.

Tör, M., Brown, D., Cooper, A., Woods-Tör, A., Sjölander, K., Jones, 
ERECTA 
gene encodes a receptor-like protein kinase with extracellular 

Toubart, P., Desiderio, A., Salvi, G., Cervone, F., Daroda, L., and De 
the endopolygalacturonase-inhibiting protein (PGIP) of Phaseolus 
vulgaris L. Plant J. 2, 367–373.

ERECTA gene encodes a putative receptor protein kinase with extracellular 

Toubart, P., Desiderio, A., Salvi, G., Cervone, F., Daroda, L., and De 
ERECTA 
gene encodes a receptor-like protein kinase with extracellular 

ERECTA gene encodes a putative receptor protein kinase with extracellular 

Vancanneyt, G., Schmidt, R., O’Connor-Sanchez, A., Willmitzer, L., 

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 

Van der Hoorn, R.A.L., Rivas, S., Wulff, B.B.H., Jones, J.D.G., and 
and Cf-9 resistance proteins is an intrinsic property of Cf proteins and not because of their association with high-molecular-weight proteins. Plant J. 35, 305–315.

Cf-4 allows construction of a Cf-9 mutant that confers recognition of 

Vinatzer, B.A., Patocchi, A., Gianfranceschi, L., Tartarini, S., 
receptor-like genes homologous to the Cladosporium fulvum resis-
tance gene family of tomato with a cluster of genes cosegregating with 

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


BR1 is a critical component of a plasma-membrane receptor for plant 

Wengier, D., Valsecchi, I., Cabanas, M.L., Tang, W.H., McCormick, 
S., and Muschietti, J. (2003). The receptor kinases LePRK1 and 
LePRK2 associate in pollen and when expressed in yeast, but 
USA 100, 6860–6865.

Worrall, D., Elias, L., Ashford, D., Smallwood, M., Sidebottom, C., 
leucine-rich-repeat protein that inhibits ice recrystallization. Science 
282, 115–117.

Genetic variation at the tomato Cf-4/Cf-9 locus induced by EMS 

Wulff, B.B.H., Thomas, C.M., Smoker, M., Grant, M., and Jones, 
J.D.G. (2001). Domain swapping and gene shuffling identify se-
quencies required for induction of an Avr-dependent hypersensitive 

Yamamuro, C., Ihara, Y., Wu, X., Noguchi, T., Fujioka, S., Takatsuto, 
function of a rice brassinosteroid insensitive 1 homolog prevents 
internode elongation and bending of the lamina joint. Plant Cell 12, 
1591–1605.


MICROSPORECYTES1 gene encodes a putative leucine-rich repeat 
receptor protein kinase that controls somatic and reproductive cell 
Structure–Function Analysis of Cf-9, a Receptor-Like Protein with Extracytoplasmic Leucine-Rich Repeats
Renier A.L. van der Hoorn, Brande B.H. Wulff, Susana Rivas, Marcus C. Durrant, Anke van der Ploeg, Pierre J.G.M. de Wit and Jonathan D.G. Jones

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eTOCs Sign up for eTOCs at: http://www.plantcell.org/cgi/alerts/ctmain
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