Research Note

The C-terminal Dilysine Motif for Targeting to the Endoplasmic Reticulum Is Not Required for Cf-9 Function

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The tomato resistance gene *Cf-9* encodes a membrane-anchored, receptor-like protein that mediates specific recognition of the extracellular elicitor protein AVR9 of *Cladosporium fulvum*. The C-terminal dilysine motif (**KKRY**) of Cf-9 suggests that the protein resides in the endoplasmic reticulum. Previously, two conflicting reports on the subcellular location of Cf-9 were published. Here we show that the AARY mutant version of Cf-9 is still functional in mediating AVR9 recognition, suggesting that functional Cf-9 resides in the plasma membrane. The data presented here and in reports by others can be explained by masking the dilysine signal of Cf-9 with other proteins.

Additional keywords: avirulence gene, CLAVATA, ER retention, ER retrieval, high-affinity binding site.

The interaction between tomato and the pathogenic biotrophic fungus *Cladosporium fulvum* is a well-established model in which to study molecular aspects of host resistance and pathogen avirulence (Joosten and De Wit 1999). In this interaction, gene-for-gene-based recognition events result in a hypersensitive response (HR) and host resistance. Avirulence gene *Avr9* of *C. fulvum* encodes a race-specific elicitor that is secreted by the fungus during its growth in the extracellular space of the tomato leaf (Van Kan et al. 1991). *Cf-9*, the matching resistance gene of tomato, encodes a receptor-like protein with leucine-rich repeats (LRRs), a transmembrane domain, and a short C-terminal domain that lacks motifs for downstream signaling (Jones et al. 1994). Transformation of tobacco, potato, petunia, and several *Nicotiana* species with *Cf-9* showed that this gene is essential and sufficient to confer specific recognition of AVR9 (Hammond-Kosack et al. 1998; Van der Hoorn et al. 2000).

Considering the key role of Cf-9 in mediating perception of the extracellular AVR9 protein, it is anticipated that Cf-9 resides in the plasma membrane with its LRRs protruding in the extracellular space. The presence of a C-terminal dilysine motif (**KKRY**), however, predicts a different location for the

Cf-9 protein. As seen in yeast and mammals, this type of motif confers endoplasmic reticulum (ER) localization of membrane-anchored proteins through retrieval and retention mechanisms (Andersson et al. 1999; Teasdale and Jackson 1996). To elucidate the role of Cf-9 in AVR9 perception and subsequent activation of HR, it is crucial to know the exact location of the Cf-9 protein within the plant cell. Recently, two conflicting reports on the subcellular location of Cf-9 were published. One report shows that Cf-9 is localized in the plasma membrane (Piedras et al. 2000), whereas another demonstrates that the dilysine motif of Cf-9 is functional and the protein resides in the ER (Benghezal et al. 2000). The latter study also shows that the ER retrieval–retention mechanism is saturable, raising serious doubts over the conclusions drawn by Piedras et al. (2000), who used *Cf-9* transgenes that were driven by the strong CaMV 35S promoter. Thus far, the requirement of the KKRY motif for the function of Cf-9 has not been examined.

In this study, we examined the effect of mutations in the KKRY motif on Cf-9 function. Coexpression of *Cf-9* with *Avr9* by agroinfiltration of tobacco leaf sectors results in an HR that is visible as necrosis (Van der Hoorn et al. 2000) and was used as an assay to test the function and activity of mutant Cf-9. To determine whether the dilysine motif is essential for Cf-9 function, we changed the KKRY sequence of Cf-9 into AARY (Fig. 1A). Significantly, in the presence of AVR9, agroinfiltration of Cf-9-AARY showed that this mutant is still functional in AVR9 recognition (Fig. 1B).

If Cf-9 is functional in the ER, then activity of the Cf-9-AARY mutant that lacks the ER retrieval–retention signal could be the result of overexpression, by which the secretion pathway is saturated and part of the Cf-9-AARY proteins remains in the ER. We therefore examined the activity of the Cf-9-AARY mutant at lower expression levels. During agroinfiltration, *Agrobacterium tumefaciens* cells are present in excess compared with plant cells, which results in the transformation of nearly every cell of the infiltrated tissue (Kapila et al. 1997). Dilution of the cultures likely results in a decrease in the number of T-DNA transfers to each cell, resulting in decreased expression levels. This quantitative assay was introduced previously (Van der Hoorn et al. 2000) and was validated further by demonstrating that Cf-4 mutants carrying

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single amino acid substitutions showed a slightly reduced activity when compared with wild-type Cf-4 (Van der Hoorn et al., *in press*). Quantification of necrotic areas obtained from infiltration of dilution series showed that the Cf-9-AARY mutant is only slightly less active as wild-type Cf-9 (Cf-9-KKRY) at lower expression levels (Fig. 1C). Therefore, the activity of the Cf-9-AARY mutant is not likely to result from overexpression.

These data indicate that the dilysine motif in Cf-9 is somehow ignored by the ER retrieval-retention mechanism. Studies on translocation of proteins in yeast showed that phenylalanine (F) or tyrosine (Y) residues at the C terminus can weaken the ER retrieval-retention mechanism (Itin et al. 1995). The presence of a tyrosine in the KKRY motif may

therefore reduce the effect of the dilysine motif. To study the role of the two C-terminal amino acid residues in Cf-9, we changed the KKRY motif into KKAA (Fig. 1A). This Cf-9-KKAA mutant appeared to be fully functional (Fig. 1B), and its activity is only slightly reduced when compared with wild-type Cf-9 (Cf-9-KKRY) (Fig. 1C), demonstrating that weakening the effect of the KKxx motif by the C-terminal tyrosine is not compromising Cf-9 function.

Another reason why the Cf-9-AARY mutant is still functional could be the absence of the dilysine motif in the mature Cf-9 protein. LRR proteins that are not anchored in the membrane can still be functional in recognition. Examples are the resistance gene product Xa21D (Wang et al. 1998) and polylacturonase inhibitor proteins (De Lorenzo and Cervone

Fig. 1. Functionality and activity of Cf-9 mutants. **A**, Schematic representation of the Cf-9 protein encoded by open reading frames (ORFs) of the (mutant) Cf-9 constructs used in this study. Genes are present on the T-DNA of binary plasmids and ORFs encoding (mutant) Cf-9 are driven by the CaMV 35S promoter. The four C-terminal amino acid residues are shown. Two mutations in the KKRY motif are boxed. The C-terminal ISWQ sequence of the Cf-9-ΔTMG mutant represents the C-terminal end of the E domain (Jones and Jones 1997). Wild-type Cf-9 (Cf-9-KKRY) is encoded by binary plasmid pRH27, which was generated by cloning a fragment encoding LRRs 1–17 of Cf-9 into binary plasmid pRH22 that contains Cf-9 lacking the sequence encoding LRRs 1–17 with the *Cla*I and *Bam*HI restriction sites (Van der Hoorn et al. 2001). Cf-9 mutants were generated by polymerase chain reaction with pRH27 as template and forward primer 5'-GCATTATTGGAGATCTTGGTTGG-3' (*Bgl*III site underlined) and reverse primers 5'-AGCTCTGCAGCTAATATCTTGGCGGTGCTTTTTCATTTTCGTAG-3', 5'-AGCTCTGCAGCTAAGCTGCTTTCTTGTGCTTTTTCATTTTCGTAG-3', and 5'-AAACTGCAGCTACTGCCAACTGATCATTGGTG-3' (*Pst*I sites underlined), thereby generating mutants Cf-9-AARY, Cf-9-KKAA, and Cf-9-ΔTMG, respectively. Amplified fragments were cloned into pRH18, which encodes Cf-9 lacking LRRs 1–17 (Van der Hoorn et al. 2001), with the *Bgl*III and *Pst*I restriction sites. Fragments with the correct sequence were cloned into pRH27 with the *Bam*HI and *Eco*RI restriction sites, resulting in pRH386, pRH377, and pRH380, respectively. These vectors and pRH27 were transferred to *Agrobacterium tumefaciens* (strain MOG101), which was used for agroinfiltration. SP, signal peptide; B, B domain; LRRs, domain containing 27 leucine-rich repeats; D, D domain; E, E domain; TM, transmembrane domain; G, short C-terminal domain. **B**, Transient expression of (mutant) Cf-9 proteins in tobacco. Agroinfiltration of transgenic cultures of *A. tumefaciens* carrying pRH27 and various (mutant) Cf-9-encoding constructs into *Nicotiana tabacum* cv. Petite Havana SR1 was performed as described (Van der Hoorn et al. 2000). For transient coexpression of Cf-4 and (mutant) Cf-9 genes with *Avr9* or *Avr4*, *A. tumefaciens* cultures containing each of the genes were mixed in a 1:1 ratio and infiltrated into a leaf sector of tobacco. Photograph was taken at 7 days postinfiltration (dpi) and represents four independent assays. **C**, Quantitative comparison of the necrosis-inducing activity of wild-type Cf-9 (Cf-9-KKRY) with Cf-9-AARY (upper panel) and Cf-9-KKAA (lower panel) mutants. *A. tumefaciens* cultures carrying the constructs were mixed in different ratios with a culture of equal density carrying an AVR9-encoding plasmid and infiltrated into sectors of opposite tobacco leaf halves (Van der Hoorn et al. 2000). The percentage of the infiltrated area that had become necrotic at 7 dpi was measured and plotted against the concentration of *A. tumefaciens* that carries the plasmid-encoding (mutant) Cf-9. Curve represents nine curves obtained from three independent triplicate experiments.

1997). Interestingly, heterologous expression of the Cf-9-NNRY mutant in tobacco BY-2 cells results in a similar truncated protein consisting of domains B to E (Fig. 1) that remains associated with the membrane fraction (Benghezal et al. 2000). In order to test whether Cf-9 protein lacking the trans-membrane domain is still functional, we deleted the TM and G domains from Cf-9, resulting in mutant Cf-9-ΔTMG (Fig. 1A). This mutant was no longer functional in AVR9 recognition (Fig. 1B), indicating that the membrane anchor is required for Cf-9 function.

The most plausible explanation for the dilysine motif not to be essential for Cf-9 function is that other proteins mask this motif. A similar escape from the ER retrieval-retention machinery has been described for the human receptor complex for immunoglobulin E, in which the α -chain contains a dilysine motif that is sterically masked after association with the γ -chain on the ER membrane (Letourneur et al. 1995). This masking is thought to result in quality control at the level of the ER, discriminating between assembled and unassembled receptors. Only fully assembled receptor complexes in which the dilysine motif is masked can leave the ER. The dilysine motif of Cf-9 might function in a similar way, assuring that only Cf-9 protein present in a functional signaling complex integrates in the plasma membrane. Thus, nonfunctional Cf-9 protein remains in the ER, whereas functional Cf-9 protein present in a complex is transported to the plasma membrane. The slightly reduced activity of the Cf-9-AARY mutant might be a result of a quicker exit from the ER of this protein, thus interfering with the formation of functional complexes.

We suggest two candidate proteins that could mask the KKRY motif of Cf-9 in a complex. Because Cf-9 does not appear to bind directly to AVR9 (R. Luderer, S. Rivas, T. Nürnberger, and B. Mattei, *unpublished results*), it seems likely that perception of AVR9 requires the high-affinity binding site (HABS) for AVR9 that is present in plasma membranes of solanaceous plants (Kooman-Gersmann et al. 1996). This HABS possibly could mask the dilysine signal upon association with Cf-9 on the ER membrane. A second candidate protein is a receptor-like kinase (RLK), similar to CLAVATA-1 (CLV1). The CLV perception complex determines cell fate in shoot and floral meristems of Arabidopsis and Brassica species (Torii 2000). The CLV2 protein, which has a structure that is very similar to Cf-9, functions within a complex that includes CLV1 (Jeong et al. 1999). By analogy, Cf-9, similar to CLV2, does not contain a signaling domain and could require association with an RLK, like CLV1, to function. Because this type of RLK carries a cytoplasmic kinase domain, it may mask the dilysine motif of Cf-9.

This “masking model” could explain the results presented by Piedras et al. (2000). The CaMV 35S-driven Cf-9 transgene likely overproduces Cf-9, without increasing expression of the masking protein. This probably results in the accumulation of Cf-9 in the ER in addition to its presence in the plasma membrane. With the use of two-phase partitioning, Piedras et al. (2000) showed that Cf-9 is present in the plasma membrane, although the authors ignored a significant signal in the ER fraction. Moreover, labeling of protoplasts showed that Cf-9 is present at the cell surface, although the chosen experimental conditions were not suitable to detect signals in the ER. Thus, the signal in the ER that was ignored by Piedras et

al. (2000) could represent an inactive form of Cf-9 that accumulates as a result of Cf-9 overexpression. Interestingly, Piedras et al. (2000) also noted that insertion of cMyc epitope tags in the G domain of Cf-9 renders the protein less active. A similar reduced activity was observed upon insertion of a FLAG epitope tag in this domain (R. A. L. Van der Hoorn, *unpublished*). It is likely that insertion of epitope tags in the G domain increases the distance between the membrane and the KKRY motif, which results in a less efficient masking, thereby reducing the activity of Cf-9.

Benghezal et al. (2000) used transgenic BY-2 tobacco cells containing 35S-driven Cf-9 for two-phase partitioning to show that Cf-9 is absent from the plasma membrane fraction, whereas it is present in high amounts in the fraction containing ER membranes. It is unknown, however, whether Cf-9 is functional in these cells. Functionality of Cf-9 can be developmentally regulated (Honée et al. 1998), and the absence of the masking protein in these cells would result in complete localization of Cf-9 to the ER. Alternatively, only a small, undetectable fraction of Cf-9 might be present in the plasma membrane of BY-2 cells, where it is functional. The authors also found endoproteolytic cleavage in the E-domain of Cf-9, which was observed only for the Cf-9-NNRY mutant and not for wild-type Cf-9 (Benghezal et al. 2000). This mutant protein is not retained in the ER and is likely exposed to proteolytic enzymes during transport to the plasma membrane. Functional Cf-9 may be resistant to these enzymes because the endoprotease recognition site can be buried in the assembled complex.

This “masking hypothesis” also could apply to Cf-4 and Hcr9-4E proteins that carry a C-terminal dilysine motif and mediate recognition of the extracellular elicitors AVR4 and AVR4E, respectively (Takken et al. 1999; Thomas et al. 1997). A plasma membrane location also is expected for Cf-2 and Cf-5, which are similar receptor-like proteins but do not contain a dilysine motif (Dixon et al. 1996; Dixon et al. 1998). These Cf proteins mediate recognition of the extracellular elicitors AVR2 and AVR5, respectively. In conclusion, all cloned tomato genes that confer resistance to the extracellular leaf pathogen *C. fulvum* encode receptor-like proteins that are likely anchored in the plasma membrane and mediate recognition of extracellular elicitor proteins. The exact process of elicitor perception on the plasma membrane is an exiting topic for further investigation.

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