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# ARABIDOPSIS CRINKLY4 Function, Internalization, and Turnover Are Dependent on the Extracellular Crinkly Repeat Domain [W]

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**The study of the regulation and cellular dynamics of receptor kinase signaling in plants is a rapidly evolving field that promises to give enormous insights into the molecular control of signal perception. In this study, we have analyzed the behavior of the L1-specific receptor kinase ARABIDOPSIS CRINKLY4 (ACR4) from *Arabidopsis thaliana* in planta and have shown it to be present in two distinct compartments within cells. These represent protein export bodies and a population of internalized vesicles. In parallel, deletion analysis has shown that a predicted β-propeller–forming extracellular domain is necessary for ACR4 function. Nonfunctional ACR4 variants with deletions or point mutations in this domain behave differently to wild-type fusion protein in that they are not internalized to the same extent. In addition, in contrast with functional ACR4, which appears to be rapidly turned over, they are stabilized. Thus, for ACR4, internalization and turnover are linked and depend on functionality, suggesting that ACR4 signaling may be subject to damping down via internalization and degradation. The observed rapid turnover of ACR4 sets it apart from other recently studied plant receptor kinases. Finally, ACR4 kinase activity is not required for protein function, leading us to propose, by analogy to animal systems, that ACR4 may hetero-oligomerize with a kinase-active partner during signaling. Plant and animal receptor kinases have distinct evolutionary origins. However, with other recent work, our study suggests that there has been considerable convergent evolution between mechanisms used to regulate their activity.**

## INTRODUCTION

Plant receptor-like kinases (RLKs) represent one of the largest protein super families identified in *Arabidopsis thaliana*, with >600 potential members (Shiu and Bleecker, 2001a, 2001b, 2003). They generally have a typical receptor kinase structure being composed of an extracellular domain, a transmembrane span, and a cytoplasmic domain containing conserved kinase motifs. The RLK clade in plants is distinct from that of animals in that the catalytic cytoplasmic domains appear to represent a monophyletic group descended from an ancestor related to the PELLE/IRAK-like cytoplasmic kinases in animals. Over the course of plant evolution, the RLK cytoplasmic domain has become associated with diverse extracellular domains, giving a wide range of distinct subgroups. By far the largest of these subgroups is represented by the Leu-rich repeat (LRR) RLKs (Dievart and Clark, 2004). These proteins have been relatively widely studied and implicated in a wide variety of processes, ranging from developmental patterning and growth substance perception to the interaction of plants with symbiotic and pathogenic bacteria. Of the other smaller but very distinct subgroups, only the S-LOCUS RECEPTOR KINASE (SRK)–like group and WALL-ASSOCIATED KINASE (WAK) group have been studied in any great detail at the cellular level (Cabrillac et al., 2001; Kohorn, 2001; Wagner and Kohorn, 2001; Vanoosthuyse et al., 2003; Murase et al., 2004).

In animals, modulation of signaling by receptor kinases is brought about by many mechanisms, including protein modifications, regulatory interactions at the membrane, and internalization before recycling or turnover. Although PELLE/IRAK-like kinases in animal systems are associated with receptor kinase–containing complexes, it is impossible to draw meaningful direct parallels about the behavior and interactions of plant RLKs from the behavior of PELLE and related cytoplasmic kinases in animals. In addition, because of their apparently evolutionarily distinct origins, it also seems probable that many aspects of the control of localization and activity of plant RLKs at the membrane have evolved independently of mechanisms associated with animal receptor kinases, although the basic cellular machinery evolved is likely to be similar (Cock et al., 2002). To complicate the issue further, it also seems likely that, as in animals, a wide variety of mechanisms localize and regulate the activity of plant RLKs and that these could differ dramatically between different

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subfamilies. The study of RLK regulation in plants could therefore produce novel examples of convergent evolution in regulatory mechanisms at the protein level.

Receptor kinase activation in animals generally occurs in response to ligand binding, with downstream signaling being mediated by phosphorylation of the cytoplasmic domain. Ligand binding often mediates or stabilizes receptor oligomerization; thus, ligand-mediated changes in phosphorylation status can be due to either receptor autophosphorylation or to transphosphorylation between kinase domains within an oligomeric complex. There is considerable evidence that, as in animal systems, many plant RLKs autophosphorylate in response to ligand binding (Cock et al., 2002; Tichtinsky et al., 2003). There is also evidence that, as in animal systems, oligomerization of plant receptors is common and that *trans*-phosphorylation within receptor complexes can occur (Jeong et al., 1999; Li et al., 2002; Nam and Li, 2002; Russinova et al., 2004). Activation in plant RLK complexes can also require interactions with receptor-like molecules lacking transmembrane and extracellular domains, the so-called receptor-like cytoplasmic kinases (Murase et al., 2004).

In both animal and plant receptor kinases, the phosphorylation status of the cytoplasmic domain is critical for activation and downstream signaling. Several mechanisms appear to have evolved to repress kinase activity when ligands are not present and to downregulate, or dampen down, signaling after ligand binding. Such mechanisms are crucial in maintaining responsivity to changes in ligand availability, and in animals, they include the action of phosphatases as well as internalization and turnover or recycling of receptors within the cell (Ostman and Bohmer, 2001; Gonzalez-Gaitan and Stenmark, 2003). Interestingly, animal early endosomal compartments can participate in signaling by activated receptors. In addition, it has been shown that, for some animal receptor kinases, molecules can be endocytosed and recycled to the membrane in the absence of ligand binding, possibly to aid in their redistribution within the membrane. Thus, endocytosis per se cannot be used as a gauge of receptor activation or inactivation (reviewed in Gonzalez-Gaitan, 2003).

In plants, some parallel mechanisms for receptor regulation appear to have evolved. KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP) has been found to interact with many RLKs, including CLAVATA1, WAK1, SRK, SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 (SERK1), and FLAGELLIN SENSITIVE2, and has been shown to negatively regulate at least a subset of these molecules by dephosphorylating them (Braun et al., 1997; Williams et al., 1997; Stone et al., 1998; Gomez-Gomez et al., 2001; Park et al., 2001; Shah et al., 2002; Vanoosthuyse et al., 2003). In some cases, the activity of KAPP may in turn be regulated by its phosphorylation by the active form of the kinase in question (van der Knaap et al., 1999). Interesting recent work has also linked the activity of KAPP to internalization. In cowpea (*Vigna unguiculata*) protoplasts, stable interaction between KAPP and SERK (revealed by fluorescence resonance energy transfer) occurs in internalized vesicles rather than at the plasma membrane. Moreover, the presence of KAPP appeared to be required for internalization of SERK in this system (Shah et al., 2002). Thus, it seems possible that dephosphorylation by KAPP and internalization could be integrally linked methods of downregulating at least a subset of plant RLKs. In addition, it has also been shown that BRASSINOSTEROID INSENSITIVE1 (BRI1) and AtSERK3 (BAK1) heterodimerize and are endocytosed in cowpea protoplasts and moreover that heterodimerization leads to accelerated endocytosis compared with homodimerization of BRI1. This endocytosis has not been linked to ligand binding and may represent some form of constitutive protein redistribution mechanism, as is undergone by animal receptors, such as members of the EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) family (Russinova et al., 2004).

How plant RLKs are targeted for internalization, and to their subsequent fates, remains largely unknown. In animals, several receptor kinases, including the EGFR family, have been shown to undergo ubiquitylation catalyzed by ubiquitin ligases like the RING finger protein CASITAS B LYMPHOMA (CBL). CBL activation appears to require phosphorylation by the activated kinase domain. Ubiquitylation, by CBL and possibly other proteins, is a necessary signal for internalization but also seems to play an important role in deciding whether the final fate of the internalized receptor will be degradation by the lysosome or proteasome or a default pathway involving recycling back to the membrane (Dikic and Giordano, 2003; Marmor and Yarden, 2004). Interestingly, a protein shown to interact with the plant RLK SRK, ARM REPEAT CONTAINING1, has a U domain structurally related to the RING finger domain and shows ubiquitin ligase activity, raising the interesting possibility that it, and related proteins, could be involved in the ubiquitylation of plant receptor kinases (Stone et al., 2003). Another plant RLK, the chitinase-related CHRK1, has also been shown to interact with a similar U domain containing protein in tobacco (*Nicotiana tabacum*) (Kim et al., 2003). In addition, SRK has been shown to interact with a sorting nexin (SNX1), another element implicated in downregulation of receptor kinase signaling by internalization in animal systems (Vanoosthuyse et al., 2003; Merino-Trigo et al., 2004).

Aside from a link with KAPP and potentially ubiquitylation, very little is known about the molecular mechanisms regulating internalization of plant receptor kinases. It is not even certain that internalization is a regulatory mechanism for all RLKs in plants or if ligand binding and protein activation are required for it to occur. A few potential components of pathways involved in the internalization of other plant plasma membrane proteins have been identified, including the brefeldin A (BFA)–sensitive ARF GDP/GTP exchange factor GNOM and members of the Rab family of small GTPases (Steinmann et al., 1999; Bonifacino and Jackson, 2003; Geldner et al., 2003). However, links between these proteins, or indeed internalization per se, and receptor kinase function have not yet been made. Evidence that plant receptor kinases are internalized comes from studies in heterologous cellular systems, as performed for SERK and BRI1 (above), as well as the fact that the localization of several receptor kinases is BFA sensitive, indicating that they may be present in internalized vesicles (Gifford et al., 2003; Russinova et al., 2004).

Here, we present evidence that the ARABIDOPSIS CRINKLY4 (ACR4) protein, which is required for normal L1 cell layer organization, is internalized from the plasma membrane via a BFA-sensitive pathway when expressed at physiological concentrations under its own promoter. We also present a functional analysis of ACR4 that shows that part of the ACR4 extracellular domain is absolutely required both for signaling and for normal

protein internalization to take place. Finally, we show that protein functionality and internalization are linked to a rapid protein turnover, which is lost when the extracellular domain is mutated.

## RESULTS

### Subcellular Behavior of a C-Terminal Green Fluorescent Protein Fusion to the ACR4 Protein Expressed under the ACR4 Promoter

The ACR4 protein is an RLK that is required for normal epidermal development and organization in Arabidopsis (Gifford et al., 2003; Watanabe et al., 2004). The *acr4* mutant phenotype, which includes disorganized growth of ovule integuments and sepal margins, can be complemented by the introduction of the ACR4 open reading frame under the control of the *ACR4* promoter. In addition, C-terminal fusions of green fluorescent protein (GFP) to ACR4 have been shown to complement the *acr4* mutant phenotype and to produce protein that localizes to the plasma membranes of L1/epidermal cells in all shoot meristems, ovule integuments, and root primordia. Cytoplasmic bodies containing GFP were also observed in ACR4:GFP expressing roots (Gifford et al., 2003). Treatment with BFA led to the appearance of large GFP-containing bodies in the root cells of ACR4:GFP-expressing plants. BFA is a commonly used inhibitor of vesicle movement in both animals and plants and is thought to act by targeting and inhibiting the action of proteins involved in vesicle formation. BFA inhibits a subset of GDP/GTP guanine-nucleotide exchange factors (Geldner et al., 2001, 2003; Nebenfuhr et al., 2002). The exact basis for BFA effects is not clear for either animal or plant cells, and the effects of BFA seem to vary between different plant cell types. It is, however, widely accepted that BFA disrupts the trafficking of internalized vesicles in Arabidopsis root cells (Nebenfuhr et al., 2002; Russinova et al., 2004; Samaj et al., 2004).

To understand further the nature of the cytoplasmic bodies observed in ACR4:GFP-expressing plants, colocalization studies were performed using the amphiphilic steryl dye FM4-64 (Bolte et al., 2004). FM4-64 inserts into one side of the plasma membrane bilayer and fluoresces only when in a hydrophobic environment. The amphiphilic nature of the dye means that it is thought only to be able to enter the cell via internalization of membrane vesicles. In our tests, plasma membranes in the epidermal meristematic regions of roots incubated in 17 μM FM4-64 were well labeled after 15 to 20 min, with internal vesicles clearly visible after 30 min. After 80 min, large parts of the endomembrane system and vacuolar membrane started to become visible, eventually obscuring the smaller vesicles (data not shown). Several authors have reported that extended treatment with FM4-64 can lead to labeling of vesicles derived from the Golgi apparatus and targeted to the plasma membrane or to vacuoles (Ueda et al., 2001). Because we wanted to distinguish between internalized plasma membrane–derived vesicles and vesicles derived from the endomembrane system, our observations were made in a window between 30 and 50 min after the start of treatment.

Observations in small, unexpanded cells near to the root tip of plants carrying the ACR4:GFP construct and treated with FM4-64 showed the presence of two distinct populations of GFP-containing bodies, one labeled with FM4-64 and the other only with GFP (Figures 1A to 1C). Occasional colocalization of GFP fluorescence with FM4-64 labeling was observed, but GFP fluorescence in bodies showing colocalization was always considerably weaker than in GFP-only bodies. To determine whether the distribution and nature of bodies changed in more mature cell types, cells within the elongation zone (just distal to the zone where the bulges of root hair initiate) were also examined. In this zone, although GFP-only bodies were still observed, they appeared relatively less abundant than in more distal zones. However, many more of the FM4-64–labeled bodies also showed GFP fluorescence, although this was still weak compared with GFP-only–labeled bodies (Figures 1D to 1F).

Because we observe two independent populations of vesicles, we were intrigued as to whether these populations responded differently to treatment with BFA. Roots from plants expressing ACR4:GFP were treated with FM4-64 and 50 μM BFA or with FM4-64 and 0.5% DMS0 (control) for 1 h, and then the distribution of both GFP and FM4-64 were noted (Figures 1G to 1I). BFA had a considerably greater effect on FM4-64 distribution than on GFP distribution. Most cells contained relatively few small FM4-64 staining vesicles compared with controls and one to three very large cytoplasmic bodies, which were densely stained with FM4-64. These BFA-induced bodies were also found to contain GFP fluorescence (Figures 1G to 1I). Interestingly, GFP fluorescence was still observed in a population of very bright, small GFP-only bodies, which appeared identical to those observed in control cells. The intensity of GFP fluorescence in the BFA-induced bodies tended to be lower than that in the GFP-only staining bodies, especially in meristematic regions. It seems clear, therefore, that FM4-64–labeled compartments and GFP-only bodies behave very differently in the presence of BFA, with GFP-only bodies showing little or no perturbation in their behavior.

One explanation for the presence of numerous and bright GFP-only bodies, with little or no sensitivity to BFA, is that these are outbound compartments delivering ACR4:GFP to the plasma membrane. To investigate this possibility, treatments with the protein synthesis inhibitor cycloheximide were performed. Roots of ACR4:GFP-expressing plants were subjected to treatments with 30 μM cycloheximide. Roots were incubated for 1.5, 2.5, or 3.5 h in cycloheximide (Figures 1K to 1M). We observed a steady decrease in the intensity of GFP-only bodies over time compared with 0.1% ethanol controls (Figure 1J). By 2.5 h, there seemed to be an increased population of weakly marked GFP-only bodies and only a few bright bodies resembling those seen in control roots. By 3.5 h, the GFP-only bodies that could be observed were almost all only weakly fluorescent. In addition, membrane-associated GFP fluorescence intensity in these samples was generally lower than in control roots. To determine whether roots were capable of recovering from cycloheximide treatments, seedlings that had been treated with cycloheximide for 3.5 h were subjected to several washes in water, incubated in a large volume of water for 4 h, and observed (Figure 1N). Strongly fluorescing GFP-only bodies were once more observed in the cytoplasm of root epidermal cells. In many cases, these bodies appeared even more strongly fluorescent than in control plants. Because cycloheximide is a general protein transport inhibitor,

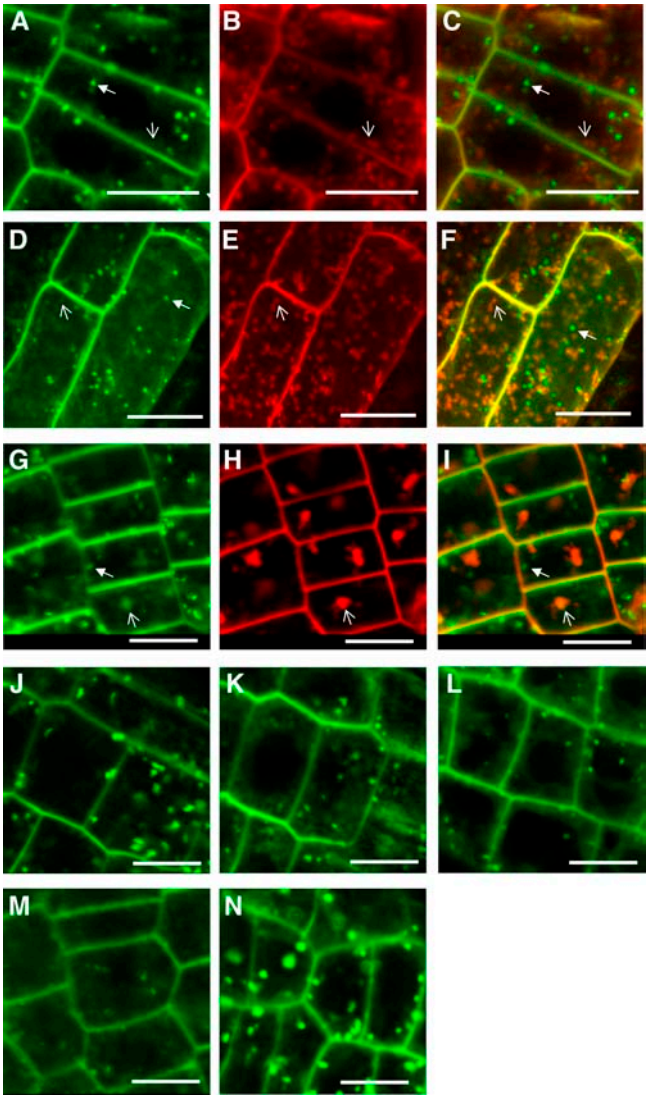


**Figure 1.** Behavior of ACR4:GFP in Root Cells.

**(A)** to **(I)** Root cells of plants carrying the complementing *ACR4:GFP* fusion protein and treated with FM4-64. Cells were simultaneously observed using the GFP channel (**[A]**, **[D]**, and **[G]**) and the FM4-64 channel (**[B]**, **[E]**, and **[H]**), and images were then merged (**[C]**, **[F]**, and **[I]**).

**(A)** to **(C)** Cells near the root tip. Closed arrows indicate GFP-only compartments, and line arrows indicate GFP/FM4-64 colocalizing vesicles.

**(D)** to **(F)** Cells in the elongation zone of the root. Colocalization is more pronounced in this region.

**(G)** to **(I)** BFA-treated meristematic cells. FM4-64 fluorescence is concentrated into large bodies that show GFP colocalization. GFP-only compartments appear unaffected by BFA treatment.

**(J)** to **(N)** GFP images of a cycloheximide treatment time course. **(J)** shows a 3.5-h control. **(K)** to **(M)** show treatment with cycloheximide for 1.5, 2.5, and 3.5 h, respectively. **(N)** shows treated cells after a 4-h recovery period.

Bars = 5 μM.

the effects of cycloheximide might have been attributable to a disruption of protein trafficking in general. However, when FM4-64 was added to roots treated with cycloheximide for 3.5 h, internalized vesicle were observed after 20 to 30 min, as in noncycloheximide-treated controls, suggesting that membrane internalization at least is not noticeably inhibited by treatments of this length (see Supplemental Figure 1 online).

Our interpretation of the above results is that BFA-insensitive GFP-only bodies may represent outgoing compartments carrying freshly made ACR4:GFP to the membrane. Cycloheximide inhibits de novo ACR4:GFP synthesis, and this is visible as a gradual reduction in the fluorescence of GFP-only compartments. BFA-sensitive bodies showing colocalization of FM4-64 and weak GFP fluorescence probably represent internalized membrane vesicles.

Several animal receptor kinases are subject to ligand binding–mediated internalization via BFA sensitive pathways. In some cases, this internalization leads to protein degradation, whereas in others, internalized proteins are recycled to the plasma membrane. To ascertain whether the ACR4:GFP protein is subject to cleavage or degradation, protein gel blots were performed with an anti-GFP polyclonal antibody using inflorescence material from plants carrying the $P_{ACR4}$:*ACR4*:*GFP* fusion construct. Surprisingly, full-length fusion protein (predicted $M_r$ 125 kD) proved almost very difficult to detect (shown as part of Figure 6). Shorter protein products were often observed, but their lengths depended on the protein isolation conditions used (data not shown). The inability to detect full-length proteins appears not to be due to nonspecific protein degradation because other *ACR4* variants expressed under the *ACR4* promoter produced proteins that were easily detectable (results below). It therefore seems likely that ACR4:GFP is rapidly turned over at the membrane. Rapid degradation would be supported by the observed disparity in intensity of internalized vesicles compared with GFP-only labeling export bodies.

## Functional Analysis of the ACR4 Extracellular Domain

To ascertain the roles of the various protein domains of ACR4 for protein function, localization, and turnover, a complementation approach was undertaken. A series of ACR4 derivatives (Figure 2) in which specific protein domains were deleted or modified were expressed from the *ACR4* promoter in an *acr4* null background (*acr4-2*). Constructs encoding identical protein derivatives fused C-terminally with GFP were transformed into wild-type and *acr4* mutant plants to test localization.

The two major domains within the extracellular region of ACR4 are a domain consisting of seven repeats termed "crinkly repeats" (Cao et al., 2005) and a domain with homology to the three Cys-rich repeats of the tumor necrosis factor receptor (TNFR) extracellular domain. In construct ΔREPEAT, 4.5 of the 7 crinkly repeats had been deleted, and in construct ΔTNFR, the entire TNFR homology domain was removed. Constructs ΔREPEAT:GFP and ΔTNFR:GFP included C-terminal GFP fusions. Another construction, ΔTM/KIN/C-TER:GFP, in which the 39–amino acid repeats and TNFR homology region were retained, but the transmembrane and cytoplasmic domains were removed, was created to

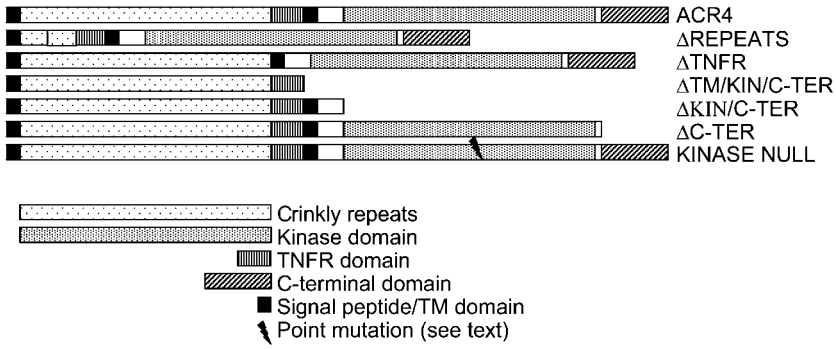


**Figure 2.** ACR4 Derivatives Used for Complementation Analysis.

confirm that the crinkly repeat and TNFR homology domains were targeted to the extracellular space.

Complementation studies were performed by transforming constructions directly into *acr4-2* homozygous plants and screening for complementation in at least 20 T1 individuals. Complementation was confirmed in subsequent generations and was assessed based on silique filling and seed phenotype. Noncomplemented lines showed >40% ovule abortion, heterogeneous seed development including embryo/seed abortion at around the heart stage, and mature seeds with an irregular surface and an abnormal rounded shape, as previously described (Gifford et al., 2003). Partially complemented lines tended to show decreased ovule and/or seed abortion but maintained abnormal seed shape. Fully complemented lines showed full siliques, with wild-type–shaped seeds. Details are presented in Supplemental Figure 2 and Supplemental Table 1 online. Of the five mutant proteins expressed, only ΔTNFR and ΔTNFR:GFP were found to complement the *acr4* mutant phenotype. ΔREPEAT:GFP and ΔTNFR:GFP were localized to the same membranes as the ACR4:GFP protein fusion, confirming that the lack of complementation by ΔREPEAT:GFP was not due to mislocalization. Interestingly, fluorescence in the membranes of ΔTNFR:GFP-expressing lines generally appeared slightly stronger than in ACR4:GFP lines, whereas fluorescence in ΔREPEAT:GFP lines was usually considerably stronger than that in ACR4:GFP lines (data not shown). Fluorescence in ΔTM/KIN/C-TER:GFP lines was not only stronger than in ACR4:GFP expressing lines but also localized to the cell wall rather than to the plasma membrane. Fluorescence signal appeared stronger in regions with a thicker wall, for example, at the corners of cells, suggesting that the protein might be relatively free to move within the apoplast. This was particularly obvious in developing ovules where protein accumulates in the large apoplastic compartments between the inner and outer cell layer of the outer integument (Figure 3).

These complementation studies suggest that the extracellularly localized crinkly repeat domain is necessary for ACR4 function. As previously reported, it is predicted to fold into a β-propeller type structure (McCarty and Chory, 2000; Cao et al., 2005). A core C(X~10)CWG sequence motif is highly conserved amongst the repeats. Modeling of the ACR4 extracellular crinkly repeats was undertaken based on the x-ray structures of (1) two seven-bladed β-propeller folds the β-Lactamase Inhibitor Protein-II (BLIP-II) from *Streptomyces exfoliatus* (Lim et al., 2001) and (2) the Regulator of Chromosome Condensation (RCC1) from human (Renault et al., 1998), as described in Methods and Supplemental Figure 3 online. The regularly spaced Cys residues in the extracellular crinkly repeat domain (absent in template structures) are likely to form stabilizing disulfide bridges because they occur on neighboring antiparallel β-strands within each repeat, close in space within the folded three-dimensional model

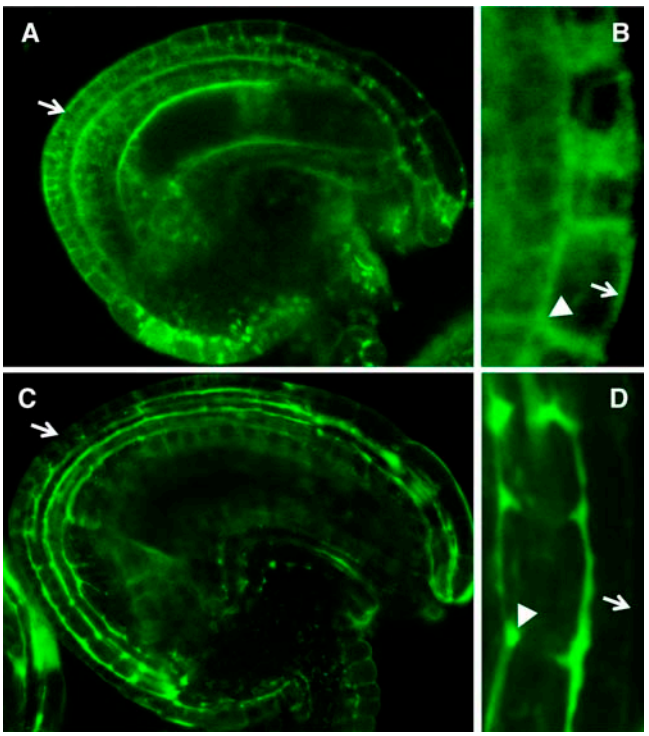


**Figure 3.** The ACR4 TNFR-Like Domain and Crinkly Repeats Are Targeted to the Extracellular Space.

Ovules expressing ACR4:GFP (**[A]** and **[B]**) and ΔTM/KIN/C-TER:GFP (**[C]** and **[D]**). Arrows indicate the ovule surface. Accumulation of fluorescence at cell junctions (arrowheads) and in the apoplastic space between integument cell layers is evident in **(C)** and **(D)** but not in **(A)** and **(B)**.

structure, and could stabilize the β-propeller structure in the oxidizing extracellular environment (Figure 4A). Interestingly, similarly spaced Cys residues in putative RCC1-like β-propeller sequences (with the exception of other plant CR4-like proteins) were found only in proteins from two bacteria; *Actinoplanes teichomyceticus* (one protein; data not shown) and *Bdellovibrio bacteriovorus* (four proteins, one example shown in Supplemental Figure 4 online).

To test further the importance of the crinkly repeats for ACR4 protein function, a TILLING analysis was performed. This analysis identified 16 single base pair changes within the crinkly repeat domain that were predicted to alter amino acids conserved either between the ACR4 and CR4 proteins and/or between the conserved crinkly repeats (see Supplemental Table 2 online). Of these changes, 12 were aphenotypic (or showed phenotypes that were too weak to be detected). Four alleles showed a phenotype indistinguishable from *acr4-2* (see Supplemental Table 3 online). Of these, three introduced stop codons and so would be predicted to produce drastically truncated proteins (*acr4-8*, *acr4-9*, and *acr4-10*). The fourth (*acr4-7*) changed a single Cys from the core C(X～10)CWG of the fourth repeat to a Tyr (Y(X～10)CWG) and thus might be expected to disrupt folding of this repeat within the β-propeller structure (Figure 4A). To verify that the null phenotype of *acr4-7* was not simply due to complete destabilization of the protein, the open reading frame, including the single base pair change, was amplified out, fused to GFP, placed under control of the *ACR4* promoter, and transformed into wild-type plants (ACR4C180-Y:GFP). Plants were examined for protein expression and localization by studying GFP fluorescence (Figure 4B). Protein was localized normally to the plasma membrane of root epidermal cells, ovules, and other meristematic regions, indicating that loss of function in this allele is not due to a total lack of protein stability or abnormal localization. To confirm that ACR4C180-Y:GFP was not functional, the same construction was transformed directly into *acr4-2* mutant plants. As predicted, no complementation of the *acr4* mutant phenotype was observed (see Supplemental Table 2 online).

A final indication of the functional importance of the extracellular domain came from two constructs that were originally designed to follow the fate of the N-terminal region of ACR4. In these constructions, a signal peptide and GFP were fused N-terminally to full-length ACR4, either alone (GFP:ACR4) or in combination with the existing C-terminal fusion (GFP:ACR4:GFP). Although these fusion proteins localized correctly to the membrane, they were unable to complement the *acr4* mutant phenotype and were therefore nonfunctional. It is possible that adding a GFP moiety to the N terminus of ACR4 prevents the normal interactions and/or folding of the crinkly repeat domain from taking place.

### Functional Analysis of the ACR4 Cytoplasmic Domain

To ascertain the roles of the cytoplasmic domains of ACR4, constructs were made that lacked the kinase domain and C-terminal domain (ΔKIN/C-TER:GFP), that lacked just the C-terminal domain (ΔC-TER and ΔC-TER:GFP), or that had a single Lys–Met amino acid change in the activation loop of the kinase domain, which has previously been shown to eliminate kinase activity in vitro (KIN-NULL and KIN-NULL:GFP) (Gifford et al., 2003). ΔKIN/C-TER:GFP did not complement *acr4.* However, no detectable GFP fluorescence was observed in ΔKIN/C-TER:GFP plants, and no signal was detected on protein gel blots other than a weak band of the size of GFP (data not shown), suggesting that this deletion derivative may be unstable. By contrast, both ΔC-TER and KIN-NULL complemented the *acr4* mutant phenotype, and both proteins, when fused to GFP, were

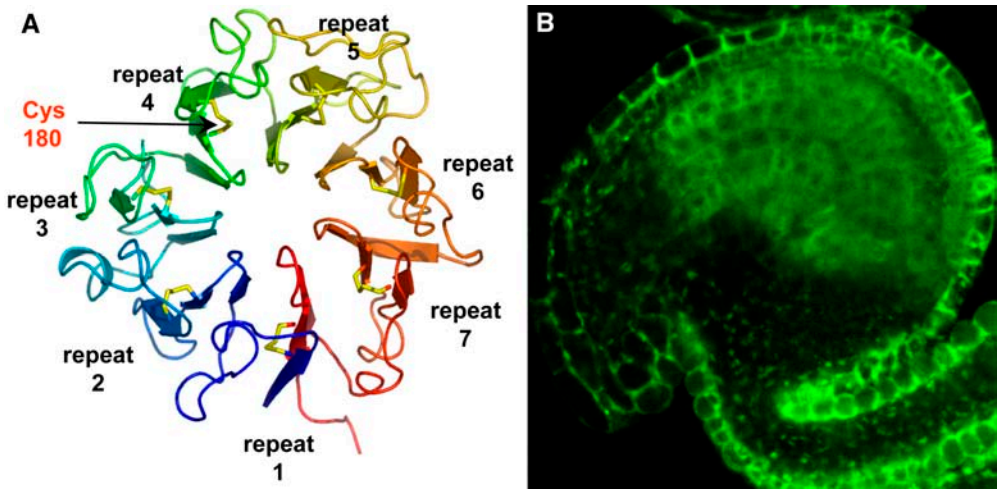


**Figure 4.** Three-Dimensional Model of the ACR4 Crinkly Repeat Domain.

PyMol (http://www.pymol.org) illustration of the three-dimensional model structure of the crinkly repeat domain of ACR4, with secondary structure assigned by the standard settings within PyMol. The model figure depicts a seven-bladed β-propeller fold, and the crinkly repeats are labeled. The seven putative disulfide bridges (shown as sticks) are colored yellow, and Cys 180, which is mutated to Tyr in *acr4-7*, is indicated **(A)**. The full-length *ACR4* open reading frame from *acr4-7* was amplified, fused to *GFP*, and expressed under the *ACR4* promoter **(B)**. The predicted protein product of *acr4-7* localizes normally to cell membranes in ovules.

found to localize normally to the plasma membrane. KIN-NULL:GFP fluorescence was slightly reduced compared with that of ACR4:GFP (data not shown). These results suggest that neither the highly conserved C-terminal domain nor the kinase activity of ACR4 is required for its signaling function.

### Subcellular Behavior of Modified Proteins

To follow up the implications of our functional analysis at the cellular level, BFA treatments were performed on the roots of plants expressing ACR4 derivatives fused to GFP. All complementing GFP fusion derivatives showed localization sensitivity to BFA in exactly the same manner as ACR4:GFP. However, neither ΔTM/KIN/C-TER:GFP, ΔREPEAT:GFP, nor ACR4C180-Y:GFP entered BFA bodies upon treatment. These constructs are exported normally to the membrane, again suggesting that export of ACR4 protein to the membrane is not BFA sensitive in roots. This was investigated in greater depth by looking at colocalization between GFP fluorescence and FM4-64 fluorescence in the roots of plants carrying ACR4C180-Y:GFP or ΔREPEAT:GFP. In these plants, GFP-containing bodies in young meristematic cells looked identical to those of ACR4:GFP plants, and the same two populations of bright GFP-only–labeled compartments and FM4-64–labeled vesicles were observed (data not shown). Colocalizing vesicles were, however, hard to find. This was borne out by observations in older cells (elongation zone) where considerably less colocalization between GFP and FM4-64 was seen than in ACR4:GFP plants, although bodies showing colocalization were still occasionally observed (Figures 5A to 5C). Upon treatment with BFA, little or no GFP fluorescence was found in FM4-64–labeled BFA bodies, but the same population of GFP-only–labeled compartments as in non-BFA–treated controls was observed (Figures 5D to 5F). Thus, localization of nonfunctional ACR4 derivatives in internalized vesicles is diminished compared with that of functional ACR4 derivatives.

### Behavior of Deleted Proteins at the Protein Level

To investigate whether protein internalization is linked to some form of posttranslational modification of ACR4, proteins from plants carrying ACR4:GFP derivatives were analyzed by protein gel blots. Inflorescences from plant expressing ACR4:GFP, GFP:ACR4, GFP:ACR4:GFP, ΔREPEAT:GFP, ΔTNFR:GFP, ΔTM/KIN/C-TER:GFP, KIN-NULL:GFP, and ΔC-TER:GFP were either prepared as total extracts or immunoprecipitated from triton soluble fractions using an anti-GFP antibody and subjected to protein gel blot analysis. In a separate experiment, a protein gel blot was also performed on total extracts from plants expressing ACR4C180-Y:GFP and control plants expressing ΔREPEAT:GFP (Figure 6). Unlike the situation in plants carrying ACR4:GFP, predicted full-length protein products were detected in plants expressing each of the seven constructions ΔREPEAT:GFP (predicted 101 kD), ΔTNFR:GFP (predicted 118 kD), ΔTM/KIN/C-TER:GFP (predicted 73 kD), ΔC-TER:GFP (predicted 115 kD), ACR4C180-Y:GFP (predicted 125 kD), GFP:ACR4 (predicted 128 kD), and GFP:ACR4:GFP (predicted 155 kD). Full-length ΔREPEAT:GFP and ACR4C180-Y:GFP were highly stabilized and detectable without immunoprecipitation, in agreement with

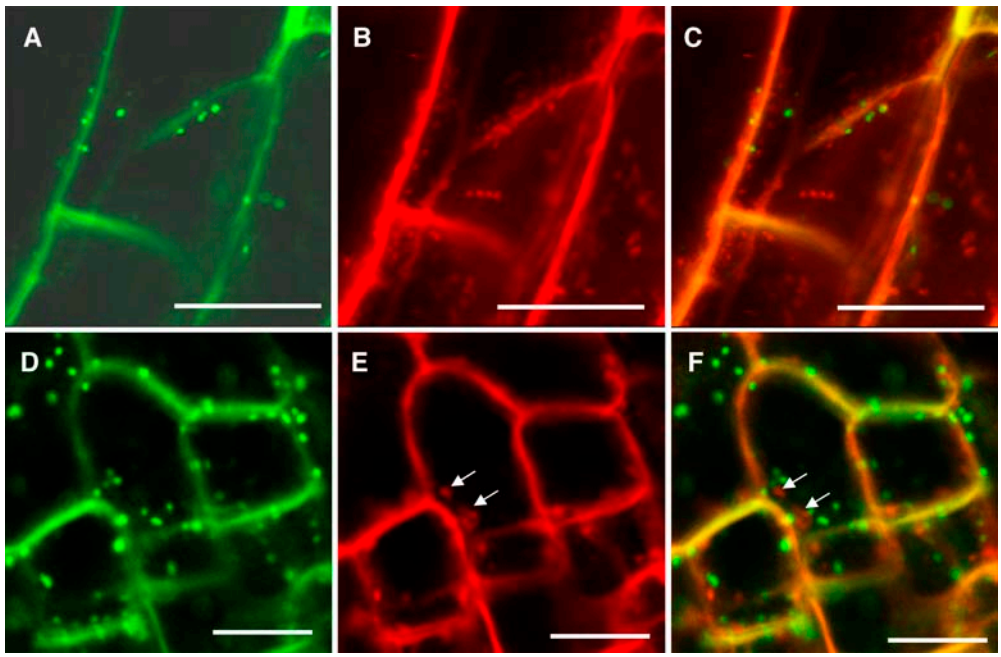


**Figure 5.** Cellular Behavior of Nonfunctional ACR4 Variants.

Visualization of GFP (**[A]** and **[D]**), FM4-64 (**[B]** and **[E]**), and merged images (**[C]** and **[F]**) of root cells from plants carrying the ΔREPEAT:GFP construct. **(A)** to **(C)** show cells from the elongation zone, and **(D)** and **(E)** show BFA-treated meristematic cells. Little or no colocalization of GFP and FM4-64 is observed in these plants. BFA bodies are indicated with arrows. Bars = 5 μM.

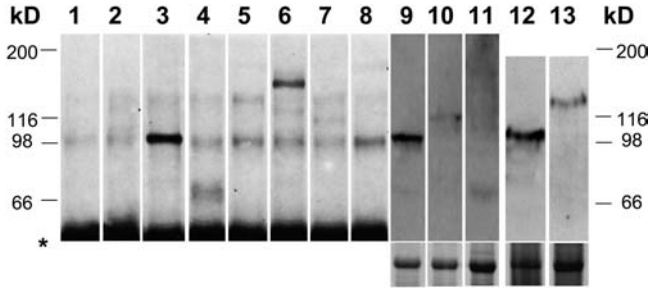


**Figure 6.** Protein Gel Blot Analysis of GFP Protein Fusions.

Protein gel blot analysis of proteins isolated from plants carrying GFP-tagged *ACR4* derivatives using an anti-GFP polyclonal antibody. Lanes 1 to 8 were immunoprecipitated with anti-GFP polyclonal antibody, and lanes 9 to 13 show total extracts. Lanes are as follows: 1, Columbia-0 (untransformed); 2, ACR4:GFP; 3, ΔREPEAT:GFP; 4, ΔTM/KIN/C-TER:GFP; 5, GFP:ACR4; 6, GFP:ACR4:GFP; 7, ΔC-TER:GFP; 8, KIN-NULL:GFP; 9, ΔREPEAT:GFP; 10, ΔTNFR:GFP; 11, ΔTM/KIN/C-TER:GFP; 12, ΔREPEAT:GFP; 13, ACR4C180-Y:GFP. The asterisk indicates the immunoglobulins remaining after elution of the immunoprecipitation. Protein loading controls (from a stained gel with identical aliquots of protein samples) are shown below lanes 9 to 13.

live observations showing increased protein abundance in lines expressing these protein derivatives (Figure 6). Although full-length ΔTNFR:GFP was detectable without immunoprecipitation, the amount detected was considerably less than for ΔREPEAT:GFP. This result is interesting because it implies that the TNFR region may play a role that was not uncovered by our complementation analysis. A similar situation was observed for ΔC-TER:GFP, although in this case full-length protein could only be detected in immunoprecipitated samples. Full-length KIN-NULL:GFP (predicted 125 kD), as for ACR4:GFP, was difficult to detect on protein gel blots, even when samples had been immunoprecipitated. The nonfunctional GFP:ACR4 and GFP:ACR4:GFP proteins were also detectable in their full-length forms, supporting the hypothesis that loss of function is to some measure linked to protein stabilization of ACR4 derivatives.

## DISCUSSION

We have investigated the functional significance of various domains within the ACR4 protein and have found the only domain tested that is absolutely required for protein function to be the extracellular crinkly repeat domain. We have, moreover, shown that nonfunctional variants of ACR4-containing deletions or mutations of the crinkly repeat domain are considerably more stable than wild-type protein, supporting initial indications that suggested that full-length ACR4:GFP is difficult to detect by protein gel blots because it is rapidly turned over. Finally, we have shown that loss of function and stabilization correlate with an absence of mutated protein variants in the BFA bodies of BFA-treated root cells, where wild-type ACR4 is found. Recent studies have shown that BFA bodies are partially composed of endocytosed vesicles from the plasma membrane, especially in roots (Bonifacino and Jackson, 2003; Geldner et al., 2003; Grebe et al., 2003). This result in combination with observations suggesting that FM4-64–labeled internalized vesicles also contain a considerably lower concentration of nonfunctional mutant protein variants, than of wild-type ACR4, leads us to propose that nonfunctional protein variants are neither endocytosed nor turned over in the same manner as wild-type ACR4. Despite this, some internalization of nonfunctional protein variants can occasionally be observed, especially in more distal zones of the root where internalization of functional protein appears greater. The significance of this low level internalization of nonfunctional variants is unclear. We believe that changes in protein behavior observed for nonfunctional variants are unlikely to be due to differences in protein export to the membrane for two reasons: First, nonfunctional variants are normally localized within the plasma membrane, and second, putative export bodies appear normal in plants expressing these variants.

### Functional Significance of ACR4 Domains

The fact that the domain altered in our nonfunctional ACR4 variants is extracellularly localized means that the lack of internalization and degradation observed in these variants is not due to an intrinsic physical change in the cytoplasmic domain preventing its normal processing. It therefore seems likely that processing and internalization are intrinsically linked to wild-type function. There are many examples in animal systems where ligand binding–induced activation of receptor kinases results in rapid internalization and either degradation or recycling of receptor molecules (Waterman and Yarden, 2001; Oved and Yarden, 2002). This is thought to be a biologically crucial mechanism for the damping down of signaling that allows cells to remain responsive to changes in the concentration or distribution of incoming signal molecules (ligands). One possibility, then, that would explain the observed results could be that the crinkly repeat domain of ACR4 is involved either directly in ligand binding or in a protein–protein interaction that permits ligand binding (such as oligomerization). The structure of the crinkly repeat domain does suggest a potential role in interaction with a peptidic partner. The predicted β-propeller structure of this domain resembles most closely that of the β-lactamase inhibitor BLIP-II, a secreted protein produced by the soil bacterium *S. exfoliatus* (Lim et al., 2001), and of the human RCC1 (Renault et al., 1998). Both of these proteins bind other proteins via their β-propeller domains.

The functional importance of the conserved Cys residues in the crinkly repeats has been demonstrated by our tilling approach, and we hypothesize that they may have been recruited to stabilize the extracellular domains of the CR4-like proteins in plants in the apoplast. The only other predicted putative β-propeller–forming protein sequences in the databases that showed a similar distribution of Cys residues were predicted proteins from bacteria such as *B. bacteriovorus* (Rendulic et al., 2004) (see Supplemental Figure 4 online). This appears to be an interesting case of convergent evolution in protein sequence because the bacterial proteins, like the RCC1 protein, contain four predicted antiparallel β-strands in their repeats (according to PsiPred), rather than the three that are found in the repeats of CR4-like proteins and the non-Cys–containing BLIP-II protein

(see Supplemental Figure 4 online; D.C. Soares, unpublished results).

The TNFR-like region of ACR4 contains three Cys-rich repeats, of which two (by analogy to the TNFR in animal systems) could bind a ligand, whereas the third might be responsible for receptor oligomerization (trimerization in the case of TNFR) (Chan et al., 2000). To date, no molecules unambiguously resembling TNFs (the TNFR ligands) have been identified in plants. However, these molecules are very diverse even within the animal kingdom, and so their existence in plants certainly cannot be excluded. The fact that deletion of the TNFR-like region of ACR4, while not apparently compromising the ability of the protein to functionally complement *acr4* mutants, does stabilize it somewhat, could indicate a role for the TNFR domain in ACR4 protein dynamics. However, attribution of actual functions to the TNFR-like domain in ACR4 awaits more detailed analysis.

Our results regarding the functional significance of the intracellular domains of ACR4 also present some interesting questions, possibly the most intriguing of which regards the fact that the kinase activity of ACR4, at least in our hands, appears not to be necessary for protein function. One explanation for this observation could lie in the possibility that ACR4 may oligomerize with another kinase-active receptor kinase or be phosphorylated by a cytoplasmic kinase. Interesting new work has indeed shown that the ACR4 kinase domain is capable of phosphorylating the kinase domain of AtCRR2, one of four Arabidopsis RLKs closely related to ACR4, in vitro (Cao et al., 2005). AtCRR2 is kinase dead because of the deletion of a critical section of its kinase domain. However, it cannot be excluded that ACR4 heterodimerizes with another similar, but kinase active, protein. In animal systems, examples exist where kinase-deficient receptor kinases signal efficiently because of their heterodimerization with, and transphosphorylation by, a kinase-active member of the same protein family in response to ligand binding (Kim et al., 1998). Interaction with a membrane-bound cytoplasmic kinase has recently been shown to be necessary for SRK signaling (Murase et al., 2004). At least three receptor-like cytoplasmic kinases showing similarity to the ACR4 kinase domain are also predicted to exist in Arabidopsis (Shiu and Bleecker, 2003). In animal systems, the activity of dead or fractured RTKs has also been shown to be functionally compensated by cytoplasmic kinases (Kroiher et al., 2001). Another interesting possibility is that part of ACR4 signaling may pass via a route independent of ACR4 kinase activity. The ability of the maize atypical receptor kinase to directly stimulate the kinase activity of a potential downstream signaling component MARK INTERACTING KINASE could represent a novel means of signaling through receptor kinases (Llompart et al., 2003).

Our complementation result contradicts that of Watanabe et al. (2004), who have expressed a slightly different kinase-null version of ACR4 under the 35S promoter and found that this does not complement the *acr4* mutant phenotype. One attractive explanation for this discrepancy could be that the use of the 35S promoter in the Watanabe et al. study, which would lead to overexpression of their kinase-null variant, might actually lead to a dominant-negative phenomenon. If homooligomerization of ACR4 as well as heterooligomerization of ACR4 with another kinase can take place, overexpression of a kinase-null version of ACR4 could lead to mopping up of available ligand by an overrepresented population of homooligomers of kinase-null ACR4 and therefore block signaling by active hetero-oligomers.

Removal of the C-terminal domain of ACR4 produced a protein that was able to complement *acr4* mutants but that, like ΔTNFR, appeared more stable than ACR4. The increase in stability was less marked than for ΔTNFR but could, as hypothesized for ΔTNFR, be due to a role for this domain in protein dynamics. The C-terminal of ACR4, being cytoplasmically located, could be directly involved in protein turnover. Indeed, several animal receptors have been shown to be targeted for internalization/turnover by ubiquitylation on Lys residues near to their C termini (Marmor and Yarden, 2004). The C-terminal domain of ACR4 is highly conserved with maize (*Zea mays*) CR4 and contains conserved Lys residues. This domain is absent from all other homologous RLKs. Our result again contradicted results reported by Watanabe et al., who generated a similar C-terminal deletion construct expressed under control of the 35S promoter that did not complement *acr4* mutants.

### The Dynamics of ACR4 Protein Localization

ACR4 appears to be unique amongst plant RLKs studied to date, in the fact that it is so rapidly turned over. This rapid turnover is borne out by several observations, the most compelling being that full-length nonfunctional protein variants expressed under exactly the same promoter are easily detected on protein gel blots. Another indication of rapid turnover comes from the large

**Table 1.** Primer Pair Combinations Used for Construction of Deletion Constructs

| Construct | N-Terminal End Amplified with: | C-Terminal End Amplified with: |
|---|---|---|
| pΔ39aa | CR5/CREC5 (see text) | CREC3 (see text)/CR3 |
| pΔ39aa:GFP | CR5/CREC5 | CREC3/GFP3STOP |
| pΔTNFR | CR5/CRTN5 (5′-AGATCTATACAGAGTCCTGGTG-3′) | CRTN3 (5′-GGATCCCAGTGGAGGCAAGGAAAAAG-3′)/CR3 |
| pΔTNFR:GFP | CR5/CRTN5 | CRTN3/GFP3STOP |
| pΔTM/K/C-ter | CR5/CRNM5 (5′-GGATCCTTTTTCCTTGCCTCCACTGG-3′) | – |
| pΔTM/K/C-ter:GFP | CR5/CRNM5 | CRCT3 (5′-AGATCTAGAATGAGTAAAGGAGAAGAAC-3′)/GFP3STOP |
| pΔC-ter | CR5/CRCTstop (5′-GAGCTCTATAGCTGTGCAAGCGCTCG-3′) | – |
| pΔC-ter:GFP | CR5/CRCT5 (5′-GGATCCCATTAGCTGTGCAAGC-3′) | CRCT3/GFP3STOP |

quantities of protein that are observed in putative export compartments. This suggests that the supply at the plasma membrane is in constant need of replenishment. This is particularly true after cycloheximide recovery, where very large quantities of protein appear to be being exported. By comparison, the fluorescence observed in internalized vesicles is relatively weak, suggesting that proteins may be being rapidly degraded upon internalization.

Little has been published regarding the in planta control of trafficking and intracellular turnover of plant receptor kinases. In addition, the majority of kinases studied to date have belonged to the LRR plant RLK superfamily, and it is possible that such receptors are regulated differently from the ACR4 class. This certainly appears to be the case for BRI1, where a constitutive endocytosis and recycling of the protein in root cells appears to occur (Russinova et al., 2004). This may explain why, in most cases studied, LRR RLK proteins can be detected as full-length proteins in vivo. However, because of the fact that ACR4 is so rapidly turned over, we feel that the internalization observed in our study likely does not represent this type of recycling. Interestingly, we have confirmed that ACR4, like its homolog in maize CR4, does not interact with KAPP in vitro (Braun et al., 1997) (our unpublished results). The same is also true for ZmPK1, an S-family RLK from maize (Braun et al., 1997). This lack of interaction with KAPP could indicate that dephosphorylation is not a major means of downregulation for these proteins. One possible explanation of the fact that ACR4 appears to be turned over so rapidly could therefore be that immediate targeting for degradation plays a greater role in damping down ACR4-mediated signaling than the signaling of KAPP-dephosphorylated RLKs. However, it cannot be excluded that phosphatases other than KAPP dephosphorylate ACR4 at the membrane.

A study of SERK1, an LRR RLK from Arabidopsis, has shown that it can be internalized, at least in cowpea protoplasts, in response to the activity of KAPP (Shah et al., 2002). However, to gain a meaningful insight regarding the biological control of internalization in response to ligand binding, it is necessary either to study receptors in planta and in tissues where their ligands are likely to be present (under their own promoters in the case of developmentally important receptors) or to identify and be able to provide the natural ligand of the receptor. We have performed this study under the native *ACR4* promoter so that the quantities and localizations of proteins produced are as near physiological as possible. In situ hybridization and promoter studies have shown that the promoter is active only in meristematic L1 cells and is, moreover, relatively weak (Gifford et al., 2003). This may well compound our difficulty in detecting unmodified ACR4:GFP protein. However, using a stronger promoter, or a promoter active in other cell layers, would undoubtedly provide unphysiological and possibly artifactual data.

In summary, we have performed a functional analysis of the ACR4 RLK and shown that its extracellular domain is absolutely required for function and for wild-type turnover of this protein at the membrane. The fact that wild-type ACR4 is so rapidly turned over at the membrane is intriguing and may well indicate that the regulation of its activity differs from that of other previously characterized plant RLKs. Further studies regarding the interactions of ACR4 should help elucidate this observation further.

## METHODS

### Modeling the Extracellular Crinkly Repeats

Modeling of the ACR4 extracellular crinkly repeats was undertaken based on the x-ray structures of two seven-bladed β-propeller folds, predicted to be significant hits by the fold-recognition server 3D-PSSM (Kelley et al., 2000): (1) BLIP-II from *Streptomyces exfoliatus* (PDB ID: 1JTD) (Lim et al., 2001) and (2) RCC1 from human (PDB ID: 1A12) (Renault et al., 1998) using the program Modeller release 7v7 (Sali and Blundell, 1993). These proteins share 22 and 19% pairwise identity, respectively, with the query sequence.

The alignment between the target sequence of the crinkly repeats and the template structures was based on an initial multiple sequence alignment using the program T-Coffee 1.42 (Notredame et al., 2000). The multiple sequence alignment between the target and templates was manually edited to ensure the most plausible alignment of conserved amino acids and also secondary structure elements guided by the secondary structure prediction server, PsiPred 2.4 (McGuffin et al., 2000). It should be noted that in the two template β-propeller structures, the first half of the first structural repeat, is made from the C-terminal end of the sequence, and the second half of the first structural repeat is made from the N-terminal end of the sequence (Renault et al., 1998; Lim et al., 2001). This was appropriately adjusted in the alignment with respect to the target sequence of the first crinkly repeat (see Supplemental Figure 3 online). The seven putative disulfide bridges within the seven repeats were restrained during model building. Twenty models were generated, and the one with the lowest objective function score (Sali and Blundell, 1993) selected as the representative model. The loop in repeat 3, for which no template-derived restraints were available, was deleted from the final model. The representative model structure was checked for valid stereochemistry using PROCHECK 3.5.4 (Laskowski et al., 1993).

### Visualization and Pharmacological Techniques

GFP-labeled proteins and FM4-64–labeled vesicles were visualized in roots and ovules using a Bio-Rad Radiance 2100 confocal microscope (Hemel Hempstead, UK). FM4-64 fluorescence was detected using a 620-nm long-pass filter, and GFP fluorescence was detected with 500-nm long-pass and 530-nm short-pass filters. Some bleedthrough from the FM4-64 channel to the GFP channel was observed when wild-type (untransformed) plants were treated with FM4-64 (see Supplemental Figure 1 online); however, we are confident that this is limited to plasma membrane regions because no bleedthrough from more weakly fluorescent endocytosed vesicles was observed using our standard GFP detection conditions. In addition, the same two populations of strongly and weakly fluorescent GFP-labeling bodies were detected in non-FM4-64–labeled plants. Images were exported and treated using ImageJ software. FM4-64 (T3166; Molecular Probes, Eugene, OR) treatments were performed as described in the text, following the method outlined by Bolte et al. (2004). BFA (B7651; Sigma-Aldrich, St. Louis, MO) treatments were performed as described in the text following the methods described by Geldner et al. (2003). Cycloheximide (C7698; Sigma-Aldrich) treatments were performed as described in the text.

### DNA Constructs

The construction of plasmid pMD5 (containing the *ACR4* open reading frame [ORF] under control of the *ACR4* promoter), used for complementation analysis of *acr4* mutants, and the complementing fusion of the full-length *ACR4* ORF with the *mGFP6* variant (pMD11) were as described by Gifford et al. (2003). To make construct ΔK/C-TER:GFP the *ACR4* ORF lacking both kinase and C-terminal regions was amplified using CR5

(Gifford et al., 2003) and 5′-CTCGAGGAGCACCTACAATTCCTCAATC-3′, fused C-terminally to *mGFP6*, and placed under the control of the *ACR4* promoter as described for pMD11. The resulting binary vector was designated pMD12. To create variants containing N-terminal *GFP* sequences, the *mGFP6* ORF was amplified from pBSmGFP6 (Gifford et al., 2003) using primers 5′-ATCTAGAATGAGTAAAGGAGAAGAAC-3′ and 5′-AGGTACCAGGTGTTTGTATAGTTCATCC-3′, cloned into pGEMT-easy and then removed using *Kpn*I and *Sac*I. This was inserted in frame, upstream of the *ACR4* ORF in pMD5, to create pMD58. The *GFP* ORF was also cloned into pMD11 to create a line with GFP tags at both termini of the ACR4 protein (pMD59). To ensure correct targeting of ACR4, the *ACR4* signal peptide was placed upstream of the N-terminal *GFP* sequences. The signal peptide was amplified from pL92 (Gifford et al., 2003) using CR5 and 5′-TCTAGACATTGAACCAAGAGCTG-3′ and cloned into pGEMT-easy (Promega, Madison, WI). The insert was removed by digesting with *Eco*RI, cloned into *Eco*RI-cut pBluescript KS vector (Stratagene, La Jolla, CA), and cloned with the appropriate orientation digested with *Xba*I to remove the signal peptide–containing insert. This was inserted into *Xba*I-digested pMD58 and pMD59, creating pMD61 and pMD60, respectively. pMD61 is referred to as pGFP:ACR4 and pMD60 as pGFP:ACR4:GFP.

To create a construct where 4.5 of the seven crinkly repeats had been removed, CREC5 (5′-AGATCTGACCAGGAGTCCCATCG-3′) and CR5 were used to amplify the appropriate N-terminal region of *ACR4* from pL92. This product was cloned into pGEMT-easy (pMD30). CREC3 (5′-GGATCCCAGGTATCGGCTTTTATGATC-3′) and CR3 (5′-GAGCT-CAGAAATTATGATGCAAGAACAAGC-3′) were used to amplify the appropriate C-terminal region. This C-terminal product was cloned into pGEMT-easy, removed by digesting with *Bam*HI and *Sac*I, and ligated in frame into *Bgl*II/*Sac*I-cut pMD30. This *ΔREPEAT* ORF was then removed using *Kpn*I/*Sac*I and cloned into *Kpn*I/*Sac*I-cut (*ACR4* ORF removed) pMD5 downstream of the *ACR4* promoter, creating pΔREPEAT. In a similar fashion, pΔTNFR, pΔTM/K/C-ter, and pΔC-ter constructs were made by amplifying with the primer combinations listed in Table 1. The N-terminal and C-terminal ends (where required) were then ligated and cloned into pMD5 as above. To create *GFP*-tagged variants of these deletion constructs, the required C-terminal fragment of the *ACR4* ORF fused to *GFP* was amplified from pMD11 using the primer GFP3STOP (5′-TCTAGTGTTTGTATAGTTCATCCATG-3′) in the place of CR3 (as listed in Table 1), ligated to the appropriate N-terminal fragment end, and cloned into pMD5. To create the kinase-null ACR4 variants, pL92 and pMD9 (containing the *ACR4* ORF fused to the *mGFP6* ORF) were subjected to site-directed mutagenesis using the same approach and primers as described by Gifford et al. (2003). This mutates the conserved Lys 540 in the ACR4 kinase domain to Met. *Kpn*I/*Sac*I insertions from the resulting plasmids were then cloned into *Kpn*I/*Sac*I-cut pMD5 as previously. Predicted protein molecular weights were calculated in EditSeq (Lazergene, Madison, WI).

To create pACR4C180-Y:GFP, the *ACR4* ORF was amplified from *acr4-7* homozygous plants and cloned into pGEMT-easy. An internal *Hin*dIII fragment containing the required mutation was excised and used to replace the corresponding wild-type *Hin*dIII fragment in pMD9 (above). The insert from this vector was then cloned downstream of the *ACR4* promoter in pMD5 as described above.

Plant transformations were performed as described by Gifford et al. (2003), and complementation of the *acr4* mutant phenotype was investigated in the *acr4-2* null allele as described by Gifford et al. (2003). Complementation was ascertained by screening primary transformants by eye for the absence or presence of characteristic ovule and seed shape defects and seed development defects seen in *acr4* mutants (see Supplemental Figure 2 and Supplemental Table 1 online). Representative examples of plants showing complementation were subjected to a PCR-based verification that they were not wild-type contaminants.

### Analysis of Protein Extracts from Plants

Proteins were extracted from inflorescence tips comprising all unopened flowers and the first open flower. Fifty to seventy such tips were used for each extraction. Material was collected into liquid nitrogen and then ground to a fine powder while frozen in a pestle and mortar. Two hundred microliters of extraction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.1 M DTT, and 1% [w/v] Triton, pH 7.5) with freshly added EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN) were added. Samples were then either immunoprecipitated using a polyclonal rabbit anti-GFP antibody (Molecular Probes) and Protein-A coupled Dynabeads (Dynal, Oslo, Norway) following the manufacturers' instructions with room temperature elution in 15 μL of 1× protein loading buffer (Sigma-Aldrich) or 0.2 volumes of 6× protein loading buffer (0.35 M Tris-HCl, 10.28% [w/v] SDS, 36% [w/v] glycerol, 0.6 M DTT, and 0.012% [w/v] bromophenol blue, pH 6.8) were added to the total sample. Samples were boiled for 1 min (immunoprecipitations) or 5 min (total extracts) and centrifuged for 1 min, and then 15 μL aliquots were loaded onto 10% acrylamide gels. Gels were subjected to protein gel blotting onto nitrocellulose membranes (Protran; Schleicher and Schuell, Dassel, Germany) using standard procedures, and GFP-labeled proteins were then detected using a rabbit polyclonal anti-GFP primary antibody (Molecular Probes) and a horseradish peroxidase–linked donkey anti-rabbit secondary antibody (Amersham Biosciences, Little Chalfont, UK). Peroxidase activity was detected using the ECL protein gel blotting analysis system (Amersham Biosciences).

## TILLING

Full information regarding TILLING alleles *acr4-7* to *acr4-22* is presented in Supplemental Table 2 online. TILLING of the extracellular region–encoding domain of *ACR4* was performed as described by Till et al. (2003a) (2003b). In this study, we describe alleles *acr4-7* to *acr4-10*. The nature of these mutations and the strategy for their genotyping by cleaved-amplified polymorphic sequences is shown in Supplemental Table 3 online.

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